Comprehensive molecular approaches to explore Bacteriophage Insensitive Mutants (BIMs) generated by phage infections in *Salmonella* Enteritidis S37 and S49

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By

Mridula V.G.

Reg. No. 4834

Under the guidance of

Dr. SARITA G. BHAT

Microbial Genetics Laboratory
Department of Biotechnology
Cochin University of Science and Technology
Cochin - 682 022, Kerala, India.

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DEPARTMENT OF BIOTECHNOLOGY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COCHIN - 682 022, KERALA, INDIA.

Ph: 0484 – 257667 | Email: saritagbhat@gmail.com | Fax: 91-484-2576267, 2577595

Dr. Sarita G. Bhat
Professor

Date: /10/2015

CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Comprehensive molecular approaches to explore Bacteriophage Insensitive Mutants (BIMs) generated by phage infections in *Salmonella* Enteritidis S37 and S49"** is based on the original research work carried out by Ms. Mridula V.G. under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

DR. SARITA G. BHAT
(Supervising Guide)

DECLARATION

I hereby declare that the thesis entitled **"Comprehensive molecular approaches to explore Bacteriophage Insensitive Mutants (BIMs) generated by phage infections in *Salmonella* Enteritidis S37 and S49"** is the authentic record of research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology for my doctoral degree, under the supervision and guidance of Dr. Sarita G. Bhat, Professor, Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has previously formed the basis for the award of any degree or diploma, associate ship or other similar titles or recognition.

Cochin-22 **Mridula V.G.**

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Buddhy[i]-Aatmanaa Vaa Prakrteh Svabhaavaat |
Karomi Yad-Yat-Sakalam Parasmai
Naaraayannayeti Samarpayaami ||

Whatever I do with the body, speech, mind or the sense organs, either by discrimination of the intellect, or by the deeper feelings of the heart, or by the existing tendencies of the mind, I Do them All (i.e. Whatever work is to be done) without Ownership, And I Surrender them at the feet of Sri Narayana.

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Mridula V.G.

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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
APS	-	Ammonium persulfate
ATP	-	Adenosine tri phosphate
BHI	-	Brain heart infusion broth
BIMs	-	Bacteriophage Insensitive Mutants
BLAST	-	Basic Local Alignment Search Tool
Bp	-	Base pair
CFU	-	Colony Forming Units
CRISPR	-	Clustered Regularly Interspaced Short Palindromic Repeats
cm	-	Centimetre
CTAB	-	Cetyl trimethyl ammonium bromide
Da	-	Dalton
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
EtBr	-	Ethidium bromide
FDA	-	Food and Drug Administration
Fig	-	Figure
g	-	Grams
h	-	Hours
H_2S	-	Hydrogen sulphide

ie.	-	that is
kb	-	Kilobase
kDa	-	Kilo Dalton
L	-	Litre
LB	-	Luria Bertani
LIA	-	Lysine iron agar
Log	-	Logarithm
LPS	-	Lipopolysaccharide
M	-	Molar
M	-	Metre
$MgCl_2$	-	Magnesium chloride
MLST	-	Multilocus sequence typing
mg	-	Milligram
MHB	-	Mueller Hinton broth
MIC	-	Minimum Inhibitory Concentration
Min	-	Minutes
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
N	-	Normality
NA	-	Nutrient agar
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NB	-	Nutrient broth
NCBI	-	National Center for Biotechnology Information

ng	-	Nanogram
Nm	-	Nanometer
No.	-	Number
OD	-	Optical density
OD_{600}	-	Absorbance at 600nm
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
pH	-	Power of Hydrogen
pI	-	Isoelectric point
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
s	-	Seconds
SDS	-	Sodium dodecyl sulphate
SGI	-	*Salmonella* genomic island
SPI	-	*Salmonella* Pathogenecity island
sp.	-	Species
TAE	-	Tris-acetate-EDTA
Taq	-	Thermus aquaticus
TE	-	Tris-EDTA
TEMED	-	N-N-N’-N’-Tetramethyl ethylene diamine
TSI	-	Triple sugar iron
TTSS	-	Type three secretion system
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-Visible

V	-	Volts
v/v	-	Volume/volume
viz.	-	Namely
XLD	-	Xylose lysine desoxycholate
w/v	-	Weight/volume
µg	-	Microgram
µL	-	Microlitre
µM	-	Micromole
µM	-	Micromolar
µm	-	Micrometer

Chapter 1. Introduction

Thousands of microorganisms impact health, safety, and economic stability of populations. Infectious microorganisms include bacteria, viruses, fungi, and protozoa (Ecker *et al*., 2005). Bacterial food-borne agents have been most investigated and monitored, especially causes of enteric communicable disease. Food is a superb vehicle by which several pathogens (bacteria, viruses/prions and parasites) can reach an appropriate colonization site in a new host. Although food production practices change, the well-recognized food-borne pathogens, such as *Salmonella* spp. and *Escherichia coli* appear able to evolve using novel opportunities, and even generate new public health challenges (Newell *et al*., 2010). According to Center for Disease Control and Prevention (CDC) every year, *Salmonella* is estimated to cause a million sicknesses within the United States, with 19,000 hospitalizations and 380 deaths (CDC May 2015). Most persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection.

Salmonella enterica serovar Enteritidis (SE) is the world's leading reason behind human salmonellosis (Guard *et al*., 2015). The reservoir for *S*. Enteritidis is mainly poultry usually adopting asymptomatic infection (Bäumler *et al*., 2000) particularly hen house conditions, the birds, the eggs as well as the human host; and through the food production chain they will consequently pass to humans (Guard – Petter, 2001).

Antibiotics are not only used to treat human illness, but also have been additionally utilized in livestock and poultry for more than half a century to control and treat diseases; and in sub-therapeutic doses in animal feeds, to promote growth and improve production of animal products (Stokstad *et al*., 1953; Pagel and Gautier, 2012). Unfortunately, this inappropriate and excessive use of antibiotics in animal husbandry is

threatening their efficacy (Jassim and Limoges, 2014) and has resulted in the development of antibiotic-resistant bacteria (Lu, 2006). *Salmonella* have evolved many virulence and antimicrobial resistance mechanisms that continue to challenge the public health infrastructure (Foley and Lynne, 2008). The emergences of infectious disease caused by drug resistant bacteria definitely need alternatives to traditional antibiotics (Barrow and Soothill, 1997; Alisky *et al*., 1998; Carlton, 1999; Sulakvelidze *et al*., 2001).

Search for new alternative anti-microbials, resulted in the use of bacteriophage as potential candidates effective against bacterial pathogens and became more and more relevant for both human and veterinary applications (Boyle *et al.,* 2007). Bacteriophages are natural antibacterials, able to regulate bacterial populations by the induction of bacterial lysis. They are active against Gram-positive, (Matsuzaki *et al.,* 2003; Biswas *et al.,* 2002) as well as Gram-negative bacteria, (Vinod kumar *et al.,* 2005; Wang *et al.,* 2006) including Multi Drug Resistant (MDR) pathogens. As mechanism of action of phage lysis is completely different from antibiotics, retaining activity against bacteria exhibiting multiple mechanisms of antibiotic resistance (Hanlon, 2007) is not uncommon. Because of its specificity, phage therapy has a narrow antibacterial spectrum with control restricted to one single species or in some cases a single strain among a species. This limits the "pressure" and the heavy collateral damage done to bystander, non-targeted bacteria from antibiotics. The entire micro biome of the patient is altered by antibiotics, not simply the intended target infectious agent (Chibani-Chennoufi *et al.,* 2004).

One of the foremost issues due to the use of phages as therapeutics is the development of phage-resistant mutants (Smith and Huggins, 1983; Gill and Hyman,

2010) also known as Bacteriophage insensitive mutants or BIMs. To protect against the invading phages, bacteria have developed several defense mechanisms such as prevention of adsorption, blocking injection, cleaving phage nucleic acid, and aborting infection (Labrie *et al*., 2010).

Recently, a completely unique defense system, clustered regularly interspaced short palindromic repeats (CRISPR) loci, has been identified as a form of acquired immunity against invading foreign DNA including bacteriophage and plasmid DNA (Barrangou *et al*., 2007; Marraffini and Sontheimer, 2008; Brouns *et al*., 2008). CRISPR loci are found in almost all archaea and approximately 40% of sequenced bacterial genomes. They comprise a short repeat sequence (21–47 bp) separated by a unique variable sequence known as a spacer (Sorek *et al*., 2008; Bolotin *et al*., 2005; Godde and Bickerton, 2006). The repeat sequence is highly conserved within a particular CRISPR locus. In contrast, the spacers vary greatly and their sequences have similarity to phages and plasmids and sometimes to host chromosomal sequences (Stern *et al*., 2010). Acquired immunity involving CRISPR/Cas systems is divided into two stages: the acquisition stage for uptake of the foreign element as a spacer into the leader-proximal end of CRISPR, and also the immunity stage involving interference with the targeting of DNA in a very sequence-specific manner (Deveau *et al*., 2010; Hovarth and Barrangou, 2010).

Noting the quickly growing resistance, the advocates of phage therapy typically refer to the advantages of phage cocktails, in which many different types of phages infect the same species or strains, and thus make the emergence of a resistant bacterial cell substantially less likely (Örmälä and Jalasvuori, 2013). One obvious advantage of a cocktail could be a collective host range that may obviate the requirement to characterize phage sensitivities of the infecting pathogenic bacteria. A second attainable advantage is in thwarting resistance: if multiple phages target the same bacterium,

evolution of resistance to all such phages is also needed, before treatment fails. A third possible mechanism is dynamical: two phages may collectively kill the bacterial population more rapidly or more completely than either phage alone (Schmerer *et al*., 2014). Phage cocktails used for reduction of *Salmonella* Enteritidis *in vivo* and *in vitro* (Andreatti Filho *et al*., 2007) might have prevented the emergence of phage-resistant mutants as phages using different receptors had found their host and multiplied. Therefore, it appears that the potential obstacles to the use of this treatment including narrow host range of phage, resistance of host bacteria to phage, potential for inactivation by the patient's immune system, and safety of phage preparations in humans, can be partially overcome by using unique receptor-specific phage cocktail (Donlan, 2009).

OBJECTIVES OF THE STUDY

One of the potential replacement choices for antibiotics is the use of bacteriophages as antimicrobial agents. Phage therapy is an important alternative to antibiotics in the current era of drug-resistant pathogens (Golkar *et al.,* 2014). A major concern of bacteriophage therapy is that the emergence of BIMs (Filippov *et al*., 2011).

The development of phage resistance can be forestalled altogether if phages are utilized in cocktails (Kutateladze and Adamia, 2010). To address this concern it is important to study the host mutants generated after infection and understand the changes that occur in the host. Hence this study aims to isolate and characterize the BIMs generated from *Salmonella* Enteritidis infected by their specific bacteriophages, and study them with respect to their antibiogram, CRISPR regions, MLST (Multilocus sequence typing) sequences, their LPS (Lipopolysaccharide) profile and virulence gene

pattern. In addition the study also addressed the issue of combating these mutants by using phage cocktails.

The specific objectives of the study are

1. Isolation of BIMs generated from *Salmonella* Enteritidis strain S37 and S49 by phage infection, and their biochemical and molecular characterization.
2. Characterization and analysis of CRISPR regions in *Salmonella* Enteritidis S37 and S49 and their BIMs using bioinformatics tools after PCR amplification.
3. LPS and virulence gene profiling of *Salmonella* Enteritidis S37 and S49 and their BIMs.
4. MLST pattern analysis of *Salmonella* Enteritidis S37 and S49 and their BIMs.
5. Application of phage cocktails to combat the BIMs.

CHAPTER 2. REVIEW OF LITERATURE

2.1 *Salmonella,* the extreme insider

Diarrheal infections around the world are due to enteric bacterial pathogens. Frequently, contaminated food items is responsible for outburst of gastroenteritis. Hence foodborne gastrointestinal illnesses have major socioeconomic effects in both developed and developing countries (McWhorter and Chousalkar, 2015).

Salmonellae are facultative anaerobic Gram-negative rod-shaped bacteria generally 2–5 microns long by 0.5–1.5 microns wide and motile by peritrichous flagella. Genome sizes of *Salmonella* vary among serovars with ranges from 4460 to 4857 kb. *Salmonellae* belong to the family *Enterobacteriaceae* and are a medically important pathogen for both humans and animals (Andino and Hanning, 2015).

The genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist Salmon, who alongside Theobald Smith discovered the organism that causes hog cholera, *Salmonella enterica* var. Choleraesuis also known as swine fever (Salmon and Smith, 1886). The significance of this discovery was neglected when the viral etiology of swine fever was discovered, and a number of years elapsed before *Salmonella* Choleraesuis was identified as a primary pathogen causing several different disease syndromes (Somyanontanagul *et al*., 2009).

Despite the fact that the organism has been intensively studied during the last century, much remains to be learnt about this pathogen. The perplexing nomenclature system of *Salmonella* has long been a subject of discussion. At present, the nomenclature system used at the CDC (Center for Disease Control and Prevention) for the genus *Salmonella* is established on recommendations from the (World Health

Organisation) WHO Collaborating Centre. According to the CDC system, the genus *Salmonella* encompasses two species, *S. enterica*, the type species, and *S. bongori.* On March 18, 2005, a new species, "*Salmonella subterranean*" was accepted by the Judicial Commission (Shelobolina *et al*., 2004); CDC may include the species in their system in the near future (Su and Chiu, 2007). *S. enterica* comprises of six subspecies (Grimont and Weill, 2007; Brenner *et al*., 2000): I-*S. enterica* subsp. *enterica*; II-*S. enterica* subsp. *salamae*; IIIa-*S. enterica* subsp. *arizonae*; IIIb-*S. enterica* subsp. *diarizonae*; IV-*S. enterica* subsp. *houtenae*; and VI-*S. enterica* subsp. *indica*.

Salmonella enterica subspecies I is primarily isolated from warm-blooded animals and accounts for more than 99% of clinical isolates though remaining subspecies and *S. bongori* principally isolated from cold-blooded animals, account for less than 1% of clinical isolates (Ferede, 2014). *Salmonella enterica* is the largest group with more than 2500 serovars (Guibourdenche *et al*., 2010).

Salmonella isolates are serotyped as per the Kaufmann-White scheme utilizing somatic (O), capsular (Vi or K) and flagellar (H) antigens that are present in the cell surface. The O factors decide the serogroup and the H factors characterize the serotype of a *Salmonella* strain (Thong and Lin, 2009). O antigen is highly polymorphic polymer with recurrent units of three to six sugars in *Salmonella* serogroups A to E. The basis of the variation in O antigen structure is depicted by the different types of sugar present, the organization of sugars, the addition of branch sugars, and altering side groups; such variation is utilized to serogroup *Salmonella* isolates (Fitzgerald *et al*., 2003; Luk *et al*., 1993).

Another classification of serotypes is based on antigenicity of flagellar H antigens which are specially for *Salmonella* (Scherer and Miller, 2001). H antigens are heat-labile proteins associated with the peritrichous flagella and can be conveyed in one or two phases. The phase 1 H antigens are specific and

related with the immunological identity of the particular serovars, while phase 2 antigens are non-specific enclosing different antigenic subunit proteins shared by many serovars. K antigens are heat- sensitive surface-bound polysaccharide capsular antigen of *Salmonella* serovars (Hu and Kopecko, 2003; Yousef and Carlstrom, 2003).

By newer convention, names are maintained only for subspecies *enterica* serovars, and are no more italicized. The first letter is a capital letter "*S*" followed by the serovar names of subspecies *enterica* (e.g. Typhimurium or Montevideo) (Meneses, 2010).

The prime niche of *Salmonella* serovars is the intestinal tract of humans and livestocks. It can likewise be present in the intestinal tract of wild birds, reptiles, and at times insects (Le Minor, 1992). It is an intracellular pathogen, relying upon both host species; and serovars can bring about disease in both humans and animals extending from mild diarrhea to typhoid fever. Humans generally acquire *Salmonella* through the consumption of polluted foods, including fruits, vegetables, nuts, dairy, meat, eggs and poultry meat (Carrasco *et al*., 2012).

2.1.1 *Salmonella enterica* serovars linked to food poisoning

Thousands of cases of salmonellosis are consistently reported worldwide every year. However, the real number of infections may be entirely different and many times greater, since many benign cases are not diagnosed or reported (Hurley *et al*., 2014). *Salmonella enterica* infection is a noteworthy public health problem, causing an estimated 1 million domestically acquired foodborne illnesses and >350 deaths each year in the United States and an expected 93.8 million illnesses and 155 000 deaths each year worldwide (Chai *et al*., 2012). The estimates in India would therefore be much higher.

Salmonellosis causes significant morbidity and mortality on a global scale, due to ingestion of food or water that are contaminated by fecal or urinary excretions of animals that are reservoirs of *Salmonella* (Raffatellu *et al*., 2008). After infection with *Salmonella*, a broad range of clinical indications are presented depending on the susceptibility of the host. These comprise bacteremia, enteric fever, enterocolitis and chronic asymptomatic carriage. Typhoid and Paratyphoid fever termed as enteric fever, are due to infection with *S. enterica* serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi), respectively. Conversely, gastroenteritisis commonly associated with NTS (non typhoidal *Salmonella*) serovars such as Typhimurium (*S*. Typhimurium) and Enteritidis (*S*. Enteritidis) (Crump and Mintz, 2010; Majowicz *et al*., 2010).

The two most frequently reported serovars are *Salmonella* enterica serovar Typhimurium (*S*.Typhimurium) and *S*. Enteritidis that influence both human and animal health (Omwandho and Kubota, 2010). Human ailment brought on by infection with *Salmonella* enterica serovar Enteritidis (*S*. Enteritidis) expanded worldwide beginning as early as the mid-1970s and by 1990, this serovar replaced *Salmonella* enterica serovar Typhimurium (*S*. typhimurium) as the key cause of salmonellosis in the world (Guard-Petter, 2001).

2.1.2 Pathogenesis of *Salmonella*

Human salmonellosis is predominantly foodborne and is contracted through ingestion of contaminated food of animal origin such as meat, milk, poultry and eggs. Dairy products including cheese and ice cream were also entangled in the outbreak. Although, fruits and vegetables such as lettuce, tomatoes, cilantro, alfalfa-sprouts and almonds have also been associated in recent outbreaks (Kemal, 2014).

Acute gastroenteritis is normally acquired from consumption of food

which are directly or indirectly polluted with *Salmonella*. A wide variety of animal species are equipped for harboring the organisms and in the developed world turkey, chicken, swine and cattle are observed to be infected carriers in the studies carried out in the abattoirs. These carriers promptly shed *Salmonella* during transportation to the abattoir and defile abattoir workers or equipment during slaughter. The dynamic pattern of advanced mass processing and distribution of food products has been an essential element in the increase incidences of *Salmonella* foodborne infections. Person to person spread has been exhibited on numerous events and may take place in young children and groups living under poor finanacial conditions, where effective sanitation is lacking (Wray, 1994). Direct or indirect contact with animals colonized with *Salmonella* is another root of infection, including contact during visits to petting zoos and farms (Friedman *et al*., 1998).

Serovar Enteritidis can colonize both humans and chicken. In humans, the infection is displayed with non-bloody diarrhea with abdominal pain, nausea, vomiting and fever. The disease (non-typhoidal fever) is usually self-limiting and recovery follows within a few days to a week but, once in a while systemic infection may occur invulnerable human patients such as infants and elderly people, leading to serious syndromes (Zhao, 2002).

Salmonella species can tramp over the epithelial barrier by passive transport aided by dendritic cells, which spread out pseudopods between local epithelial cells, or by active invasion. On arriving at the lower intestine, the bacteria will adhere to the mucosal membranes and attack epithelial cells (Rescigno *et al*., 2001). One such site where this happens is the microfold (M) cells of Peyer's patches situated in the small intestine, where bacteria shift across the epithelial barrier to the underlying follicles and mesenteric lymph nodes of the lymphoid tissue (Jones *et al*., 1994). During extended bacteremia, secondary infections can occur due to the dispersal of bacteria to other organs such as the gallbladder, liver and spleen. Infection by invading bacteria can

stem from both the blood and/or the bile. These incidents roll out a cycle of infection, wherein bacteria basolaterally reinvade epithelial cells of the intestinal wall or are shed in feces. In time, the manifestations of salmonellosis will resolve. On the other hand, asymptomatic carriage of the bacteria can occur in patients for considearble months or years with the potential to relapse in the future (Crawford *et al*., 2010; Gonzalez-Escobedo *et al*., 2011).

After taking contaminated food, these bacteria settle in the intestines by invading dendritic cells and enterocytes of the intestinal epithelial barrier. *Salmonella* species, which are fruitful in passing this hindrance are stood up to by proximal macrophages and may be phagocytosed, or effectively attack the macrophages, using T3SS-1 and fimbriae, among other bacterial surface adhesins (de Jong *et al.,* 2012).

Salmonella pathogenicity islands (SPI), historically obtained through horizontal gene transfer events, contain clusters of genes encoding the systems through which *Salmonella* act as a virulent pathogen (Knodler *et al*., 2005; Ibarra and Steele-Mortimer, 2009). These genetic islands are situated on the bacterial chromosome or on plasmids. Be that as it may, not all serovars possess every known SPI. SPI-1 through SPI-5 are regular in all *S. enterica* serovars. To date, 23 SPI have been portrayed despite the fact that the functions of the genes contained within each island are not totally elucidated (Sabbagh *et al*., 2010; Hayward *et al*., 2013). SPI-1 and SPI-2 are of specific significance in *in vivo* infection. The SPI encode effector proteins that are translocated specifically in to host cells over the plasma membrane type III secretion systems (T3SS-1 and T3SS-2) that furnish *Salmonella* with the biochemical machinery to endeavor this intracellular niche. T3SS can additionally be utilized to secrete effector proteins into the surrounding environment to influence host cell physiology (Galán, 1999; Hensel *et al*., 1998).

After being internalized by macrophages, *Salmonella* then inhabit membrane bound compartment distinct from the phagosome and lysosome

known as the SCV (*Salmonella* containing vacoule). In this cellular compartment, *Salmonella* can survive and replicate without host antimicrobial defense mechanisms, there by evading endosomal fusion with the NADPH oxidase complex (Gorvel and Méresse, 2001).

From within the SCVs, SPI-2 are expressed encoding T3SS-2, which empowers *Salmonella* to translocate a range of effector proteins in to the cytoplasm of the host cell such as, SigD/SopB, SipA, SipC, SodC-1, SopE2 and SptP, prompting the arrangement of the actin cytoskeleton (Coombes *et al*., 2005a). These proteins communicate with the cytoskeleton and motor proteins, leading to the formation of *Salmonella*- induced filaments (SIFs), which venture out of the SCV. The SIF encourages fusion of SCVs with other vesicles in the cell and may assume a part in *Salmonella* replication; the exact role of SIFs in *Salmonella* infections remains unclear (Foley *et al*., 2013).

Clinical manifestations of *Salmonella* Enteritidis are not normaly seen in chickens, however they can be seen in young birds. Symtoms of infection are non-specific and include depression, poor growth, weakness and diarrhea. Mortality is typically in the first few weeks of life. Expeditiously developed septicaemia can bring about high mortality in chicks with few or no clinical signs (Keery, 2010). Spreading of infection to chicks is likewise a high concern and is the reason why tracking hatcheries for Enteritidis is a high priority in most control programs. Vertical transmission of *S.* Enteritidis is conceivable (Methner *et al*., 1995; Berchieri *et al*., 2001); and chicks are extremely vulnerable to Enteritidis colonization as they lack an entrenched microbiota in their intestines (Duchet-Suchaux *et al*., 1995).

In a newly hatched chicken, Enteritidis can cause diarrhea and septicemia with invasion and infection of a variety of internal organs of the hen covering liver, spleen, peritoneum, ovaries and oviducts (Gast and Beard, 1990). When the animals become infected with serovar Enteritidis, immense interstitial oedema of the lamina propria and submucosa can be observed within

one day, trailed by quick influx of granulocytes and macrophages (Desmidt *et al*., 1996). In general, contracting of inflammatory cells is much rapid and strong in day-old chicken than in month-old chicken. Once the bacteria have arrived at the macrophages, they may use these cells as vehicles to propagate to distinct organs. In this regard, the course of Enteritidis infection in young chicken resembles that in susceptible humans (Zhao, 2002).

As stated by many reports, eggs are the most plausible source of *S*. Enteritidis infections in humans both in outbreaks and in isolated incidences (Mumma *et al*., 2004; Patrick *et al*., 2004; Braden, 2006). Feces from hens shedding bacteria may pollute eggs externally. If the eggs are poorly washed at the egg processing plant, *S*. Enteritidis is able to survive on the surface and potentially cross-contaminate the fluid portion of the egg when it is cracked for consumption (Davies and Breslin, 2003).

2.1.3 Prevention measures for *Salmonella*

Salmonella are widely distributed in nature although their native habitat is the intestinal tract of humans and animals. The capacity of *Salmonella* to cycle between host and non host environments and to survive for extended periods on diverse materials means that *Salmonella* may be found just about anywhere (Jones, 2011). Owing to their ubiquitous nature, persistent survival, and adaptability, it was proposed that every available tool to control the organism must be employed and control efforts must be sustained by including such efforts in standard management practices (Ricke *et al*., 2005).

There are various methods for controlling *Salmonella* contamination in feed (Maciorowski *et al*., 2007). For feed degradation, shortening storage time to prevent browning and caking of the feed, and the supplementation with soybean oil to prevent fat losses would be of first importance, before implementing other prevention methods. Moreover, rapid drying is widely used to preserve raw feed ingredients (ICMSF, 2005). Considering the addition of

various antimicrobial agents, disinfectants such as acids (mineral acids, short-chain fatty acids), isopropyl alcohol, aldehydes, and trisodium phosphate may decrease the risk of contamination with *Salmonella*, through inactivation of this pathogen during the storage of feed (Galiş *et al*., 2013).

In feeds *Salmonella* killing may involve pelleting (thermal processing), chemical addition, or both (Tabib *et al*., 1984). The pelleting process comprises 3 major steps or stages: mixing steam with mash feed (conditioning), pressing conditioned feed through metal dies (pelleting), and removing heat and moisture via large volumes of air (cooling) (Riley, 1969).

Biocides play a key role in limiting the spread of infectious disease. Triclosan, chlorhexidine, hydrogen peroxide, and benzalkonium chloride are some active biocidal agents. It has been indicated in *Salmonella enterica* that sublethal exposure to biocidal agents can lead to the emergence of tolerant isolates (Condell *et al*., 2012).

One of the most potent sanitizers known, ozone is active against all forms of microorganisms at relatively low concentrations (Khadre *et al*., 2001). High ozone concentrations (12% to 14% wt/wt O_3 in O_2 mix) inactivate ≥ 5 log units *S*. Enteritidis effectively on the surfaces of shell eggs (Rodriguez-Romo *et al*., 2007).

Produced by radioactive substances, called radioisotopes like cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs), gamma rays are apart of irradiation on microrganisms (Riganakos, 2010). The radiation dose required to decrease the *Salmonella* spp. load on the eggshells by one log cycle (D10) was 448 Gy (Al-Bachir and Zeinou, 2006). Using ultraviolet pulsed light (3 times per second, with pulse duration of 360 μs) of 3800 V input voltage, 1.27 J/cm^2/pulse of radiant energy was generated at 1.5cm below the lamp surface. Samples consisted of shell-eggs artificially contaminated with *S*. Enteritidis were subjected to different treatment regimens (1, 3, 5, 10, 15, 20, and 30 s at 9.5 and 14.5 cm) which were successful in reducing the *Salmonella* load (Keklik *et al*.,

2009).

The efficacy of gas plasma technology for superficial decontamination was evaluated by exposing shell eggs artificially inoculated with *S*. Enteritidis and *S*. Typhimurium to gas plasma for different times: 0, 10, 20, 30, 45, 60, and 90 min. For *S*. Enteritidis, an exposure of 10 to 20 min resulted in a decrease of 1.0 to 1.6 log CFU/eggshell, compared to untreated samples (Ragni *et al*., 2010). Perforations are induced by plasma in the membranes of microorganisms and provoke a marked acidification of the medium (Laroussi *et al*., 2003; Laroussi and Leipold, 2004).

A non-thermal technology, Pulsed light (PL) treatment applies pulses of short duration of an intense broad-spectrum light (200 to 1000 nm) (Woodling and Moraru, 2005; Wekh of *et al.,* 2001). The inactivation of *S*. Enteritidis by using PL delivered in 100 μs, with 30% of the spectral output corresponding to UV light was demonstrated (Hierro *et al*., 2009).

Oscillating electromagnetic waves, microwaves have frequencies in the 300 MHz to 300 GHz range. They can be used to reduce the load of bacteria found on the eggshell including *S*. Enteritidis. At the end of the microwave processing, a CO_2 treatment for 30 s was performed. The maximum diminution of *S*. Enteritidis on the eggshell was of approximately 2 log cycles, this value being considered by the authors as appropriate to eliminate *S*. Enteritidis in most naturally contaminated eggs (Lakins *et al*., 2008).

Ultrasound treatment of food products is a successful tool to minimal processing, as the transfer of acoustic energy is instantaneous and distributed throughout the whole volume of the products (Ulusoy *et al*., 2007). By shell eggs treatment, in combination with thermal treatment ultrasonic method was applied efficiently on *Salmonella* Enteritidis and their count was reduced (Cabeza *et al*., 2011).

Vaccination is frequently used for the control of *Salmonella* spp. infection in hen eggs among various preventive measures (Van Immerseel *et al*.,

2005). In U.S.A. (FDA 2009) and Canada, the application of vaccines to increase the resistance of birds against *Salmonella* spp. is encouraged. In Canada, the vaccination of the layer flocks introduced into a new house is highly recommended if the former flock was tested *S*. Enteritidis positive (Keery, 2010). Immunization with antigens from selected microorganisms (for example, *S*. Enteritidis and *S*. Typhimurium) in laying hens; react by producing high quantities of specific antibodies (IgY), which are transported from the blood into the egg yolk. The eggs containing high levels of antigen-specific IgY are called hyperimmune eggs and can be administered as a feed additive (usually in the form of whole yolk powder) to other species to provide them with passive immunity (Chalghoumi *et al*., 2009). It was demonstrated that it is possible to produce IgY against *S*. Enteritidis and *S*. Typhimurium OMP in the same egg with concentrations of 429 ± 20 mg/g (Chalghoumi *et al*., 2008).

Recently a review published the inhibitory effects of several plant extracts on *Salmonella* spp. The phenolic compounds are responsible for the bactericidal effects as they interact by permeabilizing the membrane; and their biological activity seems to depend also on the solvent used for extraction (Norajit and Ryu, 2012).

Other natural antimicrobial agents are bacteriophages, which are viruses that specifically attack and kill their host bacteria, in a process regarded as a possible treatment method for combating bacterial infectious diseases (Cao *et al*., 2015).

2.2 Bacteriophages

The year 2015 marks 100 years since Dr. Frederick Twort found the "filterable lytic component" later autonomously found and named "bacteriophage" by Dr. Felix d'Herelle (Wei, 2015).

Upon the launch of the new *Bacteriophage* journal, established in early 2011, Alexander Sulakvelidze defined bacteriophages as "the most ubiquitous

organisms on Earth, assuming a significant role in maintaining microbial balance on this planet" (Wittebole, 2014). Certainly, bacteriophages or phages are wherever their bacterial host is present; it has been established that the population of phages in aquatic systems is in the range of 10^4 to 10^8virions per mL and about 10^9virions per g in the soil (Weinbauer and Rassoulzadegan, 2004), with an expected total number of 10^{32} bacteriophages on the planet (Hanlon, 2007).

Since time immemorial, there have been recorded reports of river waters with the potential to cure infectious diseases such as leprosy. But, in 1896, the British bacteriologist Ernest Hankin reported antibacterial activity against *Vibrio cholerae*, which he noticed in the Ganges and Jumna rivers in India. He proposed that an unidentified substance was accountable for this phenomenon and for restricting the spread of cholera epidemics. Two years later, Gamaleya, the Russian bacteriologist, detected a similar phenomenon while working with *Bacillus subtilis* (Golkar *et al*., 2014).

It was not until 1914, that British bacteriologist Frederick Twort put forward the hypothesis by proposing that it may have been because of a virus. For different reasons, counting financial difficulties, Twort did not continue this finding. A French-Canadian microbiologist Felix d'Herelle, intially noticed in 1910 the bacteriophage phenomenon while considering microbiologic methods of controlling locusts in Mexico. In the lab, when he spread some cultures on agar, he detected round zones without growth, which he called plaques, and claimed they were caused by viral parasites.After six years, he proposed the name "bacteriophage," or bacterium-eater (Chanishvili, 2012).

In 1917, d'Herelle started testing his phage in human patients. Under the clinical guidance of Professor Victor-Henri Hutinel at the Hospital des Enfants-Malades in Paris, he confirmed the safety of his phages by ingesting them. The following day, he demonstrated their efficacy by administering them to a 12-year-old boy with acute dysentery. The patient's symptoms dropped

after a single treatment. Dr. d'Herelle's anti-dysentery phage was then alloted to three additional patients, all of whom began to recover within 24 hours of treatment (Häusler, 2006). In 1923, two physicians from Baylor University's College of Medicine reported fruitful results from one of their phage therapy trials conducted in United States, and inferred that "the bacteriophage holds enormous possibilities as a new weapon for fighting infectious disease" (Ho *et al*., 2001).

Even as bacteriophage pioneers proposed phage utilization as natural antibacterial agents, at least seven commercial phage preparations to tackle skin abscesses, ulcers and other topical infections had been produced. Woefully, the lack of knowledge of phage biology, non-standardized assembling procedures and storage of phage products made them greatly unstable, which gave rise to distrust among physicians and clinicians (Fischetti *et al*., 2006; Housby and Mann, 2009). Alexander Fleming interrupted studies on bacteriophages in Western Europe and the United States when the era of antibiotics started with the discovery of penicillin. Drug therapy introduced in the large scale was astoundingly effective therefore, the enthusiasm for phage research/applications diminished. On the other hand, drug extensive application brought about enormous increase of bacterial drug resistance and scientists swung back to the idea of phage utilization as potential replacement tool against pathogens. Numerous publications mostly from Eastern Europe and the Soviet Union have demonstrated the potency of phages in eradication of most common bacteria resulting various infections in humans and animals (Drulis-Kawa *et al*., 2015).

Due to the high specificity of host receptor identification by phage particles, bacterial viruses have also been fruitfully utilized as a clinical diagnostic tool and in diagnosis of potential foodborne pathogens (Schofield *et al*., 2012; Schmelcher and Loessner, 2014). Briskly progressing synthetic biology makes new possibilities in the design of genetically modified phages or recombinant phage derived particles to boost their application in therapy,

diagnostics and biotechnology (Lu *et al*., 2013; Lu and Koeris, 2011).

2.2.1 Phage classification

Phages come in an array of shapes, sizes, capsid symmetries, and structures, all made of nucleic acid (genome) encapsulated by a protein coat (capsid). Phage genomes can be double or single-stranded DNA or double or single-stranded RNA. Capsids are recognized in numerous forms, ranging from small 3D hexagon-like structures to filaments, to highly complex structures comprising a head and a tail (Elbreki *et al*., 2014). It is assessed that approximately 5,500 bacteriophages have been viewed by electron microscopy since 1959. Of those studied from a morphological point of view, 96.3% had a tailed morphology (Ackermann and Prangishvili, 2012; Klumpp *et al*., 2010). Through out the years, a sophisticated phage classification system has been drawn up to account for the diversity, i.e. the International Committee for Taxonomy of Viruses (ICTV). Initially the taxonomy of phages was organised according to their morphological characteristics, type of nucleic acid, and presence or absence of envelope or lipid (Ackermann, 2001; 2004; 2009; Matsuzaki *et al*., 2005).

In recent times, the significance of phage genome sequences comparisons has also been recognized. Some phage families have been grouped into orders; for example, the three tailed phage families (*Myoviridae*, *Siphoviridae*, and *Podoviridae*) belong to the Caudovirales order, and the Archaea-infecting *Lipothrixviridae* and *Rudiviridae* phages belong to the Ligamenvirales order. It is noticable that many of the other families have not yet been assigned an order. The inoviruses (*Inoviridae* family) have nonenveloped rod of protein filaments surrounding a circular ssDNA genome. The microviruses (*Microviridae* family) have a linear, ssDNA genome and a nonenveloped, icosahedral capsid. The tectiviruses (*Tectiviridae* family) and corticoviruses (*Corticoviridae* family) both have external icosahedral capsids

with a lipid membrane lying directly beneath. These two families vary in terms of capsid and genome organisation. While the corticovirus genome is circular and highly supercoiled, the tectivirus genome is linear with terminal inverted repeats. By distinction, the plasmaviruses (*Plasmaviridae* family) have an external lipid envelope, pleomorphic geometry, and a circular genome. They are only known to infect the mycoplasmal genus Acholeplasma. The cystoviruses (*Cystoviridae* family) possess a linear, segmented, dsRNA genome. They are charactarised by a double capsid with a surrounding lipid envelope. The leviviruses (*Leviviridae* family) possess a linear, positive-stranded, ssRNA genome and a nonenveloped, spherical capsid (Ackermann and Prangishvili, 2012; Elbreki *et al*., 2014).

FAMILY	PROPERTIES	SHAPE
Myoviridae	Contractile tail	
Siphoviridae	Noncontractile long tail,	
Podoviridae	Short tail	
Microviridae	ssDNA (C), 27 nm, 12 knoblike capsomers	
Corticoviridae	dsDNA (C), complex capsid, lipids, 63 nm	
Tectiviridae	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	
Leviviridae	ssRNA (L), 23 nm, like poliovirus	
Cystoviridae	dsRNA (L), segmented, lipidic envelope, 70–80 nm	
Inoviridae	ssDNA (C), filaments or rods, 85–1950 x 7 nm	
Plasmaviridae	dsDNA (C), lipidic envelope, no capsid, 80 nm	

Fig 2.1 Bacteriophage classification Adapted from Karthik *et al.* (2014).

2.2.2 Phage life cycle

In order to value the role of phages in nature, a perception of their possible interactions with their hosts is necessary. Phages have different possible life cycles, which, along with interaction with their physical environment, dictate their role in bacterial/archaeal biology (Clokie *et al*., 2011). Adsorption is the initial step in phage infection. It is a strain-specific event, involving host recognition of the bacterial surface (reversible binding) trailed by irreversible fixation of another tail protein to a secondary receptor molecule (Mercanti *et al*., 2015). The interactions between phages and hosts occur between phage tail proteins and bacterial receptors, which are proteins or LPS. These interactions decide host specificity and range (Chaturongakul and Ounjai, 2014).Known receptors of *Salmonella* phages are flagella proteins (e.g.,FliC and FljB (Shin *et al*., 2012); and FliK, (Choi *et al*., 2013), outer membrane porin or osmoregulatory protein OmpC (Ho and Slauch, 2001b; Marti *et al*.,2013), outer membrane protein for vitamin B12 uptake BtuB (Kim and Ryu, 2012), outer membrane protein for drug efflux pump TolC (Ricci and Piddock, 2010), outer membrane transport protein FhuA (Casjens *et al*., 2005), O-antigen of LPS (Shin *et al*., 2012) and Vi capsular antigen (Pickard *et al*., 2010).

After attachment, the next important process is the penetration of nucleic acid. Penetration is accomplished either by contraction of the tail or by the vicinity of some particular enzymes that cause distortion of the bacterial cell membranes. The hollow tail of the phage shapes a pore over the cell membrane through which the phage nucleic acid enters the bacterial cell. The phage devoid of nucleic acid remains attached to the cell wall of bacteria as a ghost. Majority of the phages are host specific and sometimes a bacterium can be made vulnerable to a phage through artificial transfection. After gaining entry into the host, the nucleic acid has two ways to propagate itself (Karthik *et al*., 2014).

Taking in to account their subsequent propagation cycle, most phages can be in general divided into two major groups: virulent and temperate Virulent phages promptly divert the host metabolism toward the generation of new phage virions, which are discharged upon cell death within several minutes to hours after the initial phage attachment event. This is termed the lytic cycle. Virulent phage infection results in clear plaques on the respective host bacterial lawns. Temperate phages can reproduce either by the lytic cycle as mentioned above or by building up a stable long-term viable relationship with their host bacteria. In this state, the phage DNA is replicated together with the host's chromosome. This is termed the lysogenic cycle, during which viral genes that are undesirable to the host are not expressed (Deresinski, 2009; Strauch *et al*., 2007; Summers, 2001).

Filamentous phages are competent in forming long-term associations with bacterial cells, through lysogeny and pseudo lysogeny, while phages with a solely lytic lifestyle are obligate killers of their host, requiring lysis to transmit to the next host cell (Koskella and Brockhurst, 2014).

2.2.2.1 Lytic infection

In the event of lytic cycle, phage causes bacterial cell lysis once the replication cycle is completed. They assume control over the cellular machinery to make phage components. Right after the entry of nucleic acid into the bacterial cell, eclipse period starts when not even a single infectious phage particle can be obseved either outside or inside the bacterial cell. Replication cycle is alike the replication cycle of all viruses in that once the nucleic acid is produced; it is packaged into the head, trailed by the assembly of tail. The cell wall of the bacteria is debilitated by the phage enzymes which lead to the crack of the membrane and ultimately the mature phages make their discharge from the infected bacterial cell (Skurnika and Strauch, 2006).

2.2.2.2 Lysogenic infection

Temperate phages have the same lytic ability as virulent phages, however they can opt for a non-lytic lifestyle. Within minutes after phage DNA infusion into the bacterial cytoplasm, and relying upon the metabolic state of the bacterial cell, the phage can "choose" to initiate a lytic cycle or to integrate its DNA into the bacterial chromosome of its host to become a prophage. If the last mentioned option is chosen, a specific phage repressor (CI in phage λ) inhibits transcription of most of the phage genes, including those needed for the lytic cycle, and the prophage becomes quiescent. Its DNA is replicated correspondingly with the bacterial chromosome during cell division, and this cycle can keep going for an infinite period of time. A bacterial cell carrying one or more prophages is called a lysogen and for the most part, it is "immune" toward lytic or lysogenic infection by other phages of the same group (Fortier and Sekulovic, 2013).

2.2.2.3 Pseudolysogenic infection

Pseudolysogeny can be explained as the phase of stalled development of a bacteriophage in a host cell without either multiplication of the phage genome (as in lytic development) or its replication synchronized with the cell cycle and stable maintenance in the cell line (as in lysogenization), which continues with no viral genome degradation, thus permitting the subsequent restart of virus development. This phenomenon is normally brought on by unfavorable growth conditions for the host cell (such as starvation) and is ceased with initiation of either true lysogenization or lytic growth when growth conditions improve. Pseudolysogeny assumes to play an important role in phage survival, as bacteria in a natural environment are starved or their growth is very slow. This phenomenon can be an important aspect of phage-dependent

bacterial mortality and may impact the virulence of some bacterial strains (Łoś and Węgrzyn, 2011).

2.2.2.4 Chronic or continuous infection

The chronic infection lifestyle is observed in some archaeal viruses, in filamentous phages (rod shaped single stranded DNA phages), and in plasmaviruses, which infect *Mycoplasma*. In this life cycle phages are slowly shed from the cell for quite a while without obvious cell death (Clokie *et al*., 2011).

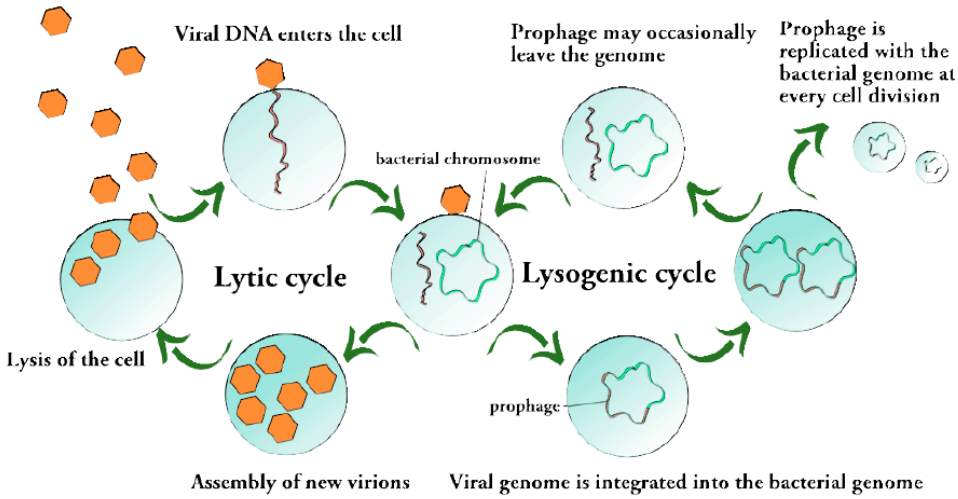

Fig 2.2 Lifecycle of bacteriophage. Adapted from Örmälä-Odegrip, 2015.

2.2.2.5 Restricted infection

Injection of phage DNA can likewise be obstructed in some cases. Once inside the bacterial cells, phage DNA is subject to the well-studied restriction/modification systems that degrade foreign DNA. These depend on differences in methylation status to establish self-nonself recognition and block

the activity of sequence-specific nucleases toward endogenous DNA, while focusing the invaders (Karginov and Hannon, 2010).

2.2.2.6 Abortive infection

Abortive infection (Abi) 'innate immunity' is a post-infection defense mechanism that interferes with phage proliferation and result in the death of the infected bacterium, a form of 'bacterial apoptosis'. The 'programmed cell death' that is activated by Abi systems provides viral protection by constraining phage spread via 'altruistic cell suicide' (Dy *et al*., 2014). There are more than 20 Abis (predominantly plasmid-encoded lactococcal systems) and, with the exception of a few, the molecular basis for phage defence is unclear (Chopin *et al*., 2005).

2.2.3 Mechanism of bacteriolysis by phages

Phage exit infected bacterial host cells by expressing two proteins – holin and lysin. Toward the end of the phage replicative cycle within the bacterial cell, the holin creates a pore in the cytoplasmic membrane permitting the lysin to pass through and interact with the peptidoglycan matrix. The lysin enzymically cleaves specific peptidoglycan bonds, disrupting the integrity of the structure. The disrupted peptidoglycan matrix can no more withstand the internal osmotic pressure, resulting in extrusion and rupture of the cytoplasmic membrane through hypotonic lysis, and release of progeny phage particles (Pastagia *et al*., 2013; Wang *et al*., 2000).

The muralytic action of endolysins permits their classification according to the bond of the peptidoglycan on which the enzymes act. At least four types of endolysins have already been recognized: (i) lysozymes and (ii) transglycosidases act on the glycosidic bond that connects the amino sugars in

the cell wall, and (iii) amidases and (iv) endopeptidases act on the amide and peptide bonds of the cross-linking oligopeptide stems and interpeptide bridges (Fischetti, 2010; Loessner, 2005; Young, 1992). Most frequently, lysins showed a typically modular structure of at least two distinct domains. That is an N-terminal CD (CDs - catalytic domains) and a C-terminal CBD (CBDs - cell wall binding domains), corresponding to their two basic functions: enzymatic hydrolysis and substrate recognition (Yang *et al*., 2014).

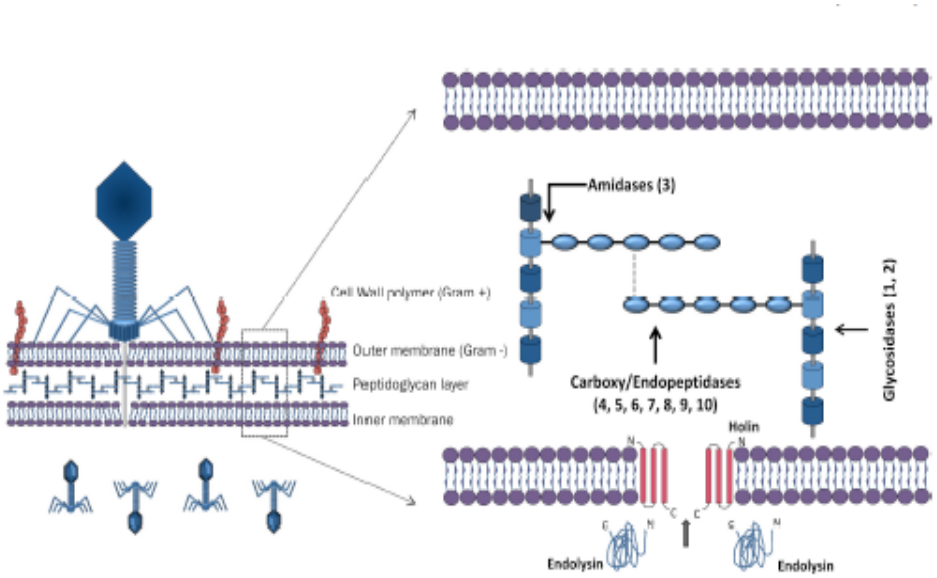

Fig 2.3 Schematic representation of how phage endolysins gain access to the peptidoglycan through the most common holin-endolysin lytic system. Adapted from Oliveira *et al*., 2013.

By and large, the CD becomes acquainted with and specifically cleaves the major bonds in the peptidoglycan *via* the specific identification of the CBD.The substrates of CBDs are hypothesized to be unique and conserved molecule in the cell walls that are crucial for bacterial viability, usually neutral polysaccharides that are confined to particular species or even strains. For

instance, pneumococcal phage lysin targets choline, an essential cell wall molecule for anchoring in *S. pneumonia* (Garcia *et al*., 1988; Hermoso *et al.,* 2003). The CBDs of *Listeria* phage lysins can even recognoze various serotypes of *Listeria* species in liquid or food samples (Loessner *et al*., 2002; Schmelcher *et al*., 2010; Eugster *et al*., 2011; Eugster and Loessner, 2012).

Filamentous phages do not reproduce by lysing bacteria; rather, they are secreted into the environment without killing the host. They are discharged by secretion, using a dedicated filamentous phage assembly secretion system. Filamentous phage secretion-assembly needs the proton-motive force and ATP (Rakonjac, 2011).

An alternate method is utilized by small single-stranded genome phages. However the mechanisms are not all understood. In studied cases the phage encodes a single lysis protein belonging to a family referred to broadly as 'amurins'. The most well characterized amurin is the protein encoded by gene E from the microvirus ΦX174, hereafter referred to simply as 'E (Tanaka and Clemons, 2012). E is a small 91-residue protein (Langeveld *et al*., 1978) whose over expression from a plasmid can result in host cell lysis (Langeveld *et al*., 1978; Henrich *et al*., 1982). It contains a conserved N-terminal transmembrane domain and accomplishes lysis by inhibiting the transmembrane protein, MraY, which is a key enzyme in peptidoglycan synthesis of bacteria (Buckley and Hayashi, 1986; Bläsi and Lubitz, 1985; Bernhardt *et al*., 2000; Bernhardt *et al*., 2001).

2.2.4 Phage therapy

Phage therapy is the application of phages essentially as drugs to treat bacterial infections (Abedon, 2015). In 1917, d'Herelle demonstrated the phage therapy when he utilized phages to treat Shigella strains isolated from patients

with dysentery after which small and clear areas appeared on the agar plates. He proposed that the phenomenon was because of parasitizing of bacteria by virus infection. The name "bacteriophage" was also suggested by d'Herelle; it was derived from the words "bacteria" and "phage in" (Xu *et al*., 2015).

Another important offering by d'Herelle was that, he firmly advanced the thought that phages were living viruses, but not "enzymes" as many researchers thought in his era. The first report of phage therapy came in 1921 when Bruynoghe and Maisin used bacteriophages to treat staphylococcal skin disease (Bruynoghe and Maisin, 1921; Payne and Jansen, 2000).

In 1925 d'He'relle reported treatment of plague (four types) by antiplague phages that drew attention towards phage therapy. Later on he visited India and worked on phage therapy of plague at the Haffkine Institute, Bombay (Mumbai) (Gandham, 2015). Ensuing phage therapy work in humans is reviewed in 1931 by d'Hérelle, who also portrayed the first use of intravenous bacteriophage, used in the treatment of cholera by Asheshov in India. Further more, at his suggestion, Dr. Davioud in France was able to cure a "hopeless" case of staphylococcal bacteremia (Abedon *et al*., 2011).

In 1932, East European researchers identified the scientific dosage of phage based on abundant animal and human tests. After that, several companies, such as the Parke-Davis Company and Eli Lilly and Company, started active commercial production of phages against various bacterial pathogens (Chhibber and Kumari, 2012).

Scientific uncertainties, along with the discovery and global marketing of antibiotics, relegated phage therapy to the history books in the western world. As such, the current 'knowledge' of the therapeutic effect of phages is principally in light of theoretical grounds, basic laboratory observations, animal models safety studies in healthy humans and decades of empirical medical experience (Pirnay *et al*., 2012).

These empirical data were for the most part accumulated in the former

Soviet Union and its eastern European satellite states, with a chief role for the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi (Georgia), several institutes in Russia and the Hirszfeld Institute in Wroclaw (Poland). Phage therapy remained a sound therapeutic component in France until the early 1990s. Sadly, regulators do not consider the historical clinical data because it has not been accepted according to current western regulatory standards (Dublanchet, 2009).

The overall antibiotic crisis has prompted a renewed interest in phage therapy. Since ancient times, phages have controlled bacterial populations on our planet, locked in an evolutionary arms race with their hosts (consisting of the repeated emergence of new phage infectivity and bacterial defense mutations). The ability of bacteriophages to rapidly overcome bacterial defence mechanisms makes them suitable for flexible therapeutic applications (Bush *et al*., 2011; Borysowski, *et al*., 2014). To maximally make use of this key advantage of phages over conventional 'static' drugs such as traditional small molecule-type antibiotics, it is vital that sustainable phage products are not submitted to the conventional long medicinal product development and licensing pathway (Pirnay *et al*., 2011). A key objective for the modern phage therapy community must be the development and validation of an expedited product development and licensing pathway in consultation with policymakers and competent authorities (Pirnay *et al*., 2015).

Phages are part of both gastrointestinal and environmental ecosystems (Topley *et al*., 1990), providing an advantageous natural, nontoxic approach for controlling several human pathogens (Alisky *et al*., 1998). The safety of phage therapy, mainly in immunocompromised individuals has also been investigated (Borysowski and Górskiin 2008), where the researchers reviewed the possible negative interactions with the immune system and the relative safety of the therapy compared to its effectiveness; as phage resistant bacteria and some

phage preparations, particularly lysates, were found to exert immunostimulatory activity. This is of great importance in phage therapy as immune response mediated antibacterial activity may be substantially suppressed in immune compromised patients (Jassim and Limoges, 2014).

Further safety aspect is that, phages replicate at the site of infection or where the host bacteria are present; while phages are absent in sterile areas, thus assuring an optimal self-adjusting dose of phages which is not found in other modes of non-biological antimicrobial agents (Mizoguchi *et al*., 2003). These disputes have helped pave the way for phage therapy/biocontrol to become a broadly relevant technology, for veterinary, agricultural, and food microbiology applications.

Phage practice in agriculture has revealed promise for treating numerous plant pathogens, and some formulations of phages have been sold for large-scale environmental application (e.g., AgriPhage™, Omnilytics) (Frampton *et al*., 2012). The recent USFDA 2006 (United States Food and Drug Administration**)** consent of *Listeria*-specific phage preparations (Listshield™, Intralytix) for food additives has opened the door to new applications of these natural bacterial killers. It is clear that phages only infect and lyse bacterial cells that are harmless to mammalians (USFDA, 2006). This has ultimately led to the evolution of a phage related product which received regulatory approval from the FDA in 2011, as a natural antimicrobial for use in agro-food industry as GRAS (Generally recognized as safe) and by US-FSIS (US-Food Safety and Inspection Service) as safe for use in animals (Tiwari *et al*., 2014, Sillankorva *et al*., 2012; Klumpp and Loessner, 2013).

Another effective avenue for phage therapy may be aquaculture, an industry that has increased 10-fold globally in the last 30 years (Food and Agriculture Organization (FAO), 2012). Microbial diseases symbolize a severe threat to aquaculture productivity; consequently, phages efficient of lysing

pathogens such as *Flavobacterium psychrophilum*, the causative agent of bacterial cold-water disease, have been isolated and tested as therapeutic agents (Kim *et al*., 2010).

2.2.4.1 Benefits of phage therapy upon antibiotics.

Bacteriophages are natural antibacterials capable of regulating bacterial populations by the induction of bacterial lysis. They are active against Gram-positive and Gram-negative bacteria, including MDR pathogens. In fact, mechanism of action phage lysis is entirely different from antibiotics, retaining activity against bacteria displaying multiple mechanisms of antibiotic resistance (Wittebole *et al*., 2014). As bacteriophages are very specific to their hosts, it minimizes the chance of secondary infections, but antibiotics do target both pathogens and normal flora of patients, which can cause the secondary infections or sometimes-super infections (Golkar *et al*., 2014). Further, bacteriophages replicate at the site of infection at which point they are mostly needed to lyse the pathogens, but antibiotics travel throughout the body and do not concentrate at the site of infection. No adverse effects have been reported during or after phage application, but resistant bacteria, allergies (sometimes even fatal anaphylactic reaction), and secondary infections are the common side effects of antibiotics treatment (Sulakvelidze *et al*., 2001).

As phages are natural products**,** public resistance to laboratory synthesized drugs or genetically modified organisms should not apply to non-engineered phage products (Loc-Carrillo and Abedon, 2011). The production of phages especially involves a combination of host growth and subsequent purification (Gill and Hyman, 2010). Although the cost of host growth varies depending upon bacterial species, the cost of phage purification appears to be coming down as technology improves (Kramberger *et al*., 2010). Normally these costs of phage production, per unit (Kutter *et al*., 2010), are not out of line with the costs of pharmaceutical production whereas the costs of discovery

(isolation) and characterization can be relatively low (Skurnik *et al*., 2007).

Contrary to chemical antibiotics, only a single phage is needed to kill a single bacterium (Carlton, 1999). Often fewer "units" of phages thus are required per treatment, however high multiplicities of phage adsorption to bacteria are still required to substantially reduce target bacterial densities (Abedon and Thomas Abedon, 2010).

Phages versus many pathogenic bacteria are easily discovered, often from sewage and other waste materials that contain high bacterial concentrations. Isolation can be more technically demanding, nevertheless, if host bacteria themselves are difficult to culture (Clokie and Kropinski, 2009) and bacteria may vary in terms of the number of phage types to which they are susceptible (Krylov, 2001). Distant from antibiotics, which can be toxic, phages that display little or no toxicity can be isolated against most target bacteria (Bentley and Bennett, 2003).

In addition, though bacteria can become resistant to phages, phage resistance is not nearly as worrisome as drug resistance. Like bacteria, phages mutate and therefore can evolve to counter phage-resistant bacteria (Ho, 2001; Matsuzaki *et al*., 2005).

2.2.4.2 Hindrances in phage therapy

Even though discovered as potential therapeutic agents over 80 years ago, and there is continual use in Russia and Georgia ever since few clinical trials of so-called "phage therapy" have been conducted in Western medicine (Wright *et al*., 2009; Sarker *et al*., 2012). Since, no clinical phage therapy products are presently available in the West, and regulatory burdens may have dampened pharmaceutical interest, as years of research and clinical trials can cost millions of euros, presenting a formidable hurdle (Meaden and Koskella, 2013).

Despite being an advantage, the tremendous specificity of phages can be burdensome when the exact species of bacteria is unknown or if there are multiple infections. Besides, phages are larger than the chemical molecules of antibiotics and hence the sites in the body that can be affected by phage must be specified. Consequently, it appears that phage therapy is most effective in treating wounds or other easily accessible sites of infection. Eventually, infections whose agents are on the interior of human cells may be inaccessible to phages (Jeney, 2013). Because some phages are injected directly into the bloodstream they can be excreted and immediately recognized by the immune system and antibodies may be produced as an immune response. Phages are also more difficult to administer than antibiotics. Proper administration demands a physician with special training in order to prescribe and use phage therapy (Häusler, 2006).

Lytic phages are efficient of killing bacteria by invading and propagating within the host cell and then lysing open the cell to "burst" out, thus killing the bacteria. This is contrary to temperate phages, which integrate into the genome of their hosts and can be transmitted vertically, serving as a refuge for phages in harsh environments (Svircev *et al*., 2011). Phages facilitate horizontal gene flow through the process of transduction and it is known that beta-lactam resistance genes have been isolated from environmental phage genomes (Colomer-Lluch *et al.,* 2011), there is clear need to be cautious in our application of phages in the environment.

Various investigations indicated that natural phages are well adapted to their local bacterial populations (Vos *et al*., 2009; Koskella *et al*., 2011) and that bacteria in turn adapt to resist their local phages (Kunin *et al*., 2008; Koskella, and Meaden, 2013). Although, a recent review of phage resistance as a result of prolonged phage therapy (Ormälä and Jalasvuori, 2013) concludes that, it is possible to isolate phages infective to bacteria from different geographical

locations and evolutionary histories (Flores *et al*., 2011); as a diverse set of phages efficient in infecting newly resistant strains will always be available, long-term resistance need not be a concern. Since local phage diversity is often high (Breitbart and Rohwer, 2005), infective phages should be easy to isolate from just a few environmental samples.

Phage therapy provides a complement in treating infections where it is theoretically and practically possible to apply large enough doses of phages, and where immunological complications but will never totally replace conventional antibiotic treatment. It might be a welcome addition to the treatment options for infections caused by antibiotic-resistant bacteria. It might additionally be possible to isolate phages with a broader host range or to alter virulent phages whose tail fibres bind to specific surface receptors to bind to a more general receptor of pathogenic bacteria. It is important to develop theoretically well-founded treatment methods if the goal is to reduce the emergence of resistance and to gain synergies even if there are great advantages to treatment with phage cocktails (Nilsson, 2014).

2.2.5 *Salmonella* phages

Many phages infecting *Salmonella* have been isolated. Félix d'Hérelle gave the first report of a *Salmonella* phage and it dates back to 1918. Afterwards, *Salmonella* phages have been isolated from different sources: wastewater plants, sewage, manure, faeces and caecal contents from different animals (e.g. poultry, turkey, pig, humans), zoo ponds, nests from poultry farms and many others. The hunt for *Salmonella* phages from different sources may be attributed to the interest prompted by the medical and veterinary significance of their pathogenic host (Andretti Filho *et al*., 2007; Santos *et al*., 2010; Sillankorva *et al*., 2010).

Phylogenetic investigation of the sequenced phages, considering the related protein content using core genes, discloses that these viruses fall into five groupings (P27-like, P2-like, lambdoid, P22-like, and T7-like) and three outliers (epsilon15, KS7, and Felix O1). The P27 group is only represented by ST64B; the P2 group contains Fels-2, SopEΦ, and PSP3; the lambdoid *Salmonella* phages include Gifsy-1, Gifsy-2, and Fels-1. The P22-like viruses include epsilon34, ES18, P22, ST104, and ST64T. The only member of the T7-like group is SP6 (Kropinski *et al*., 2007).

2.2.5.1 *Salmonella* phage groups

2.2.5.1.1 P27- like *Salmonella* phages

Phages P22 and P27 join with group C in Bradley's morphological classification (Bradley, 1967), with hexagonal head symmetry and a short, noncontractile tail terminating in a baseplate (Wollin *et al*., 1981).

The ST64B bacteriophage (hereafter termed $ST64B_{DT64}$) induced from *S.* Typhimurium DT64 is portrayed as a genetic mosaic, which has derived significant portions of its genome from sources outside the genus *Salmonella* (Hiley *et al*., 2014). $ST64B_{DT64}$ has similarities to lamboid phages but is grouped with P27-like phages (Mmolawa *et al*., 2003a). The genome of ST64B composed of putative genes with similarities to genes from phages of diverse bacterial groups, including Gram-positive organisms and is clearly a mosaic. Mosaicism is obviously seen in the tail genes, with the majority of ST64B tail genes having identity to those of phage Mu and the remaining two putative tail genes having similarity to those of phages Fels-1, Fels-2, and Gifsy-2. ST64B might have or has played a role in the transfer of virulence determinants in the past (Mmolawa *et al*., 2002).

2.2.5.1.2 P2-like *Salmonella* phages

P2 is classified in the family *Myoviridae* and the order Caudovirales. It is a temperate phage with a tail and a contractile sheath (Bowden and Modrich, 1985). It consists of a double stranded DNA genome packaged in an icosahedral capsid with a diameter of 60 nanometers that is connected to a 135-nanometer long tail (Dearborn *et al*., 2012). P2-like phages isolated from other γ - proteobacteria (Nilsson and Haggård-Ljungquist, 2006) are less similar to P2 but have many homologous genes in common. For instance, the two Salmonella phages Fels-2 and SopEΦ are 63–67% identical, and phage ΦCTX isolated from *Pseudomonas aeruginosa* is 53% identical, to P2 at the protein level (Nilsson *et al*., 2011).

2.2.5.1.2.1 SopEΦ

The P2-like phage SopEΦ was identified in serovar Typhimurium strain DT49/DT204 that caused an epidemic in the United Kingdom and the former East Germany during the 1970s and 1980s (Pelludat *et al*., 2003). The tail fiber regions of SopEΦ encode a moron (extra gene cassettes) encoding the type III effector protein SopE (Mirold *et al*., 2001; Mirold *et al*., 1999). SopE modulates host cellular signaling and leads to dramatic responses like membrane ruffling and invasion of host cells after injection into animal cells (Wood *et al*., 1996; Hardt *et al*., 1998a). Lysogenic conversion of virulent serovar Typhimurium strain ATCC 14028 (normally SopEΦ^{-}) with SopEΦ enhances its invasiveness in cultured cells and its enteropathogenesis in a bovine infection model (Zhang *et al.,* 2002).

2.2.5.1.2.2 Fels-2

Phage Fels-2 having a long tail with a contractile sheath and carrying no genetic region related to P22 is morphologically similar to *E. coli* T-even

phages (Yamamoto, 1969). It resides as a prophage in the genome of *Salmonella enterica* serovar Typhimurium strain LT2. Flagellar phase variation is controlled by the inversion of two DNA segments hin and fin in LT2. A search of the complete genomic sequence revealed that LT2 possesses DNA invertase gene fin located adjacent to another invertible DNA segment within a resident prophage, Fels-2 (Kutsukake *et al*., 2006).

2.2.5.1.2.3 PSP3

Phage PSP3 was isolated from *Salmonella potsdam* but can lysogenize *E. coli.* PSP3 consists of both circular and linear DNA molecules and is about 31 kb. Linear PSP3 DNA molecules possess single-stranded cohesive termini (cos). Sequencing of the fragment anticipated to contain cos disclosed a 19-base sequence identical to cos of phage 186. Of the 107 bp to the right of cos, 94 were identical in 186 DNA (88% similarity) and of the 370 bp to the left, 229 were identical (62% similarity) (Bullas *et al*., 1992).

2.2.5.1.3 Lambdoid group *Salmonella* phages

Gifsy-1 and Gifsy-2, which contribute to the virulence of *Salmonella enterica* serovar Typhimurium, are lambdoid prophages. The nucleotide sequence of the replication region is similar in organization to the replication region of bacteriophage lambda and in both prophages it is identical (Słomiński *et al.,* 2007).

2.2.5.1.3.1 Gifsy-1

The Gifsy-1 phage that infects *Salmonella enterica* serovar Typhimurium is a temperate bacterial virus. The phage integrates site-specifically into a single bacterial attachment sequence in the *Salmonella* chromosome during lysogeny. The reaction involves recombination between the phage attachment sequence *(attP*) and the bacterial attachment sequence (*attB*).

Gifsy-1 integrase (Int), the phage-encoded enzyme, catalyzes recombination reaction, between the phage chromosome and the bacterial host chromosome. Gifsy-1 carries a number of potential virulence modulating genes including *gogB* (Flanigan, 2008).The gifsy-1 phage encodes gipA, which helps the bacteria to colonize the small intestine and invade the intestinal epithelium (Stanley *et al*., 2000).

2.2.5.1.3.2 Gifsy-2

Gifsy-2 phage carries unidentified virulence factors and the periplasmic superoxide dismutase gene, sodCI (Ho and Slauch, 2001a). The gene *sodCI* encodes one of two periplasmic Cu/Zn superoxide dismutases of *Salmonella* (De Groote *et al*., 1997; Farrant *et al*., 1997). SodCI catalyzes the conversion of superoxide radical to hydrogen peroxide in the periplasm. Superoxide and nitric oxide production by macrophages is an important component of host defense against *Salmonella (*De Groote *et al.,* 1996).

2.2.5.1.3.3 Fels-1

Phage *Fels*-1 of *Salmonella* serovar Typhimurium LT2 carries *nanH* and *sodCIII* (Hermans *et al*., 2005). This prophage can be induced to produce infectious virions by various environmental signals including DNA damage, antibiotics such as mitomycin C and hydrogen peroxide (Bearson *et al*., 2014).

2.2.5.1.4 P22-like *Salmonella* phages

The P22-like bacteriophages with short tails bind to their polysaccharide receptors through six trimeric tail spike proteins that surround the tail tip. A trimeric needle protein of these short tails extends beyond the tail spikes from the center of the tail tip, in a position that suggests that it should make first contact with the host's outer membrane during the infection process

(Leavitt *et al*., 2013). The P22-like viruses comprises of ε34, ES18, P22, ST104, and ST64T (Kropinski *et al*., 2007). Studies of the genomes of these P22-like phages implies that morphogenesis-related genes are highly conserved, but other genes are variable, hinting that even though they have similar phage morphologies, the host specificity of these P22-like phages may vary among them (Sabour and Griffiths, 2010).

2.2.5.1.4.1 P 22

Salmonella targeting phage P22 has been well characterized to develop genetic transfer tools via lysogenization and belongs to the family of *Podoviridae* morphologically (Shin *et al*., 2014). It can grow in a lytic cycle on *Salmonella* Typhimurium, or it can lysogenize this host. The DNA found in phage particles is a single linear duplex molecule (26 to 27 million mol wt), which is terminally repetitious and circularly permuted in its nucleotide sequence (Botstein and Levine, 1968). Studies on host receptors have revealed that the phage tail spike protein plays a role in the interaction with the host by interacting with the O antigen of LPS in *S*. Typhimurium (Baxa *et al*., 1996; Venza-Colón *et al*., 2010).

2.2.5.1.4.2 ST104

Phage ST104 exists as prophage in the genome of *Salmonella* Typhimurium DT104. The entire DNA sequence of 41,391 bp, includes 64 open reading frames, and displayed high similarity to P22 and to phage type conversion phage ST64T (Tanaka *et al*., 2004). The selected genes of ST104 phage (*ae2, g45, g62, gtrA, gtrC, sieB* and *g8*) were detected in each DT104 strain. None of these genes are exclusive for this phage type, as homologues of these genes are present in other related phages (P22, ST64B and ST64T) (Drahovská *et al*., 2007).

2.2.5.1.4.3 ES18

ES18 that naturally infects *Salmonella enterica* serovar Typhimurium, as well as some serovar Enteriditis, Dublin, Pullorum, Gallinarum, and Paratyphi B strains is a temperate, generalized transducing double stranded DNA (dsDNA) phage (Casjens *et al*., 2005). It infects both rough and smooth *Salmonella enterica* strains and has an estimated genome size of about 46,000 bp, and consists of an icosahedral head and a long noncontractile tail (Schicklmaier and Schmieger, 1997). Most of its integration-excision, immunity, Nin region, and lysis genes are nearly identical to those of the short-tailed *Salmonella* phage P22, whereas other early genes are nearly identical to *Escherichia coli* phages λ and HK97, *S. enterica* phage ST64T, or a *Shigella flexneri* prophage. Some of the ES18 late genes are unique, while others are most closely related to phages HK97, lambda, or N15. Hence, the ES18 genome is mosaically related to other lambdoid phages, as is typical for all group members (Toth *et al*., 2003; Cheng *et al*., 2004; Casjens *et al*., 2004).

2.2.5.1.4.4 *ε34*

Salmonella phage ε34 modifies the host cell surface lipopolysaccharide upon lysogenization in a process called "lysogenic conversion"; is a temperate phage isolated in the 1950s (Villafane *et al*., 2008). It has both the conversion genes and virion O-antigen polysaccharide receptor-recognizing tail spike encoding gene (Zayas and Villafane, 2007; Iwashita and Kanegasaki, 1975) and ε34 will only adsorb to and thus infect its *Salmonella enterica* serovar Anatum host cell if the latter carries a ε15 prophage (Barksdale, 1959; Uetake *et al*., 1955).

2.2.5.1.4.5 ST64T

A temperate bacteriophageST64T was induced from *S. enterica* serovar Typhimurium DT 64. It could mediate generalized transduction (Mmolawa *et al*., 2003b). The genomic sequence was 40,679 bp in size with an overall GC content of 47.5%, similar to the GC content of P22 (47.1%) (Altschul *et al*., 1997). ST64T mediates phage type conversion and is heteroimmune to P22 (Mmolawa *et al*., 2002). Sequence analysis of ST64T has confirmed that this bacteriophage has an immunity region different from that of P22. The presence or absence of potential receptors on the bacterial cell surface or the carriage of a template phage primarily determines the phage type, and it was suggested that integration of ST64T into the chromosome results in phage type conversion by changing immunity to the panel of the typing phage (Tanaka *et al*., 2004).

2.2.5.1.5 T7-like *Salmonella* phages

T7-like viruses are members of the family *Podoviridae* and have short noncontractile tails. Lipopolysaccharide (LPS), have been reported to be the receptors of T7-like viruses but different phages bind different moieties of LPS (Molineux, 2005).

2.2.5.1.5.1 φSG-JL2

φSG-JL2, newly discovered *Salmonella* T7-like virus is lytic to serovar Gallinarum biovar Gallinarum and has a double-stranded DNA of 38,815 bp with 55 putative genes (Kwon *et al*., 2008). The constrained specificity and broad lytic activity of φSG-JL2 may be useful for differentiation of serovar Gallinarum biovar Gallinarum from *S. enterica* serovar Enteritidis and serovar Gallinarum biovar Pullorum, and the prophylactic efficacy of φSG-JL2 against

fowl typhoid was tested with a respiratory model of fowl typhoid (Kwon *et al*., 2000; Park *et al*., 1996).

2.2.5.1.5.2 SP6

Bacteriophage SP6 that infects *Salmonella enterica* serovar Typhimurium LT2 is a small double-stranded DNA tailed phage (Dobbins *et al*., 2004). A single-subunit RNA polymerase that is very similar to the T7, T3, and K11 RNA polymerases is encoded its genome (Shin *et al*., 2000). In addition the lytic *Salmonella* phage SP6 encodes a tail protein with a high degree of sequence similarity to the tail protein of the biologically unrelated lysogenic *Salmonella* phage P22 (Baxa *et al*., 1999). An upstream region that encompass a promoter and a downstream region that contains a putative Rho-independent transcription terminator, giving it a cassette or modular structure almost identical to the structure of the tail genes of coliphages K1E, K5, and K1-5, flank the SP6 tail gene (Scholl *et al*., 2001).

2.2.5.1.6 Three outlier *Salmonella* phages

2.2.5.1.6.1 Felix O1

Bacteriophage O1 (also called phage Felix O1, 01 or 0–1) with an icosahedral head 73 nm in diameter and a contractile tail (17x113nm) terminating in six straight tail fibers is a member of the A1 group of the *Myoviridae* (Whichard *et al*., 2010). Felix and Callow discovered and first used it in 1943 in England in the original scheme for the identification and typing of *Salmonella* Typhi (Van Oye, 2013). It is fairly unique among *Salmonella* bacteriophages due to its relative *Salmonella*-specificity. Approximately 98.2% % of *Salmonella* and less than 1.4% of other *Enterobacteriaceae* thought to have lysed by this phage (Welkos *et al*., 1974). Lipopolysaccharide is the somatic receptor for phage Felix O1 (Hudson *et al*., 1978). As this phage infects

almost all *Salmonella* isolates, it has been suggested as a therapeutic or decontaminating agent (Whichard *et al*., 2003), and has used as a diagnostic reagent (Kuhn, 2007). A derivative of Felix O1 carrying the *luxAB* genes has been set up to detect *Salmonella* bacteria in food samples (Kuhn *et al*., 2002).

2.2.5.1.6.2 KS7

A group at Korea National Institute of Health, Laboratory of Enteric Infections, in Seoul (South Korea) sequenced the 40.8 kb genome of this phage (NC_006940). Albeit named an "unclassified bacteriophage" at NCBI (Kim *et al*., 2005), the information section recommends that this is an individual from the P22-like viruses.

2.2.5.1.6.3 εs15

Bacteriophage epsilon15 (εs15) Group E1 *Salmonella*-specific phage that belongs to the Order "Caudovirales" and the Family "*Podoviridae*" is a temperate phage (Ackermann, 1999). It was a popular experimental model for Japanese and US investigators in the 50's, 60's and 70's, both because of its ability to cause serotype conversion and because of its enzymatically active tail spikes, which display endorhamnosidase activity towards the host cell O-polysaccharide structure and was among the first *Salmonella*-specific phages to be discovered (Guichard *et al*., 2013). gp15 and gp17 proteins likely constitute the central tube portion of the epsilon 15 adsorption apparatus, with gp17 being more distally positioned than gp15 and dependent upon both gp15 and gp16 for its attachment, and that tail spike proteins comprised of gp20 can assemble onto nascent virions that encompass gp7, gp10, gp4 and packaged DNA, but which lack both gp15 and gp17 (Baker *et al*., 2013; Jiang *et al*., 2006; Chang *et al*., 2010).

2.3 Bacteriophage Insensitive Mutants (BIMs)

Foremost concern dealing with the use of phages in the treatment of infectious diseases still remains the emergence of phage-resistant mutants (Filippov *et al*., 2011). Phages are the most plentiful and diverse nucleic acid-based entities on Earth (Aziz *et al*., 2015) and they proliferate by a series of events: adsorption of the virion to the host's cell wall, injection of the viral genome (DNA, RNA) through the cell membrane(s), expression of viral genes, replication of the viral genome and assembly of viral protein capsids, and finally release of progeny virions (Jore *et al.,* 2012). Their diversity is mostly fulfilled by their dynamic adaptation when facing selective pressure such as phage resistance mechanisms, which are widespread in bacterial hosts. This resistance can crop up due to the alteration or loss of the bacterial cell surface receptor, blocking the receptor by the bacterial extracellular matrix, inhibition of phage DNA penetration, production of modified restriction endonucleases degrading phage DNA, or inhibition of phage intracellular development **(**Labrie *et al*., 2010).

The most frequent cause of phage resistance is the mutations affecting phage receptors (Heller, 1992). Diverse bacterial surface-exposed molecules including many outer membrane proteins, sugar residues in the O antigen or lipopolysaccharide (LPS) core, teichoic acids, polysaccharides of the capsule or slime layer, or components of flagella and pili are the phage receptors (Lindberg, 1973). Many phage receptors are essential virulence factors; some identified through the selection of phage-resistant mutations are in pathogenic bacteria. These receptors include capsular polysaccharides (Smith and Huggins, 1982; Smith *et al*., 1987; Pickard *et al*., 2010), adhesion and invasion factors (Pruzzo *et al*., 1983; Ricci and Piddock, 2010; Begum *et al*., 2010), a protein involved in intracellular growth (Spears *et al*., 2008), and, very commonly, different components of LPS (Zhang and Skurnik, 1994). If a phage receptor lies in a surface structure significant for virulence, the phage-resistant mutants

resulting from a loss or alteration of the receptor will be avirulent or attenuated (Petty *et al*., 2007).

The recently recognized CRISPR is a molecular-level component of the ongoing struggle between viruses and hosts, and part of the dynamic process by which their genomes coevolve. Besides, the CRISPR system applies selective pressure on phage populations, perhaps explaining the relatively high mutation rates of phage genomes in those populations where CRISPR loci are active. This can lead to the emergence of Bacteriophage Insensitive Mutants (BIMs) (Barrangou and Horvath, 2010).

The application of molecular genetics for the analysis of phage-host relationships has contributed enormously to the unraveling of specific events, which dictate insensitivity to bacteriophage infection, and has revealed that while they are complex and intricate in nature, they are also extremely effective. Besides, the strategy has laid solid foundations for the construction of phage resistant strains for use in commercial applications and has provided a sound basis for continued investigations into existing, naturally derived and novel, genetically engineered defence systems. Of course, it has also become clear that phage particles are highly dynamic in their response to those defence systems which they do encounter and that they can readily adapt to them as a consequence of their genetic flexibility and plasticity (Forde and Fitzgerald, 1999).

The first negative effect of bacteriophages on dairy fermentation was reported in the mid 30s of the previous century (Whitehead and Cox, 1935). Inspite of sanitary precautions, starter strain rotations and constant development of new phage-resistant bacterial strains, phages remain one of the main and economically most serious sources of fermentation failures. Due to their natural presence in the milk environment, bacteriophages cause problems in industrial dairy fermentations worldwide. Their short latent period, relatively large burst

size and/or resistance to pasteurization makes them difficult to eliminate (Daly *et al*., 1996).

Phage-induced bacterial cell lysis leads to failed or slow fermentation, decrease in acid production and reduction of milk product quality (e.g. nutritive value, taste, texture, etc.), which in effect cause profound economical losses (Lawrence, 1978). An intriguing high number of bacteriophages of *Lactococcus* and *Streptococcus* bacteria reflect the biotechnological interest and engagement of the dairy industry in research on biology of these phages (Brüssow and Hendrix, 2002).

Selection of BIMs is a way to obtain phage-resistant strains without genetic manipulations. The idea of obtaining such cells is to infect a starter strain culture and select for mutants which have sustained phage attack (Mc Grath *et al*., 2007).

The first report on isolating CRISPR-containing lactic acid bacteria came from Barrangou *et al*. (2007) who described the an approach of obtaining spontaneous *Streptococcus thermophilus* BIM cells by providing selection pressure due to phage infection. Protocols of isolating such strains have been later developed for dairy *S. thermophilus*, applied in the manufacturing of cheese and yoghurts (Szczepankowska *et al*., 2013).

Exploiting these BIMs collectively provides a population of isogenic derivatives, differing in only the CRISPR region, with increased probability of surviving a phage infection. However, based on the current environment of the bacterium, it is proposed that only those cells carrying the relevant spacer regions will survive the attack. It may therefore be beneficial to isolate individual BIMs (colonies) that have unique spacer content towards the leader region of the CRISPR locus, which can be subsequently used as part of a starter

rotation strategy and hence use CRISPR sequencing as the primary screen to select variants that differ in their phage susceptibilities. Hence, upon phage infection, BIMs 'in reserve' can be assayed for phage sensitivity, and those that evade phage attack can be slotted into the rotation scheme. This ensures that the manufacturer has a constant supply of phage-resistant derivatives from a single strain (Mills *et al*., 2010).

The benefits of CRISPR based variants seem to be infinite and promissory in industrial level. Examples of applications of this inventions are countless, includes phage resistance, for diagnostics (to predict or determine the sensitivity of microbes to bacteriophages, resistance to plasmid transfer, antibiotic resistance genes and genes encoding virulence factors among others. At this time there are available in the market some commercial starter cultures for pizza cheese manufacture (CHOOZIT™ SWIFT, DuPont ™), composed three to six *Streptococcus thermophilus* strains, with increased phage resistance besides allowing reliable acidification during the cheese process (Carminati *et al*., 2015).

2.4 Clustered regularly interspaced palindromic repeats (CRISPR)

Phage-host connections have been mulled over seriously since the beginning of molecular biology. In the late 1970s, while viruses were found to be omnipresent, it was assumed that they were present in relatively low numbers and that their effect on microbial communities was low (Stern and Sorek, 2011). In seawater, an environment in which phage abundance has been widely concentrated on, it has been evaluated that there are 5-10 phages for every bacterial cell (Sorek *et al*., 2008). Thus, bacteria are confronted with a constant threat of phage predation.

The Red Queen hypothesis also termed the evolutionary arms race

hypothesis suggested that in tight co-evolutionary interactions, for example those in a prey-predator relationship, changes (e.g., running quicker) on the one side might prompt close eradication of the other side. The only way the second side can keep up its wellness is by counter-adaptation (running considerably quicker). This will prompt an uneasy harmony between the prey and predator, where species need to always evolve to stay at the same wellness level. The illustration of an evolutionary arms race is relevant for many biological processes, however in no place is this analogy as adept as in host-parasite relationships (Van Valen, 1974). More or less 10^{25} phage infections per second are thought to occur on Earth, thereby imposing a strong selection pressure on bacteria and a resultant bacterial lysis and turnover that effects global nutrient cycling (Dy *et al*., 2014).

Prokaryotes are highly diverse. One of the clarifications of this diversity is the high extinction rate, due to genetic aggression, which leads to the clearance of ecological niches and accordingly may permit new prokaryotic species to emerge. In the absence of host defense, viral infection of prokaryotic colonies results in colony extinction or the fixation of a fraction of the invader's genetic material in the host genome, significantly influencing the life cycle of the host (Sorokin *et al*., 2010). Regardless of being outnumbered by phages, bacteria proliferate and avoid extinction by using various innate phage-resistance mechanisms, such as restriction enzymes and abortive infection (Sturino and Klaenhammer, 2006). The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system is explained in the forthcoming pages.

2.4.1 History of CRISPR

Prokaryotes have evolved an adaptive immune system called CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR associated protein) that empower them to counter invasions from viruses and plasmids (Swarts *et al*., 2012). Ishino and colleagues made the first

explanation of a CRISPR array in 1987; they observed 14 repeats of 29 base pairs (bp) that were interspersed by 32-33 bp non-repeating spacer sequences and were adjacent to the isozyme-converting alkaline phosphatase (*iap*) gene in *Escherichia coli* (Ishino *et al*., 1987). Later, similar CRISPR arrays were noticed in *Mycobacterium tuberculosis* (Hermans *et al.*, 1991), *Haloferax mediterranei* (Mojica *et al*., 1995), *Methanocaldococcus jannaschii* (Bult *et al*., 1996), *Thermotoga maritima* (Nelson *et al*., 1999) and other bacteria and archaea. Currently computational analyses revealed that CRISPRs are found in approximately 40% and 90% of sequenced bacterial and archaeal genomes (Kunin *et al*., 2007).

Along side with this initial analysis of the abundance of CRISPRs, Jansen and co-workers spotted four CAS genes that were in most cases found adjacent to the repeat arrays (Jore *et al*., 2012). Subsequent studies detected 25-45 additional CAS genes in close proximity to the arrays. The same set of genes is not present in genomes that lack CRISPRs (Semenova *et al*., 2011).

2.4.2 Structural features of CRISPR systems

The CRISPR-Cas module inside the genome of archaea and bacteria is in two parts, i.e., arrays of repeat sequences known as clustered, regularly interspaced, short palindromic repeats (CRISPRs) and genes encoding CRISPR-associated (Cas) proteins (Fig 2.4) (Takeuchi *et al*., 2012).

2.4.2.1 Repeats

The repeats of one locus are mostly identical regarding size and sequence, while repeats of different loci differ in sequence, length as well assecondary structure of their transcripts. Comprehensive studies of prokaryotic CRISPR arrays have categorized repeats based on their sequence and noticed that most bacterial repeats were palindromic, while most archaeal repeats were

not (Hrle, 2014).

Palindromes are inferred to contribute to RNA stem-loop secondary structure of the repeat, while the repeat-spacer arrays are transcribed to RNA to maintain the stem (Kunin *et al*., 2007).

Besides the structural features, many repeats have a conserved 3′ terminus of GAAA(C/G) which act as binding sites for one or more of the CRISPR-associated proteins (Jansen *et al*., 2002a).

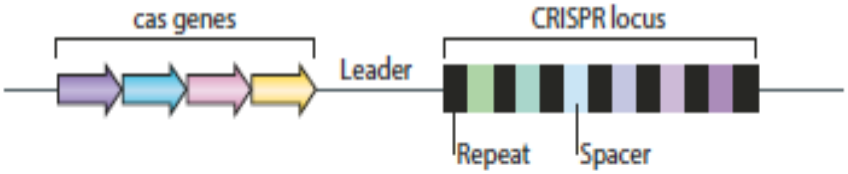

Fig 2.4 The CRISPR locus. An A/T-rich leader sequence is followed by a series of repeats (black rectangles), separated by variable spacer sequences derived from invading genetic elements (green-purple). CRISPR-associated (*cas*) genes encode the protein machinery. Adapted from van der Oost *et al*., 2014.

2.4.2.2 Spacers

Spacers are heritable, and can also be acquired through the incorporation of foreign DNA sequences (Hargreaves *et al*., 2014). Analyses of bacterial, archaeal and viral genome sequences have led to the understanding that the variable spacer elements are virus-derived and confer resistance to the corresponding viruses (Mojica *et al*., 2005; Bolotin *et al*., 2005). This means the CRISPR/Cas system is considered to be a form of adaptive immunity, and the spacer content of CRISPR arrays is a record of past infections (Barrangou *et al*., 2007). Examining spacers can provide insights into phage-host dynamics that have occurred within bacterial populations (Kuno *et al*., 2012; Stern *et al*., 2012).

2.4.2.3 Leader

This is a 550bp AT-rich sequence located at the 5' end of the CRISPR (Fig 2.4) gene adjacent to the first repeat (Jansen *et al*., 2002b; Lillestøl *et al*., 2006) and the new repeat-spacer inserted between leader and the previous unit also acts as a promoter of CRISPR gene during transcription (Tang *et al*., 2002, 2005).

2.4.2.4 The CRISPR-associated protein machinery

An extremely diverse cassette of so called *CRISPR-associated* (*cas*) genes forms the final building block of the CRISPR locus (Fig.4). Diverse group of *cas* genes adjacent to CRISPR locus encode proteins (generally called Cas proteins) which are required for mediating the adaptive immune response (Dhawan *et al*., 2015; Haft *et al*., 2005). *Cas* genes exhibit an unusual degree of variation and add to the complexity of the system (Godde and Bickerton, 2006). Twenty-five *cas* gene products have been defined to date, of which six are generally conserved (*cas1–6*) and only two (*cas1* and *cas2*) are present in all CRISPR loci (Jansen *et al*., 2002; Haft *et al*., 2005). *Cas* genes encode a large group of proteins with functions ranging from nucleolytic or helicase enzymatic activity to unique RNA binding properties.

RNA-binding proteins are often known as repeat associated mysterious proteins (RAMPs) (Makarova *et al*., 2002). They possess one or more domains that are similar to the RNA recognition motif (RRM, also known as the ferridoxin-like fold) a structural motif that appears to be involved in nucleic acid interactions in many protein classes (Wang *et al*., 2012; Maris *et al*., 2005). Regarding RAMPs, the RRM-like β1-α1-β2-β3-α2-β4 topology is interferred by various secondary structure elements. A common characteristic of all RAMP RRM-like domains is a conserved glycine-rich loop between α1 and β2, which has been connected in RNA binding (Judith *et al*., 2013). On the contrary, they

have lost the conserved consensus sequences on β1 and β3, which often mediate protein–RNA binding in most ribonucleoprotein particles (RNPs). Other loops and peripheral domains differ between different RAMPs and serve as a basis for classification (Wang *et al*., 2012).

2.4.3. Classification of CRISPR-Cas systems

Considering phylogenetic features of Cas1 and Cas2 proteins, the CRISPR repeat sequences, the cas gene sequences and cas operon arrangement, Makarova *et al.* (2011) grouped the CRISPR-Cas systems into three major types, type I, II and III, that are further divided into at least 11 subtypes (I-A to I-F, II-A to II-C, III-A and III-B) (Table 2.1).

A signature protein designates each major type of CRISPR system. All type I systems encompass a Cas3 protein, type II systems contain a Cas9 protein and every type III system contains a Cas10 protein. Moreover, type III CRISPR systems and I are grouped into subtypes, each featured with a signature protein (Makarova *et al*., 2011).

Complementary ways of classifying the different CRISPR machineries are based on features of the repeat sequences themselves or on functional characteristics (van der Oost *et al*., 2014).

• Type I: targets DNA using the 'Cascade' interference complex.
• Type II: targets DNA using a single protein, Cas9.
• Type III-A: targets DNA using the Csm interference complex.
• Type III-B: targets RNA using the Cmr interference complex.

An overview of subtype classification and predicted as well as experimentally determined Cas protein functions is given in Table 2.1 and Fig 4.5.

Cas protein family	Subtype	Name	RRM-like domain	Function
Cas1	I, II, III	Cas1	No	Adaptation
Cas2	I, II, III	Cas2	Yes	Adaptation
Cas4	I, II		No	Adaptation
Csn2	II	Csn2	No	Adaptation
Cas6	I, III	Cas6	Yes	Processing: metal-independent ribonuclease
Cas7	I, III	Subtype-specific nomenclature I-A: Csa2 I-D: Csc2 I-B/C/E: Cas7 I-F:Csy3 III-A: Csm3 III-B: Cmr4	Yes	Interference: backbone of the crRNP complex. Target Cleavage (RNA): catalytic Cas7-like protein Cmr4
Large subunit	I, III	I: Cas8 (I-D: Cas10) III: Cas10 (Csm1/ Cmr2)	Yes	Interference: interaction with Cas7/5, capping of 5′ crRNA end, PAM recognition
Small subunit	I, III	I-A: Csa5 I-E: Cse2 III-A:Csm2 III-B:Cmr5	No	Interference: interaction with Cas7
Cas5	I, III	I:Cas5(a-f) III-A:Csm4 III-B:Cmr3	Yes	Interference: (non-catalytic homologs) interaction with Cas7/8/1c Processing: (catalytic homolog) (I-C) Cas6 substitute
Cas3	I	Cas3 (helicase) Cas3HD	No	Target cleavage (DNA): superfamily 2 helicase and HD-nuclease domain
Cas9	II	Target recognition and nuclease lobes with two active sites (NHN, RuvD)	No	Processing, Interference, Target cleavage (DNA)

Table 2.1 Overview of major Cas protein families. Adapted from Hrle, 2014

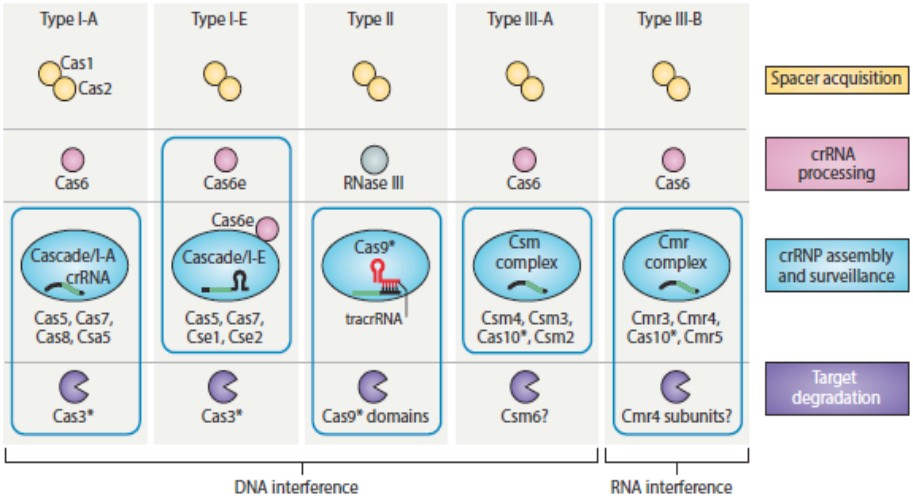

Fig 2.5 Cas systems. Based on the presence of a signature Cas gene (indicated by an asterisk) the Cas proteins can be divided into three general types, which coincide with their functional purpose (Type I/II/III-A DNA targeting, III-B RNA targeting). These are further divided into distinct sub-types (a representative selection shown in the five panels). While type I and type III systems contain multiple subunits, the type II system contains a minimalistic set of proteins. Boxes highlight the variation of potential components of the crRNP complexes for each system. Adapted from van der Oost *et al*., 2014.

2.4.4 The CRISPR/Cas mechanism

The CRISPR–Cas systems impart immunity to invading genetic elements via a three-stage process-adaptation, expression and interference, all of which are mediated by single Cas proteins or multiple Cas protein containing complexes (Makarova *et al*., 2011).

2.4.4.1 Adaptation phase

The adaptation stage (Fig 2.6) is the spacer acquisition phase where short pieces of DNA homologous to virus or plasmid sequences are integrated into the CRISPR loci (Barrangou *et al*., 2007; Garneau *et al*., 2010; Sontheimer and Marraffini, 2010).

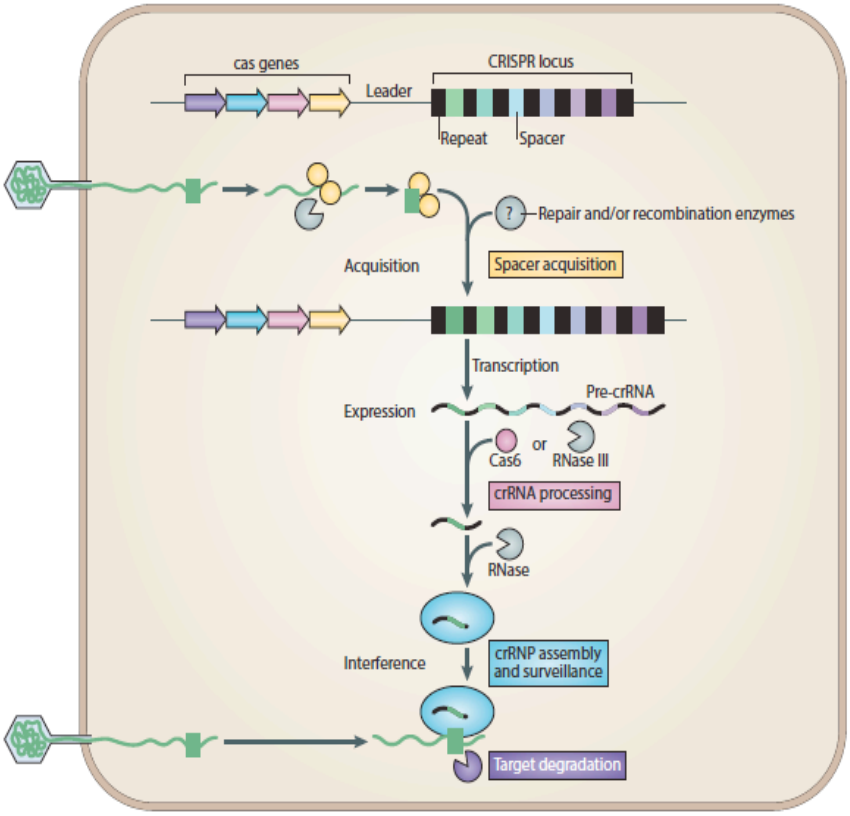

Fig 2.6 The CRISPR pathway. Cas genes located adjacent to the repeat-spacer sequences encode a protein machinery (Cas proteins), which administrate the general phases of the immune response. Cas genes, Cas proteins as well as other involved proteins (grey) are colored based on their functional contribution to spacer acquisition (yellow), crRNA biogenesis processing (pink), crRNP assembly and target binding (blue), and degradation (purple). In the initial step, invading foreign DNA is recognized and processed fragments (protospacers-green box) are integrated between a repeat of the CRISPR locus. Spacer acquisition is mediated by the universally conserved proteins Cas1 and Cas2 (yellow circles) and has been linked to DNA repair/recombination enzymes (grey circle). As a response to new invasion, the second phase of the CRISPR pathway is initiated: the expression of long primary crRNA transcripts. These are then endonucleolytically processed into mature crRNAs that serve as a template for the assembly of the crRNP. In the final interference step, the fully assembled complex detects the complementary target and initiates its nucleolytic degradation. Adapted from van der Oost *et al*., 2014.

Viral challenge typically activates insertion of a single virus-derived resistance-conferring spacer, with a characteristic length of approximately 30 bp, at the leader side of a CRISPR locus; acquisition of multiple spacers from the same phage is often less.

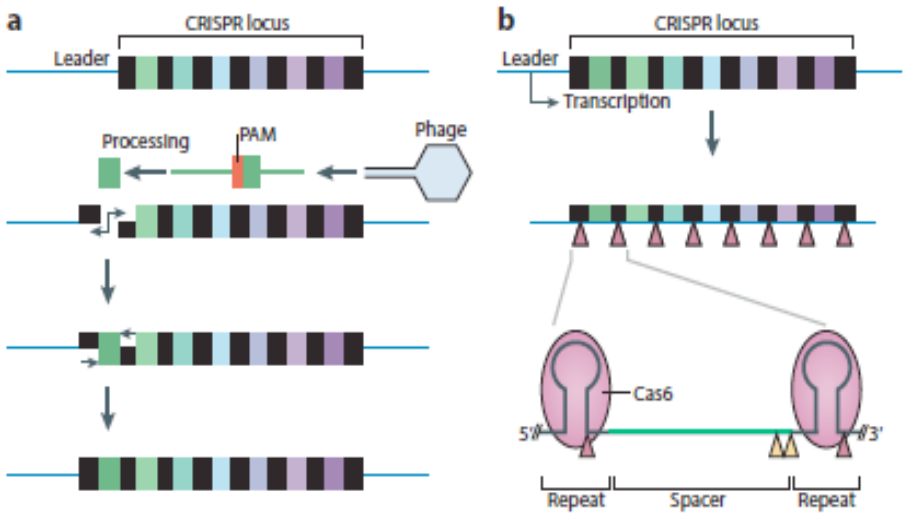

Fig 2.7 Spacer acquisition, biogenesis and processing. a. In types I and II, protospacers (green) from the invasive genetic material, in this case phage DNA, are recognized via a short three-nucleotide protospacer adjacent motif (PAM-red). The protospacer is further processed and the leader end repeat is opened in order to allow spacer integration. During this process the initial repeat is duplicated. b. The transcription of the CRISPR array produces a long pre-CRISPR RNA (pre-crRNA), which is primarily processed within the repeat sequences (pink triangles). In typeI and III systems, these steps are catalyzed by the endoribonuclease Cas6 (pink circle). The product is a crRNAs in which spacers are flanked by repeat-derived handles: a 5′ handle of 8 nucleotides, and a longer 3′ handle. The 3′ handle is either a stable hairpin structure or, if unstructured, it is subject to additional processing (yellow triangles) by unknown ribonucleases. Adapted from van der Oost *et al*., 2014.

Each integration event is followed by the duplication of a repeat and thus produces a new spacer-repeat unit. The selection of spacer precursors (protospacers) from the foreign DNA seems to be determined by the recognition of protospacer-adjacent motifs (PAMs) (Fig 2.7). PAMs are normally several

nucleotides long and differ between variants of the CRISPR-Cas system (Mojica *et al*., 2009; Deveau *et al*., 2008). There is presently no direct evidence for a mechanism of spacer acquisition, although the extremely conserved Cas proteins, Cas1 and Cas2, are the prime candidates for proteins with key roles in this process (Barrangou *et al* 2007; Brouns *et al*., 2008).

In type I systems, PAMs are located at the 3′ end of the protospacer, whereas in type II systems they are located at the 5′ end of the protospacer. Type III systems lack this principle of selection (Marraffini and Sontheimer, 2010).

2.4.4.2 Expression phase

The second phase is expression (Fig 2.6), in CRISPR-Cas-mediated immunity during which the long primary transcript of a CRISPR locus (pre-crRNA) is produced and processed into short crRNAs. Endoribonucleases catalyse the processing step, which either operates as a subunit of a larger complex (such as the CRISPR-associated complex for antiviral defence (Cascade) in *Escherichia coli* or as a single enzyme like Cas6 in the archaeon *Pyrococcus furiosus*. An intriguing variant was discovered in *Streptococcus pyogenes* recently, in which a trans-encoded small RNA (tracr RNA) acts as a guide for the processing of pre-crRNA, catalysed by RNase III in the presence of Csn1 (also known as Cas9) (Deltcheva *et al*., 2011). The mature crRNA remains associated with the complex after the initial endonuclease cleavage in the Cascade complex of type I CRISPR-Cas systems (Brouns *et al*., 2008; Haurwitz *et al*., 2010) whereas in the Cascade complex of type III systems, Cmr type *Pyrococcus furiosus* the crRNA, processed by Cas6, is passed on to a distinct Cas protein complex where it is processed further at the 3′ end by unknown nucleases (Carte *et al*., 2008; Hale *et al*., 2009; Wang *et al*., 2011).

2.4.4.3 Interference phase

During the interference phase (Fig 2.6), the foreign DNA or RNA is

targeted and cleaved within the proto-spacer sequence (van der Oost *et al*., 2009; Garneau *et al*., 2010; Sontheimer and Marraffini, 2010). The crRNAs guide the respective complexes of Cas proteins, such as the *E. coli* Cascade complex, to the complementary virus or plasmid target sequences that match the spacers. The HD endonuclease domain of Cas3 protein in *E. coli* probably catalyses the cleavage. (Brouns *et al*., 2008). Besides, the PAMs seem to play a significant part in the interference process (Deveau *et al.,* 2008; Marraffini and Sontheimer, 2010). Targeting either strand of the phage DNA confers immunity to the cognate phage in *S. thermophilus* and *E. coli*, an observation that is best compatible with DNA being the target (Barrangou *et al*., 2007; Brouns *et al*., 2008; Haurwitz *et al*., 2010). In addition, insertion of a self-splicing intron into the proto-spacer sequence of the target gene renders the corresponding plasmid resistant to CRISPR-mediated immunity in *Staphylococcus epidermidis*, implying that it is the invading DNA instead the corresponding mRNA that is targeted in this species (Marraffini and Sontheimer, 2010). Furthermore, the hyperthermophilic archaeon *Sulfolobus solfataricus* targets the DNA of *Sulfolobus* spindle-shaped virus 1 (SSV1), as CRISPR mediated immunity does not rely upon transcription of the target gene (Manica *et al*., 2011). *In vitro* experiments with the CRISPR–Cas system from *P. furiosus* indicated that in this species the crRNA targets the foreign mRNA instead (Hale *et al*., 2009). These observations emphasize the remarkable mechanistic and functional diversity of CRISPR-Cas systems, however the full range of their activities remains to be determined. Various Cas proteins might be involved in either one stage or multiple stages of CRISPR-Cas system action, perhaps as protein complexes (van der Oost *et al*., 2009).

2.4.5 CRISPRFinder

Although CRISPRs' *in silico* analyses started in 1995 (Mojica *et al*., 1995) no specific stand-alone CRISPR software tool was created. Different

authors used several software to identify these particular repeats but usually a manual discard of background was necessary, and generally some CRISPR clusters were missed or neglected, especially the shortest one (less than three motifs) (Benson, 1999).

The program CRISPRFinder, that allows the identification of structures with the principal characteristics of CRISPRs, the smaller being composed of a truncated or diverged Direct Repeat (DR), a spacer and a complete DR. It is accessible on the web and submission is very simple. The CRISPRFinder web server is an interface to extract with precision and to further analyze CRISPRs from genomic sequences. Four main advantages may be cited: (i) short CRISPR-like structures are detected, they are labelled questionable but may be of great interest if later confirmed; (ii) DRs are accurately defined to single base pair resolution; (iii) summary files may be uploaded (CRISPR properties summary and spacers file in Fasta format) and (iv) flanking sequences or spacers can be easily extracted and blasted against different databases (Grissa *et al*., 2007). The very small candidates will need to be typed across different isolates within the same species or very closely related species to search for variations. This strain-dependent polymorphism is especially interesting for epidemiological and phylogenetic studies (Kamerbeek *et al*., 1997; Hoe *et al*., 1999). A tool to easily create a dictionary of spacers from different strains is proposed in a CRISPR-dedicated web database (http://crispr.u-psud.fr/crispr/).

2.5 Lipopolysaccharide and its structure

Lipopolysaccharide (LPS), the surface structure encountering the surrounding environment is the major component of the outer membrane of gram-negative bacteria (Zhao *et al*., 2013). It comprises of lipid A, core sugars, and O-antigen (Maeshima and Fernandez, 2013). LPS is referred to as an endotoxin in Gram-negative bacteria (Wang and Quinn, 2010). It provides resistance to bile salts and hydrophobic antibiotics, and it protects the pathogen

from the complement system and from killing by macrophages (Kong *et al*., 2012).

LPS may be present either in the smooth form (S, possessing the polysaccharide region) or in the rough form (R, lacking the polysaccharide, also called lipooligosaccharide, LOS). Both forms include lipid A (Holst and Molinaro, 2009) and a core oligosaccharide (OS) that comprises up to15 sugar residues. The core OS region is substituted by a polysaccharide in the S-form LPS, which most often is an O-specific polysaccharide (O-antigen), and in other cases is the enterobacterial common antigen (only in *Enterobacteriaceae*) or a capsular polysaccharide (Kuhn *et al*., 1988).

Richard Pfeiffer recognized this endotoxin in 1892 (Beutler and Rietschel, 2003). Around 60 years was taken to establish the appropriate extraction protocol (the hot phenol–water procedure by which the isolation of rather pure endotoxin was possible). It became obvious after that the endotoxin comprises of sugars, phosphates, and fatty acids, and is a lipoglycan termed "lipopolysaccharide"(Westphal, 1965).

2.5.1 Structural components of LPS

2.5.1.1 The core region

The particular 3-deoxy-D-manno-oct-2-ulopyranosonic acid namely Kdo residue that links the core region to the lipid A is only one structural element, which is present in all core regions. The core region include L-glycero -D -manno -heptose (L, D -Hep) and an L-a -D -Hep-(1→3)-L-a -D -Hep-(1→5)-[a -Kdo-(2→4)]-a -Kdo tetrasaccharide (Hep II, Hep I, Kdo II, and Kdo I, respectively), that may be further substituted by other sugars or phosphate residues, or sometimes by acetyl groups or amino acids. In addition to L, D -Hep, several LPS contains its biosynthetic precursor, D -glycero -D -manno heptose (D, D -Hep). There are other LPS that encompass only D, D -Hep or even lacksany heptose (Holst, 2011; Unger, 1981).

Enterobacterial lipopolysaccharides core regions can be grouped into two types: the *Salmonella* type and core region different to the *Salmonella* type. In the first, the common structural element L, D-Hep-(1→ 7)-L, D-Hep-(1→3)-L, D-Hep-(1→5)-Kdo is present, which is substituted at O-3 of the second heptose by glucopyranose (Glcp). Heptose residues II and I are phosphorylated and O-4 of Hep I is not substituted by a saccharide (Müller Loennies *et al*., 2002).

Core oligosaccharides distinct from the *S*. enterica type possess a common partial structure L,D-Hep-(1 → 7)-L, D-Hep-(1→ 3)-L,D-Hep-(1→ 5)-Kdo, which is not substituted at O-3 of Hep II by Glc and in which heptose residues are not generally phosphorylated. Position O-4 of Hep I is substituted by a hexose residue or oligosaccharide (Knirel *et al*., 2005).

2.5.1.2 Lipid A

Lipid A, a hydrophobic moiety, anchors LPS into the asymmetric outer membrane. It is necessary for growth and is an important barrier that imparts resistance against antimicrobial peptides and environmental stresses that affect cell viability. Lipid A functions as an immunomodulatory molecule for pathogens that stimulates a strong innate immune response via the Toll-like receptor 4 (TLR4)-MD2-CD14 pathway resulting in the activation of nuclear factor κB (NF-κB) and up regulation of co-stimulatory molecules and inflammatory cytokines (Ghosh *et al*., 1998).

The structure of lipid A is almost conserved among different pathogenic bacteria. In *E*. coli and *S*. Typhimurium lipid A is a β, 1′-6-linked disaccharide of glucosamine phosphorylated at the 1 and 4′ positions and acylated at 2, 3, 2′, and 3′ positions with R-3-hydroxymyristate. The OH groups of the R-3-hydroxymyristate chains that are affixed at positions 2′ and 3′ are therefore further acylated with laurate and myristate (Kawasaki *et al*., 2004).

2.5.1.3 The O-antigen

The O-antigen component of the lipopolysaccharide (LPS) symbolizes a population of polysaccharide molecules with nonrandom (modal) chain length distribution (Kalynych *et al*., 2011). The O-antigen polysaccharide (OAg) is organized as an undecaprenyl-phosphate (Und-P-P)-linked intermediate and ligated to lipid A-core OS with the release of Und-P-P. Although lipid A and core OS structures are relatively conserved, the OAg is highly diverse, giving rise to thousands of O-specific serotypes among Gram-negative bacteria. The O Ag plays a role in pathogenesis by influencing macrophage recognition and resistance to the lytic action of the complement system (Clay *et al*., 2008; Murray *et al*., 2003; Murray *et al*., 2006; Saldías *et al*., 2009), epithelial cell invasion (Duerr *et al.,* 2009; West *et al*., 2005), and intracellular survival in certain bacteria (Paixão *et al*., 2009).

2.5.2 Activity of LPS Constituents

Lipopolysaccharide (LPS) of *Salmonella* is essential for functions, including swarming motility, intestinal colonization, serum resistance, invasion/intracellular replication, and resistance to killing by macrophages, all of which are critical for successful infection by the pathogenas it is a recognized virulence determinant (Kong *et al*., 2011). Its role in virulence has been widely discussed and this molecule is a potent endotoxin (León and Bastías, 2015).For numerous bacteriophages LPS is also a receptor (Ishiguro *et al*., 1983; Michel *et al*., 2010; Kiljunen *et al*., 2011). Direct interaction between LPS and phage proteins has been demonstrated, including by X-ray crystallography evidence (Steinbacher *et al*., 1997; Inagaki *et al*., 2000).

The loss of LPS in *Salmonella* produces changes in bacteriophage susceptibility and the loss of virulence in mice (Chart *et al*., 1989). Contemporary work has shown that strains of *Salmonella enterica* serovar Enteritidis resistant to the lytic phages *f2*αSE, *f3*αSE and *f18*αSE are avirulent

in the nematode *Caenorhabditis elegans*. The Bacteriophage Resistant (BR) strains lack the O-polysaccharide from LPS and show abnormal colony morphology implying that modifications in this structure may be involved in resistance and the loss of virulence (Santander and Robeson, 2007).

A similar context was noticed with the strain Salp572$^{\varphi 1S}$ of *Salmonella enterica* serovar Paratyphi B and bacteriophage φ. Gene expression analysis revealed that Salp572$^{\varphi 1R}$, a BR strain under expresses several genes related to virulence, such as *cmE, sthE,* and *cheY* (periplasmic heme-dependent peroxidase, a putative major fimbrial subunit, and a chemotaxis regulator, respectively) and lacks the *O*-polysaccharide from LPS. Further, Salp572$^{\varphi 1R}$was completely avirulent in mice;whereas the parental sensitive strain Salp572$^{\varphi 1S}$ killed the mice after 48 h of infection. These illustrations strongly suggest that modifications in LPS can produce bacteriophage resistance and decrease virulence simultaneously (Capparelli *et al*., 2010).

O-specific chain is distinguished by extremely high structural variability even within a given bacterial species, which establishes the chemical basis for the serological classification of individual wild-type bacterial strains corresponding to their O-antigenic determinants. LPS without O-specific chains in bacterial mutants are able to grow and multiply *in vitro*, indicating that the O-chain in principle is dispensable for bacterial viability (Raetz and Whitfield, 2002). On the basics of characteristic colony morphology, distinct from the smooth (S)-form of wild-type enterobacterial species, these mutants have been termed as rough (R)-mutants leading to a corresponding general sub-classification into S- and R-form LPS. There is extensive heterogeneity in the size of the molecules due to variations in the chain length of the O-polysaccharides when LPS molecules extracted from any S-LPS-containing strain are separated by SDS-PAGE. This is noticeable in the classical "ladder" pattern in SDS-PAGE, where each "rung" up the ladder represents a lipid A-core molecule substituted with an increment of one additional O-unit. The

spacing between the rungs is decided by the size of the O-unit (Matsuura, 2013).

The primary role (s) of the O-polysaccharides appears to be protection for bacteria. O-polysaccharides may contribute to bacterial evasion of host immune responses particularly the alternative complement cascade in animal pathogens. Assembly of the membrane attack complex is affected by the chemistry of the O-polysaccharide, its chain length, and the relative amounts of long chain S-LPS (Rautemaa and Meri, 1999; Murray *et al*., 2006).

Compared to the hyper variable O-polysaccharides the core oligosaccharide has much less structural variation and has limited variation within a given bacterial genus. Neither the core oligosaccharide nor O-antigen is required for the immunostimulatory activity of LPS, but rather the lipid A portion is responsible for the activity (Moran, 2008; Skoglund *et al*., 2009).

The hexa-acylated *E. coli* type among natural lipid A is relatively conserved in a wide variety of Gram-negative bacteria, however some bacterial species have different types of lipid A. Some variants of lipid A frequently coexist and their structures are sometimes modified under different environmental conditions even in a single species of bacteria (Raetz and Whitfield, 2002).

Several studies have indicated that the composition or size of the O-antigen might be a reliable indicator of virulence potential and that these important features often differ within the same bacterial strain so understanding LPS structural variation in bacterial pathogens is important. O-antigen modifications seem to play an important role at several (at least two) stages of the infection process, including the colonization (adherence) step and the ability to bypass or overcome host defense mechanisms in general. There are several reports of modifications of O-antigen in bacterial pathogens, resulting either from altered gene expression, from lysogenic conversion or from lateral gene transfer followed by recombination (Lerouge and Vanderleyden, 2002).

2.6 Virulence genes of *Salmonella*

The facultative intracellular pathogenic bacteria, *Salmonella* can invade macrophages, dendritic and epithelial cells. The culpable virulence genes for invasion, survival, and extra intestinal spread reside in *Salmonella* pathogenicity islands (SPIs). SPIs are thought to be obtained by horizontal gene transfer (Sırıken, 2013).

2.6.1 *Salmonella* pathogenicity islands (SPIs)

These genetic islands are positioned on the bacterial chromosome or on plasmids, although, not all serovars possess every known SPI. SPI-1 through SPI-5 are common among all *S. enterica* serovars (Hurley, 2014). 23 SPI have been described to date however the functions of those genes contained within each island have not yet been completely elucidated (Sabbagh *et al*., 2010: Hayward *et al*., 2013). The complete distribution of the SPIs identified in the iNTS (Non Typhoidal) strains could be grouped into three categories: (i) intact (or mostly intact) SPIs with universal presence in all of the iNTS isolates (SPIs 1–5, 9, 13 and 14); (ii) SPIs that are totally absent from this iNTS collection (SPIs 7, 8 and 15); and (iii) SPIs with variable or mosaic presence across the serovars (SPIs 6, 10–12 and 16–19) (Suez *et al*., 2013).

SPI-1 and SPI-2 are of special importance in *in vivo* infection. The effector proteins encoded by SPI are translocated directly into host cells across the plasma membrane typeIII secretion systems (T3SS-1 and T3SS-2) which provide *Salmonella* with the biochemical machinery to exploit this intracellular niche. T3SS secrete effector proteins in to the surrounding environment to influence hostcell physiology (Galán, 1999; Hensel *et al*., 1998). During the systemic phase of the disease SPI3 is necessary for survival within macrophages and growth in low Mg^{2+} environments. SPI4 may also contribute to toxin secretion and is suspected to be required for intramacrophage survival. Similar to SPI1, SPI5 appears mainly associated with enteropathogenesis, being

involved in inflammation and chloride secretion (Soto *et al*., 2006).

2.6.2 Functions associated with virulence factors

2.6.2.1 *lpf operon, agf operon and sef operon*

Certain virulence factors are associated with the cellular structure of the bacteria, such as fimbriae. The affinity of the bacteria for Peyer's patches and adhesion to intestinal M cells is contributed by the long polar fimbria (*lpf operon*) (Borges *et al*., 2013). One of the key functions of aggregative fimbria (*agf operon*) is to favor the initial interaction of the bacteria with the intestine of the host and stimulate bacterial self-aggregation, resulting in higher rates of survival (Collinson *et al*., 1992; 1993). The *Salmonella*-encoded fimbria (*sef operon*) boosts a better interaction between the bacteria and the macrophages (Collinson *et al*., 1996).

2.6.2.2 HilA, InvA, AvrA and *spv*

Type III secretion system (TTSS) is capable of injecting bacterial effector proteins through bacterial and host membranes to interact with host cells (Marcus *et al*., 2000). The central regulator HilA encoded by the *hilA* gene is necessary for the expression of the TTSS components. HilA are also essential for invading epithelial cells and to induce apoptosis of macrophages (Bajaj *et al*., 1996).

For epithelial invasion, the protein InvA is essential (Galán & Curtis III, 1989) and AvrA is an effector protein of the TTSS complex that provides virulence to the *Salmonella* spp. by restricting the host's inflammatory responses through the inducement of cell apoptosis, especially of macrophages, and by the inhibition of IL-8 and TNF-α (Collier-Hyames *et al*., 2002; Ben-Barak *et al*., 2006).

The invasion of these bacteria through the generation of membrane deformations (Hardt *et al*., 1998b) and the rearrangement of the cytoskeleton of

the host cells (Galán and Zhou, 2000) is contributed by *Salmonella* spp.'s outer proteins (Sops). The *sivH* gene encodes an outer membrane protein associated with intestinal colonization (Kingsley *et al*., 2003).

Some important *Salmonella* spp. virulence factors are found on virulence plasmids. A highly conserved region designated *spv* RABCD (*Salmonella* plasmid virulence) is shared by all virulence plasmids. The *spv* region boosts rapid growth and survival of *Salmonella* spp. within the host cells and it is significant for systemic infection (Libby *et al*., 1997). Diarrhea caused by *Salmonella* is a complex phenomenon involving several pathogenic mechanisms, including production of enterotoxin. Production of this enterotoxin is mediated by the *stn,* thus it plays a significant role in causing gastroenteritis (Chopra *et al*., 1987).

2.6.2.3 PhoP/Q and *slyA*

The ability of *Salmonellae* to set up and maintain an intracellular replication niche is vital to their pathogenesis. For survival and proliferation inside phagolysosomes require the induction of systems that stimulates resistance to cationic antimicrobial peptides (CAMPs), oxygen and nitrogen radicals, and other specific stresses, such as acidic pH (Prost and Miller, 2008). Within these sensory systems is the *Salmonella* PhoP/Q virulence system, which regulates enzymes that modify lipid A to increase the outer membrane permeability barrier to CAMP and provoke bacterial resistance (Dalebroux *et al*., 2014).

slyA was initially identified as a gene necessary for *Salmonella* virulence and for survival in macrophages and was thereafter shown to belong to a family of low-molecular-weight transcriptional regulators (Buchmeier *et al*., 1997). It was formerly shown that a *slyA* mutant is unable to survive in the tissues of the reticuloendothelial system. The mechanism by which *slyA*

facilitates *Salmonella* survival and replication in macrophages is not well understood (Daniels *et al*., 1996).

2.6.2.4 Tetrathionate reductase (Ttr)

Certain genera of Enterobacteriaceae including *Salmonella* have the ability to respire tetrathionate (Richard, 1977). The occurrence of tetrathionate respiration is phylogenetically wide spread although it has not been so extensively studied in other bacterial families (Barrett and Clark, 1987), permitting bacteria to take advantage of the availability of tetrathionate in many environments (Barbosa-Jefferson *et al*., 1998). The tetrathionate reductase (Ttr) is a membrane-bound enzyme in *Salmonella* containing molybdopterin guanine dinucleotide cofactor (MGD) as a prosthetic group. The ttr genes reside within *Salmonella* Pathogenicity Island 2 at centisome 30.5. *ttrA*, *ttrB* and *ttrC* are the tetrathionate reductase structural genes **(**Hensel *et al*., 1999). Sequence analysis conveys that ttrA consists of a molybdopterin guanine dinucleotide cofactor and a [4Fe–4S] cluster (Breton *et al*., 1994; Boyington *et al*., 1997), that ttrB binds four [4Fe–4S] clusters (Berks *et al*., 1995), and that ttrC is an integral membrane protein containing a quinol oxidation site. It is predicted that ttrC anchors ttrA and ttrB to the periplasmic face of the cytoplasmic membrane implying a periplasmic site for tetrathionate reduction **(**Barrett and Clark, 1987).

2.6.2.5 MgtC

There are three transport systems for Mg^{2+}, CorA, MgtA, and MgtB in *Salmonella*. Constitutively expressed CorA gene encodes a 40kDa protein product, which mediates both the influx and efflux of Mg^{2+}. Putative P-type ATPases is encoded by mgtA and mgtCB loci (Moncrief and Maguire, 1998). These loci are under the control of the two-component PhoPQ regulatory system. Unlike CorA, mgtA and mgtB deal only the influx of magnesium and are produced under Mg^{2+}-limiting conditions (Hmiel *et al*., 1989; Tao *et al*.,

1998). Two proteins are encoded by the mgtCB locus. A hydrophobic protein, MgtC with a predicted molecular mass of 22.5 kDa (25, 28) and MgtB, the P-type ATPase, has a molecular mass of 102 kDa. The mgtCB locus has been recognized as part of a new pathogenicity island, SPI-3. It is vital for long-term survival within the macrophage and for virulence (Smith *et al*., 1998; Blanc Potard and Groisman, 1997).

2.6.2.6 *spi4 and sopE*

It is predicted that SPI-4 carries genes (*spi4R* and *spi4D*) which encode a type I secretion system while *pipAB* and *sopB* genes which encodes for TTSS-1 and TTSS-2 effector proteins are located on SPI-5 (Kaur and Jain, 2012). SPI-7, another important island which is responsible for the production of the Vi polysaccharide capsule also carries genes for potential virulence factors such as a phage encoding the *sopE* effector protein of SPI-1, a type IV pilus and a putative type IV secretion system (Seth-Smith, 2008).

2.6.2.7 GogB

By providing an extended repertoire of virulence determinants that have integrated into ancestral regulatory networks of the bacterial cell through acquisition of phage genes by lysogenic conversion contributed to the genetic diversity of *Salmonella* (Pilar *et al*., 2012). A secreted effector GogB encoded in the Gifsy-1 prophage and is a substrate of both T3SS-1 and T3SS-2 is found in some *S. enterica* strains (Coombes *et al*., 2005b). It was intially shown that GogB is a chimeric protein containing a C-terminal domain with similarity to known proteins and an N-terminal canonical leucine-rich repeat domain (LRR). Although its function and host cell target(s) were not known *Salmonella* translocates GogB into the host cytoplasm. It has sequence similarity to a group of effectors that function as novel E3 ubiquitin ligases (NELs). The transcriptional activator, SsrB influences the genetic regulation of *gogB* in

Salmonella, under SPI-2-inducing conditions, but the modular nature of the *gogB* gene permits for autonomous expression and type III secretion following horizontal gene transfer into a heterologous pathogen (Golubeva *et al*., 2011). GogB functions to limit tissue damage from enhanced inflammatory host response during *S.* Typhimurium infection are reported by most recent findings (Quezada *et al*., 2009; Singer *et al*., 2008; Pilar *et al*., 2013).

2.6.2.8 SODs

Superoxide dismutases (SODs) catalyzing the conversion of oxygen radical (O_{2}-) into hydrogen peroxide and oxygen are virtually ubiquitous in aerobic bacteria (McCord and Fridovich, 1989). Of the three types of SODs found in bacteria, two are cofactored by manganese or iron and are lodged in the cytosol and catalyse dismutation of O_{2}-generated during aerobic metabolism and a third SOD has been identified in the periplasm of a wide range of Gram-negative bacteria namely *Caulobacter crescentus* (Steinman, 1982), *Escherichia coli* (Benov and Fridovich, 1994; Imlay and Imlay, 1996), *Salmonella* Typhimurium, *S.* Choleraesuis and *S.* Dublin (Canvin *et al*., 1996; Farrant *et al*., 1997), and in some of the Gram-positive bacteria like *Mycobacterium tuberculosis* is cofactored by copper and zinc (Cu, Zn-SOD) (Wu *et al*., 1998).Cu, Zn-SODs or SodC have been shown to contribute to the pathogenicity of these bacterial species and also protect Gram-negative bacteria against oxygen damage (Sanjay *et al*., 2010).

2.6.2.9 *gtgE*

Salmonella enterica serovar Typhimurium that carries Gifsy-2 as its prophage contributes significantly to the pathogenicity of strains. For the full virulence of *S. enterica* serovar Typhimurium Gifsy-2 lysogens *gtgE*, is necessary (Ho *et al*., 2002). A putative protein of 228 amino acids with no

significant homologs in other bacterial species found in the National Center for Biotechnology Information database is encoded the *gtgE* gene. The GtgE protein is acidic (pI 4.6) with no apparent transmembrane domains or N-terminal signal sequence, implying a cytoplasmic protein. GtgE produced by *S. enterica* serovar Typhimurium cells grows in vitro as well as intracellularly in HEp-2 epithelial cells is indicated in epitope-tagging experiments (Uzzau *et al*., 2001). Therefore, GtgE represents a novel bacterial virulence factor. It is conventionally possible that the SPI-2 type III secretion system secretes the protein, for instance, given that the signals for type III secretion are not easily recognized. It is also expected that GtgE is a type III chaperone. If it is atype III chaperone, then its target protein is not carried on Gifsy-2 or Gifsy-1. Infact, the phenotype conferred by loss of GtgE is probably more severe than that conferred by loss of any previously identified SPI-2 effector except SifA (Ruiz Albert *et al*., 2002).

Certainly, adaptation of *Salmonella enterica* to host milieu is associated with sensing of environmental changes and subsequent co-ordinated expression of virulence genes. A significant induction of virulence genes involves the cumulative action of global gene regulators and specific virulence gene regulators. Functional inactivation of virulence gene regulation might prompt avirulence (Clements *et al*., 2001).

2.7 Antibotic resistance in *Salmonella*

One of medicine's greatest achievements in the first half of the previous century has been the discovery and production of antibiotics. Antimicrobial agents has reduced morbidity and mortality of humans and added considerably to human's increased life span (van Hoek *et al*., 2011). At the time of introduction of antibiotics it was expected that the evolution of antibiotic resistance (AR) was unlikely. This was based on the impression that the

frequency of mutations generating resistant bacteria was negligible (Davies, 1994).

Worldwide antimicrobial-resistant salmonellosis is a significant public health concern. However intestinal infection caused by nontyphoid *Salmonella* serotypes is usually self limiting, effective antimicrobial therapy is necessary if spread beyond the intestine occurs. The large-scale use of antimicrobials in humans and animals has led to an increase in multidrug resistance among several bacterial strains (Adesiji *et al*., 2014; Dione *et al*., 2011).

Globally *Salmonella* Enteritidis is one of the most prevalent serovars with an increased display of antimicrobial resistance (Khumalo *et al*., 2014). It is presently the world's leading cause of non-typhoidal salmonellosis (Center for Disease Control and Prevention (CDC) 2011; Ngoi and Thong, 2013), principally associated with eggs, egg products, poultry, and the farm environment and cross-contamination of other foods from eggs (Perry and Yousef, 2011). It has adapted to be host specific and is still a major public health risk in developing nations (Betancor *et al.,* 2010; Okeke *et al.,* 2007).

The application of medicated feeds in intensive animal husbandry systems and sub-therapeutic doses and unplanned use of antimicrobials that are often administered through the feed or drinking water for therapy, prophylaxis or growth promotion (Aarestrup, 2005) have been suspected in the emergence of antibiotic resistance. This increase confines the empirical therapeutic options available for clinical cases that demand antimicrobial treatment (Tamma et *al*., 2012).

Through a number of mechanisms bacteria have become resistant to antimicrobials (1) Permeability changes in the bacterial cell wall, that restricts antimicrobial access to target sites, (2) Active efflux of the antibiotic from the

microbial cell, (3) Enzymatic modification of the antibiotic, (4) Degradation of the antimicrobial agent, (5) Acquisition of alternative metabolic pathways to those inhibited by the drug, (6) Modification of antibiotic targets, (7) Overproduction of the target enzyme (Spratt, 1994; McDermott *et al*., 2003; Magnet and Blanchard, 2005; Wright, 2005).

Increased resistance to multiple antibiotics in bacteriais contributed significantly by the multidrug efflux systems. To identify compounds that can restrain the efflux functions is a major challenge in developing efficacious antibiotics against drug resistant pathogens (Lin *et al*., 2015). Even a single bacterium may harbor multiple efflux transporters of different families, with the overlapping substrate spectra (Morita *et al*., 2012). Transcriptional regulators that either repress or activate the transcription of the multidrug efflux genes usually control the expression of bacterial multidrug efflux system. The relevance of multidrug efflux system may not be exaggerated for a specific antibiotic or organism (Baucheron *et al*., 2014).

Resistance to β-lactam antibioticshas become a world wide health care problem. A major and threatening resistance mechanism toward β-lactam antibiotics isproduction of β-lactamases. Emergence of β- lactamase-mediated cefotaxime resistance in *Salmonella enetrica* Serovar Infantisis reported in a recent epidemiological work (Chuma *et al*., 2013).

Production of aminoglycoside-modifying enzymes is a major mechanism of aminoglycoside resistance. Aminoglycoside kinase and aminoglycoside 6^{1}-*N*-acetyltransferase typeIb aretwo enzymes with aminoglycoside-modifying activities (Shi *et al*., 2013; Ramirez *et al*., 2013).

Mutations within the genes that encode the enzymes such as *gyrA*, *gyrB*, *parC* and *parE* result in resistances to quinolones and fluoroquinolones.

Maximum of these mutations occur in the quinolone resistance determining region (QRDR), which is a conserved site in these enzymes focused by these antimicrobials. Resistance to nalidixic acid and then to fluoroquinolones establishes in a stepwise process of mutations in the QRDR region yielding an enzyme with a target region that quinolones cannot bind to (Chen *et al*., 2007).

In humans nalidixic acid resistance among *S*.Enteritidis isolates hiked from 0.9% in 1996 when NARMS (The National Antimicrobial Resistance Monitoring System) testing began to 5.2% in 2010. Within all nontyphoidal *Salmonella* isolates from humans, nalidixic acid resistance was 2.0% (49/2474) in 2010, and 55% (27/49) of the nalidixic acid-resistant isolates were *S*. Enteritidis (CDC, 2010). Resistance to nalidixic acid correspond to reduced susceptibility to the fluoroquinolone ciprofloxacin and might be correlated with treatment failure, so identifying sources of resistant infections is important (O'Donnell *et al*., 2014).

Mechanisms of phenicol resistance noticed in U.S. NARMS animal isolates have been *floR*, *cmlA*, and *cat1*. Besides, the chloramphenicol resistance gene *floR* is often observed in the class I integron located in *Salmonella* Genomic Island 1(SGI-1)(Frye and Jackson, 2013).

Resistance to sulfonamides and trimethoprim develops by procurement of genes encoding enzymes that do not bind these compounds. These encompass the *sul* genes, *sul1*, *sul2*, and *sul3*, which encode an insensitive DHPS (dihydropteroate synthase) enzyme and are observed in *Salmonella* worldwide (Prescott, 2000). Trimethoprim resistance is by DHFR encoding genes, either *dhfr* or *dfr*, both of which have been observed in *Salmonella* animal isolates in the U.S (Glenn *et al*., 2011; Frye *et al*., 2011).

Tetracycline resistance mechanisms involve efflux, modification of the rRNA target, and inactivation of the compound. Although, in *Salmonella*, active efflux systems are most commonly detected and include *tet* (A), *tet* (B), *tet*(C), *tet* (D), *tet* (G), and *tet* (H). In U.S. *Salmonella* animal isolates, *tet* (A), *tet* (B), *tet*(C), *tet* (D), and *tet* (G) are commonly detected and are primarily accompanied by the *tetR* regulator (Frye and Fedorka-Cray, 2007; Frye *et al*., 2008, 2011; Zou *et al*., 2009; Glenn *et al*., 2011; Lindsey *et al*., 2011).

Multi-drug-resistant (MDR) *Salmonella* serotypes emergence has a great impact on the efficacy of antibiotic treatment. An increasing prevalence of MDR strains may associate with an increase in mortality rates of *Salmonella* infections. MDR *Salmonella* serotypes are more virulent than susceptible strains, as reflected by increased severity and more prolonged disease in patients infected by MDR strains indicated by epidemiological studies. Preventive measures have been suggested to eliminate the spread of *Salmonella* infection. Although the maintenance of effective food hygiene and water sanitation remains the cornerstones, additional measures such as restriction of unplanned use of antibiotics in food animals are important (Eng *et al*., 2015).

2.7 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a broadly used method to verify the phylogeny of a bacterial population based on sequence analysis of representative genes of the bacterial core genome (Janßen *et al*., 2015). As accurate strain identification is indispensable for anyone working with bacteria, MLST is regarded as the "gold standard" of typing (Larsen *et al*., 2012). It was proposed in 1998 for *Neisseria meningitidis* as a portable sequence-based method to determine clonal relationships among bacteria (Maiden *et al*., 2013).

This highly discriminatory method characterize bacterial isolates on the basis of the sequences of approximately 450-bp internal fragments of seven

housekeeping gene amplified by PCR (Szabó, 2014). In most MLST schemes, seven 'MLST loci' are indexed, for which each unique sequence for each locus is alloted an arbitrary and unique allele number. The designations for each of the loci are incorporated into an allelic profile (for example, 2-3-4-3-8-4-6), or a sequence type (ST), which is also assigned a numerical designation (for example, ST11). The ST and allelic designations are linked to their respective allelic profiles and sequences in the MLST databases (Maiden, 2006) and each ST thus compiles thousands of base pairs of information. In those bacteria that were broadly examined by MLST, many hundreds of alleles at each locus and thousands of STs (see the PubMLST database collection) have been identified. However an ST symbolize only a tiny percentage of the 'conserved' parts of the genome in question, the large number of STs in many bacterial populations indicates the significance of an expandable means of compiling and comparing data. The allele and ST designations can be utilized for definitions of strains or grouped into clonal complexes or lineages as an enhanced understanding of the biological population structure emerges (Pérez-Losada *et al*., 2013; Maiden, 2006). Concept of MLST had been used formerly by multilocus enzyme electrophoresis (MLEE) which examines metabolic enzyme variation, but the indexing of gene sequence variation by MLST dramatically enhanced resolution, reproducibility and portability (Selander *et al*., 1986).

The progress of curated online MLST reference data sets (such as those found in the PubMLST database collection) contributed both portable nomenclature schemes and the feasibility of analysing the sequences to interpret evolutionary relationships (Maiden *et al*., 1998; Pérez-Losada *et al*., 2013). The demand for effective data repositories is well recognized, as is obvious from the interest generated by initiatives such as the Global Microbial Identifier (Köser *et al*., 2012; Aarestrup *et al*., 2012; Carriço *et al*., 2012) and the power of such infrastructure is embellished by the success of the 16S rRNA sequence and

MLST databases (Maiden, 2006; Harmsen *et al*., 2002). For instance, there are many MLST databases available on a number of websites (see PubMLST, the MLST homepage, the MLST databases at the ERI, UCC (Environmental Research Institute, University College Cork, Ireland) and the Institute Pasteur MLST databases), and these databases facilitate data generated in different laboratories to be efficiently compared (Chan *et al*., 2001; Jolley *et al*., 2004; Aanensen and Spratt, 2005).

Of the 79 MLST databases that are publicly available (http://pubmlst.org/databases.shtml), the *Salmonella enterica* MLST database (http://mlst.ucc.ie) ranks fourth in number of isolates. This publicly accessible and actively curated web-based MLST database facilitates the global exchange of information. In particular, new alleles and new STs depend on user submissions rather than decisions by a central reference laboratory, and are immediately made publicly accessible. Similar global exchange of information at the strain level does not exist for serotyping. The database currently provides data for >500 of the 1,500 existing serovars in subspecies *enterica*, including all common serovars and many that are rare. These data have been accumulated through a decentralized global effort since 2002 and with time, it is anticipated that representatives of all 1,500 serovars will be tested, thus providing a reasonably complete mapping between serovar and eBG/ST (Achtman *et al*., 2012). eBG is an acronym for eBurstGroup, and is equivalent to ST Complex or Clonal Complex in other bacterial MLST schemes. STs were assigned to a common eBG if an ST contained 10 or more isolates or there were at least 2 STs linked by identity at 6/7 alleles (so-called Single Locus Variants). Some double locus variants STs were affiliated with an existing eBG if they contained the same serovar (Didelot *et al*., 2011). Now it has been moved to University of Warwick. One of the schemes based on seven housekeeping genes that has been

widely used and provides access to global data from a publicly accessible database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) (Sun *et al*., 2014)

2.7.1 Advantages and disadvantages

MLST uses alleles as the unit of comparison, on behalf of nucleotide sequences. In allele-based comparisons between isolates, each allelic change is counted as a single genetic event, disregarding the number of nucleotide polymorphisms involved. This maintains a simple and effective correction for the fact that in many bacteria, common horizontal genetic transfer events account for many more polymorphisms among specimens than limited point mutations (Didelot and Maiden, 2010). The MLST approach owns information at all loci and avoids the need to categorize which changes are recent point mutations and which are because of recombination. As MLST schemes trace the sequences of allelic variants, MLST data can also be utilized for sequence-based analyses when this is relevant (Pérez-Losada *et al*., 2013; Maiden, 2006; Turner and Feil, 2007). MLST permits high levels of discrimination between isolates because many alleles can be accurately and directly identified in a single locus (Cornelius *et al*., 2012). Further advantages of using MLST as a genotyping method are the DNA sequences can be determined using automated technology and demands minimal subjective interpretation of data (Maiden *et al*., 1998). Besides, the portability of MLST data permits results from different laboratories to be compared. Moreover, the data acquired by MLST can be used to address basic questions about the evolutionary and population biology of bacterial species (Enright *et al*., 2000).

MLST uses only seven loci, which restrict the ability to detect some switches, and can be considered as a disadvantage of this technique. It requires performing PCR and sequencing of the PCR products using an automated sequencer, which is not accessible in most clinical laboratories. PCR

amplification is sensitive to contaminating DNA, and sequence diversity may lead to the failure of particular primers to amplify gene fragments (Maiden, 2006). Over all, MLST is not convenient for routine infection control or outbreak investigation due to high cost, labor intensity, and lack of broad access to high-throughput DNA sequencing (Enright *et al*., 2000; Szabó, 2014).

Lamentably, the variability of housekeeping genes between different bacteria makes it impossible to develop MLST schemes for anything but closely related bacteria. Therefore, even within genera (for example, in the genus *Streptococcus* (Do *et al*., 2009; Webb *et al*., 2008; Coffey *et al*., 2006; Enright & Spratt, 1998), it is mandatory to have more than one MLST scheme, focusing different loci. Moreover, MLST does not provide ample discrimination for all typing purposes, together with resolving differences among variants of single-clone, low diversity, asexual pathogens such as *Bacillus anthracis* (Priest *et al*., 2004) and *Yersinia pestis* (Achtman *et al*., 2004) or isolates of more diverse pathogens that fit to the same lineage. For instance, at least two distinct sublineages within the ST11 clonal complex of *Neisseria meningitides* are indistinguishable by MLST (Jolley *et al*., 2012). Hence, MLST alone is not always enough for applications like contact tracing in epidemics or for distinguishing single-clone pathogens (Holt *et al*., 2008), and in these cases MLST can be enriched with additional typing schemes that indicate more variable loci, like antigen genes (Jolley *et al*., 2007; Dingle *et al*., 2008) or variable-number tandem repeats (VNTRs) (Adair *et al*., 2000).

2.8 Phage cocktail

An important aspect of phage functioning as biological antibacterials is their efficacy to be applied directly to living tissues without causing harm; likewise antibacterial agents such as antiseptics and antibiotics that is, they demonstrate selective toxicity. However not always emphasized, particularly

historically, a crucial component of selective toxicity is an ability to avoid impairing the often-useful normal microbiota that are associated with mammalian bodies. Hence, exhibiting a narrow spectrum of activity can be a useful quality for an antibiotic or equivalent antibacterial (Rea *et al*., 2011; Relman, 2012; Fischbach and Walsh, 2009). Moreover, the hosts range of phages, as equivalent to their spectrum of activity, is likely to be relatively narrow, often consisting of only a subset of strains molding a single bacterial species (Hyman and Abedon, 2010). This same characteristic can be restraining, although, in terms of the ability of specific phage products to impact bacterial infections (Chan *et al*., 2013).

To ensure that phage formulations yield a phage that possesses a host range that includes target bacteria, multiple phage types retaining a diversity of host ranges are often combined into mixtures called 'phage cocktails' (Goodridge, 2010; Chan and Abedon, 2012). Monophage therapy employs the application of only a single phage type, while polyphage therapy is the application of a phage cocktail, that is, therapy involving the concurrent use of more than one phage type (Hall *et al*., 2012; Levin and Bull, 2004).

Research using phage cocktails commenced to expand at the start of the millennium due to a number of insufficient to modest outcomes realized while evaluating single phage preparations (Skurnik and Strauch, 2006). This is not to allude that monophage therapy successes have not been seen, but relatively that a number of strategies exist by which phage therapy outcomes may be sharpened. One such approach is phage formulation into cocktails (Carlton, 1999).

Several studies have suggested techniques to improve the phage cocktails being refined. A procedure was developed by Kelly *et al.* which was based on *Staphylococcus* phage K, requiring multiple passages on previously

phage-resistant strains to enrich for broad host-range spontaneous phage mutants. These authors identified phage resistance systems in 29 S. aureus strains. Among them, 24 had restriction modification mechanisms, three had an adsorption inhibition mechanism and for two, the underlying resistance mechanisms were not recognized (Labrie *et al*., 2010; Hyman and Abedon, 2010). Along with the original phage K six of the most potent phage derivatives, were chosen to make up the resulting cocktail, which was then tested against a panel of *S. aureus* strains to validate the breadth of their combined spectrum of activity (Kelly *et al*., 2011).

A phage cocktail was prepared by isolating phages using both wild type bacteria and phage-resistant variants as hosts similar to the selective approach used by Kelly and colleagues. Their three phage cocktail was applied for potency by treating mice suffering following a lethal dose of *Klebsiella pneumoniae*. A single intraperitoneal dose applied 1h postbacterial inoculum resulted in 100% recovery, which was reproducible for a delay of 3h if a higher phage dose was applied. The phage cocktail was found to lyse 88% of *K. pneumoniae* strains tested *in vitro* (Gu *et al*., 2012).

To determine phage receptors using a more molecular approach, Filippov *et al.* applied site-directed mutagenesis and trans complementation to nine phages in a case of *Yersinia pestis*. Six receptors were identified for eight of the phages in the lipopolysaccharide core, speculating that a combination of these phages could be formulated into a therapeutic cocktail. Testing in mice displayed that bacteria that had mutated to cultivate resistance against these engineered phages had become attenuated, resulting in a higher 50% lethal dose and longer survival times (Filippov *et al*., 2012).

It might be practicable to design phage cocktails that are applicable to all possible bacterial targets, including targets that might fluctuate over time, in

application, for a variety of reasons it is usually desirable to generate less complex cocktails. Although, the coverage of less complex cocktails may be incomplete, especially given the limited host range of many phage isolates, as the phage susceptibility of prominent pathogenic strains of bacteria may change over time; for instance, as one can notice within the context of phage typing (Williams and LeJeune, 2012).

Phage therapy is a form of personalized medicine at its best because specific phages (generally multiple phages combined as a multivalent cocktail) are carefully chosen to treat a patient's specific bacterial infection. Hence the use of personalized phage cocktails has historically been crucial for effective treatment as success rates from these customized phages are five to six fold higher than that of standardized phage products (Keen, 2012).

2.8.1 Phage cocktail applications

2.8.1.1 Cocktails for Food Safety

Intralytix a Biotech Company that focuses on production and marketing of bacteriophage products successfully received approval by the U.S. Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) sells two phage products. Each product incorporates a "cocktail" of phages that focus and kill the same bacterium. Intralytix's first phage product, ListShield™ certified by the FDA as food additive, aims *Listeria monocytogenes* in ready to eat meat and poultry (Scallan *et al*., 2011). EcoShield™, a second product, is sprayed on red meat before grating into hamburger to wipe out *Escherichia coli* O157:H7, the cause of 62,000 foodborne diseases annually in the United States. In analysis with government investigators, Sulakvelidze *et al*. (2001) showed that EcoShield wiped out 95–100% of *E. coli* O157:H7 within 5 minutes (Abuladze *et al*., 2008). SalmoFresh™, a third product that will target *Salmonella* in poultry and other foods is pending FDA approval. The company

is working on other phage treatments for oral health; wound healing and veterinary care (Potera, 2013).

2.8.1.2 Cocktails for Medical Applications

Currently phages are now being investigated in medical applications in the United States. The FDA approved the first phase 1 clinical trial in 2008 to evaluate an unnamed cocktail of eight phages to treat venous leg ulcers prepared by Intralytix. The phages aim *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *E. coli*. The treatment was confirmed safe, the major goal of phase 1 trials (Rhoads *et al*., 2009).

At Rockefeller University in New York City Nelson and colleagues have purified lysins that in animal studies killed *Streptococcus* was able for scarlet fever, rheumatic fever, necrotizing fasciitis ("flesh-eating disease"), and pneumonia (Nelson *et al*., 2001; Fischetti *et al*., 2007). Recently this team solved the X-ray crystal structure of PlyC, the most potent lysin known, contributing clues to its superior potency. It is 100 times more effective at killing than other lysins and chemical disinfectants. Precisely 10 ng of PlyC kills 10^7 bacteria in 5 seconds (McGowan *et al*., 2012).

From the sebaceous follicles of people (where *Propionibacterium acnes* concentrates) with and without acne, genomes of phages were obtained and sequenced (Kim *et al*., 2002), where a variety of phages that kill *P. acnes* to varying degrees were reported as "a great therapeutic opportunity" as a topical phage treatment for acne (Marinelli *et al*., 2012).

2.8.1.3 Cocktails to control *Salmonella*

To control *S.* Enteritidis PT4 in broilers Fiorentin *et al*. (2005) tested a cocktail of three phages. Administered phage dose was also very high (10^{11}

PFUs of each phage). In this analysis phage was administered only seven days post infection and a 3.5 $\log_{10}$ CFU decline per gram of caecal content was reported five days later. This level was maintained for 25 days. However a high dose and a cocktail of phages were used with the intention of reducing the emergence of phage resistant bacteria; the presence of such BIM cells was not evaluated (Fiorentin *et al*., 2005).

Two days after infection when a similar dose (10^{11} PFUs) was given orally, Atterbury *et al*. (2007) were able to attain a decline of the caecal colonization of both *S*. Enteritidis and *S*. Typhimurium in broiler chickens by up to 4.2 $\log_{10}$ CFU/g (Atterbury *et al*., 2007).

In the control of *Salmonella in vivo* and *in vitro* in 2007, Andreatti Filho reported on the isolation and testing of two different phage cocktails (one with 4 phages and another with 45 phages). *In vitro* test of these two cocktails at concentrations of 10^5 to 10^9 PFU/mL in simulated crop environment brought about a 1.5 or 5-$\log_{10}$ reductions of *Salmonella* Enteritidis, subsequently for the 4-phage and 45-phage cocktails in two hours after treatment. The 4-phage cocktail did not produce a reduction at six hours post-treatment, however the 45-phage cocktail was efficient to reduce bacterial counts by 6 $\log_{10}$. This study apparently indicates the advantage of a cocktail with a large number of phages, perhaps enabling complementary host range between phages and broader action in bacteria (Andreatti Filho *et al*., 2007).

Pretreatment often day old broiler chicks were done by Borie *et al*. (2008) by coarse spray or drinking water containing a cocktail of three phages 24 hours before administering roughly 10^6 CFUs of *Salmonella* Enteritidis (calculated multiplicity of infection (MOI) of 10^3). Phages were recovered from the intestinal and other organs after ten days of infection. A meaningful deduction in *Salmonella* Enteritidis was achieved at that time for both routes of administration with a deduction of more than 1 $\log_{10}$ CFU/mL in challenged bacterial numbers. By this study it indicates that not only phages are efficient to

reduce *Salmonella* bacterial loads in broiler chickens but also that aerosol spray and drinking water are possible routes of administration in the application of a phage product, which will surely expedite application and establishment of phage biocontrol in an industrial environment (Borie *et al*., 2008).

The association of phage therapy and competitive exclusion was tested in the treatment of chickens infected experimentally with *Salmonella* (Toro *et al*., 2005), where in phage treatment that included a cocktail of three different phages with different host ranges was given orally. Altogether in the treated groups, with phage or competitive exclusion alone or together, a decline in the *Salmonella* counts was noticed with a reduction to marginal levels in the ileum and a six-fold reduction in the caeca. Additionally, there was a marginal improved weight gain in the treated animals. Both approaches were able to reduce the *Salmonella* counts; unlike the previous study (Borie *et al*., 2009) however a synergistic effect was not observed (Toro *et al*., 2005).

The ability to reduce carcass contamination was recently tested using a cocktail in pigs infected with *S*. Typhimurium experimentally shortly before processing. Application of the phage brought about a 2-3 $\log_{10}$ reduction of *Salmonella* colonization (Wall *et al*., 2010).

It might be that the dangers connected with disruption of the human microbiome due to use of relatively broad-spectrum antibacterial agents could bring about a greater role for phage therapy as an alternative, narrow-spectrum antibacterial treatment. It will be particularly within this context that we anticipate a role for prêt-à-porter phage cocktails that however are almost modifiable instead somewhat fixed in their formulation (Sommer and Dantas, 2011).

Chapter 3

Isolation of Bacteriophage Insensitive Mutants (BIMs) generated by *Salmonella* Enteritidis S37 and S49 by phage infection and their biochemical and molecular characterization

3.1 Introduction

Globally reported human *Salmonella* contaminations are brought on by numerous serotypes. However at present, the most noteworthy frequency is represented by *Salmonella* enterica serotype Enteritidis (Retamal *et al*., 2015). The changing epidemiology of *S*. Enteritidis infection in the recent 20 years has permitted this serotype to turn into the most pervasive among *S*. enterica serotypes, and is accordingly viewed as an emergent pathogen (Jackson *et al*., 2013). Poultry is supposed to be the single largest reservoir of *S*. Enteritidis and most risk attribution studies have recognized poultry and poultry products as the major source of human infection. *S*. Enteritidis is transferred to humans mainly via handling and consumption of contaminated poultry meat and eggs (Shah *et al*., 2011). Human *Salmonella* infections and food poisoning manifest gastroenteritis, which can result in death in highly, affected individuals (O Ishola *et al*., 2010). *Salmonella* serovars are able to sustain in various niches and they can be also be harbored sub clinically in livestock and poultry as asymptomatic commensal microorganisms; thus prevailing in the environment for long periods of time, and are therefore hard to control without detailed knowledge of organisms in that distinct niche (Ricke *et al*., 2014). Apart from good hygiene and bird's husbandry practice, the few strategies utilized now to reduce *S.* Enteritidis infections in poultry farms include using preventative feed medication or antibiotic growth promoters, employing competitive exclusion products like probiotics

and development of vaccines (El-Ghany *et al*., 2012). Since the widespread use of antimicrobial agents by humans in the late 1940s, its utilization in food animal production systems has brought about an increase of antibiotic resistant zoonotic bacteria that can be transmitted to humans through the food chain (de Freitas Neto *et al*., 2010). The appearance of *S*. Enteritidis strains that are resistant to most currently procurable antimicrobials and the confined use of antibiotics have increased the requirement or novel and effective *S*. Enteritidis control strategies (Lim *et al*., 2012). In this way, to meet the vital goal of any food safety program, i.e., consumer protection, new food preservation techniques must be constantly be created to meet demands, enabling the control of emerging pathogens and their impact on a global scale (Lewis, 2008). Towards a food safety outlook, strictly lytic phages are conceivably one of the most harmless antibacterial approaches accessible (Sillankorva *et al*., 2012). The adoption of host-specific bacteriophages as a bio control is one possible mediation by which *Salmonella* colonization could be scaled down (Atterbury *et al*., 2007) but the rise of Bacteriophage-Insensitive Mutants (BIMs) has long been recognized as a major impediment of phage therapy (Connerton *et al*., 2005).

Microbes formulate various tactics enabling them to persist exposure to foreign genetic elements. Despite out-populated and preyed upon by copious and ubiquitous viruses, microbes routinely survive, hold on, and occasionally thrive in inhospitable and aggressive environments (Horvath and Barrangou, 2010). The continuous exposure to exogenous DNA via transduction, conjugation, and transformation have compelled microbes to establish an array of defense mechanisms that allow the cell to identify and extricate incoming "foreign" DNA, from "self " DNA and to survive exposure to obtrusive elements (Mc Garty, 2015). These systems retain genetic integrity; yet seldom allow exogenous DNA uptake and conservation of

genetic material favorable for adaptation to the environment (Storz and Hengge, 2010). Certain approaches, for instance prevention of adsorption, blocking of injection, and abortive infection, are fruitful against viruses; other defense systems particularly focus invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases (Szczepankowska *et al*., 2013).

Entirely different, analogous but not homologous, RNA-based adaptive immune system are organized in bacteria called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), a system that provides them with "memory" of past invasions as readying for future encounter (Koonin and Makarova, 2009). Host genomes that have procured spacers homologous to phage sequences impart resistance to that particular phage and hence titled Bacteriophage Insensitive Mutants (BIMs) (Young *et al*., 2012).

Specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 were previously isolated (Augustine and Bhat, 2012) and characterized for potential use in phage therapy. In this study, we take a look at the Bacteriophage Insensitive Mutants that emerged due to the interaction of *Salmonella* Enteritidis S49 with ΦSP-1 and ΦSP-3 and S37 with ΦSP-2. Biochemical and molecular testing were done to characterize BIMs and their antibiotic profiling was also performed.

3.2 Materials and Methods

3.2.1 Host bacteria and their specific phages

The host bacteria *Salmonella* Enteritidis strains S49 and S37 and their respective specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 used for this study were already available in the lab. *Salmonella* Enteritidis S49 and S37 were isolated from the intestinal contents of the broiler chicken, collected from the retail markets in

Ernakulam, Kerala. *Salmonella* specific phages were also isolated from the same samples (Augustine and Bhat, 2012). The bacteria were revived first and the procedures used are described in detail; these tests were also used for the confirmation of BIMs as *Salmonella*. Phage lysate that were stored in sterile 40mL polypropylene screw cap tubes at 4ºC were revived for the study.

3.2.2 Bacteriophage revival

3.2.2.1 Enrichment method

For the enrichment process, a portion of the crude lysate was mixed with the host bacteria (in log phase), incubated at 37ºC for 12 hours. It was centrifuged at 4000 xg (Sigma, 3K30, Germany) for 10 minutes at 4ºC, to make them bacteria-free. Then it was filtered through 0.22 μm membrane filter (Millipore, USA) and this filtrate was screened for the presence of phage.

3.2.2.2 Double agar overlay method

The filtered lysate was then analyzed according to the double-agar overlay method of Adams (1959) with modification. Host bacterial strains in nutrient broth (HiMedia, Mumbai, India), at the logarithmic phase (1 mL) were mixed with 1 mL of the serially diluted lysate and were incubated at 37ºC in a water bath (Scigenics, Chennai, India) for 1 hour. Following incubation 3 mL of sterile soft agar (nutrient broth containing 0.8% agarose) was added to this mixed well and was instantly overlaid on nutrient agar plates. After that the plates were incubated at 37ºC for 16 hours. Host-free cultures (containing only phage) and phage-free cultures (containing only bacterial host) were used as controls. When plaques were observed on the bacterial lawn in the plates it was recorded positive for phages.

3.2.2.3 Phage purification

From the plate a single plaque was picked with a sterile toothpick, inoculated into 3 mL of a log phase culture of the *Salmonella* host in nutrient broth (HiMedia). It was then incubated at 37ºC in an environmental shaker (Orbitek, Scigenics, India) at 120 rpm for 12 hours. Then it was centrifuged at 10000xg (Sigma, 3K30, Germany) accompanied by filtration through 0.22 µm membrane (Millipore). The lysate obtained after filtration was used for double agar overlay. This process was repeated 6 times until uniform sized plaques were obtained on the plate.

3.2.2.4 Large-scale production of phage lysate

The plates that contained plaques were washed with SM buffer (Appendix -2). It was overlaid with 10mL of SM buffer and was incubated at 4ºCin a gel rocker that provides gentle rocking so that phages could easily diffuse into the buffer. After incubation the phage suspension was collected from all the plated and pooled. To this pooled mixture chloroform was added to a final concentration of 5 % (v/v), using a vortex mixer mixed well and incubated at room temperature for 15 minutes. By centrifugation at 5000xg for 10 minutes (Sigma, 3K30, Germany) the cell debris were then removed and the supernatant was transferred to sterile polypropylene tube. To this chloroform was added to a final concentration of 0.3 % (v/v) and was stored at 4ºC until use. After serial dilution the titer of this lysate was noted (Sambrook *et al.,* 2000).

3.2.2.5 Phage concentration

Using polyethylene glycol (PEG) 6000 phage was concentrated as illustrated in Sambrook *et al*. (2000). To 200 mL nutrient broth (HiMedia), 1% (v/v) of an overnight culture of the host bacteria was transferred and incubated for 3.5 hours in an

environmental shaker at 100 rpm (Orbitek, Scigenics, India) at 37ºC. At a multiplicity of infection (MOI) of 0.2 phage was added and the incubation was continued at 100 rpm for 12-15 hours at 37ºC. Then this broth was centrifuged at 10000 xg for 20 minutes (Sigma), the supernatant collected and filtered through 0.22 µm membrane filter (Millipore, USA). To this added DNase I (Bangalore Genei) and RNase (Genei) to a final concentration of 1 µg/mL each. It was then incubated at room temperature for 30 minutes. Then added solid NaCl to a final concentration of 1 M and was dissolved by stirring with a sterile glass rod. This blend was kept in ice for 1 hour accompanied by centrifugation at 11000xg at 4ºC for 10 minutes. To the supernatant, added solid PEG 6000 (SRL, India) at a final concentration of 10% (w/v). By slow stirring on a magnetic stirrer at room temperature it was dissolved. Then it was kept in ice overnight followed by centrifugation at 11000xg for 10 minutes at 4ºC. The pellet was resuspended in 5mL of Phosphate buffered saline (PBS) (Appendix-2) while the supernatant was discarded completely. By the addition of an equal volume of chloroform PEG and cell debris were removed from the phage suspension. It was vortexed for 30 seconds accompanied by centrifugation at 3000xg for 15 minutes at 4ºC. The aqueous phase consisting of phage particles were collected and stored at -20ºC.

3.2.2.6 Storage of phage

For long-term storage, the phage lysate was maintained as stock cultures mainly by two methods namely, storage at 4ºC as such and as glycerol stock. After large-scale production the phage lysate obtained was stored in sterile 40mL polypropylene screw-cap tubes at 4ºC until use. Filtered phage lysate was mixed with nutrient broth (HiMedia) containing 50% glycerol in a sterile microfuge tube of (1.5mL capacity) and the mixture was frozen at ¯80ºC, until use.

3.2.2.7 Morphological analysis by Transmission Electron Microscopy (TEM)

For TEM, initially onto a carbon-coated TEM grid one drop of high titer phage sample was spotted. It was allowed to settle for 2-3 minutes and excess of sample was wiped off by blotting. With slight modifications the protocol for staining as described by Quintarelli *et al.* (1971) was used. For negative staining, a drop of 1% phosphotungstic acid hydrate (Sigma Aldrich) was used. The process by which it adsorbs on to tissues was suggested as electrostatic force rather than by hydrogen bonding, hence the adsorption is not affected by pH. On the spot where sample was added phosphotungstic was spotted and after some 2-3 minutes for reaction, the excess stain was wiped off by touching a blotting paper strip to the edge of the grid. After that the grid was dried for 3 hours. It was examined and photographed using a Transmission Electron Microscope (Model Jeol/JEM 2100 2000X) operated at 200 kV at Sophisticated Test and Instrumentation Centre, Kalamassery, Kerala. From the micrographs obtained, phage morphology was observed.

3.2.2.8 Estimation of optimal multiplicity of infection

The number of virus genomes of a given virus species that infect individual cells is the multiplicity of cellular infection (MOI) (Gutiérrez *et al*., 2015). By dividing the number of phage added (volume in mL x PFU/mL) by the number of bacteria added (volume in mL x colony forming units/mL) MOI could be calculated. According to Lu *et al*. (2003) optimal MOI was calculated. In short bacteria were infected at different MOI (0.01, 0.1, 0.5, 1, 5 and 10 PFU/mL) and were incubated at 37^0Cfor one hour. After incubation the mixture was centrifuged (Sigma, 3K30,) at 8000xg for 10 minutes and supernatant was passed through 0.22 μm membrane filter (Millipore). To determine the phage titer, the lysate was assayed applying the double agar overlay method described earlier. Host-free cultures (containing only phage) and

phage-free cultures (containing only bacterial host) were used as controls. All assays were carried out in triplicates. The MOI providing maximum yield was thought out as optimal MOI.

3.2.3 Isolation of Bacteriophage Insensitive Mutants

Bacteriophage Insensitive Mutants were obtained when *Salmonella* Enteritidis serotype S49 and S37 was challenged by phages ΦSP-1, ΦSP-3 and ΦSP-2. Briefly 1 mL of overnight culture of *S*. Enteritidis strain S49 and S37 was mixed with 10 µL of phage lysate (10^{12}pfu/mL) (Augustine *et al*., 2013), added1mL of normal saline and incubated at 40^{o}C for one hour in a water bath (Scigenics). After incubation, 3mL of soft agar, (Nutrient broth (HiMedia) containing 0.8% agarose) was added, mixed well and immediately overlaid on nutrient agar plates (Adams, 1959), and incubated for 16 h at 37^{o}C. The colonies that appeared in the top agar layer were picked from the plate as bacteriophage insensitive mutants, inoculated in nutrient broth and stored on nutrient slants at 4^{o}C until use. This procedure was done to isolate three sets of BIMs. The BIMs isolated were confirmed as phage resistant as they were reinfected with the phages again, and there were no visible plaque formation.

In order to confirm that the BIMs were not lysogenic, the host bacteria *Salmonella* Enteritidis S49 and S37 were subjected to mitomycin C induction to induce prophage excision. Mitomycin C was used for induction of lysogenic phages as per protocol described by Yee *et al*. (1993) with modifications. *Salmonella* strains were grown in Luria broth (LB) (HiMedia) at 37 °C until an absorbance of 0.5 at 600 nm. Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added at 1 µg/mL and incubated overnight at 37 °C. The culture supernatants were passed through 0.22-µm membrane (Millipore, USA). The filtrate was used as putative phage lysate to screen for plaque forming ability by double agar overlay method. The nutrient agar used for

double agar contained 1 µg/mL mitomycin. The filtrates were assayed for phage after appropriate dilution (Faruque *et al*., 1998). This experiment was repeated thrice.

3.2.3.1 Confirmation of BIMs as *Salmonella*

The guidelines of Food and Drug Administration (FDA) in the Bacteriological Analytical Manual (BAM) (Andrews *et al.,* 2007) were used for the confirmation of BIMs as *Salmonella.*

3.2.3.2 Selective plating

The BIMs that were stored on nutrient slants were inoculated into nutrient broth. From that, a loopful of culture was streaked onto xylose lysine deoxycholate (XLD) agar (Difco, USA), Hektoen enteric (HE) agar (Difco, USA) and Bismuth sulfite (BS) agar (Appendix-1) and incubated at 35°C for 24 hours.

Xylose-Lysine Deoxycholate Agar

Xylose-Lysine Deoxycholate Agar is a selective as well as differential medium recommended for the isolation and enumeration of *Salmonella.* Deoxycholate, ferric ammonium citrate and sodium thiosulphate are selective agents in this medium that inhibit Gram-positive microorganisms. Yeast extract provides the essential nutrients, and growth factors for growth. Xylose, sucrose and lactose are the fermentable sugars in this medium. *Salmonellae* metabolize the xylose and decarboxylate lysine and thus change the pH to alkaline. Sodium thiosulphate helps in reactivation of sulphur containing compounds and prevents the desiccation of these compounds during storage. It also forms the substrate for enzyme thiosulphate reductase, which breaks it to form hydrogen sulfide. Thiosulphate and ferric ammonium citrate are the hydrogen sulfide indicators in the medium. Sodium

thiosulphate is also in activator of halogens, mercurial and aldehyde and can minimize its toxicity in the testing sample, if any during microbial limit tests. Sodium chloride maintains the osmotic equilibrium in this medium. Phenol red is the pH indicator.

Degradation of fermentable sugars proceed simultaneously and generates acids, which cause pH indicator to provide various shades of color, causing a color change in the colonies and in the medium from red to yellow on prolonged incubation. Under alkaline conditions hydrogen sulfide production results in colonies with black centers, which can be inhibited by acid production by carbohydrate fermentation. Alkaline condition causes the color of the medium to change back to red.

Hektoen Enteric Agar

Both a selective and differential medium, Hektoen Enteric Agar is designed to isolate and differentiate members of the species *Salmonella* and *Shigella* from other *Enterobacteriaceae.* Compared to other differentiating media Hektoen Enteric Agar is efficient in increasing the isolation rate of *Salmonella* sp. Bile salts, bromthymol blue and acid fuchsin inhibit the growth of most Gram positive organisms. Lactose, salicin and sucrose, serves as fermentable source of carbohydrates to encourage the growth and differentiation of enteric bacteria. In this medium, by increasing the carbohydrate and peptone content of the medium, the inhibitory effect of bile salts and indicators are countered. Proteose peptone supply nitrogen, carbon, and amino acids required for organism growth. Yeast Extract is a vitamin source. The osmotic balance of the medium is maintained by sodium chloride. Sodium thiosulfate lends a source of sulfur. Hektoen Enteric Agar can also detect the production of hydrogen sulfide gas, which turns parts of the medium black. Ferric ammonium citrate serves as iron source, which cause production of hydrogen sulfide from sodium thiosulphate and also helps

in the visualization of hydrogen sulfide production by reacting with hydrogen sulfide gas to form a black precipitate.

Non-fermenters will produce blue-green colonies. Organisms that reduce sulfur to hydrogen sulfide will produce black colonies or blue-green colonies with a black center. *Salmonella* reduce sulfur to hydrogen sulfide, producing a black precipitate.

Bismuth Sulphite Agar

Bismuth Sulphite Agar is recommended for the selective isolation of *Salmonella*. *S.* Typhi, *S.* Enteritidis and *S.* Typhimurium typically grow as black colonies with a surrounding metallic sheen resulting from hydrogen sulfide production and reduction of sulphite to black ferric sulfide. *Salmonella* Paratyphi A grows as light green colonies. Bismuth Sulphite Agar may be inhibitory to some strains of *Salmonella* species and therefore should not be used as the sole selective medium for these organisms.

3.2.3.3 Biochemical characterization of *Salmonella*

KBMOO2 HiMotility^{TM}Biochemical kit for *Salmonella* (HiMedia) was used for the biochemical characterization of *Salmonella*.KBM002is a comprehensive test system that can be used for identification of Gram-negative *Salmonella* species.

Each KBM002 kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests including motility and four carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation, *Salmonella* exhibit metabolic changes, which are

indicated by a spontaneous color change in the media that can be either interpreted visually.

A single well-isolated colony was picked up and inoculated in 5mL Brain Heart Infusion broth (HiMedia)(Appendix 1) and incubated at 35-37°C for 4-6 hours until the turbidity was≥0.1 OD at 620nm.The kit was opened aseptically. The sealing foil was peeled off. Stab inoculated the first well, second well was not inoculated. Inoculated the remaining kit (well no.3-12) by stabbing each individual well with a loopful of inoculum. Inoculum reached the bottom of the wells. Time of incubation was 18-24 hours.

3.2.3.3.1 Motility test

Motility Test Agar (Appendix 1) is used for the differentiation of microorganisms on the basis of motility. Bacterial motility is observed macroscopically by a diffuse zone of growth spreading from the line of inoculation. Certain species of motile bacteria will show diffuse growth throughout the entire medium, while others may show diffusion from one or two points appearing as nodular outgrowths along the stab. In the kit positive reaction is noted as dark pink growth in the first well and the movement of dark pink growth from first well to second well (Cheesbrough, 2006; Perilla *et al*., 2003).

Enzymatic Digest of Gelatin and Beef Extract in Motility Test Agar provide the nitrogen, carbon, and vitamin sources. Sodium Chloride maintains the osmotic environment. Agar is the solidifying agent used at a low concentration.

3.2.3.3.2 Citrate utilization test

Simmons Citrate Agar (Appendix 1) is recommended for differentiation the members of *Enterobacteriaceae* on the basis of citrate utilization as sole carbon source.

Ammonium dihydrogen phosphate and sodium citrate serve as the sole nitrogen and carbon source respectively. Microorganisms also use inorganic ammonium salts as their sole nitrogen source. Metabolism of these salts causes the medium to become alkaline, indicated by a change in color of the pH indicator from green to blue. Bromothymol blue is the pH indicator. *Salmonella* is citrate positive (Bello, 2002).

3.2.3.3.3 Urease test

Urease activity can be described as the splitting of urea via hydrolysis by a urease enzyme. Urea Agar Base (Appendix 1) with the addition of urea for the detection of urease production, it is mainly used to detect the genus *Proteus. Salmonella* is urease negative.

Peptic digest of animal tissues is the source of essential nutrients. Dextrose is the energy source. Sodium chloride maintains the osmotic equilibrium of the medium whereas phosphates serve to buffer the medium. Urea is hydrolyzed to liberate ammonia. Phenol red indicator detects the alkalinity generated by visible color change from orange to pink.

On incubation urea is utilized to form ammonia, which makes the medium alkaline, showing a pink color by the change in the phenol red indicator. Negative

reaction is shown as orange yellow color. Prolonged incubation may cause alkaline reaction in the medium (Abdullahi, 2010).

3.2.3.3.4 Arginine utilization test

Arginine utilization test is used to differentiate bacteria on the basis of arginine decarboxylase (or dihydrolase) activity towards the amino acids. Bacteria producing arginine dihydrolase enzyme decarboxylates the arginine present in this medium (Appendix 1) to putrescine. The production of amine putrescine, elevates the pH. Bromocresol purple is the pH indicator, which forms purple color in alkaline condition. Color change from purple to yellow and then back to dark purple is positive reaction. *Salmonella* shows positive reaction.

Peptic digest of animal tissue provide the necessary nutrients to the organisms while L-arginine stimulates the arginine dihydrolase synthesis. Dipotassium phosphate buffers the medium while sodium chloride maintains the osmotic balance (de la Torre *et al*., 2005).

3.2.3.3.5 Lysine utilization test

Lysine Agar (Appendix 1) is recommended for the differentiation of enteric organisms especially *Salmonella* based on their ability to decarboxylate or deaminates lysine. *Salmonellae* are known to decarboxylate lysine rapidly. In the kit, dark purple color represents positive reaction for lysine.

Peptic digest of animal tissue and yeast extract provide essential nutrients. Dextrose is a source of fermentable carbohydrate. Lysine decarboxylation causes an

alkaline reaction (purple color) to give the amine cadaverine and the organisms, which do not decarboxylate lysine, produce acid butt (yellow color) (Phirke, 1977).

3.2.3.3.6 Hydrogen sulfide production

This test determines whether the microbe reduces *sulfur-containing compounds* to sulfides during the process of metabolism. If sulfide is produced, it combines with *iron compounds* to produce iron sulfide (FeS), a black precipitate. H2S is produced when sulfur containing amino acids is decomposed. Usually used medium sulphite indole motility (SIM) contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced as a result of ferrous ammonium sulfate reacting with H_2S gas. *Salmonella* shows a black color, which is a positive reaction (Shelef and Tan, 1998).

3.2.3.3.7 ONPG test

ONPG test is used for the rapid detection of β- galactosidase activity in microorganisms, especially to identify late lactose fermenters quickly.

The ortho- nitrophenylgalactopyranoside (ONPG) contained in the medium is hydrolyzed by the microorganisms, which contain the enzyme β- galactosidase to a yellow color orthonitrophenolic compound. Some *Enterobacteriaceae* like *E-coli, Klebsiella* etc. can produce β- galactosidase and permease and they can ferment lactose but *Salmonella, Proteus* etc. cannot produce β- galactosidase and they are non-lactose fermenters. *Salmonella* shows a colorless reaction in the kit (De Ryck *et al.,* 1994).

3.2.3.3.8 Arabinose, Lactose, Maltose and Trehalose utilization tests

The purpose is to see if the microbe can ferment these carbohydrates (Austin and Austin, 2007). If these sugars are fermented to acid end products, the pH of the medium will drop. The commonly used medium for these tests is phenol red arabinose broth, phenol red lactose broth, phenol red maltose broth and phenol red trehalose broth. Phenol red is the indicator in these mediums, which is red at neutral pH, but turns yellow at pH <6.8.The medium is a nutrient broth to which 0.5-1% arabinose, lactose, maltose and trehalose are added. A positive reaction test consists of a color change from red to yellow, indicating a pH change to acidic. *Salmonella* shows positive reactions with a yellow color in the kit for arabinose, maltose and trehalose but it is negative for lactose, which shows a red/pink color.

3.2.3.3.9 Triple sugar iron agar test

Triple Sugar Iron Agar is used for the identification of Gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulfide production. This test was not present as such in kit it was done separately.

Peptone, yeast extract and meat extract provide nitrogenous compounds, sulfur, trace elements and vitamin B complex etc. Sodium chloride maintains osmotic equilibrium. Lactose, sucrose and glucose are the fermentable carbohydrates. Sodium thiosulphate and ferric or ferrous ions make hydrogen sulfide indicator system. Phenol red is the pH indicator. Organisms that ferment glucose produce a variety of acids, turning the color of the medium from red to yellow. More amounts of acids are liberated in butt (fermentation) than in the slant (respiration). Growing bacteria also form alkaline products from the oxidative decarboxylation of peptone and these

alkaline products neutralize the large amounts of acid present in the butt. Thus the appearance of an alkaline (red) slant and an acid (yellow) butt after incubation indicates that the organism is a glucose fermenter but is unable to ferment lactose and/or sucrose. Bacteria that ferment lactose or sucrose (or both) in addition to glucose produce large amounts of acid. Thus no reversion of pH in that region is possible and thus bacteria exhibit an acid slant and acid butt. Gas production (CO_2) is detected by the presence of cracks or bubbles in the medium, when the accumulated gas escapes. Thiosulphate is reduced to hydrogen sulfide by several species of bacteria and H_2S combines with ferric ions of ferric salts to produce the insoluble black precipitate of ferrous sulfide. Reduction of thiosulphate proceeds only in an acid environment and blackening usually occurs in the butt of the tube. *Salmonella* represents an alkaline red slant with yellow acidic butt and production of hydrogen sulfide in the butt region with black color (Kumar *et al*., 2008).

3.2.3.4 Serological agglutination test

3.2.3.4.1 Polyvalent somatic (O) test

After the screening procedure the BIMs underwent a serological agglutination test. From 24-48 hour TSI slant a loopful of the culture was emulsified with 2 mL 0.85% saline on a clean glass slide. 1 drop of *Salmonella* polyvalent somatic (O) antiserum (Difco, USA) was added and mixed the culture suspension for 1 minute by back-and-forth motion. After this any degree of agglutination was considered to be positive (Russell *et al*., 2006).

3.2.3.5 Molecular identification of *Salmonella* BIMs by partial 16S rRNA gene sequence analysis

3.2.3.5.1 Extraction of DNA

The isolation of DNA was according to the protocol described by Esteban *et al.* (1993). For this first, a single colony was inoculated into Luria broth (Appendix 1) (HiMedia) and incubated at 37^{o}C overnight with constant shaking. From that 1.5 mL culture was taken in a microfuge tube and centrifuged at 8000 rpm (Sigma, Germany) for 5 minutes. Then the pellet was resuspended in 567µLTris-EDTA (TE) buffer (pH 8) (Appendix 2). 30µL of 10% sodium dodecyl sulphate (SDS) and 3µL Proteinase K (20mg/mL) were added to the suspension, and mixed well. It was then kept for incubation at 37^{o}C for 1hour in a water bath. After incubation 100µL of 5M NaCl and 80µL Hexadecyl trimethyl ammonium bromide (CTAB) (10mg/mL) were added and incubated for 10minutes at 65^{o}C in a water bath. Then the tubes were allowed to cool to room temperature and an equal volume of chloroform-isoamyl alcohol (24:1) was added. After that the contents were mixed gently and centrifuged at 12000g for10minutes. When centrifugation was over, using a sterile cut- tip the aqueous layer at the top, containing the DNA was carefully transferred to a fresh microfuge tube. To the aqueous layer an equal volume of phenol-chloroform-isoamyl alcohol mixture (25:24:1) was added and centrifuged again at 12000xg for 10 minutes. As mentioned earlier the aqueous layer was collected in a new tube and 0.6 volume of isopropanol was added to it. It was then mixed gently and centrifuged at 12000xg for 10minutes. After centrifugation, the supernatant was discarded and the pellet was washed with 70% ethanol. Then it was subjected to centrifugation at 12000xg for 10minutes. Again the supernatant was discarded and the pellet was dried at room temperature. The extracted DNA was then dissolved in 50µL TE buffer (pH 8). Using BioSpec-nano (Shimadzu, Japan) the concentration of DNA was estimated. For further use the DNA was stored at -20^{o}C.

3.2.3.5.2 Polymerase chain reaction

The BIMs were subjected to molecular characterization by PCR using a primer pair for 16S rDNA. From the genomic DNA a portion of the 16S rRNA gene (1.5kb) was amplified (Table 3.1). After PCR amplification the products were subjected to sequencing, followed by homology analysis.

Sequence	Amplicon	Refrences
16SF 5' AGTTTGATCCTGGCTCA 3' 16SR 5' ACGGCTACCTTGTTACGACTT 3'	1500bp	(Shivaji*et al*., 2000) (Reddy *et al*., 2002a; Reddy *et al*., 2002b)

Table 3.1 16S rDNA primers used for amplifying

Composition for PCR.

2 mM each dNTPs (Chromous Biotech, India)	2µL
Forward primer (10 picomoles) (Sigma Aldrich)	2µL
Reverse primer (10 picomoles) (Sigma Aldrich)	2µL
*Taq*DNA polymerase (1U/µL) (Sigma-Aldrich)	1µL
10X PCR buffer	2 µL
$MgCl_2$ (Sigma-Aldrich)	1.2 µL

Template DNA (50 ng/µL) and sterile distilled water was added to a final volume of 20 µL.

In Thermal Cycler (BioRad MJ Mini Gradient) amplifications were carried out. The program used was a hot start cycle of 94^0C for 5 minutes, followed by 30 cycles of 94^0C for 1minute, annealing at 56^0C for 1 minute and extension at 72^0C for 90 seconds and finally with an extension step of 72^0C for 10 minutes.

3.2.3.5.3 Agarose gel electrophoresis

For the analysis of the amplified products 1% agarose gels were prepared (Appendix 2). They were prepared in Tris-Acetate-EDTA (TAE) buffer. To that Ethidium bromide was added at a concentration of 0.5mg/mL.Aliquots (5µL) of PCR product was mixed with gel loading dye (Appendix 2). It was loaded into the wells. For confirmation of amplicon size DNA markers (1 kb marker, Fermentas) were run along with the products. Using Mini gel electrophoresis unit (Genei, Bangalore, India) electrophoresis was performed at a constant volt (5V/cm) and gel pictures were captured with the help of gel documentation system (Syngene, UK)

3.2.3.5.4 Sequencing of DNA

Sequencing of the PCR amplicon of 16S rRNA gene was done by the ABI XL DNA analyzer, using the big dye Terminator kit (Applied Biosystems, USA) at SciGenom Cochin, India Ltd. By comparing the sequences attained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.*, 1990) at NCBI site. (http://blast.ncbi. nlm.nih.gov) the identity of the sequences was determined. The sequences were deposited in the Genbank database and accession numbers were obtained.

3.2.3.5.5 Multiple sequence alignment and phylogenetic tree construction

All the nucleotide sequences obtained after sequencing were converted into FASTA format and multiple sequence alignment was done using the CLUSTAL W

(Thompson *et al*., 1994) program in BioEdit software (Hall, 1999). Aligned sequences were imported into MEGA 5 (Molecular Evolutionary Genetics Analysis version 5.0) (Tamura *et al*., 2011). To obtain equal lengths the ends were trimmed for all sequences and the aligned sequences were converted into MEGA format for phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

3.2.3.6 Antibiotic susceptibility test

According to disc diffusion method (Bauer *et al*., 1966) antibiotic susceptibility test was performed. The culture suspension was swabbed, on Mueller-Hinton agar (HiMedia) (Appendix 1) plate to obtain a uniform bacterial lawn. Antibiotic discs were placed, and spaced to provide room for the zone of inhibition. The plates were incubated at 37^{o}C for 24 h before the examination.

Antibiotics	Zone diameter Range (in mm)		
	Resistant	Intermediate	Sensitive
Ampicillin	≤ 11	12-14	≥ 15
Azithromycin	≤ 13	14-17	≥ 18
Cefixime	≤ 15	15-17	≥ 18
Cefuroxime	≤ 14	15-17	≥ 18
Chloramphenicol	≤ 12	13-17	≥ 18
Ciprofloxacin	≤ 15	16-20	≥ 21
Tetracycline	≤14	15-18	≥19

Gentamicin	≤12	13-14	≥15
Nalidixic acid	≤13	14-18	≥19
Trimethoprim	≤10	11-15	≥16

Table 3.2 The range pattern of antibiotics used in this study

The result was interpreted as resistant, intermediate or sensitive based on the zone diameter (mm) as per the manufacturer (HiMedia, India). The tested antibiotics included ampicillin (5 µg/disc), azithromycin (15 µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc), ciprofloxacin (5 µg/disc), chloramphenicol (30µg/disc), tetracycline (30 µg/disc), gentamicin (10 µg/disc), nalidixic acid (30 µg/disc) and trimethoprim (5 µg/disc). The experiment was independently repeated thrice and the interpretation was based on the average of the three measurements as per the manufacturers instruction as sensitive, intermediate and resistant (Table 3.2).

3.3 Results

3.3.1 Characterization of phage

3.3.1.1 Morphological analysis by TEM

The host bacteria and phages used in the study for BIM isolation were previously characterized. ΦSP-1 and ΦSP-3 were characterized using Transmission electron microscopy as belonging to the family *Podoviridae* and *Siphoviridae* respectively (Augustine and Bhat, 2012), but phage ΦSP-2 was not previously identified and hence this was done in this study.

The TEM picture of ΦSP-2 revealed typical Siphovirus morphology with an isometric capsid, 84.59 ± 1.06 nm in diameter, connected to a long, flexible and non-contractile tail of 200.58 ± 0.52 nm in length (not including baseplate structure). The phage sizes were determined from the average of three independent measurements (mean ± standard deviation)

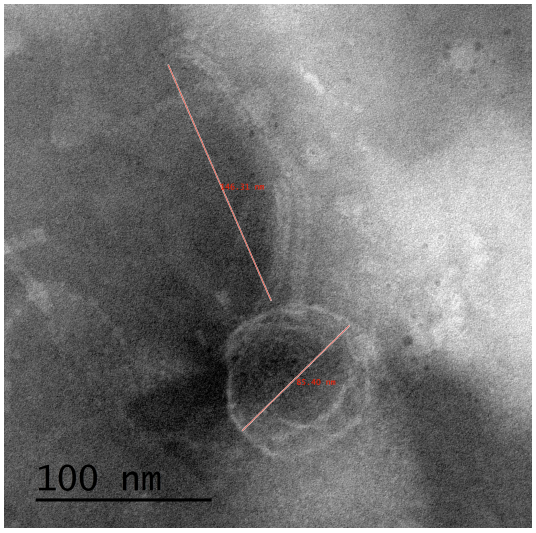

Fig 3.1 Transmission Electron micrograph image of phage ΦSP-2 stained with 1% phosphotungstic acid. Bar represents 100nm.

3.3.1.2 Optimal multiplicity of infection

The ratio of virus particle to individual host cell is defined, as multiplicity of infection or MOI and it is very significant factor for phage titer. The optimal MOI was calculated. With *Salmonella* strain S49 as host; the optimal MOI of ΦSP-1 was five phages per bacterium and that of ΦSP-3 was one phage per bacterium; while it was ten phages per bacterium for S37 with its specific phage ΦSP-2.

3.3.2 Isolation and characterization of BIMs

The challenge of *Salmonella* Enteritidis S49 by its specific lytic phage ΦSP-1 and ΦSP-3 led to generation of bacteriophage insensitive mutants. Repeated infection of these BIMs by ΦSP-1 and ΦSP-3 did not lead to cell lysis, which confirmed their phage insensitivity. Three iterations yielded three sets of BIMs. Similarly, in the case

of *Salmonella* Enteritidis S37 infected with its specific lytic phage ΦSP-2, three iterations yielded three sets of BIMS.

Initially infection of S49 with its specific lytic phage ΦSP-1 yielded five BIMs namely BIM 1, 2, 4, 8 & 9, in the second round (set 2) four BIMs namely BIM 19, 20, 21 and 22 were obtained and the third infection (set 3) finally settled to isolation of four BIMs namely BIM 36, 37, 38 and 39.

Host Bacteria	**Lytic phage used**	**BIMs obtained in first set**	**BIMs obtained in second set**	**BIMs obtained in third set**
Salmonella Enteritidis S49	Infected with phage ΦSP-1	BIM1 BIM2 BIM4 BIM8 BIM9	BIM19 BIM20 BIM21 BIM22	BIM36 BIM37 BIM38 BIM39
Salmonella Enteritidis S49	Infected with phage ΦSP-3	BIM3 BIM6 BIM7 BIM10	BIM15 BIM16	BIM40 BIM42 BIM43 BIM44
Salmonella Enteritidis S37	Infected with phage ΦSP-2	BIM5	BIM27 BIM28	BIM31 BIM32 BIM33 BIM34 BIM35

Table 3.3 BIMs obtained in three independent sets by infection of host bacteria *Salmonella* Enteritidis S49 and S37 with its specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 respectively.

Infection of S49 with another specific lytic phage ΦSP-3 yielded four BIMs in first set (BIM 3, 6, 7 and 10), two BIMs (BIM 15 and 16) in the second set, while in last set four BIMs were isolated, BIM 40, 42, 43 and 44.

When strain S37 was infected with its specific lytic phage ΦSP-2 only one BIM was isolated in first set (BIM 5), two in second set (BIM 27 and 28). In last set five BIMs were isolated, namely BIM 31, 32, 33, 34 and 35. The BIMs obtained after phage infection in each sets is shown in table 3.3.

The traditional and most common approach to study prophages or temperate phages presence is to induce lysogenic bacteria with mitomycin C treatment. In this instance no 'cloudy plaque' morphology was noted after this induction, even after three passages; ensuring that the isolated BIMs were indeed mutants and did not harbor any prophage.

The BIMs isolated were confirmed as *Salmonella* according to the selective plating method done and by their biochemical characterization. In Xylose-Lysine Deoxycholate agar plates it produced black colonies, in Hektoen Enteric Agar plates they developed a bluish green colonies with a black center and in Bismuth Sulphite Agar plates they appeared as black colonies with a metallic sheen halo. They showed all the characteristics colonies typical of *Salmonella* on the selective media.

3.3.2.1 Biochemical characterization of BIMs

Biochemical tests were performed with all 31 isolated BIMs, which also confirmed them as *Salmonella.* In the KBMOO2 HiMotility™ Biochemical kit for *Salmonella* identification the bacterial cultures produced a dark pink growth in first well, which migrated into the second well in the kit that indicating their motility. For citrate utilization test it showed a blue color, orangish yellow color in urease test as the *Salmonella* are urease negative; dark purple color indicating arginine utilization,

purple color for lysine utilization, which are typical of these bacteria.

Organisms / **Tests**	**Motility**	**Citrate Utilization**	**Urease**	**Arginine Utilization**	**Lysine Utilization**	**H_2S Production**	**ONPG**	**Arabinose**	**Lactose**	**Maltose**	**Trehalose**
Salmonella Enteritidis S49	+	+	-	+	+	+	-	+	-	+	+
ΦSP-1Mutants											
BIM 1	+	+	-	+	+	+	-	+	-	+	+
BIM 2	+	+	-	+	+	+	-	+	-	+	+
BIM 4	+	+	-	+	+	+	-	+	-	+	+
BIM 8	+	+	-	+	+	+	-	+	-	+	+
BIM 9	+	+	-	+	+	+	-	+	-	+	+
BIM 19	+	+	-	+	+	+	-	+	-	+	+
BIM 20	+	+	-	+	+	+	-	+	-	+	+
BIM 21	+	+	-	+	+	+	-	+	-	+	+
BIM 22	+	+	-	+	+	+	-	+	-	+	+
BIM 36	+	+	-	+	+	+	-	+	-	+	+
BIM 37	+	+	-	+	+	+	-	+	-	+	+
BIM 38	+	+	-	+	+	+	-	+	-	+	+
BIM 39	+	+	-	+	+	+	-	+	-	+	+
ΦSP-3Mutants											
BIM 3	+	+	-	+	+	+	-	+	-	+	+
BIM 6	+	+	-	+	+	+	-	+	-	+	+
BIM 7	+	+	-	+	+	+	-	+	-	+	+
BIM 10	+	+	-	+	+	+	-	+	-	+	+
BIM 15	+	+	-	+	+	+	-	+	-	+	+

BIM 16	+	+	-	+	+	+	-	+	-	+	+
BIM 40	+	+	-	+	+	+	-	+	-	+	+
BIM 42	+	+	-	+	+	+	-	+	-	+	+
BIM 43	+	+	-	+	+	+	-	+	-	+	+
BIM 44	+	+	-	+	+	+	-	+	-	+	+
Salmonella Enteritidis S37	+	+	-	+	+	+	-	+	-	+	+
ΦSP-2 Mutants											
BIM 5	+	+	-	+	+	+	-	+	-	+	+
BIM 27	+	+	-	+	+	+	-	+	-	+	+
BIM 28	+	+	-	+	+	+	-	+	-	+	+
BIM 31	+	+	-	+	+	+	-	+	-	+	+
BIM 32	+	+	-	+	+	+	-	+	-	+	+
BIM 33	+	+	-	+	+	+	-	+	-	+	+
BIM 34	+	+	-	+	+	+	-	+	-	+	+
BIM 35	+	+	-	+	+	+	-	+	-	+	+

+ Represents positive reaction and – represents negative reaction

Table 3.4 Biochemical characteristics of *Salmonella* Enteritidis S49, S37 and their Bacteriophage Insensitive Mutants using KBMOO2 HiMotility™ Biochemical kit for *Salmonella* (HiMedia) identification.

They produced hydrogen sulfide, and as *Salmonella* are devoid of β-galactosidase enzyme, there was no yellow color production for ONPG; positive yellow color for arabinose and red/ pink shade for lactose, as they can't ferment lactose. And finally for the last two reactions in the kit, they produced a yellow color for maltose and trehalose and indicating their fermentation, which is also typical for *Salmonella*. In Triple sugar iron agar they produced an alkaline red slant with yellow

acidic butt, with production of hydrogen sulfide in the butt region with black color. The results obtained for biochemical tests using HiMedia kit for *Salmonella* and their BIMs are listed in table 3.4 and a sample representation of the color reactions in the kit is depicted in figure 3.2.

The biochemical test, although routine were carried out to look for discernable changes in the phenotypic profile of the BIMs. However, in this study no variations were noted in the phenotypic characteristics of the BIMS. All biochemical tests answered by the wild type host were answered by the BIMs.

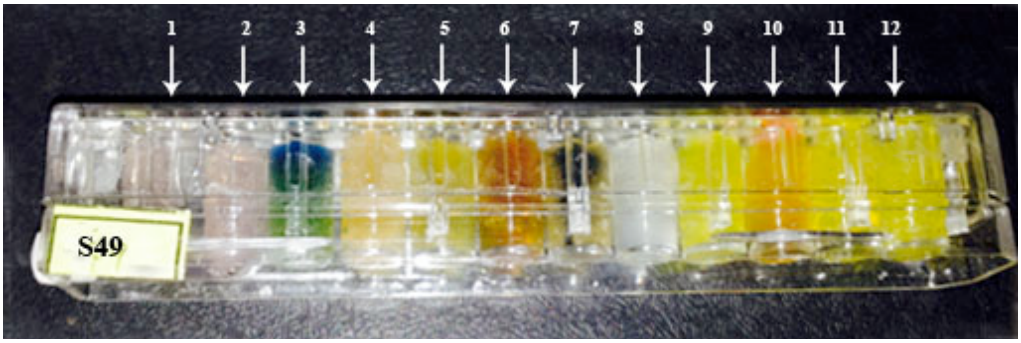

Fig 3.2 A sample representation of the biochemical results for *Salmonella* Enteritidis S49 obtained using HiMotility™ Biochemical kit for *Salmonella* (HiMedia). Lane 1 and 2-Motility, Lane 3-Citrate, Lane 4-Urease, Lane 5-Arginine, Lane 6-Lysine, Lane 7-H_2S production, Lane 8-ONPG, Lane 9-Arabinose, Lane 10- Lactose, Lane 11-Maltose, Lane 12-Trehalose.

3.3.2.2 Molecular characterization and phylogenetic tree construction

The PCR amplification of the 16S rDNA gene and subsequent sequence analysis using BLAST indicated the sequence similarity between the BIMs and their

hosts and showed that they were all *Salmonella* Enteritidis. Accession numbers were obtained after submission of the sequences to GenBank (included in list of publications).

Based on the 16S rRNA gene sequences, the relatedness of the host bacteria S49 and S37 and its BIMs were studied by constructing a phylogenetic tree as depicted in Fig 3.3 and Fig 3.4. From the phylogenetic tree, it can be observed that the in both cases, the host bacteria and their corresponding BIMs were grouped together to form a single clade separated away from the out-group, pointing to the fact that they were related and derived from the same parent strain.

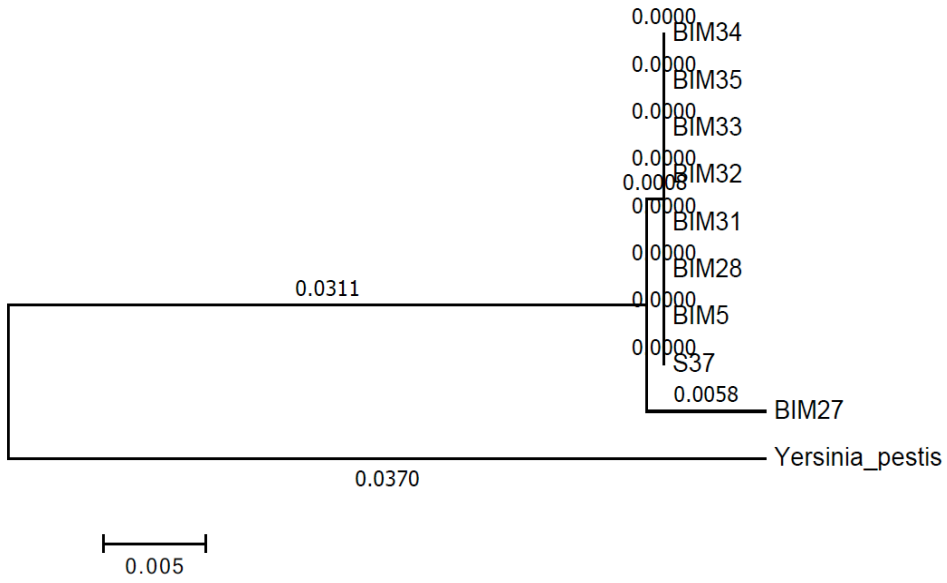

Fig 3.3 Phylogenetic tree indicating the relationship of S37 and its BIMs based on 16SrDNA sequences. The numbers at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on Neighbour-joining analysis of 1000 resampled data set. *Yersinia pestis* was used as the out-group.

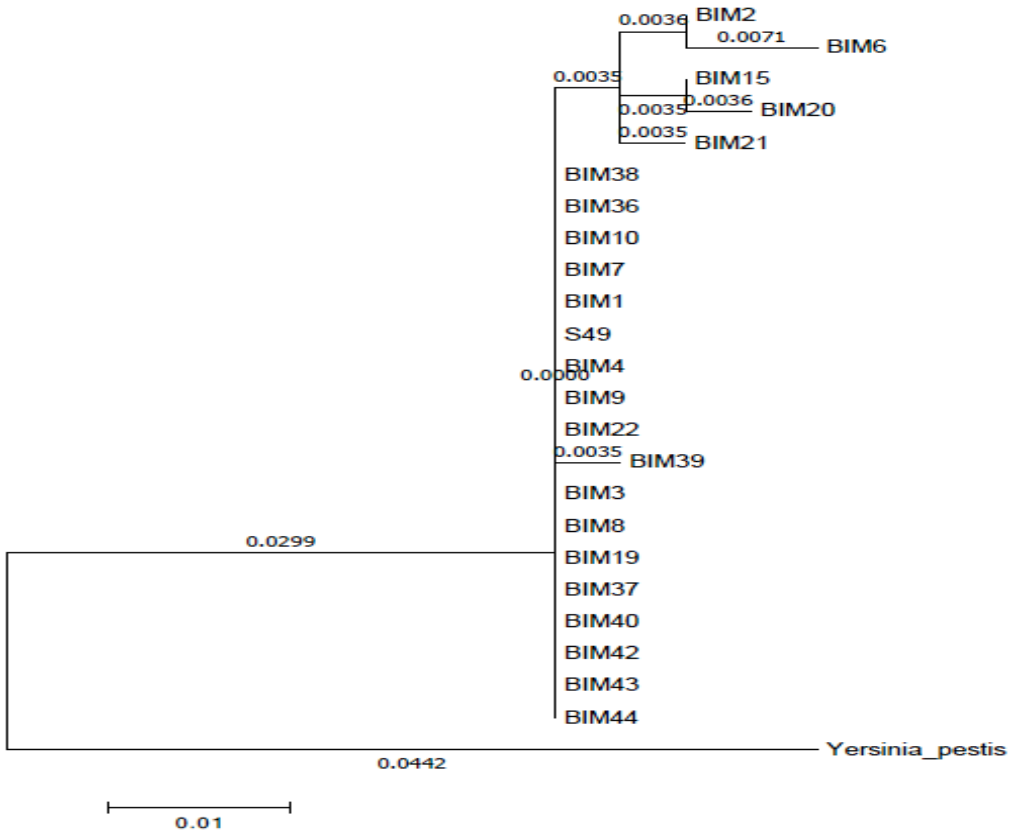

Fig 3.4 Phylogenetic tree indicating the relationship of S49 and its BIMs based on 16SrDNA sequences. The numbers at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on Neighbour-joining analysis of 1000 resampled data set. *Yersinia pestis* was used as the out-group

3.3.3 Antibiotic susceptibility of BIMs

The antibiotic sensitivity was interpreted as per the manufacturers instruction as sensitive, intermediate and resistant. The antibiogram of S49 and its BIMs is shown

in table-3.5.

Organisms / Antibiotics	Ampicillin	Azithromycin	Cefixime	Cefuroxime	Chloramphenicol	Ciprofloxacin	Tetracycline	Gentamicin	Nalidixic acid	Trimethoprim
Salmonella Enteritidis S49	S	I	S	I	S	I	R	S	I	S
ΦSP-1Mutants										
BIM 1	S	I	S	I	S	S	I	S	S	S
BIM 2	S	I	S	I	S	S	R	S	S	S
BIM 4	S	I	R	S	S	S	R	S	S	S
BIM 8	S	I	R	S	S	S	R	S	S	S
BIM 9	S	I	S	I	S	S	R	S	R	S
BIM 19	S	I	I	S	S	I	S	S	S	S
BIM 20	I	I	I	S	S	R	S	S	I	S
BIM 21	S	I	R	S	S	R	S	S	S	S
BIM 22	S	I	R	S	S	R	S	S	S	S
BIM 36	S	S	S	I	S	S	I	S	I	S
BIM 37	S	S	S	S	S	I	I	S	S	S
BIM 38	S	S	S	S	S	S	I	S	S	S
BIM 39	S	I	S	S	S	I	I	S	S	S
ΦSP-3Mutants										
BIM 3	S	I	S	S	I	S	I	S	S	S
BIM 6	S	S	S	S	S	S	I	S	S	S
BIM 7	S	I	I	S	S	S	R	S	I	S
BIM 10	S	S	S	S	S	S	I	S	S	S
BIM 15	S	S	S	I	S	S	R	S	S	S
BIM 16	S	S	S	I	S	S	R	S	S	S
BIM 40	S	S	S	I	S	S	I	S	I	S
BIM 42	S	I	S	I	S	I	R	S	R	S
BIM 43	S	I	S	I	S	S	I	S	I	S
BIM 44	S	S	R	R	S	S	I	S	I	S

Salmonella Enteritidis S37	I	S	S	S	S	S	I	S	S	S
ΦSP-2 Mutants										
BIM 5	I	S	S	I	I	S	I	S	S	S
BIM 27	S	S	S	S	I	S	I	S	S	S
BIM 28	S	S	S	S	S	S	I	S	S	S
BIM 31	S	S	S	S	I	S	I	S	S	S
BIM 32	S	S	I	S	S	S	I	S	S	S
BIM 33	S	S	S	S	S	S	I	S	I	S
BIM 34	S	S	S	S	I	S	I	S	I	S
BIM 35	S	S	I	S	S	S	I	S	S	S

S- Sensitive, I- Intermediate, R-Resistant

Table 3.5 Antibiotic sensitivity profile of *Salmonella* Enteritidis S49, S37 and their three sets of BIMs.

Strain S49 was resistant only to tetracycline and showed intermediate sensitivity for azithromycin, cefuroxime, ciprofloxacin and nalidixic acid. The antibiotic sensitivity profile of ΦSP-1BIMs was different from that of its host. In the case of first set BIMs all exhibited resistance to tetracycline, while BIM 4 and 8 showed resistance to cefixime and in contrast they were sensitive to cefuroxime whilst the host was intermediate. Besides BIM 9 was observed to be resistant to nalidixic acid whereas the host was intermediate.

In the second set BIMs, BIM 21 and BIM 22 showed resistance to cefuroxime while its host S49 indicated intermediate sensitivity. All exhibited resistance to tetracycline, but BIM 19 showed intermediate sensitivity. The BIMs 20, 21 and 22 showed resistance to ciprofloxacin where the host strain was intermediately sensitive. Third set BIMs displayed intermediate sensitivity to tetracycline while host was resistant. BIMs 36, 37 and 38 showed sensitivity to azithromycin while S49 was intermediate to it. Except BIM 36 all others showed sensitivity to cefuroxime. BIM 37

and 39 were intermediate to ciprofloxacin. And conversely except BIM 36 all were sensitive to nalidixic acid.

From the antibiogram of ΦSP-3 mutants, the first set BIM 6 and 10 were sensitive to azithromycin, whereas the host showed intermediate sensitivity. Except BIM 7 all were showed intermediate sensitivity to cefixime like the host. All BIMs were sensitive to cefuroxime where as host displayed intermediate zone. BIM 3 was intermediate to chloramphenicol however all others were sensitive like host bacteria. On the contrary all the BIMs exhibited sensitivity towards ciprofloxacin where host showed intermediate susceptibility. Except BIM7 all others showed intermediate sensitivity to tetracycline and in addition it displayed intermediate zone like S49 while all others were sensitive.

The second set BIMs, BIM 15 and 16 were sensitive to azithromycin and ciprofloxacin whereas host was intermediate to both. They also showed sensitivity towards nalidixic acid while host showed intermediate sensitivity. Like host bacteria they were also resistant to tetracycline.

In the case of third set BIMs except BIM 42 all others showed intermediate sensitivity towards tetracycline. BIM 42 also exhibited resistance towards nalidixic acid and moreover it showed intermediate sensitivity towards ciprofloxacin. BIM 44 exhibited resistance to cefixime and cefuroxime. BIM 40 and 44 were showed sensitivity towards azithromycin where host displayed intermediate zone.

Considering the strain S37 it denoted intermediate sensitivity to ampicillin and tetracycline while it was sensitive to all other antibiotics used. BIM 5 the only ΦSP-2 mutant in first set indicated intermediate sensitivity to cefuroxime and chloramphenicol where host was sensitive. The two BIMs in second set 27 and 28 were sensitive towards ampicillin. BIM 27 showed intermediate sensitivity to chloramphenicol. The five BIMs in third set displayed sensitivity towards ampicillin.

BIM 32 and 35 demonstrated intermediate sensitivity to cefixime. BIM 34 was intermediate to chloramphenicol and in addition BIM 33 and 34 exhibited intermediate sensitivity to nalidixic acid.

The antibiotic sensitivity profile of the BIMs showed several changes compared to that of their parent strains S49 and S37 after the phage infection. This change could be the outcome of the phage –host interaction that caused the formation of these BIMs.

3.4 Discussion

Salmonella enterica serovar Enteritidis causes gastrointestinal disease in humans and is a major public health concern because of to its ability to be transmitted via contaminated eggs or egg and poultry meat products (Tohidi *et al*., 2014). The use of bacteriophages is an approach for the control of *S.* Enteritidis, which has proved valuable in the curtailment of *S*. Enteritidis infection in cheddar cheese (Modi *et al*., 2001), vegetables (Leverentz *et al*., 2001), poultry products (Higgins *et al.,* 2005) and the skin of chickens (Goode *et al*., 2003). Nevertheless phage resistant strains could emerge and persist (Park *et al*., 2000). In this context the study of phage insensitive mutants assumes importance.

Salmonella Enteritidis S49 and S37, which were isolated from chicken, infected by ΦSP-1, ΦSP-3, and ΦSP-2 their specific lytic phages, also isolated from the same environment. The morphology of ΦSP-1 and ΦSP-3 were earlier characterized and they belonged to the family *Podoviridae* and *Siphoviridae* respectively (Augustine and Bhat, 2012). Since ΦSP-2 was not morphologically characterized previously, TEM was used for this purpose.

Transmission electron microscopy is highly valuable criterion to assess phage morphology (Pelzek *et al*., 2013) and it greatly helps in their classification. The

transmission electron micrograph of ΦSP-2 revealed isometric heads with along non-contractile tail. The head was 84.59 ± 1.06 nm in diameter and the tail 200.58 ± 0.52 nm long. The long tail, their isometric morphology, as well as their size are the characteristic morphological features, typical of members belonging to family *Siphoviridae* (ICTV).

The study conducted by De Lappe *et al*. (2009) at Laboratory of Enteric Pathogens (Health Protection Agency, London, UK) reported *Salmonella* phages belonging to *Siphoviridae* family with icosahedral heads measuring 62.5 nm with their tails being rigid, non-contractile and 120nm. Study on the morphology of *Salmonella* Typhimurium typing phages (Eisenstark *et al.*, 2009) of the Lilleengen set, (The Lilleengen set comprises 12 viruses isolated from sewage, manure, and Typhimurium cultures (Lilleengen, 1948; Rabsch, 2007)), two phages were (phages 28 and 33) *Siphoviridae* with isometric heads of 64 nm in diameter and relatively long, thick, tapering tails of 225 nm with 57 or 58 cross-striations.

Kang *et al*. (2013) isolated a virulent bacteriophage (wksl3) that could specifically infect *S*. Enteritidis, *S*. Typhimurium and several additional serovars. Its transmission electron micrograph revealed phage wksl3 with isometric head (63 nm), and the long noncontractile tail (121 by 7.9 nm), with a 20-nm-wide baseplate with tail spikes, as belonging to the family *Siphoviridae.*

The multiplicity of infection (MOI) is the number of virus of a given virus species that infect bacterial cells. This parameter chiefly impacts the severity of within-host population bottlenecks as well as the intensity of genetic exchange, competition, and complementation among viral genotypes (Gutiérrez *et al*., 2015). As too many phages attaching to a single bacterial cell can cause cell lysis, even before the infection process can yield progeny, determination of optimal MOI is important (Augustine and Bhat, 2014). The optimal MOI of ΦSP-2 was ten phages per

bacterium. These optimal MOI resulting in highest phage titer under standard conditions were used in all subsequent studies.

Salmonella Enteritidis S49 and S37 played host in the isolation of three sets of BIMs with their specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 respectively. Accurate identification of bacteria is important for clinical care and public health surveillance to understand the pathobiology of infectious clinical syndromes and better use of specific antibiotic and infection control strategies for patients and populations (Baron *et al*., 2013).

The BIMs were characterized as *Salmonella* according to the guidelines of guidelines of Food and Drug Administration (FDA) in the Bacteriological Analytical Manual (BAM) (Andrews *et al.,* 2007). KBMOO2 HiMotility^{TM} Biochemical kit for *Salmonella* (HiMedia) was used for biochemical characterization. Furthermore the BIMs also underwent serological agglutination test (Russell *et al*., 2006). The combined results from the above tests have shown that these isolates were *Salmonella* and not contaminants.

The identity of *Salmonella* strains S49 and S37 was already confirmed by 16S rRNA sequence analysis as *Salmonella* Enteritidis (Augustine and Bhat, 2012). The identity of isolated BIMs as *Salmonella* Enteritidis were also confirmed by 16S rRNA sequence analysis.16S rRNA gene sequence information is widely used for molecular identification of bacteria and is also useful for establishing the taxonomy of novel species (Dewhirst *et al*., 2015). Moreover, Patel (2001) reported that the use of 16S rRNA gene sequence to study bacterial taxonomy has been used widely for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family or operons; (ii) the fact that the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more

accurate measure of time (evolution); and (iii) the fact that the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Suardana, 2014).

The phylogenetic tree constructed indicated the relationship of S49, S37 and their BIMs based on 16SrDNA sequences after multiple sequence alignment using Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic-tree analysis is often used as a method to classify organisms. Various genes have been examined for the analysis of phylogenetic relationships of *Salmonella*, *Shigella*, *and E. coli*. In general, 16S rRNA is most frequently used for such analyses (Fukushima *et al*., 2002). Wang *et al*. (2015) on his study on complete genome sequence of *Salmonella* enterica subspecies arizonae str. RKS2983 constructed phylogenetic tree highlighting the position of *S*. arizonae RKS2983 relative to strains of other *Salmonella* lineages using Neighbor-Joining method. This only confirmed that the BIMs were indeed the mutants of *Salmonella* S49 and S37 and not contaminants.

The capability of ΦSP-1, ΦSP-3 and ΦSP-2 as prophylactic agents, in effecting a successful and sustainable biocontrol of *Salmonella* Enteritidis in *in vitro* study using experimentally dosed cooked chicken cuts and *in vivo* study using *C. elegans* was already confirmed (Augustine and Bhat, 2012). In this study BIMs were obtained for *Salmonella* Enteritidis S49 and S37 by infection with these same lytic phages. And the growth and characteristics of mutants were almost similar to their hosts.

There have been reports of phage resistant mutants arising during phage therapy. Capparelli *et al*. (2010) reported the growth of *S. enterica* serovar Paratyphi B (Salp572$^{\phi 1S}$) in the presence of phage ϕ1 (selected from among 8 phages for its larger host range) provided a phage ϕ1-resistant bacterial strain (Salp572$^{\phi 1R}$) during

the treatment of gastroenteritis. O'Flynn *et al*. (2007) reported phage resistant mutants of *Salmonella enterica* serovar Typhimurium DPC6046 in the presence of a lytic phage, Felix 01; usually this parent strain is sensitive to this phage. Nurminen *et al*. (1976) isolated mutants resistant to bacteriophages (P221 and PH105 or PH51from an rfa strain of *Salmonella* Typhimurium. They were deficient in separate 33,000 to 36,000 dalton band proteins (major band proteins). The growth behaviors of the mutants obtained were also normal.

Santander and Robeson (2007) isolated phage-resistant strains of *S.* Enteritidis ATCC 13076 by using three lytic phages (*f2α*SE, *f3α*SE and *f18α*SE) and in those mutants they explored different virulence factors like lipopolysaccharide (LPS), virulence plasmid (Pla), motility and type I fimbriae, all of which that may have effects on virulence and could furthermore be related to phage resistance. The emergence of BIMs in the biocontrol program of *Salmonella* enterica serotype Hadar in poultry farms was reported (Atterbury *et al*., 2007).

In the phage biocontrol experiment of enteropathogenic and shiga toxin-producing *Escherichia coli* using two lytic phages (DT1 and DT6) in meat products by Tomat *et al*. (2013) *E. coli* bacteriophage insensitive mutants (BIMs) emerged in meat assays. Filippov *et al*. (2011) isolated six independent mutants of *Yersinia Pestis* resistant to L-413C, ϕA1122, Pokrovskaya and Y, in his study of bacteriophage resistant mutants in *Yersinia pestis* for identification of phage receptors and attenuation for mice.

Owing to various factors, bacteria develop phage resistance. Specific receptor sites present on the bacteria cell wall help in the initial attachment of phage to its host. Gene mutation resulting in the loss or inactivity of these receptor sites, lead to development of phage resistance in bacteria. Anyhow, according to Skurnik and

Strauch (2006), this loss of receptor site would be beneficial to decrease the virulence of the bacteria as the receptors act as virulence determinants. The other feasible form of phage resistance is via restriction-modification immunity where the restriction endonucleases in bacteria degrade and halt the activity of the injected phage nucleic acid, thereby pausing the phage lytic cycle. By formation of mucoid colonies some bacteria develop partial phage resistance, which protect the bacterial host against phage adsorption (Sawant, 2015).

The antibiotic susceptibility of the host *Salmonella* Enteritidis S49, S37 and their BIMs isolated was determined using the disc diffusion method (Kirby-Bauer, 1966) and its result is shown in table 3.5.

In this study host *Salmonella* strains as well as majority of BIMs were resistant to tetracycline. Many findings indicated that tetracycline resistance in *Salmonella* could be attributed to the production of an energy dependent efflux pump to remove the antibiotic from within the cell. The modification of the ribosomal target, enzymatic inactivation of tetracycline, and other mechanism of resistance, have been documented in other bacterial species but has yet to be reported in *Salmonella* (Bouchrif *et al*., 2009). Antibiotic gene profiling has not been performed in this study but the possibilities of tetracycline resistance are clearer in gene profiling. About 35 different bacterial tetracycline resistance genes have been described. Still the sequences of only five of them *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(G) have been reported for *Salmonella* isolates (Michael *et al*., 2006). Usually reported of these genes in *Salmonella* serotypes are *tet*(A) and *tet*(B) genes (Alcaine *et al*., 2007). This has been observed in *Salmonella* genomic island 1 (Caratolli *et al*., 2002), on integrons (Briggs and Fratamico, 1999), and plasmids (Frech and Schwarz, 2000; Gebreyes and Thakur, 2005). Chen *et al.* (2004) have noticed *tet*(A) and *tet*(B) genes

in isolates of *Salmonella* serotypes Agona, Derby, Enteritidis, Haardt, Hadar, Heidelberg, Orion, Typhimurium.

Bakeri *et al*. (2003) reported that among 65 clinical *Salmonella* Enteritidis isolated in Malaysia between 1995 and 2002, nineteen percent (19.0%) of the strains were resistant to tetracycline. These findings were lower compared to those reported by Son *et al*. (1995), who observed that among 35 Malaysian veterinary *Salmonella* Enteritidis, tested by their susceptibility to 10 antimicrobial agents 89% strains were resistant to tetracycline. Cardoso *et al*. (2006) stated that all the 80 *Salmonella* Enteritidis strains, which isolated from broiler carcasses in Brazil, were resistant to tetracycline.

It was observed that BIM 44 was resistant to cefuroxime while other BIMs were sensitive to this drug and the host bacteria showed intermediate sensitivity. Cefuroxime are beta-lactams that prevent synthesis and maintenance of the peptidoglycan component of the bacterial cell wall by mimicking one of the building blocks used by enzymes to construct peptidoglycan (Petri, 2006; Queenan and Bush, 2007). Most of the β-lactam resistance in *Salmonella* is encoded by horizontally acquired β-lactamases however; many other bacteria have an intrinsic β-lactamase, such as *ampC* found in *E. coli* (Siu *et al*., 2003). There is growing concern about more than 340 β- lactamases resistance genes, such as blaTEM, blaOXA, blaPER, blaPSE, blaSHV, blaCTX-M, and blaCMY, while some are more prevalent in *Salmonella* globally (Armand-Lefevre *et al*., 2003). The ß-lactamase genes have been recognized in *Salmonella* serotypes Typhimurium (Pasquali *et al*., 2005; Garbarg-Chenon *et al*., 1990), Agona (Mulvey *et al*., 2004), Typhi (Pai *et al*., 2003), Cubana (Miriagou *et al*., 2003), Enteritidis (Verdet *et al*., 2000), and Panama (Li *et al*., 2005).

A major difference was observed in the case of antibiotic cefixime; BIMs 4, 8, 21, 22 and 44 showed resistance to this antibiotic, while their host strain S49 was sensitive to it. Most isolates were resistant to cefixime in a cross sectional study on antibiotic resistance pattern of *Salmonella typhi* clinical isolates from Bangladesh (Mannan *et al*., 2014). It was observed that some extended-spectrum β-lactamases inactivate newer cephalosporins, including cefixime (Matsumoto *et al*., 2001). 8.57% bacteria of *Salmonella* isolates from hospitals wastes were resistant to cefixime in a study of multi-drug resistance profiling of the selected *Salmonella* isolates by Faruk *et al*. (2014). It was suggested that the most apprehensive fact of thriving multidrug resistance trait in bacteria is that the resistance attribute is transferable and eventually, there is an immense possibility of transferring the phenotype to other bacteria of same or distantly related species, if the resistant bacteria are allowed to spread in the environment. On the other hand, there is a good chance of these multidrug resistant microbes entering into the food chain, because of lack of proper sewage management system in the developing countries.

Another major difference was in the case of nalidixic acid where the host showed intermediate sensitivity to this antibiotic but BIMs 9 and 42 were resistant while most of the BIMs were sensitive. Literatures suggest that resistance to nalidixic acid may be an indicator of decreased susceptibility to ciprofloxacin (Asna *et al*., 2003; Hakanen *et al*., 1999; Kapil and Das, 2002; Threlfall *et al.,* 2001). Public health surveillance for resistance to nalidixic acid is effective in monitoring emerging fluoroquinolone resistance. Moreover fluoroquinolone treatment has failed for patients infected with nalidixic acid-resistant *Salmonella* (Ranjbar and Naghoni, 2014).

This was evident in the case of BIM 42 as it showed resistance to nalidixic acid and exhibited intermediate sensitivity towards ciprofloxacin. Study conducted by

Gorman and Adley, (2003) in a total of 195 *Salmonella* isolates from human (51), food (eight) and veterinary (136) sources in the mid-west region of the Republic of Ireland nalidixic acid resistance was observed in 2.6% (5/195) of *Salmonella* isolates; these resistant strains were *Salmonella* Enteritidis (two human isolates), *Salmonella* Typhimurium (two bovine isolates) and *Salmonella* Dublin (one bovine isolate). One porcine strain of *Salmonella* Derby expressed intermediate resistance to nalidixic acid.

Another important detection was in the case of ciprofloxacin where the host showed intermediate sensitivity to this drug while BIMs 20, 21 and 22 were resistant to this drug and majority of the BIMs were sensitive. Ciprofloxacin-resistant *S. enterica* serotype Typhi were isolated from 9 patients in the United States by Medalla *et al*. (2011). Chromosomal point mutations in the *gyrA* and *parC* topoisomerase genes are mechanisms of quinolone resistance in *Salmonella* spp. Other resistance mechanisms include efflux pumps, reduced outer membrane permeability, and plasmid-borne genes (e.g., *qnr*, *aac-6'-Ib-cr* genes) (Crump *et al*., 2008; Turner *et al*., 2006).

Major differences were observed in the antibiotic sensitivity profile of *Salmonella* Enteritidis S49 BIMs, while the patterns of S37 BIMs almost similar to their parent strain.

These changes in the antibiogram of the host bacteria and their BIMs were determined only by disc diffusion method in the present study. Emergence of BIMs is due to the interaction between bacteria and phages and therefore many factors could have contributed to this change. BIMs might have achieved this resistance via genetic mutations under the selective pressure when exposed to phage infections. However, this was not pursued as part of this work.

The misuse of antimicrobial agents as chemotherapy in human and veterinary medicine or as growth promoter in food for animals can potentially lead to widespread dissemination of antimicrobial resistance to *Salmonella* and other pathogens via mobile genetic elements. In bacteriophage therapy, the use of viruses that infect bacteria as antimicrobials has been championed as a promising alternative to conventional antibiotics. The long-term use of phages as antimicrobials may lead to phage resistance, emphasizing the need for phage cocktails for the effective phage therapy. Hence the search for more phages to add to this armoury against pathogenic bacteria is of high importance.

Chapter 4

Characterization and analysis of CRISPR regions in *Salmonella* Enteritidis S49 and S37 and their BIMs

4.1 Introduction

Viruses of bacteria, bacteriophages represent the most abundant life forms on the planet. In addition they are believed to inhabit every niche in which potential hosts exist (Pride *et al*., 2012; Breitbart *et al*., 2002). Hence, there is an adaptive pressure on bacteria to escape phage infection in order to survive. As such, bacteria have evolved various phage resistance mechanisms including restriction/modification and abortive infection systems (Labrie *et al*., 2010).

Clustered regularly interspaced short palindromic repeats (CRISPRs) represent a component of a CRISPR/Cas system that confers adaptive immunity against viruses and plasmids (Barrangou *et al*., 2007; Marraffini and Sontheimer, 2008). CRISPR loci are found in almost all archaea and approximately 40% of sequenced bacterial genomes. They consist of a short repeat sequence (21–47 bp) separated by a unique variable sequence called a spacer (Sorek *et al*., 2008; Bolotin *et al*., 2005; Goode and Bickerton, 2006). The repeat sequence is highly conserved within a particular CRISPR locus. In contrast, the spacers vary greatly and their sequences have similarity to phages and plasmids and sometimes to host chromosomal sequences (Stern *et al*., 2010). Each CRISPR is commonly followed by a conserved AT-rich sequence known as a leader sequence. Adjacent to the CRISPR loci are located CRISPR-associated (cas) genes, essential components of the system, (Deveau *et al*., 2010).

Acquired immunity involving CRISPR/Cas systems can be divided into two

stages: the acquisition stage for uptake of the foreign element as a spacer into the leader-proximal end of CRISPR, and the immunity stage involving interference with the targeting of DNA in a sequence-specific manner (Deveau *et al*., 2010; Horvath and Barrangou, 2010). CRISPR interference is assisted by a set of CRISPR associated (Cas) proteins that are encoded by the cas genes usually found immediately adjacent to the repeats. Cas proteins can be classified into 45 different types but their precise biochemical functions are largely unknown. Only one protein, Cas1, has orthologs in all CRISPR loci (Marraffini and Sontheimer, 2009).

To provide immunity, the system follows three general steps: spacer acquisition, biogenesis of small RNAs and interference. In the acquisition step, a bacterial cell (BIM, Bacteriophage-Insensitive Mutant) acquires a new repeat-spacer unit in its CRISPR locus following a phage challenge. The new spacer in the CRISPR array is acquired from the invading DNA through the involvement of spacer acquisition motif located in the vicinity of a (proto) spacer in the phage genome. The CRISPR locus is then transcribed and processed with the help of *trans*-acting RNA (tracrRNA) and the host RNase III to produce smaller RNAs (crRNAs). In the interference step, crRNAs and Cas9 proteins guide and cleave the invading DNA in a sequence-specific manner to ensure cell defense. Of interest here, phage mutants can bypass the interference activity (CEM, CRISPR-Escape Mutants) by point mutation in the protospacer (PS) sequence or adjacent motif (Martel and Moineau *et al*., 2014).

This study looked at the emergence of bacteriophage resistant mutants after host phage interaction *in vitro* and analyzed the CRISPR regions of the host and its BIMs to look for any variation in the CRISPR regions of the hosts and their BIMs.

4.2 Materials and Methods

4.2.1 Bacterial cultures and Bacteriophages

Salmonella Enteritidis serotype strain S49 and S37 were selected to study CRISPRs in phage-host interaction. For that previously isolated, purified and characterized specific lytic phages, namely ΦSP-1, ΦSP-3 and ΦSP-2 (Augustine *et al*., 2014, 2012; Augustine and Bhat, 2012) were used.

4.2.1.1 Isolation of Bacteriophage Insensitive Mutants (BIMs)

Bacteriophage Insensitive Mutants were obtained by challenging the sensitive *Salmonella* Enteritidis serotype S49 with two of its lytic phages ΦSP-1 and ΦSP-3 and *Salmonella* Enteritidis serotype S37 with its lytic phage ΦSP-2. The protocol for BIM isolation is already described in section **3.2.3.** And three sets of BIMs were isolated for each serotype (reported in section **3.3.2).**

4.2.1.2 Confirmation of BIM as *Salmonella*

The isolated BIMs were confirmed as *Salmonella* following the guidelines of the bacteriological analytical manual of the US Food and Drug Administration (Andrews *et al*., 2011) and also by 16S rRNA gene sequence analysis using universal primers (Shivaji *et al*., 2000) **(**described in sections **3.3.2.1 and 3.3.2.2** of this thesis).

4.2.2 Screening for CRISPR

The genomic DNA was isolated (Esteban *et al*., 1993) (mentioned in section **3.2.3.5.1)** and CRISPR regions were amplified.

4.2.2.1 PCR amplification of CRISPR regions

Salmonella Enteritidis strain S49 and S37 and their BIMs were subjected to molecular characterization by PCR using specific primer pairs for *Salmonella* for CRISPR regions (Table 4.1). After PCR amplification the products were sequenced.

Sequences	Amplicon	Refrences
CRISPR 1 region CR1F-GCTGGTGAAACGTGTTTATCC CR2R-ATTCCGGTAGATYTKGATGGAC **CRISPR 2 region** CR2FAACGCCATGGCCTTCTCCTG CR2RCAAAATCAGYAAATTAGCTGTTC	1300bp ,,	(Bratčikov and Mauricas, 2009).

Table 4.1 Primers used for amplifying CRISPR 1 and CRISPR 2 regions.

4.2.2.1.2 PCR composition and program

PCR mix was same as mentioned in section **3.2.3.5.2**. PCR was performed in 200 µL capacity thin walled tubes in a final volume of 20 µL, with cycle conditions being 94^{o}Cfor 5 minutes followed by 34 cycles of 92^{0}C for 1minute and extension at 72^{o}Cfor 2 min using thermal cycler (BioRad, USA). Annealing temperatures for CRISPR 1 was 44^{o}C for 1 minute and that for CRISPR 2 was 47.1^{o}C for 1minute. The nucleotide sequences of the PCR amplicons were determined as before and analyzed by the CRISPRFinder.

4.2.2.2 CRISPRFinder

CRISPRFinder is a web service tool offering to (i) detect CRISPRs including the shortest ones (one or two motifs); (ii) define direct repeats (DRs) and extract spacers; (iii) get the flanking sequences to determine the leader; (iv) blast spacers against Genbank database and (v) check if the DR is found elsewhere in prokaryotic sequenced genomes. CRISPR finder is freely accessible at http://crispr.u-psud.fr/Server/CRISPRfinder.php (Grissa *et al*., 2007).

The input query sequence must be in 'FASTA' format. After querying a genomic sequence by CRISPRFinder, results are summarized in a table showing the

number of confirmed and questionable CRISPRs. A CRISPR locus is displayed according to a color code showing DR (direct repeats) in yellow and spacers in different colors. The respective positions are portrayed, in addition to links to two files: a summary of the displayed properties (number of motifs, DR consensus, positions, etc.) and a FASTA file containing the list of spacers.

4.2.2.3 Analysis of CRISPR spacer sequences

The CRISPR identification application CRISPRFinder (Grissa *et al*., 2007) was used to retrieve and find CRISPR repeats and spacer sequences. CRISPR spacers were visualized as color combinations, as previously described. For sequence similarity analyses, comparisons to public sequences were carried out using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al*., 1990) at the National Center for Biotechnology Information (http://blast.ncbi. nlm.nih.gov). After doing the blast of each 32-nucleotide spacer sequences a graphic representation of spacers of CRISPR 1 and 2 regions in *Salmonella* Enteritidis S49, S37 and their BIMs were drawn. The figure comprises of boxes, which represent spacers; repeats are not included. The 5' ends are oriented on the left of each array; the 3' end spacers are oriented on the right side of each array. The same number and color represent identical spacers (Pleckaityte *et al*., 2012).

4.3 Results

4.3.1 Isolation of Bacteriophage Insensitive Mutants and their confirmation as *Salmonella*

Infection of the host *Salmonella* Enteritidis serotype S49 individually by its lytic phage ΦSP-1 and ΦSP-3 yielded Bacteriophage Insensitive Mutants. Similarly infection with *Salmonella* Enteritidis serotype S37 its lytic phage ΦSP-2 also produced BIMs. The isolated mutants were confirmed as *Salmonella* Enteritidis and

GenBank accession numbers obtained **(**described in sections **3.3.2.1 and 3.3.2.2** of this thesis).

4.3.2 Analysis of CRISPR regions

4.3.2.1 Amplification of CRISPR regions

Following DNA isolation and amplification of the CRISPR 1 and CRISPR 2 regions in both strains S49 and S37, 1300bp-amplicons were obtained in all cases. A representative gel picture showing the amplified regions of CRISPR1 and CRISPR 2 is represented in Fig. 4.1 and 4.2.

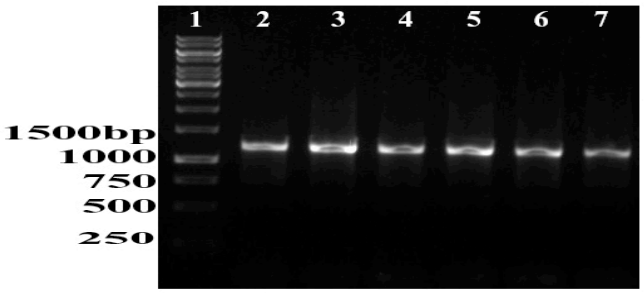

Fig 4.1 Amplified 1300bp CRISPR 1 region of *Salmonella* Enteritidis S49 and its first set BIMs. Lane 1-1kb ladder, Lane 2-S49, Lane 3-BIM4, Lane 4-BIM9, Lane 5-BIM 3, Lane 6-BIM 7, Lane 7- S37.

Among the ΦSP-1 mutants i.e. in BIMs 4, 9, 19, 20, 22 and 38 both CRISPR 1 and 2 regions were amplified, whereas in BIMs 1, 2, 8, 21, 36, 37 and 39 only CRISPR 2 regions were amplified.

In BIMs 3, 6, 7, 16, 42, 43 and 44 both CRISPR 1 and CRISPR 2 regions were amplified, but in BIMs 10, 15 and 42 CRISPR 1 region was not amplified

despite several attempts.

In BIMs 31, 32 and 35 only CRISPR 2 regions were amplified, whereas in BIMs 27, 28, 33 and 34 both the regions were amplified.

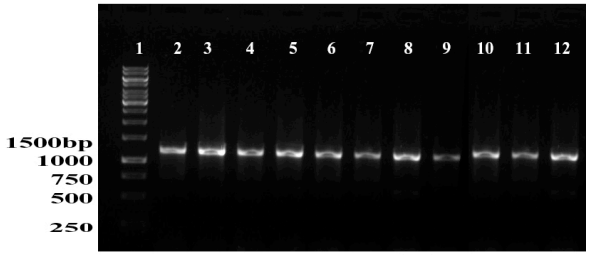

Fig 4.2 Amplified 1300bp CRISPR 2 region of *Salmonella* Enteritidis S49 and its first set BIMs. Lane 1-1kb ladder, Lane 2-S49, Lane 3-BIM 1, Lane 4-BIM 2, Lane 5-BIM 4, Lane 6-BIM 8, Lane 7-BIM 9, Lane 8-BIM 3, Lane 9- BIM 6, Lane 10-BIM 7, Lane 11- BIM 10, Lane 12- S37.

4.3.2.2. Analysis of sequences using CRISPRFinder

The 1300bp-amplified regions of both CRISPR 1 and CRISPR 2 regions from the BIMs and their hosts were sequenced and the sequence analysis was done using CRISPRFinder. A representative of the output of CRISPR 1 and 2 regions of *Salmonella* Enteritidis S49 of this finder is given in Fig. 4.2. The output of the appearance of CRISPR 1 and 2 regions of other host bacteria and all BIMs of both bacteria are included in annexure. The analysis of the sequences of both hosts and their BIMs from CRISPRFinder is shown in table 4.3. The sequenced products were deposited in the Genbank database and accession numbers were obtained KT008939-KT008942; KT070139- KT070189 (also included in list of publications).

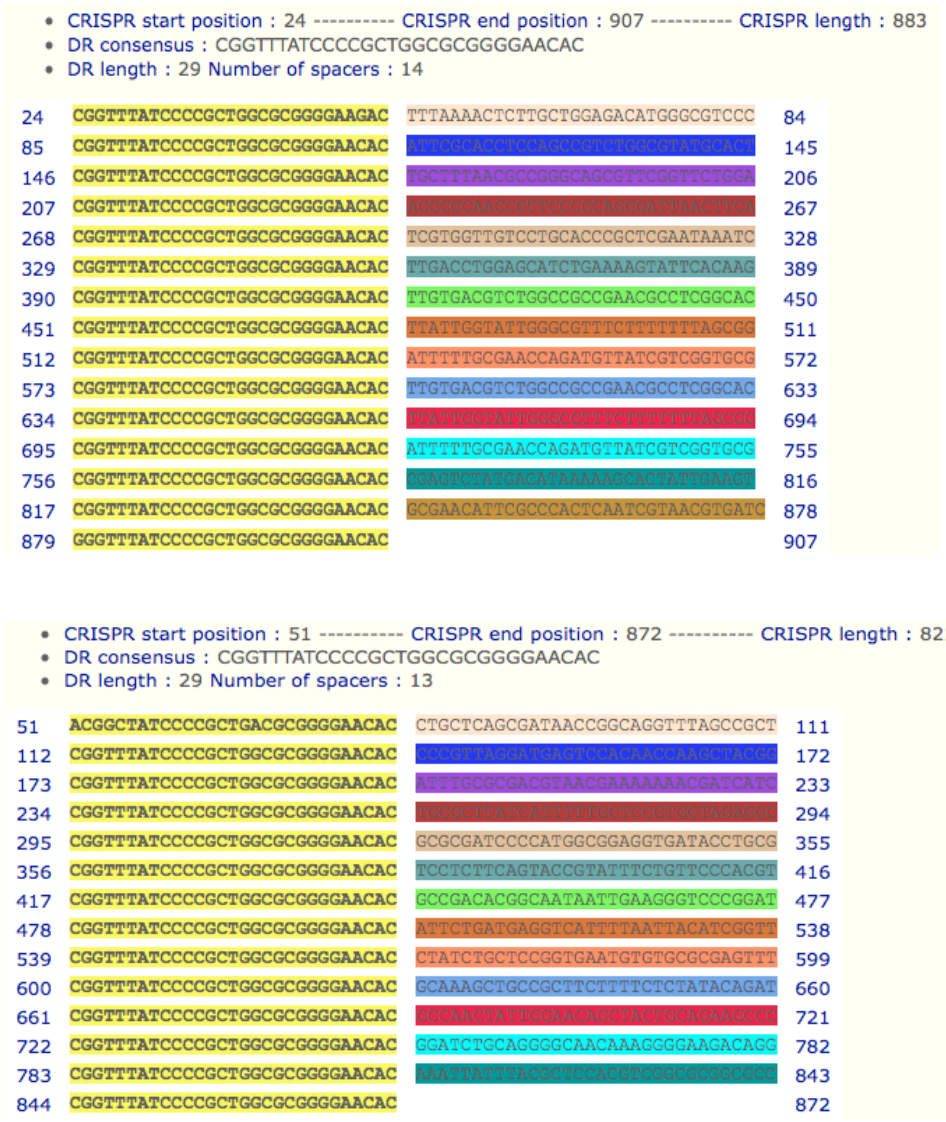

Fig 4.3 Representation of CRISPR 1 and CRISPR 2 regions of *Salmonella* Enteritidis S49 obtained from CRISPRFinder online http://crispr.u-psud.fr/Server/CRISPRfinder.php (Grissa *et al*., 2007).

From the Fig 4.3, it is clear that there are 14 spacers separated by 15 Direct repeat regions, each having 29 base pairs. Similarly the CRISPR 2 regions of S49 showed 13 spacer regions separated by 14 DR regions, also having repeat length of 29 bp. In both CRISPR 1 and 2 the DR repeat sequence were similar.

ORGANISM		**Length of CRISPR 1 (bp)**	**Length of CRISPR 2 (bp)**	**Repeat Length**		**Number of spacers**	
Bacteriophage	***Bacterium***			**CR1SPR 1**	**CRISPR 2**	**CRISPR 1**	**CRISPR 2**
	Salmonella Enteritidis S49	883	821	29	29	14	13
ΦSP-1	BIM 1	-	762	-	29		12
ΦSP-1	BIM 2	-	822	-	29		14
ΦSP-1	BIM 4	638	516	29	29	10	8
ΦSP-1	BIM 8	-	638	-	29	-	10
ΦSP-1	BIM 9	272	822	29	29	4	13
ΦSP-1	BIM 19	455	821	29	29	7	13
ΦSP-1	BIM 20	455	821	29	29	7	13
ΦSP-1	BIM 21	-	882	-	29	-	14
ΦSP-1	BIM 22	762	821	29	29	12	13
ΦSP-1	BIM 36	-	822	-	29	-	13
ΦSP-1	BIM 37	-	882	-	29	-	14
ΦSP-1	BIM 38	822	761	29	29	13	12

ΦSP-1	BIM 39	-	882	-	29	-	14
ΦSP-3	BIM 3	271	881	29	29	4	14
ΦSP-3	BIM 6	638	577	29	29	10	9
ΦSP-3	BIM 7	394	577	29	29	6	9
ΦSP-3	BIM 10	-	516	-	29	-	8
ΦSP-3	BIM 15	-	820	-	29	-	13
ΦSP-3	BIM 16	272	821	29	29	4	13
ΦSP-3	BIM 40	-	821	-	29	-	13
ΦSP-3	BIM 42	272	760	29	29	4	12
ΦSP-3	BIM 43	211	760	29	29	3	12
ΦSP-3	BIM 44	821	760	29	29	13	12
	Salmonella Enteritidis S37	943	760	29	29	15	12
ΦSP-2	BIM 27	821	760	29	29	13	12
ΦSP-2	BIM 28	883	578	29	29	14	9
ΦSP-2	BIM 31	-	210	-	29	-	3
ΦSP-2	BIM 32	-	700	-	29	-	11
ΦSP-2	BIM 33	818	760	29	29	13	12
ΦSP-2	BIM 35	-	882	-	29	-	14

- Indicates the regions CRISPR 1 and 2 that were not amplified by PCR

Table 4.2 The features of the CRISPRs from *Salmonella* Enteritidis S49, S37 and their BIMs obtained using the CRISPRFinder (Grissa *et al.*, 2007).

4.3.2.3 Examination of repeat regions

It was observed that direct repeat (DR) length of CRISPR 1 and 2 regions was 29 bp in both hosts and their BIMs. The CRISPR arrays were analyzed in *Salmonella* Enteritidis S49, S37 and its BIMs using BLAST, against the genomes in NCBI database.

The 29 bp repeat consensus CGGTTTATCCCCGCTGGCGCGGGGAACAC which was similar in hosts and BIMs both in CRISPR 1 and CRISPR 2 region showed top hit to *Salmonella enterica* subsp. *enterica* serovar Choleraesuis strain SH11G1292 CRISPR 2 repeat region which has accession number KP184385.1.

4.3.2.4 Analysis of spacer regions

On the other hand, the number of spacers present in both CRISPR 1 and 2 was different. The analysis of each spacer regions having approximately 32 nucleotides, using BLAST against the genomes in NCBI database, gave similarities to various submissions made in the database. Matches for the spacer were given continuous numbering and is as represented in table 4.2

	Spacer No:	Spacer sequence	Target region	Accession No:
CRISPR 1	1	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	Predicted *Vitis vinifera* un characterized LOC100854371(LOC100854371), mRNA	XM_003631900.1

	2	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	*Tetraodon nigroviridis* full-length cDNA	CR689712.2
	3	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	*Yarrowia lipolytica* WSH-Z06 complete genome, chromosome YALIOE	HG934063.1
	4	ACGCGCAACCGTTCCCGCAGGGATTAACTTCA	*Kutzneria albida* DSM 43870, complete genome	CP007155.1
	5	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	*Aggregatibacter actinomycetemcomitans* HK1651, complete genome.	CP007502.1
	6	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	Zebrafish DNA sequence from clone CH73-103L1 in linkage group 12, complete sequence	CU459020.8

	7	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	*Salmonella bongori* N268-08, complete genome.	CP006608.1
	8	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	*Helicobacter pylori* J166, complete genome	CP007603.1
	9	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	*Angiostrongylus cantonensis* genome assembly A_cantonensis_China , scaffold ACAC_scaffold0000011	LK945535.1
	10	CGAGTCTATGACATAAAAAGCACTATTGAAGT	Methanomethylovorans hollandica DSM 15978, complete genome	CP003362.1
	11	GCGAACATTCGCCCACTCAATCGTAACGTGATC	*Haemonchus placei* genome assembly H_placei_MHpl1, scaffold HPLM_scaffold0000048	LM583103.1

CRISPR 2	12	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	*Salmonella enterica subsp. enterica serovar* Abony str. 0014, complete genome	CP007534.1
	13	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	*Salmonella enterica* subsp. enterica serovar Enteritidis strain SARB17 CRISPR2 repeat region	JF725413.1
	14	ATTTGCGCGACGTAACGAAAAAAACGATCATC	*Salmonella enterica subsp. enterica* serovar Tennessee str. TXSC_TXSC08-19, complete genome	CP007505.1
	15	TGCGCTTATCATTTTTGCTCCGTGGTAGAGGC	*Salmonella bongori* serovar 66:z35:- strain 1900/76 CRISPR2 repeat region	JF725497.1

	16	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	*Mycobacterium kansasii* 824, complete genome	CP009483.1
	17	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	*Salmonella enterica* subsp. enterica serovar Mbandaka strain 1681K CRISPR1 repeat region	JF724861.1
	18	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	*Salmonella enterica* subsp. enterica serovar Mbandaka strain 260K CRISPR1 repeat region	JF724897.1
	19	ATTCTGATGAGGTCATTTTAATTACATCGGTT	*Cylicostephanus goldi* genome assembly C_goldi_Cheshire ,scaffold CGOC_scaffold0033355	LL428038.1
	20	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	*Cyprinus carpio* genome assembly common carp genome, scaffold LG43	LN590694.1

	21	GCCAACTATTGGAACAGCTACTGCAGAAGCCC	*Drosophila melanogaster* chromosome 3R	AE014297.3
	22	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	Predicted: Zea mays un characterized LOC103630547(LOC103630547), ncRNA.	XR_555117.1
	23	AAATTATTTACGCTCCACGTCGGCGCGGCGCC	*Aureococcus anophagefferens* hypothetical protein partial mRNA	XM_009036681.1

Table 4.3 Top hit annotations obtained for 32 nucleotide spacers of CRISPR 1 and 2 regions, for *Salmonella* Enteritidis S49, S37 and their BIMs by BLAST in NCBI database

After analyzing each spacer by blast a graphic representation of spacers of CRISPR 1 and 2 regions in *Salmonella* Enteritidis S49, S37 and their BIMs were drawn. It is represented in Fig 4.4.

In the CRISPR 1 region of *Salmonella* Enteritidis S49 (Fig 4.4 and table 4.3) there were totally 14 spacers present, among them the spacer 7, 8and 9 were tandemly repeated. These spacers showed matches to sequences in *Salmonella bongori* N268-08, complete genome, *Helicobacter pylori* J166, complete genome and, *Angiostrongylus cantonensis* genome assembly *A_cantonensis_China* respectively.

Many of the spacers in the CRISPR 2 regions were homologous to sequences of *Salmonella enterica and Salmonella bongori*.

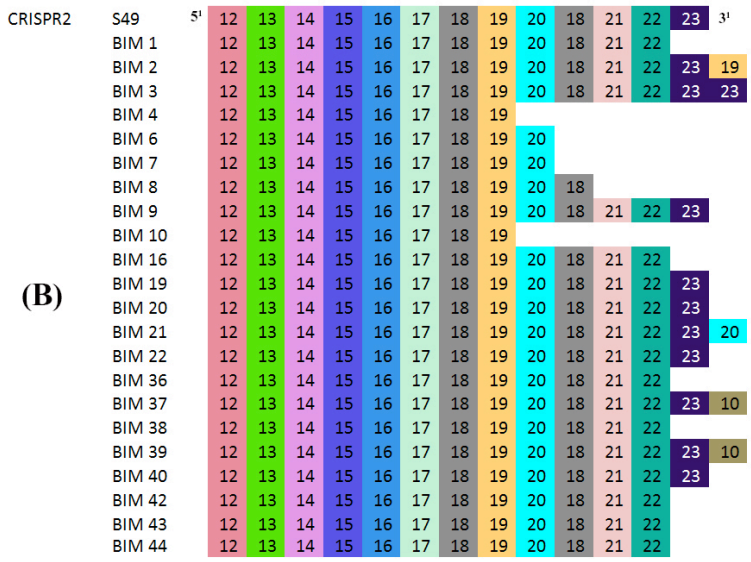

Fig 4.4 Graphic representation of spacers of CRISPR 1 and 2 regions in *Salmonella* Enteritidis S49 and their BIMs. Boxes represent spacers; repeats are not included. The 5' end are oriented on the left of each array; the 3' end spacers are oriented on the right side of each array. The same number and color represent identical spacers.

Almost the same spacers were observed among the BIMs of all sets of S49. BIM 2 and BIM 43 were different from others; in BIM 43 only three spacers (1,2 and 3) were detected. It seemed that after spacer 3 all were lost. On contrary in BIM 2, although the numbers of spacers were also three and the spacers present were not continuous; 3rd, 4th and 11th spacers were present in them. The integration of novel spacers by CRISPR1 after phage challenge in phage-resistant mutant was also

investigated. In BIM 3 there was a new spacer added at the 5' regions. Most of the spacers were found to be deleted from the 3' regions of the BIMs.

Regarding the CRISPR 2 region of *Salmonella* Enteritidis S49 and its BIMs (Fig 4.4 and Table 4.3) the spacers were totally different from those present in CRISPR 1; only13 spacers were in host. In BIM 4 and 10 the spacers after spacer 19 was absent from the 3' regions. In most BIMs studied the last spacer at the 3' regions was deleted. In BIM 21 the spacer number 20 was repeated. In BIM 37 and 39, spacer 10 was observed at the 3' regions; the spacer number 10 was present in CRISPR 1 region. The analysis of the CRISPR region showed that the BIMs were not only different from the host S49 from which they were derived, but were also different from each other.

Fig 4.5 Graphic representation of spacers of CRISPR 1 and 2 regions in *Salmonella* Enteritidis S37 and their BIMs. Boxes represent spacers; repeats are

not included. The 5' ends are oriented on the left of each array; the 3' end spacers are oriented on the right side of each array. The same number and color represent identical spacers.

Examining the CRISPR1 region of *Salmonella* Enteritidis S37 (Fig 4.5 and table 4.3), it was noted that there were a total number of 15 spacers were present. And just like in S49 the spacers 7,8 and 9 were tandemly repeated twice. In S49 at positions 14 and 15 were spacer-20 and 12, which were present in the CRISPR 2 region of S49. This indicates that there is a recombination-taking place between CRISPR 1 and 2 regions. In BIM 27 and 33 there were a total number of 13 spacers and in BIM 28 only fourteen spacers were present.

Considering the spacers of CRISPR 2 region of *Salmonella* Enteritidis S37 (Fig 4.5 and table 4.3,) of the 12 spacers present were similar to the spacers of CRISPR 2 region of *Salmonella* Enteritidis S49. Spacer number 23 was present only in BIM 32 and 35, but its location in the CRISPR locus was different in both cases. In BIM 35 there were fourteen spacers, with the last one being spacer 10, which is present in CRISPR 1 region. In BIM 31 there were only three spacers 18, 21 and 22 all other spacers were absent.

4.4 Discussion

Microbes, such as bacteria and viruses, do not exist in isolation but shape intricate ecological interaction webs (Faust and Raes, 2012). In the Red Queen hypothesis, Leigh Van Valen proposed that every positive adaptation in an organism causes a decline in fitness in those species with which it interacts. Such coevolutionary interactions create the natural cycle of adaptation and counter adaptation of ecologically interacting species, thereby driving rapid molecular evolution (Van Valen, 1974; Paterson *et al*., 2010). Nowhere is this dynamic arms

race as prevalent as in microbe-phage interactions. The constant exposure to phage infection imposes a strong selective pressure on bacteria to develop viral resistance strategies that promote prokaryotic survival. Thus, this parasite-host relationship results in an evolutionary arms race of adaptation and counter adaptation between the interacting partners. The evolutionary outcome is a spectrum of remarkable strategies used by the bacteria and phages as they attempt to coexist. These approaches include adsorption inhibition, injection blocking, abortive infection, toxin-antitoxin, and CRISPR-Cas systems (Dy *et al*., 2014).

The CRISPR locus of the recently discovered CRISPR/ Cas defense system in prokaryotes protects against invading viruses and plasmids and is a map of the "immunological memory" of the microorganism (Pleckaityte *et al*., 2012). The spacer sequences that are incorporated into the CRISPR loci provide a historical view on the exposure of the bacteria to a variety of foreign genetic elements (Horvath and Barrangou, 2010).

In the current study, the CRISPR arrays in *Salmonella* Enteritidis S49, S37 and in its BIMs were analyzed. The two CRISPR regions 1 and 2 were amplified using specific primers. Several studies have reported the presence of two CRISPR loci in *Salmonella* (Li *et al*., 2014). Within *Salmonella*, which contains the Type I-E CRISPR-Cas system (Makarova *et al*., 2011), there are two CRISPR loci (CRISPR1 and CRISPR2) that differ in both the identity and number of spacers and repeats (Jansen *et al*., 2002; Touchon and Rocha, 2010).

The amplified size of CRISPR 1 and CRISPR 2 were 1300bp in both hosts S37 and S49 and their BIMs. The PCR products of CRISPR 1 and CRISPR 2 were in between 1000bp and 3000bp for *Salmonella* Typhimuriumin in a study on CRISPR

typing and subtyping for improved laboratory surveillance of *Salmonella* Infections (Fabre *et al*., 2012). In subtyping of *Salmonella* enterica serovar Newport isolates by PCR amplification of CRISPR 1 and 2 regions the reported amplicon size of the product was 800bp to 2000bp (Shariat *et al*., 2013b).

The repeat sequences of 29 nucleotide were analyzed in the present study. It was similar in both hosts and BIMs both in CRISPR 1 and CRISPR 2 region and showed top hit annotation to *Salmonella enterica* subsp. *enterica* serovar Choleraesuis strain SH11G1292 CRISPR 2 repeat region with accession number KP184385.1. Earlier studies reported that *Salmonella* has two CRISPR loci, CRISPR 1 and CRISPR 2, which are separated by ~16 kb and which share the same consensus direct repeat sequence (29 nucleotide) and the spacers are 32nucleotide in length (Fabre *et al*., 2012; Liu *et al*., 2011a, b; Shariat *et al*., 2013a).

When spacers of CRISPRs of S37, S49 and their BIM were analyzed the numbers were different between the hosts and their respective BIMs, and among the BIMs themselves. The lowest spacer number reported was three and highest was fifteen in this study. Shariat *et al*. (2013a, b, c) analyzed the CRISPR 1 and CRISPR 2 arrays from 400 clinical *Salmonella* isolates that included 141 serovar Enteritidis, 84 Typhimurium, 86 Newport and 89 Heidelberg. They identified 179 unique spacers. The mean number of unique spacers in an array was 16 (CRISPR 1) and 20 (CRISPR 2). The smallest array seen in a single isolate contained two spacers and three direct repeats (serovar. Typhimurium, CRISPR 1 array 131). Interestingly, those two spacers represented the oldest and newest spacers. The largest CRISPR arrays contained 34 unique spacers and 35 direct repeats (four serovar. Typhimurium CRISPR 2 arrays: 164, 173, 179 and 207). On an average, in their study serovar. Enteritidis has the smallest and also the fewest number of different CRISPR arrays.

Analysis of the spacer repertoire revealed different activities of the

CRISPR/Cas loci across *S*. Enteritidis S49, S37 and their BIMs (Table. 1). Normally phage-host interactions may lead to insertions of phage nucleotides into the spacer region as protospacers (Karginov and Hannon, 2010). In an immune active system, array differences arise from spacer acquisition (Tyson and Banfield (2008). However in this study, new protospacer additions were not observed at the 5' end of the CRISPR regions except in CRISPR 1 region of BIM 3. In majority of BIMs deletion of spacers of CRISPR 1 region were noted , which may or may not be the cause for resistance in these BIMs to phage infection and therefore to cell lysis (Fig. 4.4).

Spacer acquisition itself differs dramatically among different species, and endogenous acquisition has been observed in the laboratory in only a few bacteria (Barrangou *et al*., 2007; Cady *et al*., 2012; van der Ploeg *et al*., 2009). Acquisition, along with spacer loss and duplication, makes CRISPR elements among the fastest evolving loci in bacteria (Paez-Espino *et al*., 2013; Pride *et al*., 2011).

It is well established in *Salmonella* that the overwhelming majority of CRISPR allelic polymorphisms within a serovar arise from deletion or duplication of direct repeat-spacer units, rather than acquisition of new spacers (Shariat *et al*., 2015). The low number of arrays missing the first or last spacer suggests some selection toward maintenance of these spacers and perhaps integrity of the array. Beyond this there is no selection for any particular region of the array from which spacers are lost. It is specifically noted that though spacers are lost, this occurs within the context of a spacer and its cognate direct repeat, thus maintaining the integrity of the array. This organization likely results from homologous recombination at the direct repeat sequence, thus maintaining the integrity. Such maintenance may have important implications if the CRISPR arrays provide an, as yet undetermined, alternative function that may require mature crRNAs (Shariat and Dudley, 2014).

In speculating whether the *Salmonella* CRISPR-Cas provides immunity, our

data is similar to observations made within *E. coli* species, where the CRISPR system does not exhibit typical characteristics of an active immune defense system (Touchon *et al.,* 2011).

It is becoming apparent that CRISPR-Cas systems do have alternative functions (Bondy-Denomy and Davidson, 2014; Westra *et al.*, 2014). For example, these systems have been shown to be involved in biofilm formation (Zegans *et al.*, 2009), host infection in humans and amoeba (Gunderson and Cianciotto, 2013; Sampson *et al.*, 2013), symbiotic colonization in nematodes (Veesenmeyer *et al.*, 2014) and DNA damage (Babu *et al.*, 2011).

This iterative phage challenges have typically added to CRISPR1 and CRISPR 2 locus. It helps in the separation of CRISPR mediated phage resistance from other natural antiphage defense systems. The other four systems like adsorption inhibition; DNA ejection inhibition, restriction modification systems and abortive infections are unable to generate new phage resistant derivatives in the absence of fitness cost to the host in each generation. At the same time, CRISPR mediated phage resistance can manage the acquisition of a new spacer in phage mutants even without any fitness cost.

This study design was to elucidate the changes in CRISPR regions of the host bacteria and bacteriophage insensitive mutants after infection and understand their role in the phage insensitivity. There were observable changes in the CRISPR regions not so much as acquisition of new spacers but deletion of spacers. Since there was no addition of novel spacer in this case, but only deletions, CRISPR based resistance cannot be advocated with conviction in this instance. This also suggests that there are alternative mechanisms for phage resistance. However, there were observable variations in the spacer regions, which indicates that this difference in CRISPR

regions in the wild type host and BIMs may be used as a marker to detect changes in industrial organisms. CRISPR arrays serotypes can also be analyzed to resolve intra-serotype variations.

Anyhow this enables a little understanding about the emergence of multi-phage resistant bacteria when exposed to different phages through successive challenges. It hints at the role of CRISPRs in the development of phage resistance in host, and emergence of bacteriophage insensitive mutants and also demonstrates that there may be several other mechanisms implicated in this process, needing further analysis.

Chapter 5

LPS, Virulence gene profiling and MLST pattern analysis of *Salmonella* Enteritidis S37 and S49 and their BIMs

5.1 Introduction

Lipopolysaccharide (LPS) is essential for most Gram-negative bacteria, having crucial roles in protecting bacteria from harsh environments and toxic compounds, including antibiotics (Dong *et al*., 2014). It is a complex glycolipid exclusively present in the outer leaflet of the outer membrane of gram- negative bacteria (Freinkman *et al*., 2011). LPS is one of the molecules responsible for the endotoxic shock associated with the septicemia, and is a sure indicator of infection, as the human innate system is sensitized to this molecule (Galdiero *et al*., 2012). It is comprised of three regions: lipid A, core oligosaccharide, and O-antigen polysaccharide (Wu *et al*., 2013). Lipid A, the hydrophobic anchor of LPS, is responsible for the bioactivity of LPS (Han *et al*., 2013). It is linked to the O-antigen with a 3-deoxy-D- manno-oct-2-ulosonic acid (Kdo) (Abraham *et al*., 2012). The ubiquitous nature of Kdo within LPS structures and its indispensable role in maintaining outer membrane integrity and viability of the majority of Gram-negative bacteria have led to extensive studies of its synthesis, activation and incorporation into the maturating LPS molecule (Schmidt *et al*., 2012). The core oligosaccharide consists of a hetero-oligosaccharide, with limited variability within different bacterial species. The core domain is more conserved and can contain up to 15 monosaccharides. The inner core domain is attached to the lipid A domain and consists of a more conserved carbohydrate backbone decorated with a heterogeneous set of other residues. The outer core domain attaches the O-antigen to the LPS molecule and is more variable

(Pel and Pieterse, 2013). The O-specific chain is composed of repeating oligosaccharide units that determine the serological specificity of the LPS and thereby the bacteria cell wall. Rough (R) mutants of gram-negative bacteria synthesize LPS lacking O-polysaccharides and are designated Ra-Re in the order of the decreasing complexity of the core oligosaccharide (Freudenberg *et al.,* 2008). Most wild-type bacteria synthesize smooth (S)- and R-form LPS, and thus, LPS preparations represent mixtures of S- and R-form LPS, despite their designation as the S-form (Salomao *et al*., 2012).

Virulence genes that are frequently laterally transferred include genes for bacterial adherence to host cells; type 3 secretion systems, toxins, iron acquisition, and antimicrobial resistance (Gyles and Boerlin, 2013). *Salmonella* possess a core genome plus a flexible accessory genome consisting of *Salmonella* PAIs (SPIs), bacteriophages, and plasmids (Switt *et al*., 2012). There are at least 21 PAIs in *Salmonella*, but most attention has been paid to SPI-1 and SPI-2, which are critical for invasion of nonphagocytic cells and for replication in nonphagocytic and phagocytic cells, respectively (Srikanth *et al*., 2011). MgtC, a *Salmonella*- specific gene harbored in SPI-3, is required for intracellular survival within macrophages, virulence in mice, and growth in low Mg^{2+} media (Chen, 2013). The *mgtC* gene is transcriptionally controlled by the PhoP/Q regulatory system, which is involved in the adaptation of low Mg^{2+} environment and is a major regulator of virulence functions in *Salmonella* (Groisman *et al.,* 2013). The SP-I is transcriptionally activated by virulence regulators such as SlyA during the stages of bacterial infection (Mulder *et al*., 2012; Fass and Groisman, 2009). The *slyA* has been shown to up-regulate the expression of acid-resistance proteins and cytolysin, and down-regulate several biosynthetic enzymes in *Salmonella.* It is implicated in virulence and environmental adaptation to *Salmonella* (Gao *et al*., 2014). One interesting feature of *Salmonella* physiology is the ability to

use sulphur compounds such as tetrathionate and thiosulphate as terminal electron acceptors during anaerobic respiration (Stoffels *et al*., 2012; Hinsley and Berks, 2002). The *Salmonella* tetrathionate reductase is encoded at the *ttrRSBCA* locus located on Pathogenicity Island 2. The complex has three subunits such as TtrA, TtrB and TtrC. TtrC most likely acts as a membrane anchoring subunit and quinol dehydrogenase (James *et al*., 2013). Gene products of invA, invE and spaM (invI) are required for invasion of the host cells (Varghese and Bhat, 2013). SopE is an SPI-1-dependent translocated protein that be- longs to a novel class of bacterial toxins that modulate host- cell RhoGTPase function via a non-covalent interaction (Hopkins and Threlfall, 2004). *Salmonella Enteritidis* produces thin, aggregative fimbriae, which are composed of polymerized AgfA fimbrin proteins The 453-bp *agfA* gene encodes the AgfA fimbrin **(**Collinson *et al*., 1996).

Multi locus sequence typing (MLST) is one method of genotyping bacteria based upon housekeeping genes of known function and chromosome position (Dahiya *et al*., 2013). It also allows greater discrimination between serovars. It involves detecting allelic differences in the sequences of various housekeeping genes (Bachmann *et al*., 2014). MLST can be used to accurately identify bacterial lineages. The genetic distance between two strains can be quantitatively estimated as allelic differences in the nucleotide sequences of housekeeping or virulent genes among bacterial strains (Noda *et al*., 2011). MLST data have been used in evolutionary and population analyses that estimate recombination and mutation rates and also in the investigation of evolutionary relationships among bacteria classified within the same genus (Urwin and Maiden, 2003). For each gene fragment, the different sequences are assigned as distinct alleles, and each isolate is defined by the alleles at each of the seven housekeeping loci (the allelic profile or sequence type [ST]. As there are many alleles at each of seven loci, isolates are highly unlikely to have identical allelic

profiles by chance, and isolates with the same allelic profile can be assigned as members of the same clone (Lin *et al*., 2014). Sequence data are readily compared between laboratories and a major advantage of MLST is the ability to compare the results obtained in different studies via the Internet (Thompson *et al*., 1998). MLST provides unambiguous DNA sequence data that can be easily exchanged and compared via worldwide web databases, and combines PCR and automated DNA sequencing to reduce labor and analysis time, so providing discriminatory power comparable to or higher than that provided by fragment-based methods (Maiden *et al*., 1998; Enright and Spratt, 1999).

This study aims to differentiate between *Salmonella* Enteritidis S49, S37 and their BIMs obtained by phage infection by their specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 by LPS and virulence gene profiling; and to discriminate between the host bacteria and BIMs through multilocus sequence typing. This will help to infer if there are changes occurring in the emergent BIMs due to phage infection.

5.2 Materials and Methods

5.2.1 Bacterial cultures and Bacteriophages

Salmonella Enteritidis serotype strain S49 and S37 was selected. For isolation of BIMs previously isolated, purified and characterized specific lytic phage, namely ΦSP-1, ΦSP-3 and ΦSP-2 (Augustine *et al*., 2014, 2012; Augustine and Bhat, 2012) were used. Bacteriophage Insensitive Mutants were obtained by challenging the sensitive *Salmonella* Enteritidis serotype S49 with its lytic phage ΦSP-1 and ΦSP-3 and *Salmonella* Enteritidis serotype S37 with its lytic phage ΦSP-2. The protocol for BIM isolation is already described in section **3.2.3.** And three sets of BIMs were isolated for each serotype (reported in section **3.3.2).** The isolated BIMs were

confirmed as *Salmonella* following the guidelines of the bacteriological analytical manual of the US Food and Drug Administration (Andrews *et al*., 2011) and also by 16S rRNA gene sequence analysis using universal primers (Shivaji *et al*., 2000) **(**described in sections **3.3.2.1 and 3.3.2.2** of this thesis).

5.2.2 Comparison of LPS from phage sensitive host bacteria and BIMs

5.2.2.1 LPS micro-extraction using proteinase K digestion

LPS was extracted (Hitchcock, 1984) from the parental as well as phage resistant strains in order to compare the variations in the pattern displayed.

5.2.2.1.2 Procedure

The colonies of the host *Salmonella* and BIMs grown on nutrient agar medium were picked with a sterile swab and suspended in 10 mL of cold phosphate-buffered saline (PBS, pH 7.2) to O.D $_{650}$ of 0.4. It was centrifuged for 1.5 minutes at 14000xg (Sigma, 3K30, Germany). The pellet was solubilized in 50 µL of lysing buffer (Appendix-4) and then heated at 100ºC for 10 minutes. 25 µg of proteinase K in 10 µL of lysing buffer was added to each boiled lysate to digest the bacterial proteins, incubated at 60ºC for 60 minutes. 2µL of this preparation was directly loaded for acrylamide gel electrophoresis.

5.2.2.2 LPS Acrylamide Gel Electrophoresis

5.2.2.2.1 Procedure

LPS Acrylamide Gel Electrophoresis was performed using vertical slab electrophoresis (BioRad, USA) following the procedure of Apicella, (2008). 20% resolving gel was prepared by combining 3.325 mL of 30% acrylamide, 1.25 mL of resolving buffer, 50µL of 10%SDS and 326.5µL of distilled water followed by 100µL

of ammonium persulfate solution (10%) and TEMED (10 µL) (Appendix 2). The gel was overlayed with distilled water and allowed to polymerize for 2 hour. The stacking gel was prepared by combining 333µL of 30% acrylamide, 500µL of stacking buffer, 20µL of 10% SDS and 1.135mL of distilled water followed by 40µLof ammonium persulfate and 4 µL of TEMED (Appendix 2). The layer of distilled water over the resolving gel was removed, the stacking gel was poured and the comb immediately inserted. The set up was allowed to polymerize for at least 1 hour. The gel was placed in the electrophoresis apparatus; the reservoir filled with reservoir buffer and was pre run for 1 hour at 80 V. The prepared samples were then loaded to the gel and were run at 80V until the dye front entered the resolving gel, then the current was increased to 100V. The run was stopped when the dye front reached 1 cm from the lower end of the glass plate. The gel was removed from the cast and silver stained.

5.2.2.3 Silver staining for LPS gels (Tsai and Frasch, 1982)

The gel was fixed in a fixing solution (Appendix-4) overnight, rinsed thrice with distilled water, transferred to a separate dish, followed by three additional washes with distilled water with agitation for 10 minutes each. The gel was then transferred to a separate dish with freshly prepared staining reagent (Appendix-2) with shaking for 10 minutes, followed by transfer to a separate dish, rinsed thrice with distilled water, transferred again to a clean dish and added fresh formaldehyde developer (Appendix-2). Reaction was stopped by rinsing the gel in water once the bands were developed. The image of the stained gel was captured using gel documentation system (Syngene, UK).

5.2.3 Screening for virulence /virulence related genes in the bacterial host and in BIMs

PCR based method was adopted to screen for the presence of virulence genes on the host and BIMs genome viz *invE/A, slyA, pho P/Q, ttrC, mgtC, sopE* and *agfA*. The details of the primers used are as given in Table.5.1. PCR was performed BioRad MJ Mini™Gradient Thermal Cycler. For this DNA was isolated already mentioned in section **3.2.3.5.1 (**Esteban *et al*., 1993).

Virulence gene	Sequence 5`-3`	Amplicon size	Function	References
invE/A	F - TGCCTACAAGCATGAAATGG R- AAACTGGACCACGGTACAA	457bp	Invasion to the host	Stone *et al.,* 1994
slyA	F–GCCAAAACTGAAGCTACAGGTG R - CGGCAGGTCAGCGTGTCGTGC	700bp	Cytolysin production	,,
pho P/Q	F– ATGCAAAGCCCGACCATGACG R –GTATCGACCACCACGATGGTT	299bp	Resistance within macrophages	Sara *et al*., 2006
mgtC	F-TGACTATCAATGCTCCAGTGAAT R-ATTTACTGGCCGCTATGCTGTTG	655bp	Survival within macrophages and growth in low Mg2+environments	Soto *et al*., 2006
ttrC	F- GTGGGCGGTACAATATTTCTTTT R -TCACGAATAATAATCAGTAGCGC	920bp	Vitalfor tetrathionate metabolism	,,
sopE	F-CCGTGGAACGATTGACTG R- AGCCATTAGCAGCAAGGT	~450 and 500bp	Type 111 effector involved in invasion	Susanne *et al*., 1999
agfA	F-TCCGGCCCGGACTCAACG R–CAGCGCGGCGTTATACCG	261bp	Binding to fibronectin of host	Doran *et al*., 1993

Table 5.1 List of primers used to screen for the virulence and virulence related genes in the host bacteria *Salmonella* Enteritidis S49, S37 and their BIMs genome by PCR

Composition for PCR

Template DNA (50 ng/µL) and sterile distilled water was added to a final volume of 20 µL. Amplifications were carried in Thermal Cycler (BioRad MJ Mini Gradient). The composition of the PCR mix is as follows

2 mM each dNTPs (Chromous Biotech, India)	2 µL
Forward primer (10 picomoles) (Sigma Aldrich)	2 µL
Reverse primer (10 picomoles) (Sigma Aldrich)	2 µL
Taq DNA polymerase (1U/µL) (Sigma-Aldrich)	1 µL
10X PCR buffer	2 µL
$MgCl_2$ (Sigma-Aldrich)	1.2 µL

The program used was a hot start cycle of 94^0C for 5 minutes, followed by 30 cycles of 94^0C for 1minute, annealing at 60^0C for 1 minute and extension at 72^0C for 2 minutes and finally with an extension step of 72^0C for 5 minutes.

5.2.3.1 Agarose gel electrophoresis

For the analysis of the amplified products 1% agarose gels were prepared (Appendix 2). They were prepared in Tris-Acetate-EDTA (TAE) buffer. To that ethidium bromide was added at a concentration of 0.5mg/mL. Aliquots (5µL) of PCR product was mixed with gel loading dye (Appendix 2). It was loaded into the wells. For confirmation of amplicon size DNA marker (1 kb DNA marker, Fermentas) were run along with the products. Using Mini gel electrophoresis unit (Genei, Bangalore, India) electrophoresis was performed at a constant volt (5V/cm). With the help of gel documentation system (Syngene, UK) gel pictures were captured.

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5.2.4 MLST analysis

Multi locus sequence type analysis was carried out using the MLST protocols described at the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). DNA was isolated as per (Esteban *et al*., 1993) mentioned in section **3.2.3.5.1.** The MLST primers used for amplification of seven house keeping genes are described in table 5.2.

Genes	Primers	Product size
thrA	F5'-GTCACGGTGATCGATCCGGT-3' R 5'-CACGATATTGATATTAGCCCG-3'	852bp
purE:	F 5'-ATGTCTTCCCGCAATAATCC-3' R 5'-TCATAGCGTCCCCCGCGGATC--3'	510bp
sucA	F 5'-AGCACCGAAGAGAAACGCTG-3' R 5'GGTTGTTGATAACGATACGTAC-3'	643bp
hisD	F 5'-GAAACGTTCCATTCCGCGCAGAC-3' R 5'-CTGAACGGTCATCCGTTTCTG-3'	894bp
aroC	F 5'-CCTGGCACCTCGCGCTATAC-3' R 5'-CCACACACGGATCGTGGCG-3	826bp
hemD	F 5'-ATGAGTATTCTGATCACCCG-3' R 5'-ATCAGCGACCTTAATATCTTGCCA-3'	666bp
dnaN:	F 5'-ATGAAATTTACCGTTGAACGTGA-3' R 5'-AATTTCTCATTCGAGAGGATTGC-3'	833bp

Table 5.2 MLST Primers used in the amplification of the genes and the expected product sizes used in this study (Stepan *et al*., 2011).

PCR Composition

2 mM each dNTPs (Chromous Biotech, India)	2 µL
Forward primer (10 picomoles) (Sigma Aldrich)	2 µL
Reverse primer (10 picomoles) (Sigma Aldrich)	2 µL
Taq DNA polymerase (1U/µL) (Sigma-Aldrich)	1 µL
10X PCR buffer	2 µL
$MgCl_2$ (Sigma-Aldrich)	1.2 µL

Template DNA (50 ng/µL) and sterile distilled water was added to a final volume of 20 µL.

In Thermal Cycler (BioRad MJ Mini Gradient) amplifications were carried out. The program used was a hot start cycle of 94^0C for 30 seconds, followed by 30 cycles of 94^0C for 30 seconds, annealing at 55^0C for 30 seconds and extension at 76^0C for 30 seconds and finally with an extension step of 75^0C for 2 minutes followed by hold at 4°C.

5.2.4.1 Agarose gel electrophoresis

For the analysis of the amplified products 1% agarose gels were prepared (Appendix 2). They were prepared in Tris-Acetate-EDTA (TAE) buffer. To that ethidium bromide was added at a concentration of 0.5mg/mL. Aliquots (5µL) of PCR product was mixed with gel loading dye (Appendix 2). It was loaded into the wells. For confirmation of amplicon size DNA marker (1 kb DNA marker, Fermentas) were run along with the products. Using Mini gel electrophoresis unit (Genei, Bangalore, India) electrophoresis was performed at a constant volt (5V/cm). With the help of gel documentation system (Syngene, UK) gel pictures were captured.

5.2.4.2 Sequencing of DNA

Sequencing of the PCR amplicons of MLST genes were done by the ABI XL DNA analyzer, using the big dye Terminator kit (Applied Biosystems, USA) at SciGenom Cochin, India Ltd. The sequences obtained were interrogated against the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The sequences were then deposited in the Genbank database and accession numbers were obtained (included in list of publications).

5.2.4.3 Multiple sequence alignment and phylogenetic tree construction

Allele sequences for each strain were then concatenated in the order aro C - dna N - hem D - his D - pur E- suc A - thr A (Noda *et al*., 2011). Concatenated sequences were aligned using CLUSTAL W software (Thompson *et al*., 1994) program in BioEdit software (Hall, 1999). Aligned sequences were imported into MEGA 5 (Molecular Evolutionary Genetics Analysis version 5.0) (Tamura *et al*., 2011). To obtain equal lengths the ends were trimmed for all sequences and the aligned sequences were converted into MEGA format for phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

Salmonella Enteritidis S37 has only one BIM 5 in its first set infection. A tree cannot be drawn with only two sequences so their multiple sequence alignment was conducted using CLUSTAL W software (Thompson *et al*., 1994) program in BioEdit software (Hall, 1999).

5.3 Results

5.3.1 Comparison of LPS from phage sensitive hosts and BIMs

LPS was isolated from *Salmonella* Enteritidis strains S49and S37 as well as from its BIMs from three iterations of infection, and the LPS- PAGE was carried out.

The LPS profile of the parental strain *Salmonella* S49, S37 and their BIMs are as shown in Fig 5.1, 5.2, 5.3 and 5.4.

The parental strains *Salmonella* Enteritidis S49 and S37 are smooth strains, with the typical bimodal distribution O-specific polysaccharide, visible in lane 1 of all gels.

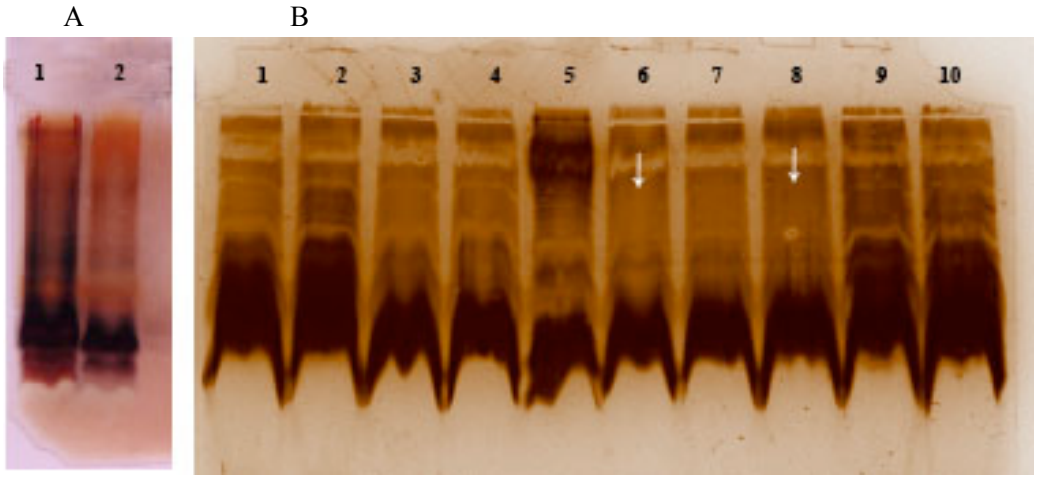


Fig 5.1 LPS profile of *Salmonella* Enteritidis S37, S49 and their first set BIMs

(A) Lane 1-S37, Lane 2-BIM 5;(B) Lane 1 –S49, Lane 2 BIM 1, Lane 3- BIM 2, Lane 4 –BIM 3, Lane 5-BIM 4, Lane 6-BIM 6, Lane 7- BIM 7, Lane 8- BIM 8, Lane 9 – BIM 9, Lane 10-BIM 10

There was not any marked difference in the patterns between the two host strains in the LPS gel. This similarity was observed between the hosts when compared with most of the BIMs. However in certain mutant strains the bands in the lanes indicated by the arrows were missing, when compared with their parent strains.

ΦSP-1 resistant mutant BIM 8 and ΦSP-3 resistant mutant BIM 6 in lanes 6 and 8 respectively, exhibited this absence of bands corresponding to the medium length chain of O- chain as indicated by the arrow. in Fig 5.1.

LPS profile of *Salmonella* Enteritidis S49 andS37 and their second set BIMs is given in fig 5.2

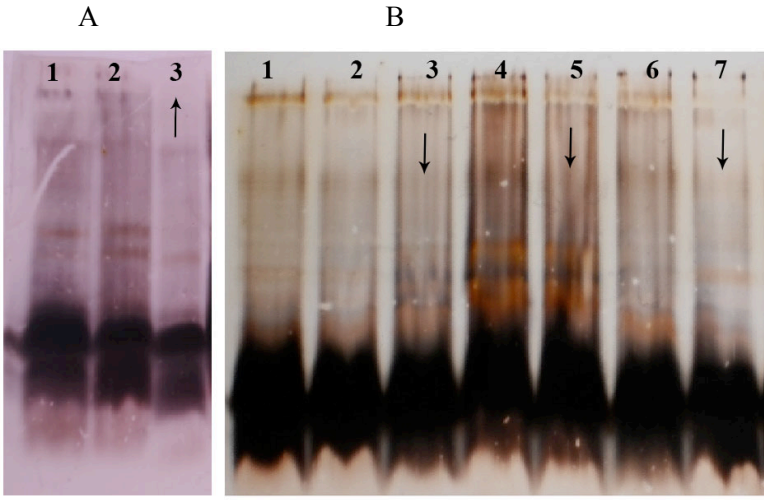


Fig 5.2 LPS profile of *Salmonella* Enteritidis S49, S37 and their second set BIMs

Lane 1-S37, Lane 2- BIM 27, Lane 3-BIM 28, Lane 1- S49, Lane 2- BIM 15, Lane 3- BIM 16, Lane 4-BIM 19, Lane 5-BIM 20, Lane 6- BIM 21, Lane 7- BIM 22.

In the case of S37, its BIM 28 (Fig 5.2(A), comparing the LPS pattern of with those of the others, band part of OAg were missing in BIM 28 was different.

Similarly in second set of BIMs, ΦSP-1 resistant mutant BIM 16 and ΦSP-3 resistant mutants BIM 20 and BIM 22 exhibited the typical rough LPS pattern, with the high molecular weight O-chain missing as indicated by the arrows (lane 3,5 and 7) (in Fig 5.2(B)).

LPS profile of *Salmonella* Enteritidis S49 and its BIMs after the third phage infection (3rd iteration) is depicted in Fig (Annexure). There was no observable change in LPS pattern of third set of BIMs of S49. The LPS profile of *Salmonella* Enteritidis S37 after the 3rd phage infection (third set) and their five BIMs is presented in figure (included in annexure).

Conversely in third set BIMs of S37 there were no differences in LPS pattern when compared to host.

5.3.2 Virulence gene profiling

Sets of seven virulence genes common among *Salmonella* Enteritidis were selected for screening (Table 5.1). Multiplex PCR screening method was used to screen for virulence genes [(*phoP/Q* (299bp), *ttrC* (920bp) and *mgtC* (655bp); *afgA* (261bp), *slyA* (700bp)], and normal PCR for *invE/A* (457bp) and *sopE (~450 and 500 bp)* in the genome of the bacterial host *Salmonella* Enteritidis strain S49, S37 and all their BIMs. The required amplication was obtained in all cases. This indicated the presence of all these virulence genes in both BIMs and their hosts confirming their pathogenic nature Fig 5.3, 5.4 and 5.5. The agarose gel pictures of the S37 and S49 and their first set BIMs only are represented here.

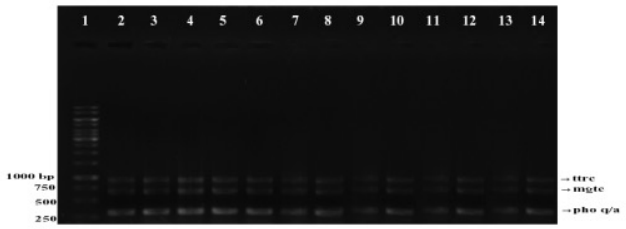


Fig 5.3 Multiplex PCR for detecting ttrc, mgtc and pho q/a in *Salmonella* Enteritidis S37, S49 and their BIMs (Set 1). (ttrc 920 bp, mgtc 655 bp, pho q/a 299 bp). Lane 1 1kb ladder, Lane 2-S37, Lane 3- BIM 5, Lane 4-S49, Lane 5- BIM 1, Lane 6- BIM 2, Lane 7- BIM 3, Lane 8-BIM 4, Lane 9- 1 kb ladder, Lane 10- BIM 6,

Lane 11-BIM 7, Lane 12- BIM 8, Lane 13- BIM 9, Lane 14- BIM 10.

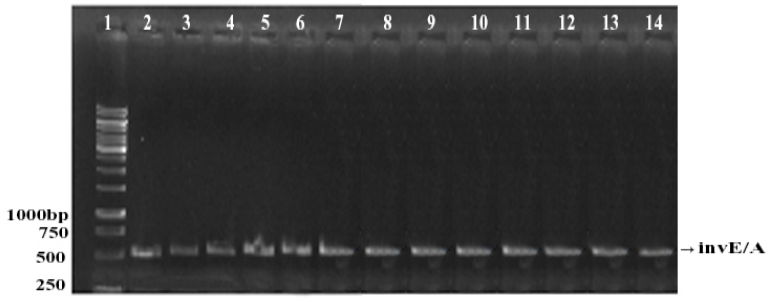


Fig 5.4 PCR for detecting inv A/E (457 bp) *in Salmonella* Enteritidis S37, S49 and their BIMs (Set 1). Lane 1 1kb ladder, Lane 2-S37, Lane 3- BIM 5, Lane 4-S49, Lane 5- BIM 1, Lane 6- BIM 2, Lane 7- BIM 3, Lane 8-BIM 4, Lane 9- 1 kb ladder, Lane 10- BIM 6, Lane 11-BIM 7, Lane 12- BIM 8, Lane 13- BIM 9, Lane 14- BIM 10.

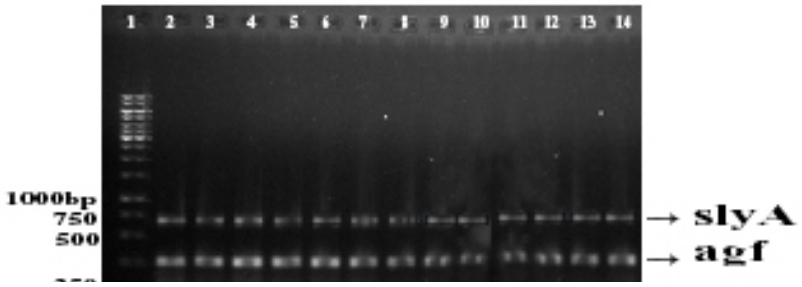


Fig 5.5 PCR for detecting sly A and agf in *Salmonella* Enteritidis S37, S49 and their BIMs (Set 1). Lane 1 1kb ladder, Lane 2-S37, Lane 3- BIM 5, Lane 4-S49, Lane 5- BIM 1, Lane 6- BIM 2, Lane 7- BIM 3, Lane 8-BIM 4, Lane 9- 1 kb ladder, Lane 10- BIM 6, Lane 11-BIM 7, Lane 12- BIM 8, Lane 13- BIM 9, Lane 14- BIM 10.

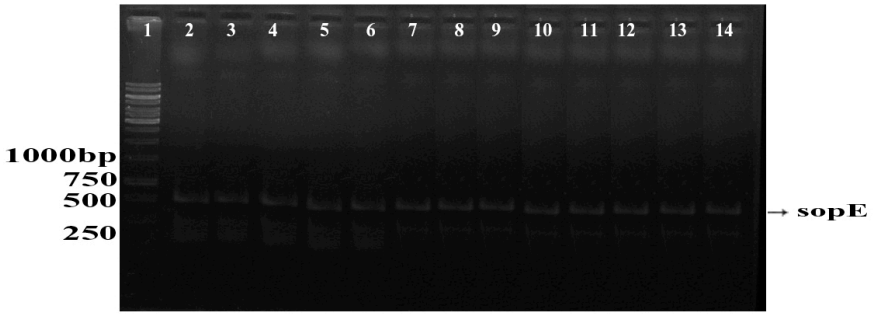
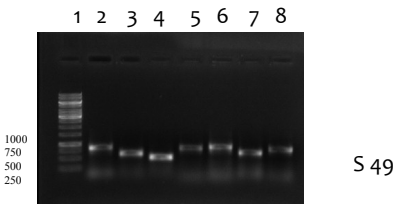


Fig 5.6 PCR for detecting sop E *Salmonella* Enteritidis S37, S49 and their BIMs (Set 1) using gene primers. Lane 1 1kb ladder, Lane 2-S37, Lane 3- BIM 5, Lane 4-S49, Lane 5- BIM 1, Lane 6- BIM 2, Lane 7- BIM 3, Lane 8-BIM 4, Lane 9- 1 kb ladder, Lane 10- BIM 6, Lane 11-BIM 7, Lane 12- BIM 8, Lane 13- BIM 9, Lane 14- BIM 10.

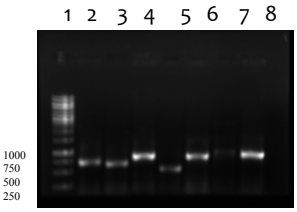
5.3.3 MLST analysis

The seven housekeeping genes used for the MLST were PCR amplified with their primers. The agarose gel for the amplicons obtained for each host and their BIMs (set I only) are shown in Fig 5.7.



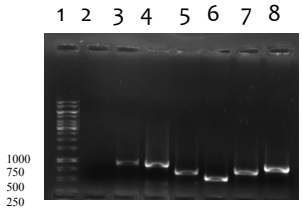
S 49

Lane 1-1kb ladder, Lane 2-thrA,Lane 3-sucA,Lane 4-purE,Lane5-dnaN,Lane6-hisD,Lane7-hemD,Lane 8-aroC



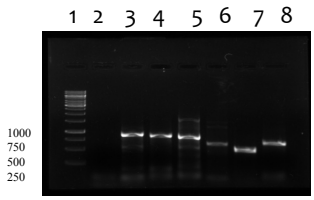
BIM 1

Lane1-1kbladder,Lane2- hemD , Lane3- sucA , Lane4 – dnaN, Lane5- purE , Lane 6- aroC , Lane7-thrA , Lane8- hisD .



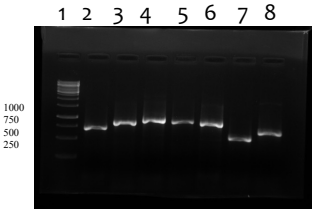
BIM 2

Lane1-1kbladder,Lane2- hisD , Lane3- thrA , Lane4 - aroC , Lane5- sucA , Lane 6- purE , Lane7- hemD Lane8- dnaN.



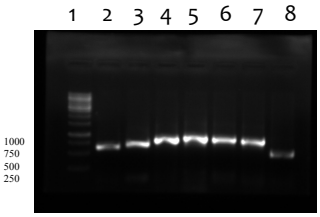
BIM 3

Lane1-1kbladder,Lane2- dnaN, Lane3- hisD , Lane4 - thrA , Lane5- aroC , Lane 6- sucA , Lane7- purE , Lane8- hemD



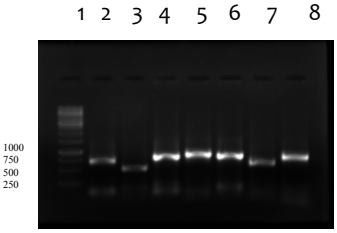
BIM 4

Lane1-1kbladder,Lane2- hemD , Lane3- dnaN, , Lane4 - hisD , Lane5- thrA , Lane 6- aroC , Lane7- purE , Lane8- sucA .



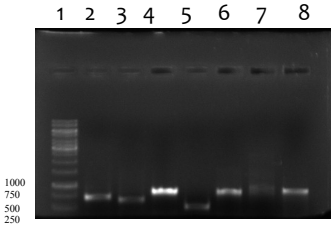
BIM 6

Lane1-1kbladder,Lane2- sucA , Lane3- hemD , , Lane4 - dnaN, Lane5- hisD , Lane 6- thrA , Lane7- aroC , Lane8- purE .



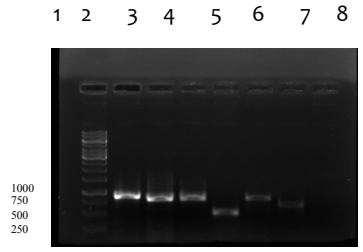
BIM 7

Lane1-1kbladder,Lane2- hemD , , Lane3- purE , Lane4 - aroC , Lane5- hisD , Lane 6- thrA , Lane7 sucA - Lane8- dnaN.



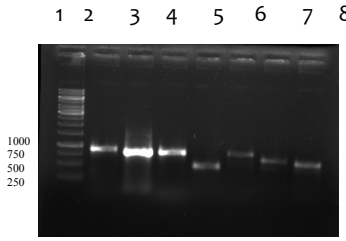
BIM 8

Lane1-1kbladder,Lane2- hemD , Lane3- sucA , Lane4 – dnaN, Lane5- purE , Lane 6- aroC , Lane7-thrA , Lane8- hisD .



BIM 9

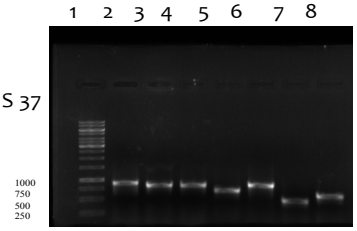
Lane1-1kbladder,Lane2- hisD , Lane3- aroC, Lane4 - dnaN,Lane5- purE , Lane 6- thrA, Lane7- hemD Lane8- sucA



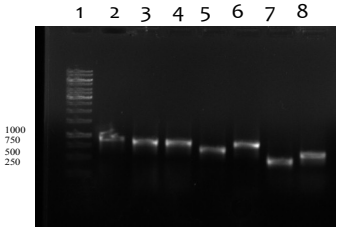
BIM 10

Lane1-1kbladder,Lane2- hisD , Lane3- aroC, Lane4 - dnaN,Lane5- purE , Lane 6- thrA, Lane7- hemD Lane8- sucA.

S 37



Lane1-1kbladder,Lane2-hisD,Lane3-dnaN,Lane4-aroC,Lane5-hemD,Lane 6-thrA,Lane7-purE,Lane8-sucA



BIM 5

Lane1-1kbladder,Lane2-hisD,Lane3-dnaN,Lane4-aroC,Lane5-hemD,Lane 6-thrA,Lane7-purE,Lane8-sucA

Fig 5.7 MLST Amplicons obtained for S49, S37 and their first set BIMs after PCR amplification.

The PCR primers designed for the *S.* Enteritidis MLST scheme are listed in table 5.2. Candidate genes were selected based on previously published genotyping schemes for members of the *S. enterica* complex. The present MLST scheme provided better discrimination of *Salmonella* serovar Enteritidis strains. The sequence obtained were anlaysed at the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). They were deposited in GenBank and accession numbers were obtained (KT855092 - KT855175) (included in list of publications).

Locus	thrA	sucA	purE	hisD	hemD	dnaN	aroC
Primer Product size (bp)	852	643	510	894	666	833	826
Allelic profile obtained (bp)							
Salmonella Enteritidis S49	501	501	399	501	432	501	501
BIM 1	501	501	399	501	432	501	501
BIM 2	501	501	399	501	432	501	501
BIM 3	501	501	399	501	432	501	501
BIM 4	501	501	399	501	432	501	501
BIM 5	501	501	399	501	432	501	501
BIM 6	501	501	399	501	432	501	501
BIM 7	501	501	399	501	432	501	501
BIM 8	501	501	399	501	432	501	501
BIM 9	501	501	399	501	432	501	501
BIM 10	501	501	399	501	432	501	501
Salmonella Enteritidis S37	501	501	399	501	432	501	501
BIM 5	501	501	399	501	432	501	501

Table 5.3 Characteristics of *Salmonella* Enteritidis S49 and S37 and their first set BIMs MLST loci.

MLST analysis did not detect any nucleotide differences among the *S.* Enteritidis strains for the seven genes analysed (*thrA*: 501 bp, *pure*: 399 bp, *sucA*: 501

bp, *hisD*: 501 bp, *aroC*: 501 bp, *hemD*: 432 bp, and *dnaN*: 501 bp). Characteristics of seven MLST loci are provided in table 5.3.

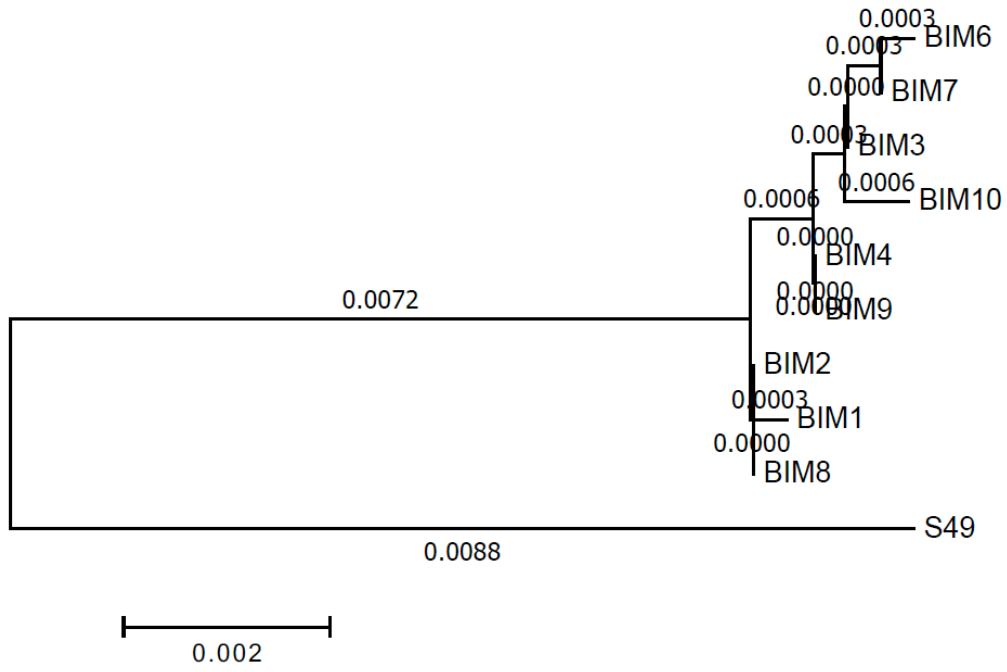


Fig 5.8 Phylogenetic tree of MLST sequences of *Salmonella* Enteritidis S49 and its first set BIMs. The numbers at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on Neighbour-joining analysis of 1000 resampled data set.

After sequence analysis, the phylogenetic tree was built following concatenation of the sequences. From the phylogenetic tree (Fig 5.8), it can be observed that the host bacteria and its BIMs were grouped together to form a single clade, and their percentage of difference is not substantial, pointing to the fact that

they were related. As it is MLST it can be said *Salmonella* Enteritidis S49 and it's BIMs all showed a single sequence type lineage. BIM 3, 6, 7 and 10 were BIMs of ΦSP-3 infection and BIM 1, 2,4,8 and 9 are BIMs of ΦSP-1 infection.

Only multiple sequence alignment was performed for BIM 5 of *Salmonella* Enteritidis S37 obtained by ΦSP-2 infection.

As expected, the MLST did not show any unusual variation, as variations in the house keeping genes could severely affect the viability of the BIM.

5.4 Discussion

5.4.1 LPS pattern of strain S49, S37 and their BIMs

Lipopolysaccharide (LPS) is a major component of Gram-negative bacterial outer membranes. It is a tripartite molecule consisting of lipid A, which is embedded in the outer membrane, a core oligosaccharide and repeating O-antigen units that extend outward from the surface of the cell (Davis and Goldberg, 2012). In *Salmonella*, the LPS itself can possess different modifications on the three components of the molecule (Herman, 2015). Resistance to complement-mediated killing is a recognized virulence trait of *Salmonella* and LPS is an important determinant of this virulence (Siggins, 2012).

Growth of sensitive bacteria in the presence of phages rapidly selects for the appearance of mutants that have developed resistance to the co-occurring phages. As has been mentioned in literatures, phage receptors are commonly protein or LPS components of the cell surface and mutations leading to phage resistance usually alter such cell surface structures. LPS has been implicated as receptors for a number of phages in many different genera (Whitton and Potts, 2012). For example, LPS is

known to act as a receptor for P22 in *Salmonella* Typhimurium, for Sf6 in *Shigella flexneri*, for T2 and T4 in *Shigella dysenteriae,* bacteriophages T3, T4, and T7 in *E. coli*, for ΦYeO3-12 and ΦR1-37 in *Yersinia enterocolitica* and for K139 in *Vibrio cholerae* (Lindberg, 1973; Prehm *et al*., 1976; Krüger and Schroeder, 1981; al-Hendy *et al.,* 1991; Baxa, 1996; Chua *et al*., 1999; Nesper *et al*., 2000; Pajunen *et al*., 2000; Kiljunen *et al.,* 2005; Pinta, 2010).

For these reasons, it was important to visualize LPS, hence to investigate the correlation between LPS and phage resistance, in the present study LPS of the parental strains *Salmonella* S49, S37 and their phage-resistant mutants were analyzed.

In this study LPS was extracted using proteinase K digestion (Hitchcock, 1984). This protocol allows for the extraction of LPS away from nucleic acids and proteins that can interfere with visualization of LPS that occurs with shorter, less intensive extraction methods. LPS prepared in this way was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Apicella, 2008) and directly stained by silver staining (Tsai and Frasch, 1982).

The outermost element of the LPS structure is the hydrophilic O-specific chain, which is built up from 1-40 repeating units (usually consisting of 2-7 sugar components) resulting in diverse composition. The repeating units are unique and characteristic for the bacteria, resulting in a large variety of antigenicity, which is the base of the serological grouping of Gram-negative bacteria. However, because of the inevitably occuring common monosaccharide components in the O- specific chain of LPSs in taxonomically different bacterial species, serological crosslinkages may appear, causing interferences in the serological identification of disease-causing bacteria (Schromm *et al*., 2000; Jerala, 2007). The presence or absence of the O-

specific chains determines the phenotype of the LPS: *R*-type, where the O-specific chain is absent, resulting in *rough* surface of the bacterial colonies. Bacterial strains with *R*-type LPSs are non-pathogenic mutant strains; *S*-type, where the O-specific chain is present with various length, resulting in *smooth* surface of the bacterial colonies. Bacterial strains with *S*-type LPSs are pathogenic wild-type strains (Bui, 2012).

The host bacteria both S49 and S37 had LPS with long O-specific polysaccharide *i.e.*, the typical smooth type LPS. And most of the BIMs didn't show a clear difference in LPS pattern when compared to hosts. However some resistant forms possessed typical bimodal distribution of O-antigen polysaccharides corresponding to the smooth phenotype (Santander and Robeson, 2007) where some of the bands corresponding to medium length O- polysaccharides were missing in the LPS of the resistant forms.

LPS-defective Gram-negative bacteria can be brought to emergence by phage treatments or phase variations. *Vibrio cholerae* phage K139, which used the O1 antigen as a receptor, selected the phage-resistant mutants that were defective in the biosynthesis of the O1 antigen or core OS and the treatment of three virulent *S.* Enteritidis phages generated the phage-resistant variants with rough phenotypes (Kim *et al*., 2014).

It was demonstrated that virus HF6S was able to bind to *Salmonella* flexineri strains containing O antigen in LPS, mutants lacking O antigen are resistant to the phage (Rakhuba *et al*., 2010).

Chart *et al*. (1989) showed that the loss of LPS in *Salmonella* produces changes in bacteriophage susceptibility and the loss of virulence in mice. More recent work has shown that strains of *Salmonella enterica* serovar Enteritidis resistant to the lytic phages *f2α*SE, *f3α*SE, and *f18α*SE are avirulent in the nematode *Caenorhabditis elegans*. The bacteriophage resistant strains show abnormal colony morphology and lack the O-polysaccharide from LPS, suggesting that modifications in this structure may be involved in resistance and the loss of virulence (León and Bastías, 2015).

A similar situation was observed with the strain Salp572ϕ1S of *Salmonella enterica* serovar Paratyphi B and bacteriophage ϕ1 (Capparelli *et al*., 2010). A bacteriophage resistant strain called Salp572ϕ1R lacks the O-polysaccharide from LPS, and gene expression analysis has revealed that Salp572ϕ1R under-expresses several genes related to virulence, such as *cmE, sthE,* and *cheY* (periplasmic heme-dependent peroxidase, a putative major fimbrial subunit, and a chemotaxis regulator, respectively). Moreover, Salp572ϕ1R was completely avirulent in mice; whereas the parental sensitive strain Salp572ϕ1S killed the mice after 48 h of infection. These examples strongly suggest that modifications in LPS can produce bacteriophage resistance and decrease virulence simultaneously.

Earlier studies indicated that the rough type (OAg negative) strains have increased efficiency of adhesion and invasion into non-phagocytic cells (Ilg *et al*., 2009). It was reported that the outer core sugars of LPS are crucial for the interaction of the bacteria with the epithelial cells, therefore, in the absence of OAg, early interaction of core sugars with epithelial cells is facilitated contributing to increased adhesion (Bravo *et al*., 2011; Hoare *et al*., 2006). Holzer *et al*. (2009) proposed that long and very long OAg interferes with the interaction of type III secretion complex with the host cell membrane and impairs the translocation of effector proteins from

bacteria to host cell. Thus, in the absence of OAg, this interference is abolished resulting in the increased efficiency of invasion.

The O-antigen negative Δ wbaV mutant of *Salmonella enterica* serovar Enteritidis showed adaptive resistance to antimicrobial peptides and elicits colitis in streptomycin pretreated mouse model (Jaiswal *et al*., 2015). These mutants showing increased invasion also showed increased adhesion. Therefore, it was proposed by the authors that increased adhesion efficiency along with the enhanced type III secretion system functionality contributed to the significantly increased invasion efficiency to OAg-negative mutants. Notably, in spite of increased invasiveness, the O-Ag negative mutants did not show increased colonization of cecum suggesting, invasiveness alone does not determine the colonization capacity of *Salmonella* (Jaiswal *et al*., 2015)..

In this study variation in pattern of LPS could be seen when compared the host bacteria with their BIMs. Moreover it was observed that rough phage-resistant mutants of *Salmonella*, are much less able to colonize the alimentary tract of poultry (Craven, 1994; Turner *et al.,* 1998). Besides, regarding the cocktail of phages used in the study (next chapter), it is proposed that cross lysing of the BIMs generated, may be by targeting different receptors, this is an effective bio control strategy.

5.4.2 Virulence gene profiling of host bacteria and BIMs

Bacterial pathogens exhibit significant variation in their genomic content of virulence factors. This reflects the abundance of strategies evolved by pathogens to infect host organisms by suppressing host immunity (Jackson *et al*., 2011). Molecular arms races have been a strong driving force for the evolution of pathogenicity, with pathogens often encoding overlapping or redundant functions, such as type III protein

secretion effectors and hosts encoding ever more sophisticated immune systems. The pathogens, frequent exposure to other microbes, either in their host or in the environment, provides opportunities for the acquisition or interchange of mobile genetic elements (Hwang *et al*., 2015).

Seven virulence genes common among *Salmonella* Enteritidis were screened in the current study in *Salmonella* strains S49, S37 and their BIMs. The PCR screening method demonstrated the presence of the following virulence genes: *invE/A*(457bp*), afgA*(261bp), *slyA*(700bp), *phoP/Q*(299bp), *ttrC*(920bp), *mgtC* (655bp) and *sopE (*~450-500bp) in the genome of the bacterial host *Salmonella* Enteritidis strain S49, S37 and their BIMs proving beyond doubt the pathogenic nature of this bacteria. The same screening also confirmed the absence of these virulence genes in the ΦSP-1, ΦSP-3 and ΦSP-2 genome (Augustine and Bhat, 2012). However full genome sequencing is obligatory to completely negate the possibility of these three phages harboring other virulence genes, this outcome takes these phages a step forward towards their candidature as biocontrol agents against *Salmonella* Enteritidis (Augustine *et al*., 2013).

In the present study *invE/A*(457bp) was detected in both bacteria and their BIMs. There have been reports of this gene in *Salmonella* Enteritidis earlier also. For detection of pathogenic *Salmonella* isolated from meat and poultry products by detecting virulence *invE/*A gene using PCR technique, all *Salmonella* isolates, (among them 1/16 were *Salmonella* Enteritidis) were positive for the presence of *invE/A* gene which is responsible for invasion of cells, have the capacity to invade and survive in macrophages (Karmi, 2013). Invasion gene was detected in all *Salmonella* Enteritidis isolated from human and animals in Iran (Amini *et al*., 2010) as this gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required

for invasion of deeper tissue.

Virulence genes *afgA*(261bp) and *slyA*(700bp) were detected by multiplex PCR in this study, this was also present in both host bacteria and their BIMs. Gene *agfA* was present in 96% (3/84) of *Salmonella* Enteritidis isolates from chicken in South of Brazil (Borges *et al*., 2013) highlighting the importance of fimbriae in the infection process. It is possible that there are additive effects of *agfA* in the colonization of the intestine and systemic virulence (Wagner and Hensel, 2011). Salehi *et al*. (2011) indicated the presence of *agfA* virulence gene in 25 *Salmonella* Enteritidis isolated from avian feces by multiplex PCR, suggesting that high expression level of fimbrial genes following the bacterial junction to surface of intestine, logically preserve *Salmonella* from fluid flow and prepares it for colonization and proliferation which is followed by activation of other virulent factors resulting in propagation of *S*. Enteritidis.

In molecular characterization of emergent multiresistant *Salmonella* enterica serotype [4, 5, 12], all were found to be positive for this *slyA* (cytolysin) gene (Guerra *et al*., 2000). A *Salmonella* gene encoding a cytolysin was detected in screening for hemolysis on blood agar from three *Salmonella* Typhimurium serotypes along with *Shigella* and *E-coli* (Libby *et al*., 1994). They suggested that *slyA* proteins encoded by this gene are selectively induced in *Salmonella* serovar Typhimurium during infection of macrophages, and it has been suggested that these proteins may stabilize bacterial macromolecular complexes after exposure to the macrophage's toxic and degradative products.

By multiplex PCR the virulence genes *phoP/Q*(299bp), *ttrC*(920bp) and *mgtC* (655bp) were screened in host bacteria and their BIMs, in all there were presence of these genes. For detection of 14 chromosomally and 1 plasmid-located virulence genes, a total of 80 strains of *Salmonella* enterica serovar Enteritidis, causing

gastroenteritis (G) or bacteraemia (B), and three control strains (C), were subjected to PCR, a number of B strains were found to carry the phoP/Q gene (Soto *et al*., 2006). It is believed that by monitoring extracellular Mg^{2+}, PhoP/Q allows *Salmonella* to sense the transition from an extracellular environment to a subcellular location and to activate a set of virulence factors, which are required for intracellular infection (Gal-Mor *et al*., 2011). In a study to determine the prevalence of *Salmonella* in bile of cattle by conventional cultivation tecniques and to verify the isolates by the detection of phoP/phoQ (phoP/Q) gene by PCR, all isolates were positive for this gene (Goncuoglu *et al*., 2013). *Salmonella* spp. can respond to bile to increase resistance and that this response likely includes proteins that are the members of PhoP regulon. These PhoP-PhoQ regulated products may play an important role in the survival of *Salmonella* spp. in the intestine or gallbladder (van Velkinburgh and Gunn, 1999).

To determine the presence of genes of the pathogenicity islands 1 to 5 (SPI-1 to 5), in *Salmonella, a* total of 125 strains of *S. enterica* belonging to different serovars, were isolated from various clinical samples, the most represented serovar was Typhimurium with 53 isolates followed by 27 isolates of Enteritidis, and detected by PCR, the strains tested positive for ttrC and mgtC gene (Sánchez-Jiménez *et al*., 2010). To determine the presence of *Salmonella* in chicken consumed in Tunisian military canteens, Gritli *et al*. (2015) screened the 24 isolates of *Salmonella* Enteritidis by PCR for virulence genes, isolated from chicken carcasses, all isolates tested positive for *ttrC* (SPI-2) and *mgtC* (SPI-3). The MgtC is a virulence factor in *Salmonella* Typhimurium that is required for growth at low-Mg^{2+} concentrations and intramacrophage survival. This gene is codified in a conserved region of the *Salmonella* Pathogenicity Island 3 (SPI-3), and is also present in the chromosome of other *Salmonella* serovars (Retamal *et al*., 2009). Tetrathionate reduction is an

important bacterial anaerobic respiratory process, *ttrC* is the tetrathionate reductase structural gene within SPI-2 and important in host invasion (Hensel *et al*., 1999).

Virulence gene *sopE (*~450-500bp) was detected in both host bacteria and their BIMs in the present study. In a study to investigate why *Salmonella* Gallinarum is less invasive in *in vitro* than *Salmonella* Enteritidis (strains were provided by the WHO Collaborating Center for *Salmonella* (Pasteur Institute, Paris, France), Rossignol *et al*. (2014) screened the virulence gene sopE along with other genes, all *S*. Enteritidis isolates were positive for this gene. This is encoded by *Salmonella* Pathogenicity Island 1 and trigger extensive rearrangements of the actin cytoskeleton leading to marked membrane ruffling and bacterial internalization (Patel and Galan, 2005). Ekateriniadou *et al*. (2015) detected and determined the distribution of sodCI, sopE and sefA genes from thirty-five *S*. Enteritidis isolates from one-day chicks, layers and broilers, all thirty-four (97,2%) isolates carried the sopE gene. Also the sopE gene has been detected in different phage types of *S*.Enteritidis and that may contribute for the expression of *Salmonella* invasion by stimulating membrane ruffling (Hopkins and Threlfall, 2004; Rahman *et al*., 2004).

Here in this study the BIMs also carried the virulence genes in their genome from their hosts, this clearly indicate that BIMs are equally virulent as their host bacteria. The study was conducted to detect whether there was any difference in virulence genes pattern of host bacteria and their BIMs, but no observable variation could be found in this study.

Bacterial evolution includes the modification of existing functions and the acquisition of new ones. Phages are a primary driving force behind the evolution of bacterial pathogens by transferring a variety of virulence genes into their hosts (Hanna *et al*., 2012). As opposed to temperate phages, lytic phages are not commonly associated with increased bacterial virulence. However, lytic phages have been shown

to select for more co-operative bacteria producing extracellular products ('public goods') that are crucial for growth and virulence (Örmälä-Odegrip, 2015).

Possible costs on virulence and the subsequent lowered virulence is especially intriguing in the context of phages having been championed as an alternative antibacterial agent in treating bacterial infections. In fact, the appearance of less virulent or avirulent phage-resistant bacteria has been observed in association with phage based antibacterial therapy trials with *Escherichia coli* and *Vibrio cholerae* (Smith and Huggins, 1983; Zahid *et al*., 2008).

5.4.3 MLST analysis

MLST is a useful method for analyzing the core genes of pathogens that are of public health importance. It can be used to analyze conserved core genes, like housekeeping genes, which are universally conserved and provide fundamental genetic information (Dixit and Shanker, 2009). MLST detects the variation that accumulates very slowly in the population and likely to be selectively natural (Kidgell *et al*., 2002). This method is now considered as a gold standard in the characterization of bacterial strains and can be used to ascertain the clonality across the geographical regions (Dahiya *et al*., 2013). In MLST, the variability in a relatively small part of the genome due to mutation or recombination events is investigated through the comparison of nucleotide base changes in multiple genes with conserved sequences, such as housekeeping genes (Hyeon *et al*., 2013). The aim in this study was to look any changes that may occur due to phage host interaction.

In the present study seven housekeeping genes of the host *Salmonella* Enteritidis S49, S37 and their BIMs were amplified using primers for these genes and

the product obtained were confirmed by using the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Then the allele sequences for each strain were then concatenated in the order aro C - dna N - hem D - his D - pur E- suc A - thr A, the concatenated sequences were then aligned using CLUSTAL W software (Thompson *et al*., 1994) and phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps. The phylogenetic tree was constructed with the MLST sequences of the host bacteria *Salmonella* Enteritidis S49 and their first set BIMs in order to find out whether there is any variation in the core seven housekeeping genes. Only multiple sequence alignment was performed for *Salmonella* Enteritidis S37 and its single BIM 5. From the phylogenetic tree it was observed that there was no substantial difference between the seven housekeeping genes of host and its BIMs.

In this study MLST was selected to study the variation of seven housekeeping genes in the host bacteria and its BIMs as conserved genes are present across bacterial genomes of the same species (or genus). A fraction of these genes, those conserved in all (or most) of the genomes of a given bacterial taxonomic group is called the 'core-genome' of that group. The core-genome can be identified either within a genus or species (Malorny *et al*., 2011) and can be used to identify the variable genes in a given genome. In addition, the conserved genes in general appear to evolve more slowly, and can be used for determining relationships among bacterial isolates (Urwin and Maiden, 2003).

Hyeon *et al*. (2013) used seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) for differentiating *Salmonella enterica* serovar Enteritidis isolates obtained from food and human sources. In previous studies using the MLST

database (Ben-Darif *et al*., 2010; Torpdahl *et al*., 2005), MLST was used to correlate *Salmonella* serotypes.

Noda *et al.* (2011) found that 30 *S*. Enteritidis isolates collected in Japan between 1973 and 2004 had homologous MLST sequences and no nucleotide differences in seven housekeeping genes. Another study targeting seven different housekeeping and virulence genes found that MLST was not able to discriminate clinically relevant serotypes of *Salmonella* well (Sukhnanand *et al*., 2005).

The limited discriminatory ability of MLST may be a result of the moderate to slow rate of mutation accumulation within the targeted housekeeping genes (Harbottle *et al*., 2006). Therefore, the discriminatory performance of MLST will be increased if more variable gene targets are examined.

For investigating genomic variation in *Salmonella enterica* core genes for epidemiological typing the seven housekeeping genes were amplified and a phylogenetic tree was constructed, they used *in silico* MLST in their study in which complete genes are used and not just the MLST alleles. However, since the alleles typically cover the majority of the genes, the difference was small. It was reported that the core genes could be divided into two categories: a few highly variable genes and a larger set of conserved core genes, with low variance. For the most variable core genes, the variance in amino acid sequences is higher than for the corresponding nucleotide sequences, suggesting that there is a positive selection towards mutations leading to amino acid changes (Leekitcharoenphon *et al*., 2012)

To the best of our knowledge this is the first study conducted in order to differentiate or find out the variations in the housekeeping genes between host bacteria and its bacteriophage insensitive mutants.

Thus this study was performed to observe whether there are any variations in the LPS pattern, virulence genes and seven housekeeping genes of host bacteria and

BIMs using MLST. Variations were observed in certain BIMs in LPS pattern, which proves the role in phage adsorption. LPS structures are a key determinant of resistance/susceptibility to coevolving phages and that coevolution with phage drives variation in LPS structure. It is not known exactly how these changes in LPS structure affect resistance, but it is possible that mutations conferring a very short LPS phenotype result in the loss of the phage receptor, whereas mutations conferring a long LPS phenotype mask the receptor site or result in structural changes that prevent access to the receptor. But it helps to explain how LPS, as the parasite-binding site, can support long-term coevolutionary dynamics in this system. No observable variations in virulence genes were noticed. However this study have confirmed the ubiquitous distribution of virulence genes amongst *Salmonella* spp irrespective of the host or its BIMs. The PCR method using target gene remains a suitable molecular tool to diagnose *Salmonella* in human, animal and plant products. These findings have important health implications to the entire populace considering the high prevalence of virulence genes in food samples studied and it also underscores the need for rapid identification of *Salmonella* virulence genes using the PCR method. And the seven house keeping genes of host and BIMs were found to be related with no substantial differences. Anyways, serotyping inferred from 7-gene MLST results derived from data is an accurate, robust, reliable, high throughput typing method that is well suited to routine public health surveillance of *Salmonella*. This approach supports the maintenance of traditional serovar nomenclature and provides further insight on the true evolutionary relationship between isolates, as well as a framework for fine level typing within eBGs for surveillance, outbreak detection and source attribution. This also supports the premise that phage therapy may not necessarily lead to more virulent mutant forms of the bacteria.

Chapter 6

Application of phage cocktails to combat the Bacteriophage Insensitive Mutants

6.1 Introduction

Salmonellosis is an important and persistent cause of diarrheal diseases among humans in developing countries (Taha *et al*., 2015). *Salmonella* spp. can colonize a wide range of hosts and all the major livestock species (poultry, cattle, and pigs) could become colonized, frequently asymptomatically, ultimately producing contaminated meat and other food products (Newell *et al*., 2010). Among over 2500 *S. enterica* serotypes, *Salmonella enterica* subsp. serovar Enteritidis (SE) is unique owing to its ability to efficiently contaminate the internal contents of eggs produced by otherwise healthy hens at a frequency associated with substantial food-borne disease (Guard *et al*., 2015). Indiscriminate use of antibiotics brought about increasing resistance to commonly used antibiotics (Alam and Bhatnagar, 2006). The development of plasmid-encoded multidrug resistant *Salmonella* has been a noteworthy evolution in antimicrobial resistance (Suh Yah, 2010). Mengistu *et al.* (2014) indicated that 27.5% of *Salmonella* isolates were multi-drug resistant (MDR) bacteria. Therefore, an escalating need is evident for finding substitute ways for combating serious cases of salmonellosis.

Phage therapy or more specifically, therapeutic use of lytic bacteriophages to cure pathogenic bacterial infections is one approach that has great potential as a solution to the severe worldwide problem of drug-resistant bacteria (Moradpour and Ghasemian, 2011). The special features of lytic bacteriophages, in particular their target specificity, rapid bacterial killing, and capacity to self-replicate, make them

especially suitable for food protection applications. Certainly, the use of bacteriophages in the biological control of different pathogens has been studied in chicken (Spricigo *et al*., 2013). One of the downsides of using phages is the emergence of Bacteriophage Insensitive Mutants (BIMs), which occurs rapidly if only one phage strain was used against a particular bacterium (Tanji *et al*., 2004). Luckily, there is an abundance of other phage species, which possess lytic ability against resistant variants (Pirisi, 2000).

The therapeutic use of phage cocktail, in which two or more phage types are combined to produce more pharmacologically diverse formulations, has been recently considered. The primary motivation for the use of cocktails is their wide spectra of activity compared to individual phage isolates. They may impact either more bacterial types or acquire effectiveness under a greater diversity of conditions. The combining of phages can also expedite better targeting of multiple strains of a bacterial species also cover multiple species that might be responsible for similar diseases. Phage cocktails in general, have greater potential for presumptive or empirical treatment (Chan and Abedon, 2012).

This study demonstrates that the BIMs which developed when *Salmonella* Enteritidis S49 and S37 was treated with its specific phage ΦSP-1, ΦSP-3 and ΦSP-2, separately could be controlled when both phages were combined and used as a phage cocktail.

6.2 Materials and Methods

6.2.1 BIMs used for study

Salmonella Enteritidis (BIM 37) previously isolated (described in **3.2.3**) from host bacteria *Salmonella* Enteritidis S49 by infecting with its specific lytic phage, namely ΦSP-1 was used a representative of the Φ SP-1 mutants for this study.

Similarly *Salmonella* Enteritidis (BIM 43) which was also isolated from *Salmonella* Enteritidis S49 by infecting with its specific lytic phage ΦSP-3 was used as candidate from ΦSP-3 based mutants. BIM 32 also isolated from *Salmonella* Enteritidis S37 targeted by its specific lytic phage ΦSP-2 was selected. The BIMs were selected randomly.

6.2.2 Bacteriophages used for the study of phage cocktail

ΦSP-1 and ΦSP-3 the specific lytic bacteriophages of *Salmonella* Enteritidis S49 was employed to study the efficacy of phage cocktail in reducing the mutant count. It was used both for *Salmonella* Enteritidis BIM 37 and BIM 43. ΦSP-2 another specific lytic phage of *Salmonella* Enteritidis S37 was used along with a new lytic phage which belong to the family *Siphoviridae* isolated from chicken gut of broiler chicken namely STP1 (already available in the lab) (unpublished data). They were both used as cocktail to reduce the mutant count of *Salmonella* Enteritidis BIM 32.

6.2.3 Phage cocktail assay using host cell lysis test

The competency of phage cocktails in *Salmonella* specific bacteriolysis was studied by the host cell lysis test (Hsieh *et al*., 2011). The experimental set up consisted of four groups (Table 6.1). Overnight cultures of BIMs were diluted 1:100 in fresh nutrient broth and grown until log phase. One milliliter of this culture (10^8 PFU mL^{-1}) was then transferred to fresh nutrient broth and infected with respective phage lysate. In case of group 2 and group 3, 10^{10}PFU mL^{-1} of lysate was added, and in case of group 4, each phage was added at 5×10^9PFU mL^{-1}. Final volume was adjusted with nutrient broth. All mixtures were incubated at 37°C at 120 rpm (Orbitek, Scigenics, India). Readings were taken at definite intervals from all the sets and was read at 600 nm spectrophotometrically (Shimadzu, Japan), to check for cell lysis. The

experiments were conducted in triplicates

	Experimental groups		
Groups	**BIM 37**	**BIM 43**	**BIM 32**
Group 1	*Salmonella* Enteritidis BIM 37 control	*Salmonella* Enteritidis BIM 43 control	*Salmonella* Enteritidis BIM 32 control
Group 2	*Salmonella* Enteritidis (BIM 37) and ΦSP-1	*Salmonella* Enteritidis (BIM 43) and ΦSP-1	*Salmonella* Enteritidis (BIM 32) and ΦSP-2
Group 3	*Salmonella* Enteritidis (BIM 37) and ΦSP-3	*Salmonella* Enteritidis (BIM 43) and ΦSP-3	*Salmonella* Enteritidis (BIM 32) and STP1
Group 4	*Salmonella* Enteritidis (BIM 37), ΦSP-1 and ΦSP-3	*Salmonella* Enteritidis (BIM 43), ΦSP-1 and ΦSP-3	*Salmonella* Enteritidis (BIM 32), ΦSP-2 and STP1

Table 6.1 Bioassay of phage cocktails with BIMs

6.2.4 Statistical analysis

Using ANOVA statistical evaluations were done accompanied by Students–Newman–Keul Test using GraphPad InStat (version 2.04a, San Diego, USA) computer program.

6.3 Results

6.3.1 Phage cocktail treatment of *Salmonella* Enteritidis BIM 37

Salmonella Enteritidis (BIM 37) was used as a candidate to study the efficacy

of phage cocktail comprising ΦSP-1 and ΦSP-3 to mitigate the mutant. Fig 6.1 shows the outcome of the phage cocktail study. The figure represents the mortality of BIM 37 when exposed to the influence of the bacteriophages individually and in combination. BIM 37 at 1 h, showedOD_{600}= 0.3 and at the end of the experiment it was 0.87 as there was no phage infection or phage lysis. Conversely in the presence of ΦSP-1 and ΦSP-3 the OD values reduced to 0.43, 0.38 respectively while in the presence of both phages it further decreased to 0.33. Values represent mean ± SD, and in all the cases, $P < 0.001$ when compared to group A, indicating high significance.

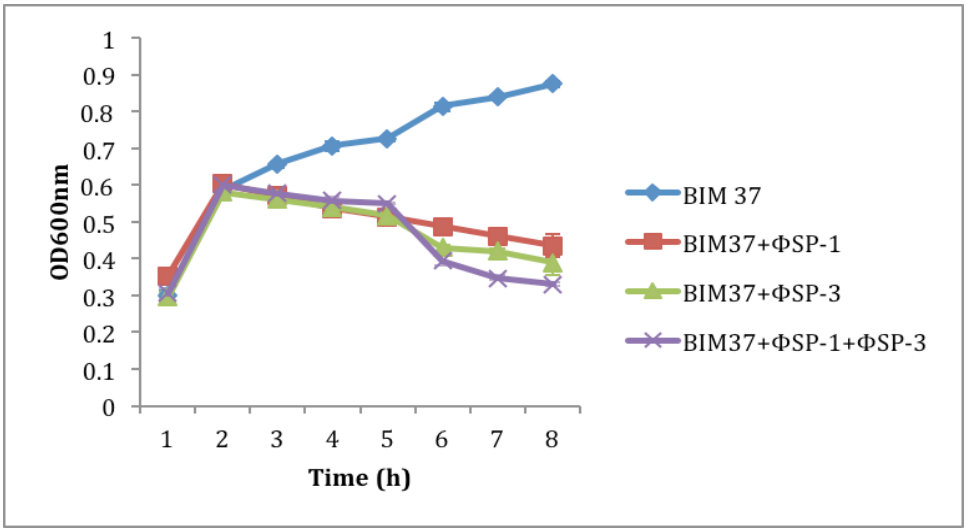


Fig 6.1 Time course of host cell lysis by Φ SP-1 and Φ SP-3. Cultures of BIM 37 were infected with Φ SP-1 and Φ SP-3 individually and in combination. OD600 nm was measured following bacterial challenges at regular time intervals. The results shown are means ± SDs from three independent experiments. In all cases, P < 0.001 when compared to control with no phages.

6.3.2 Phage cocktail treatment with *Salmonella* Enteritidis BIM 43

As a representative of the ΦSP-3 generated mutants, *Salmonella* Enteritidis

BIM 43 was used to study the adequacy of phage cocktail. A combination of ΦSP-1 and ΦSP-3 was used. Fig 6.2 shows the outcome of the phage cocktail assay. The figure represents the mortality of BIM 43 in terms of reduced OD_{600} when exposed to the influence of the bacteriophages individually and in combination. For BIM 43 in group A as there was no phage infection or phage lysis, after 1 h, the OD_{600} which was 0.33 in the beginning, increased at the end of the experiment to 0.72. Contrarily, in group B, C i.e. in the presence of ΦSP-1 and ΦSP-3 it was reduced to 0.44 and 0.5 respectively; but the cocktail of the two phages further reduced it to 0.419. Values represent mean ± SD, and in all the cases, $P < 0.001$ when compared to group A, indicating high significance.

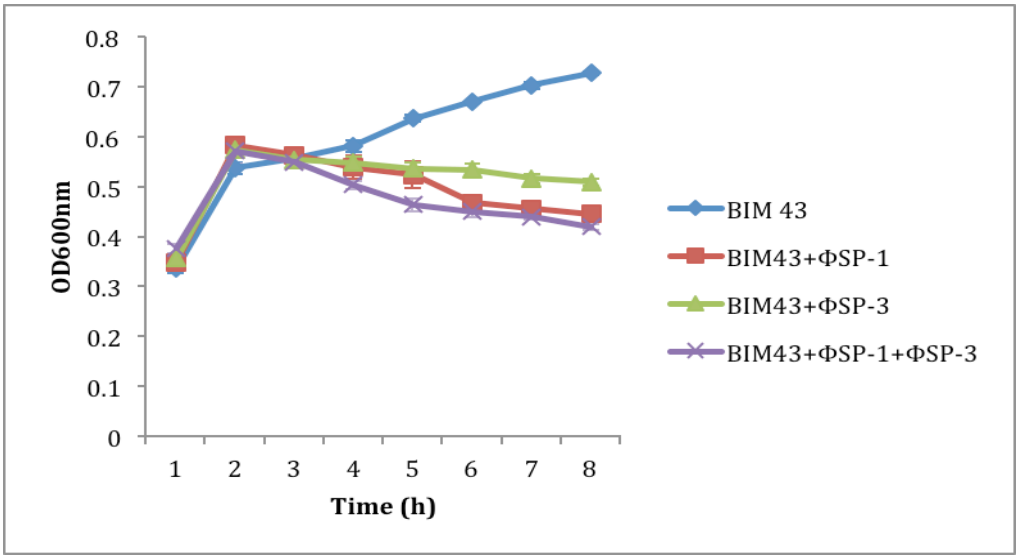


Fig 6.2 Time course of host cell lysis by Φ SP-1 and Φ SP-3. Cultures of BIM 43 were infected with Φ SP-1 and Φ SP-3 individually or in combination. OD600 nm was measured following bacterial challenges at regular time intervals. The results shown are means ± SDs from three independent experiments. In all cases, P < 0.001 when compared to control with no phages.

6.3.3 Phage cocktail treatment with *Salmonella* Enteritidis BIM 32

Salmonella Enteritidis (BIM 42) was used to study the effectiveness of phage cocktail from the group of ΦSP-2 generated mutants. Here the cocktail formulated was of ΦSP-2 and STP1. Fig 6.3 demonstrates the outcome of the phage cocktail assay. The figure indicates the declining growth of BIM 32 when exposed to the influence of the bacteriophages individually and in combination. In group A at the end of 1 h for BIM 32, the OD_{600}= 0.35 and at the end of the experiment it increased to 0.76 as there was no phage to infect and cause lysis. However in group B and C infected with phages ΦSP-2 and STP1 respectively, was reduced to 0.43 and 0.44 respectively, while the cocktail of the two phages further brought it down to 0.411. All values represent mean ± SD, and in all the cases, $P < 0.001$ when compared to group A, indicating high significance.

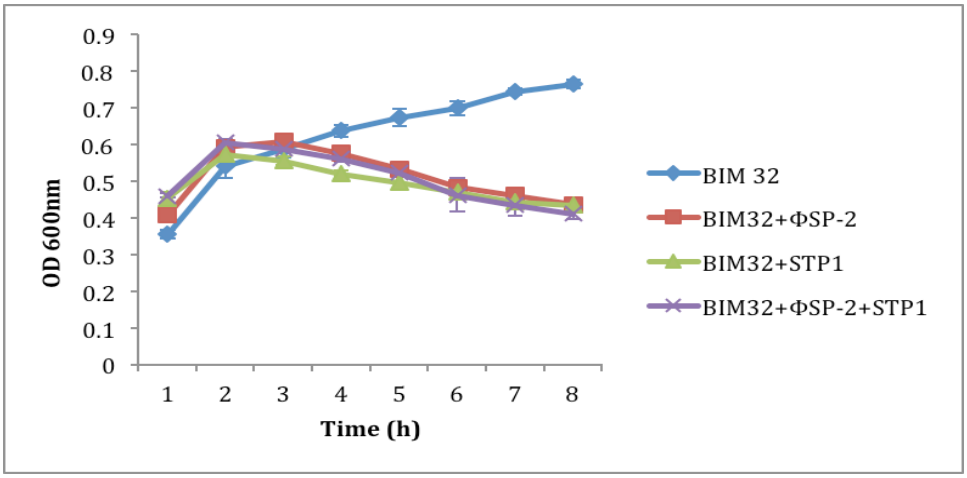


Fig 6.3 Time course of host cell lysis by ΦSP-2 and STP1. Cultures of BIM 32 were infected with ΦSP-2 and STP1 individually or in combination. OD600 nm was measured following bacterial challenges at regular time intervals. The results shown are means ± SDs from three independent experiments. In all cases,

P < 0.001 when compared to control with no phages.

In all three instances above, the individual phages as well as the phage cocktails proved efficient in controlling the growth of the bacterial host.

6.4 Discussion

Assuring food safety is a complex process that depends on the implementation of a broad range of coordinated control measures at all levels of the food production chain (based on the farm-to-fork principle). To reduce *Salmonella* levels, antibiotics are used in poultry industries at each step of the production in the farms. *Salmonellae* have developed several virulence and antimicrobial resistance mechanisms that continuously challenge our public health infrastructure (Jassim and Limoges, 2014). Bacteriophages have emerged as a novel tool among the various approaches to food safety currently under exploration for the biocontrol of bacterial contamination in foods and are thus of particular interest to the food industry (Spricigo *et al*., 2013). To use unique virulence features of phages mixed in a single therapeutic phage cocktail is the pillar of a successful phage therapy. Assembly of such mixture can result in an increased potential for phage formulations to be used doubtlessly and with an increased breadth of utility for individual formulations. The latter will apparently be crucial to the commercial as well as clinical success of phage therapy (Chan *et al*., 2013). Besides, phage cocktail therapy was more effective in counteracting bacterial mutations than monophage therapy implying that phage cocktail has great therapeutic potential for multidrug-resistant bacteria infection (Kaźmierczak *et al*., 2014). A few *Salmonella* primary phages acted as secondary phages on other bacteria. This phenomenon is significant in formulating phage cocktails in which a gang of phages can lyse together a target bacterial strain. Despite being a secondary phage to other bacterial isolates each primary phage is unique in its characteristics. Earlier various

studies formulated phage cocktails to treat certain bacterial infections (Kurosu *et al*., 2013).

The outcome of the host cell lysis test was promising. *Salmonell*a specific lytic phages ΦSP-1, ΦSP-3, ΦSP-2 and ΦStp1 were effective in lysing the BIMs used in the study, when formulated and used as a cocktail than when used individually, thereby leading to a dip in the OD600 nm.

In this study *Salmonella* phages were isolated from poultry. A similar study was done in Italy in 2007 (Andreatti Filho *et al*., 2007). As a therapeutic strategy against *Salmonella* Enteritidis S49 infection using *Caenorhabditis elegans* as model organism, *Salmonell*a specific lytic phages ΦSP-1 and ΦSP-3 lysed the experimentally infected S49 *Salmonella* thereby leading to a dip in the OD600 nm. Compared with the control, phages proved to be effective control agents in terms of their lethality, when applied individually or in combination (Augustine *et al*., 2014).

Six novel lytic bacteriophages were isolated and a cocktail was formulated with the potential to eliminate *Pseudomonas aeruginosa* PAO1 planktonic cultures (Alves *et al*., 2015). By using a suspension of the six phages in a cocktail, their OD_{600} was reduced from 0.9 to 0.1 at 24 h and a full inhibition of the bacterial culture were observed. In an experiment on phage inactivation of foodborne *Shigella* on ready to eat spiced chicken, three phages SD-11, SF-A2, SS-92 were used as cocktail and the *Shigella* count was reduced. Phages could reduce bacterial counts by up to 2 $\log_{10}$/g after 48 h incubation when treated with the cocktail, and after 72 h the host could not be detected (Zhang *et al*., 2013). Phage treatments in *Escherichia coli* O157:H7 with three phages as cocktail reduced their count. Phage treatment reduced ($P < 0.05$) the concentrations of *E. coli* O157:H7 by 1.97 $\log_{10}$ cfu/mL in ground beef. Likewise, phage treatment reduced ($P < 0.05$) *E. coli* O157:H7 by 3.28, 2.88, and 2.77 $\log_{10}$ cfu/mL in spinach when stored at room temperature for 24, 48, and 72 h, respectively

(Hong *et al*., 2014).

The efficacy of the phage cocktail (combination of PA13076 and PC2184) was studied on food samples (chicken breast, pasteurized whole milk and Chinese cabbage) that were experimentally contaminated with a mixture of equal numbers of *Salmonella* Enteritidis ATCC13076 and CVCC2184 (Bao *et al*., 2015). A significant reduction in bacterial numbers (1.5–4 log CFU/sample, $p < 0.05$) was observed in all tested foods. Spricigo *et al*. (2013) studied, the effectiveness of a bacteriophage cocktail composed of three different lytic bacteriophages (UAB-Phi 20, UAB-Phi78, and UAB-Phi87) in four different food matrices (pig skin, chicken breasts, fresh eggs, and packaged lettuce) experimentally contaminated with *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis. A significant bacterial reduction was observed when cocktail was employed. These results show the potential effectiveness of this bacteriophage cocktail as a biocontrol agent of *Salmonella* in several food matrices.

Study conducted by Taha *et al*. (2014) demonstrated the resistance rates of *Salmonella* spp. to individual phages and to phage cocktails. Compared the resistance rate of individual phage in all bacteria the resistance to phage cocktails was lower. This observation suggested that the phage cocktail was more beneficial than mono-phage therapy. They formulated and used phage cocktails, which provided evidence that phage cocktails can broaden the host range together with lowering the chances of bacteria to resist attacking phages. Anyhow, the narrow host range of phages is a good and bad yardstick at the same time. Many phages are known to be highly specific for certain bacterial receptors and hence characterized by a narrow host range, confining their infectivity to a single species or to specific bacterial strains within a species, for that reason, phages only minimally impact health-protecting normal flora bacteria (Davies, 1997; Coculescu and Flueraş, 2005). If the bacterial pathogen is well known

and well analyzed it will be beneficial. On the contrary, when not well characterized life threatening bacterial infections need highly effective and broad-spectrum antimicrobials; on that occasion, phage cocktails are much more preferred over mono-phage therapy (Kutter and Sulakvelidze, 2005).

Altogether, using therapeutic phages, in general, and phage cocktails, in particular, composed of specific lytic optimized phages against *Salmonella* Enteritidis bacteria was proved a promising substitute for antimicrobial approach. Analysis with broader phage cocktails needs to be conducted in order to standardize this approach for combating bacteria where conventional chemical antimicrobials are in vain (Gupta and Prasad, 2011).

Phage cocktail assay was conducted in order to mitigate the BIMs that emerged during the phage host interaction. For that, the BIMs which obtained when *Salmonella* Enteritidis S49, S37 was treated with their specific phages ΦSP-1 and ΦSP-3, ΦSP-2 separately, could be controlled effectively when phages were combined and used as a phage cocktail. In conclusion, monophage based therapies suffer from the drawback of easy emergence of phage resistant bacterial mutants as well as narrow host range. Moreover, the process of isolating phage specific for the particular pathogenic strain can delay the process of treatment. Thus, use of phage cocktail incorporating mixture of different phages covering broad host range can provide an effective solution that can be readily delivered without any delay.

Chapter 7. Summary and Conclusion

Salmonella colonize a broad spectrum of host organisms, efficiently infecting both animals and plants. Among the *Salmonella* species, *Salmonella* Enteritidis is the primary cause of food-borne poisoning in humans, contaminating a wide range of produce, such as pre-cut meats, poultry, eggs, vine vegetables, fruits, nuts, sprouts, leafy greens, roots and beans.

Although the development of antibiotics stands as one of the most substantial medical innovations, the ever-increasing prevalence of multidrug-resistant infections has created a global public health crisis. Therefore, the search for alternatives to antibiotics is gaining strong interest. As natural killers of bacteria, bacteriophages have great potential as alternatives to antibiotics. However, the previously widely used broad-spectrum bacteriophage treatment was unable to achieve a broad-spectrum effect, which reduced its efficacy (Fan and Tong, 2012). Currently, the international mainstream view of bacteriophage therapy is that it could be used for a personalized bacteriophage therapy program for treating multidrug-resistant bacterial infections (Miedzybrodzki *et al*., 2012).

The battle between predator and prey is perhaps the second oldest conflict on earth, and phage represents one of oldest predators on the planet. All cellular systems evolve ways to combat predators and genomic parasites. Phages form a formidable force against bacteria and archaea, being responsible for 4%–50% of their destruction. To combat this, numerous resistance mechanisms have emerged against phage leading to emergence of BIMs.

In this study, BIMs of *Salmonella* Enteritidis strains S49 and S37, developed after phage host interaction with *Salmonella* specific lytic phage ΦSP-1, ΦSP-3 and ΦSP-2 (candidate phages for phage therapy) were isolated and characterized by biochemical and molecular analysis, after reconfirmation as *Salmonella.* The *Salmonella* strains were previously isolated from chicken caecum and stocked in the lab.

The three *Salmonella* specific phage named as ΦSP-1, ΦSP-3 and ΦSP-2 were also previously isolated from chicken intestine samples via an enrichment protocol employing the double agar overlay method. These phages showed consistent lytic nature and were selected for further study. Repeated plating purified them after picking of single isolated plaques from the lawns of *Salmonella* S49 and S37 plates. The phages produced small, clear plaques indicating their lytic nature. They were concentrated employing PEG-NaCl precipitation method before further characterization.

Morphological characterization of ΦSP-1 by Transmission Electron Microscopy revealed identical hexagonal/ isomeric head outlines, with an extremely short tail, characteristic of family *Podoviridae*. Micrograph of ΦSP-3 indicated a hexagonal head and long non- contractile tail, which are typical morphological features of family *Siphoviridae* (earlier characterized). The TEM picture of ΦSP-2 revealed typical *Siphovirus* morphology with an isometric capsid connected to a long, flexible and non-contractile tail.

The BIMs developed after three independent infections of *Salmonella* Enteritidis S49, S37 by their specific lytic phages ΦSP-1, ΦSP-3 and ΦSP-2 were studied. They were identified as *Salmonella* Enteritidis by 16S rRNA gene analysis and biochemical analysis. Antibiotic sensitivity by disc diffusion method was used to detect for variation in antibiotic sensitivity of host and its BIMs. The host bacteria

were almost resistant to the antibiotic tetracycline but some of the BIMs showed intermediate sensitivity. Then much observed difference was in cefuroxime resistance in some BIMs while the host was sensitive to the antibiotic. Then another notable variation was to nalidixic acid, the host S49 showed intermediate response while most of its BIMs were sensitive to the drug, while two BIMs were resistant. The emergence of antibiotic resistant phenotypes of *Salmonella* was observed in this study after phage infection in some of the BIMs.

Another attempt was made to study the CRISPR regions in both hosts and BIMs. Not much variation was observed when comparing hosts with its BIMs. Almost all spacers that were present in the hosts were present in BIMs. In certain BIMs much of the spacers were lost. And only in one BIM (BIM 3) one new spacer addition was observed in 5' regions in proximal to leader sequences. Some of the spacers were found to be repeated in the BIMs and new addition was observed in the 3' regions in certain BIMs, although this is uncommon.

LPS profiling employing LPS acrylamide gel electrophoresis was used to compare the LPS of bacteriophage resistant mutants and the parental strains. There were some bands missing in the O-polysaccharide region in some of the BIMs. But in several others there were no visible variation in the LPS profiles of the host and the BIMs. LPS is supposed to have a role of in phage adsorption, however in this study, the lack of change in the LPS pattern of some of BIMs nevertheless indicates that diverse receptors at play here. By isolating phages that utilize diverse receptors for adsorption, the principle of cocktail is sustained; by the capacity of phages to cross-lyse each other's resistant mutants. This will help in fighting phage resistance as well as antibiotic resistances when applied together.

PCR screening of selected virulence /virulence related genes- *invE/A, slyA, pho P/Q, ttrC, mgtC, sopE,* and *agfA* was carried out in the host S49, S37 and its

BIMs. PCR screening done in phages earlier (Augustine and Bhat, 2012) indicated the absence of the tested virulence /virulence related genes in the phage genome, thus negating any chance of transfer of these genes from phage to host. The presence of virulence genes, *inv*E/A*, afg, slyA, pho P/Q, ttrC, mgtC and sopE, in* the host strain *Salmonella* S49, S37 and its BIMs indicated the pathogenic nature of these bacteria. There was no change in the virulence pattern of the mutants after phage infection.

MLST analysis was done to observe whether there are any observable changes in the seven house keeping genes (*aro C - dna N - hem D - his D - pur E- suc A - thr A*) of *Salmonella*. It was observed that the host bacteria and its BIMs grouped together in a single clade showing their relatedness. As it is MLST it can be said *Salmonella* Enteritidis S49 and BIMs all showed a single sequence type lineage.

Phage cocktail assay was conducted in order to mitigate the BIMs that emerged during the phage host interaction. For that the BIMs which developed when *Salmonella* Enteritidis S49 and S37 was treated with their specific phage ΦSP-1, ΦSP-3 and ΦSP-2, separately could be controlled when both phages were combined and used as a phage cocktail. Here one more *Salmonella* phage STP1 (unpublished data) which was already available in the lab was also used. Host cell lysis test was employed to study the competency of phage cocktails in *Salmonella*-specific bacteriolysis.

This study showed that the BIMS that emerged following phage infection were really not that different from the original hosts. The only major variations observed were in the CRISPR regions of some of the BIMs; slight variations in the antibiograms of the BIMs. It is also observed that the BIMs were not different from their hosts based on the various other molecular characteristics studied here. The fears of enhanced virulence in the emergent mutants following phage applications may be laid to rest. The silver lining is that the emergent BIMs could well be controlled using

phage cocktails.

Comprehending the eco-evolutionary aspects of phage host interactions is critical in respect of advancement in phage-based therapeutics. In this context the emphasis is on the development of bacteriophage resistance and the results of this resistance on the accomplishment of the treatment. In most cases phage-resistance was associated with lowered virulence in bacteria, most likely as a result of costs coupled with defending against phages.

In order to investigate the impact of building CRISPR-encoded immunity on the host chromosome, we determined the genome sequence of a BIM derived from the organism, after three consecutive rounds of bacteriophage challenge. Active CRISPR loci involve addition of several spacers (at the 3' end); but in this study deletions were more following exposure to bacteriophages. Overall, building CRISPR-encoded immunity does not significantly affect the genome, which allows the maintenance of important functional properties in isogenic CRISPR mutants. This criterion can be used in industrial sector as a marker and tool to protect the starter cultures from phages. This is critical for the development and formulation of sustainable and robust next-generation starter cultures with increased industrial lifespans.

MLST has played a major role in diagnosing pathogens of human disease. Rapid identification of such pathogens is crucial in our ability to identify, track, and treat disease outbreaks. Here it proved that the house keeping genes in both hosts and BIMs were found to be conserved. MLST has proven to be a high-resolution genetic approach that provides data amenable to sophisticated phylogenetic and population genetic analyses.

While this thesis has demonstrated the potential consequences of phage therapy by exploring the BIMs that arise during the phage therapy, many opportunities for extending the scope of this thesis remain. Some of these directions are antibiotic

gene sequence analysis of mutants and hosts; complete genome sequencing of host and phage so that CRISPR based mechanism can be studied in more detail. The result obtained in one dimensional page analysis of LPS could be validated using 2D page analysis. Characterization of LPS gene can also shed light to this aspect.

The aim of microbiologists working with phages will be to monitor the changes in the proportion of bacteria resistant to used phages, developing and scheduling the introduction of new phage preparations, creating new combinations of compounds that extend the lytic spectrum, all procedures that should ensure the continuity of phage therapy. If certified manufacturers of commercial phage mixtures were to participate in the preparation of proposed mono and heterospecies phage mixtures, it would both simplify and greatly diminish the response time in the preparation of personalized cocktails and thereby help phage therapy become established as a major weapon in our arsenal against bacterial infection.

Hereby from this work done, it is suggested that despite the strong selection for bacterial defense against phages, the emergence of phage-resistance should not hinder the prospects of utilizing phages as alternative antibacterial in the future.

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APPENDIX – 1

NUTRIENT MEDIUM

Ingredients		**g/L**
Peptone	-	5
Sodium chloride	-	5
Beef extract	-	1
Yeast extract	-	2

Suspended 13g of media (Hi Media, Mumbai, India) in 1000mL distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium. Final pH 7.4 ± 0.2.

MUELLER HINTON BROTH

Ingredients		**g/L**
Beef infusion	-	300
Casein acid hydrolysate	-	17.5
Starch	-	1.5

Suspended 21 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation. Final pH (at 25^{o}C) -7.4 ± 0.2.

Xylose Lysine Deoxycholate Agar

Ingredients		**g/L**
Yeast extract	-	3
L-Lysine	-	5
Lactose	-	7.5
Sucrose	-	7.5
Xylose	-	3.5
Sodium chloride	-	5
Sodium deoxycholate	-	2.5
Sodium thiosulphate	-	6.8

Ferric ammonium citrate	-	0.8
Phenol red	-	0.08
Agar	-	15

Suspended 56.68 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated with frequent agitation, until the medium boiled.

Hektoen Enteric Agar

Ingredients		**g/L**
Proteose peptone	-	12
Yeast extract	-	3
Lactose	-	12
Sucrose	-	12
Salicin	-	2
Bile salt mixture	-	9
Sodium chloride	-	5
Sodium thiosulphate	-	5
Ferric ammonium citrate	-	1.5
Acid fuchsin	-	0.1
Bromo thymol blue	-	0.065
Agar	-	15

Suspended the ingredients in 1 liter distilled water and mixed. Heated with frequent agitation, to boiling. Transferred immediately to a water bath, at 50°C. After cooling, poured into sterile Petri plates. Final pH-7.5±0.2.

Bismuth Sulfite Agar

Ingredients		**g/L**
Peptic digest of animal tissue	-	10
Beef extract	-	5
Dextrose	-	5
Disodium phosphate	-	4
Ferrous sulphate	-	0.3
Bismuth sulphite indicator	-	8
Brilliant green	-	0.025
Agar	-	20

Suspended 52.33 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the constituents completely. Plates were prepared on previous day, and stored in dark. Final pH - 7.7±0.2.

Brain Heart Infusion Medium

Bovine brain and heart tissue powder

Suspended 20 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MINIMAL MEDIA

Ingredients		**g/ 100 mL**
Sodium phosphate	-	1.28
Dipotassium phosphate	-	0.3
Sodium chloride	-	0.5
Ammonium chloride	-	0.5

Suspended the above ingredients in 100 mL distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

To this add filter sterilised

1M Magnesium sulphate	-	400 µL
1M Calcium chloride	-	20 µL
20% Glucose	-	4 mL

Christensens' Urea agar

Ingredients	**g/L**
Peptone	1
Sodium Chloride	5
Monopotassium phosphate	2
Glucose 0.1%	1
Phenol Red	0.012
Agar	15
Distilled Water	900mL
Urea	20

Distilled Water 100mL

Dissolve all the ingredients except urea in 900mL water (basal medium). Autoclaved at 15 lbs pressure (121°C) for 15 minutes. Cool to 50-55°C. Filter-sterilized urea added aseptically to cooled basal medium and mix well. Dispense in to sterile tubes and convert to slants with 2 cm butt and 3 cm slant. Final pH- 6.8 +/- 0.2 at 25ºC.

Simmons Citrate Agar

Ingredients		**g/L**
Magnesium sulphate	-	0.2
Ammonium dihydrogen phosphate	-	1
Dipotassium phosphate	-	1
Sodium citrate	-	2
Sodium chloride	-	5
Bromothymol blue	-	0.08
Agar	-	15

Suspended 24.28 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve completely, mixed well and distributed into tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and made slants. Final pH-6.8±0.2.

Lysine Iron Agar

Ingredients		**g/L**
Peptic digest of animal tissue	-	5
Yeast extract	-	3
Dextrose	-	1
L-Lysine	-	10
Ferric ammonium citrate	-	0.50
Sodium thiosulphate	-	0.04
Bromocresol purple	-	0.02
Agar	-	15

Suspended 34.56 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve completely. Dispensed into tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled the tubes in slanted position to form slants with deep butts. Final pH- 6.7±0.2.

Arginine Dihydrolase Broth

Ingredients		**g/L**
Peptic digest of animal tissue	-	1
Sodium Chloride	-	5
Dipotassium hydrogen phosphate	-	0.3
L- Arginine	-	10
Bromocresol purple	-	0.016
Agar	-	3

Suspend 19.31 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely and distribute in 13x100 mm tubes. Sterilize by autoclaving at 121°C for 15 minutes. Allow the tubes to cool in an upright position. Final pH- 6 ± 0.2.

Triple Sugar Iron Agar

Ingredients		**g/L**
Peptic digest of animal tissue	-	2
Beef extract	-	3
Yeast extract	-	3
Lactose	-	10
Sucrose	-	10
Glucose	-	1
Ferric citrate	-	0.3
Sodium chloride	-	5
Sodium thiosulphate, pentahydrate	-	0.3
Phenol red	-	0.024
Agar	-	12

Suspended 64.51 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated to boiling, to dissolve the medium completely. Mixed well and distributed into test tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for

10 minutes. Allowed the medium to set in sloped form with a butt about 1 inch long. Final pH - 7.4 ± 0.2.

Luria Bertani Broth

Ingredients		**g/L**
Casein enzymic hydrolysate	-	10
Yeast extract	-	5
Sodium chloride	-	10

Suspended 25 grams of media (Hi Media, Mumbai, India) in 1000 mL distilled water. Heated to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH is 7.5±0.2.

APPENDIX – 2

SM buffer

NaCl	- 5.8 g
$MgSO_4.7H_2O$	- 2.0 g
1M Tris HCl (pH 7.5)	- 50 mL
2% gelatin	- 5.0 mL

Ingredients were dissolved and was made up to 1 litre with milliQ water and autoclaved at 15lbs for 20 minutes and stored at 4°C until use.

Phosphate Buffered Saline (PBS)

NaCl	-	8.0 g
KCl	-	0.2 g
Na_2HPO_4	-	1.44 g
KH_2PO_4	-	0.24 g

Ingredients were dissolved in 800 mL of distilled water, pH adjusted to 7 with 1N HCl. The volume was made up to 1 litre with distilled water, autoclaved at 15lbs for 20 minutes and stored at room temperature until use.

Agarose gel (1%)

Agarose	-	1.0 g
1X TAE (see TBE buffer)	-	100 mL

Melt agarose in microwave oven and allow to cool to 50^{O}C before pouring the gel.

Bromophenol blue loading dye

Bromophenol blue	-	0.05 g
Glycerol	-	5.0 mL
EDTA	-	0.186 g
1X TAE	-	volume to 10 mL

Aliquot the dye in fresh microfuge tubes and store at 4^{O}C.

Disodium ethylenediamine tetraactete (EDTA) - 0.5 M

EDTA	-	186.1 g
Distilled water	-	1000 mL
NaOH	-	~20 g

Dissolve EDTA in 800 mL distilled water and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 using NaOH pellets and make the final volume to 1000 mL. Autoclave before use.

Ethidium Bromide (10 mg/mL)

Ethidium Bromide	-	0.1 g
Distilled water	-	10 mL

Stir the solution using a magnetic stirrer for several hours to ensure that the dye has dissolved completely. Wrap the container in aluminium foil and store at 4^{0}C. For staining agarose gels, a working solution of 0.5 µg/mL can be made.

Hexadecyltrimethylammonium bromide (CTAB) – 10 % in 0.7 M NaCl

CTAB	-	10 g
NaCl	-	4.1 g
Distilled water	-	80 mL

Dissolve 4.1 g NaCl in 80 mL distilled water and slowly add 10 g CTAB, while heating and stirring. If necessary, heat to 65OC to dissolve. Adjust final volume to 100mL.

Proteinase K (20 mg/mL)

Proteinase K	-	10 mg
Sterile distilled water	-	0.5 mL

Stored at -20^{O}C in 50 µL aliquots.

Sodium dodecyl sulphate (SDS) – 10%

SDS	-	10 g
Distilled water	-	100 mL

Tris-Acetate EDTA (50 X)

Tris base	-	242 g
0.5 M EDTA (pH 8.0)	-	100 mL
Glacial acetic acid	-	57.1 mL
Distilled water	-	to 1000 mL

The stock solution was diluted to 1X for gel runs.

Tris EDTA (TE) buffer

Tris-HCl 10 mM	-	2 mL 1M Tris-HCl (pH 8.0)
EDTA (pH 8.0)	-	1 mM 0.4 mL 0.5 M EDTA
Distilled water	-	to 200 mL

Physiological saline (0.85%)

NaCl - 0.85g

Dissolve in 100mL distilled water

APPENDIX 3

LPS Acrylamide Gel Electrophoresis

Stock acrylamide- bisacrylamide solution (30% T and 0.8% C)

Acrylamide (T)	-	30 g
Bis-acrylamide (C)	-	0.8 g
Distilled water (DW)	-	100 mL

Stored at 4°C in amber coloured bottle

Stacking gel buffer stock

Tris buffer (0.5 M)	-	6.05 g in 40 mL DW

Titrated to pH 6.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 (Whatman, England) filter paper and stored at 4°C.

Resolving gel buffer stock

Tris buffer (1.5 M)	-	18.15 g

Titrated to pH 8.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 filter paper and stored at 4°C.

Running buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g
SDS	-	1 g

Dissolved and made up to 1L with DW. Prepared in 10X concentration and stored at 4°C.

Sample buffer for Non- Reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	1.25 mL
Glycerol	-	2.5 mL
SDS (10%, W/v)	-	2 mL
Deionised water	-	3.55 mL
Bromophenol blue (0.5%, w/v)	-	0.2 mL

Samples were diluted with sample buffer prior loading into the gel

SDS (10%) - 1 g in 10 mL DW

Ammonium persulfate (10%, w/v) - 0.1 g of ammonium persulfate was dissolved in 1mL DW (prepared freshly).

LPS microextraction lysing buffer

SDS - 2%
β mercaptoethanol - 4%glycerol
1 *M* Tris–Cl (pH 6.8)

Bromophenol blue - A pinch

Silver staining

1.Fixing solution - 40% ethanol , 5% acetic acid and 0.9% periodic acid

2. Staining solution

20% Silver nitrate - 5 mL
add silver nitrate drop-wise)
0.1N NaOH - 28 mL
Concentrated NH4OH - 2.1 mL
Distilled water - 115mL

3. Developing solution

Anhydrous citric acid - 50 mg
37% formaldehyde - 0.5 mL
Distilled water - 200mL

Mixed and prepared fresh before use.

Stop solution

Sodium-EDTA	-	1.4 g
Milli Q water	-	100 mL

BIM 4 CRISPR 1

- CRISPR start position : 20 ---------- CRISPR end position : 658 ---------- CRISPR length : 638
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 10

20	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	80
81	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	141
142	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	202
203	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGCGCAACCGTTCCCGCAGCGATTAACTTCA	263
264	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	324
325	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	385
386	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	446
447	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	507
508	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	568
569	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	629
630	CGGTTTATCCCCGCTGGCGCGGGGAACAC		658

BIM 9 CRISPR 1

- CRISPR start position : 11 ---------- CRISPR end position : 283 ---------- CRISPR length : 272
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 4

11	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	71
72	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	132
133	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	193
194	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGCGCAACCGTTCCCGCAGCGATTAACTTCA	254
255	CGGTTTATCCCCGCTGGCGCGGGGAACAC		283

BIM 19 CRISPR 1

- CRISPR start position : 18 ---------- CRISPR end position : 473 ---------- CRISPR length : 455
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 7

18	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	78
79	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	139
140	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	200
201	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGCGCAACCGTTCCCGCAGCGATTAACTTCA	261
262	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	322
323	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	383
384	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	444
445	CGGTTTATCCCCGCTGGCGCGGGGAACAC		473

BIM 20 CRISPR 1

- CRISPR start position : 22 ---------- CRISPR end position : 477 ---------- CRISPR length : 455
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 7

22	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	82
83	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	143
144	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	204
205	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGGGGCAACGCTTGGGGCAGGCATTAACTTCA	265
266	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	326
327	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	387
388	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	448
449	CGGTTTATCCCCGCTGGCGCGGGGAACAC		477

BIM 22 CRISPR 1

- CRISPR start position : 20 ---------- CRISPR end position : 782 ---------- CRISPR length : 762
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

20	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	80
81	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	141
142	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	202
203	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGGGGCAACGCTTGGGGCAGGCATTAACTTCA	263
264	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	324
325	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	385
386	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	446
447	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	507
508	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	568
569	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	629
630	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	690
691	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTTGCGAACCAGATGTTATCGTCGGTGCGC	753
754	GGTTTATCCCCCGCTGGCGCGGGGAACAC		782

BIM 38 CRISPR 1

- CRISPR start position : 22 ---------- CRISPR end position : 844 ---------- CRISPR length : 822
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

22	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	82
83	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	143
144	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	204
205	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGGGGCAACGCTTGGGGCAGGCATTAACTTCA	265
266	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	326
327	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	387
388	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	448
449	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	509
510	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	570
571	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	631
632	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	692
693	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	753
754	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATAAAAAGCACTATTGAACT	815
816	CGGTTTATCCCCGCTGGCGCGGGGAACAC		844

BIM 3 CRISPR 1

- CRISPR start position : 6 ---------- CRISPR end position : 277 ---------- CRISPR length : 271
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 4

6	CGGTTTATCCCCGCTGGCGCGGGGAAGAT	TTAAAAGTCTTGCTGGAGACATGGGCGTCCC	65
66	CGGTTTATCTACGCTGGGGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	126
127	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	187
188	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGCGACGGATTAACTTCA	248
249	CGGTTTATCCCCGCTGGCGCGGGGAACAC		277

BIM 6 CRISPR 1

- CRISPR start position : 20 ---------- CRISPR end position : 658 ---------- CRISPR length : 638
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 10

20	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	80
81	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	141
142	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	202
203	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGCGACGGATTAACTTCA	263
264	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	324
325	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	385
386	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	446
447	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	507
508	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	568
569	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	629
630	CGGTTTATCCCCGCTGGCGCGGGGAACCT		658

BIM 7 CRISPR 1

- CRISPR start position : 19 ---------- CRISPR end position : 413 ---------- CRISPR length : 394
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 6

19	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	79
80	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	140
141	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	201
202	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGCGACGGATTAACTTCA	262
263	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	323
324	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	384
385	CGGTTTATCCCCGCTGGCGCGGGGAACAC		413

BIM 16 CRISPR 1

- CRISPR start position : 19 ---------- CRISPR end position : 291 ---------- CRISPR length : 272
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 4

19	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	79
80	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	140
141	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	201
202	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGCGACGGATTAACTTCA	262
263	CGGTTTATCCCCGCTGGCGCGGGGAACAC		291

BIM 42 CRISPR 1

- CRISPR start position : 15 ---------- CRISPR end position : 287 ---------- CRISPR length : 272
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 4

15	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	75
76	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	136
137	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	197
198	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGGCACCGATTAAGTTCA	258
259	CGGTTTATCCCCGCTGGCGCGGGGAACAC		287

BIM 43 CRISPR 1

- CRISPR start position : 7 ---------- CRISPR end position : 218 ---------- CRISPR length : 211
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 3

7	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	67
68	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	128
129	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	189
190	CGGTTTATCCCCGCTGGCGCGGGGAACAC		218

BIM 44 CRISPR 1

- CRISPR start position : 22 ---------- CRISPR end position : 843 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

22	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	82
83	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	143
144	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	204
205	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGGCGAACCGTTCCGGCACCGATTAAGTTCA	265
266	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	326
327	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	387
388	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	448
449	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	509
510	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	570
571	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	631
632	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	693
694	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	754
755	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATAAAAAGCAGTATTGAAC	814
815	TCGTTTATCCCCGCTGGCGCGGGGAACAC		843

S 37 CRISPR 1

- CRISPR start position: 29 ---------- CRISPR end position: 972 ---------- CRISPR length: 943
- DR consensus: CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length: 29 Number of spacers: 15

29	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	89
90	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	150
151	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	211
212	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGCGCAACCGTTCCCGCAGGGATTAACTTCA	272
273	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	333
334	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	394
395	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	455
456	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	516
517	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	577
578	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	638
639	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	699
700	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	760
761	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATAAAAAGCACTATTGAAGT	821
822	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGAACATTCGCCCACTCAATCGTAACGTGAT	882
883	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GTTTCTCACAGCCGCCAGCGCGATCTGACGGC	943
944	CGGTTTATCCCCGCTGGCGCGGGAACACC		972

BIM 27 CRISPR 1

- CRISPR start position : 22 ---------- CRISPR end position : 843 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

22	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	82
83	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	143
144	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	204
205	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ACGCGCAACCGTTCCCGCAGGGATTAACTTCA	265
266	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	326
327	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	387
388	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	448
449	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	509
510	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	570
571	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	631
632	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	693
694	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	754
755	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATAAAAAGCACTATTGAAC	814
815	TCGTTTATCCCCGCTGGCGCGGGGAACAC		843

BIM 28 CRISPR 1

- CRISPR start position : 13 ---------- CRISPR end position : 896 ---------- CRISPR length : 883
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

13	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	73
74	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	134
135	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCAGCGTTCGGTTCTGGA	195
196	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGGGCGAACGCTTGCGCGAGGGATTAACTTCA	256
257	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	317
318	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	378
379	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	439
440	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	500
501	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	561
562	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	622
623	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	683
684	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	744
745	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATAAAAAGCAGTATTCAACT	805
806	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGAACATTCGCCCACTCAATCGTAACGTGATC	867
868	GGGTTTATCCCCGCTGGCGCGGGAACACG		896

BIM 33 CRISPR 1

- CRISPR start position : 13 ---------- CRISPR end position : 831 ---------- CRISPR length : 818
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

13	CGGTTTATCCCCGCTGGCGCGGGGAAGAC	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	73
74	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCGCACCTCCAGCCGTCTGGCGTATGCACT	134
135	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCTTTAACGCCGGGCGCGTTCGGTTCTGGA	194
195	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGGGCGAACGCTTGCGCGAGGGATTAACTTCA	255
256	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGTGGTTGTCCTGCACCCGCTCGAATAAATC	316
317	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGACCTGGAGCATCTGAAAAGTATTCACAAG	377
378	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	438
439	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	499
500	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	560
561	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC	621
622	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG	682
683	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTTTGCGAACCAGATGTTATCGTCGGTGCG	743
744	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGAGTCTATGACATACAAGCAGTATTCAACT	802
803	CGGTTTATCCCCGCTGGCGCGGGGAACAC		831

BIM 1 CRISPR 2

- CRISPR start position : 57 ---------- CRISPR end position : 819 ---------- CRISPR length : 762
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

57	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	117
118	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCGGTTAGGATGAGTCCACAACCAAGGTACGG	178
179	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	239
240	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGGGCTTATCATTTTTGCTCCGTGCTAGACGG	300
301	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	361
362	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	422
423	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	483
484	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	544
545	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	605
606	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	666
667	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTCGAACAGCTACTGCACAAGCCC	728
729	GGTTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAAGGGGAAGACAGG	790
791	CGGTTTATCCCCGCTGGCGCGGGGAACAC		819

BIM 2 CRISPR 2

- CRISPR start position : 54 ---------- CRISPR end position : 936 ---------- CRISPR length : 882
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

54	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	114
115	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCGGTTAGGATGAGTCCACAACCAAGGTACGG	175
176	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	236
237	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGGGCTTATCATTTTTGCTCCGTGCTAGACGG	297
298	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	358
359	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	419
420	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	480
481	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	541
542	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	602
603	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	663
664	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTCGAACAGCTACTGCACAAGCCC	724
725	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAAGGGGAAGACAGGC	786
787	GGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGGTCGAGTCGGGCGCCCGCG	847
848	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGACGCTTGATTCGTTCACGCCTGTGACACG	907
908	CGGTTTATCCCCGCTGGCGCGGGGAACAC		936

BIM 4 CRISPR 2

- CRISPR start position : 43 ---------- CRISPR end position : 559 ---------- CRISPR length : 516
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 8

43	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	103
104	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	164
165	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATG	225
226	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGGC	286
287	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	347
348	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	408
409	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	469
470	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	530
531	CGGTTTATCCCCGCTGGCGCGGGGAACAC		559

BIM 8 CRISPR 2

- CRISPR start position : 51 ---------- CRISPR end position : 689 ---------- CRISPR length : 638
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 10

51	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	111
112	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	172
173	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATG	233
234	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGGC	294
295	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	355
356	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	416
417	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	477
478	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	538
539	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	599
600	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	660
661	CGGTTTATCCCCGCTGGCGCGGGGAACAC		689

BIM 9 CRISPR 2

- CRISPR start position : 62 ---------- CRISPR end position : 884 ---------- CRISPR length : 822
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

62	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	122
123	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	183
184	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	244
245	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGGCTTATCATTTTTGCTCCGTGGTACAGCG	305
306	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	366
367	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	427
428	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	488
489	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	549
550	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	610
611	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	671
672	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGCAACTATTCGAACAGCTACTGCAGAAGCCC	732
733	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGGC	794
795	GGTTTATCCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGGTCCACGTCGGGCGCGGCGCC	855
856	CGGTTTATCCCCGCTGGCGCGGGGAACAC		884

BIM 19 CRISPR 2

- CRISPR start position : 38 ---------- CRISPR end position : 859 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

38	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	98
99	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	159
160	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	220
221	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGGCTTATCATTTTTGCTCCGTGGTACAGCG	281
282	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	342
343	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	403
404	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	464
465	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	525
526	CGGTTTATCCCCGCTGGCGCAGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	586
587	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	647
648	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CGCAACTATTCGAACAGCTACTGCAGAAGCCC	708
709	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	769
770	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGGTCCACGTCGGGCGCGGGGGC	830
831	CGGTTTATCCCCGCTGGCGCGGGAACACA		859

BIM 20 CRISPR 2

- CRISPR start position : 43 ---------- CRISPR end position : 864 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

43	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	103
104	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	164
165	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	225
226	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGACGC	286
287	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	347
348	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	408
409	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	469
470	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	530
531	CGGTTTATCCCCGCTGGCGCAGGGAAACA	CCTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	592
593	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGATC	654
655	GGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCAACTATTGCAACAGCTACTGCAGAAGCCCG	716
717	GGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	777
778	CGGTTTATCCCCGCTGCGCGGGAACACAA	ATTATTTACGGCTGCAGGTCGGCGCGGGGG	835
836	CGGTTTATCCCCGCTGGCGCGGGGAACAC		864

BIM 21 CRISPR 2

- CRISPR start position : 50 ---------- CRISPR end position : 932 ---------- CRISPR length : 882
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

50	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	110
111	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	171
172	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	232
233	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGACGC	293
294	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	354
355	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	415
416	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	476
477	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	537
538	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	598
599	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	659
660	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGCAACAGCTACTGCAGAAGCCC	720
721	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGGATCTGCAGGGGCAACAAAGGGGAAGACAGG	782
783	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGGCTGCAGGTCGGCGCGGGGGC	843
844	CGGTTTATCCCCGCTGGCGCGGGAACACA	GACGCTTGATTTCGTTCACGGCTGTGACACG	903
904	CGGTTTATCCCCGCTGGCGCGGGGAACAC		932

BIM 22 CRISPR 2

- CRISPR start position : 55 ---------- CRISPR end position : 876 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

55	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	115
116	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	176
177	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	237
238	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCG	298
299	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	359
360	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	420
421	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	481
482	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	542
543	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	603
604	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	664
665	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGCAACAGCTACTGCACAAGCCC	725
726	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	786
787	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTGCACGTGGGCGCGGGCGC	847
848	CGGTTTATCCCCGCTGGCGCGGGGAACAC		876

BIM 36 CRISPR 2

- CRISPR start position : 64 ---------- CRISPR end position : 886 ---------- CRISPR length : 822
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

64	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	124
125	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	185
186	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	246
247	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCG	307
308	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	368
369	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	429
430	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	490
491	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	551
552	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	612
613	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	673
674	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGCAACAGCTACTGCACAAGCCC	734
735	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	795
796	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTGCACGTGGGCGCGGGCGC	857
858	GGGTTTATCCCCGCTGGCGCGGGGAACAC		886

BIM 37 CRISPR 2

- CRISPR start position : 65 ---------- CRISPR end position : 947 ---------- CRISPR length : 882
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

65	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	125
126	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	186
187	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	247
248	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	308
309	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	369
370	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	430
431	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	491
492	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	552
553	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	613
614	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	674
675	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGCAACTATTGGAACAGCTACTGCAGAAGCCC	735
736	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	796
797	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTAATTTACGGTGCACGTCGGCGCCGTGCC	857
858	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGACGCTTGATTTCGTTCACGCCTGTGACACG	918
919	CGGTTTATCCCCGCTGGCGCGGGGAACAC		947

BIM 38 CRISPR 2

- CRISPR start position : 51 ---------- CRISPR end position : 812 ---------- CRISPR length : 761
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

51	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	111
112	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	172
173	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	233
234	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	294
295	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	355
356	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	416
417	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	477
478	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	538
539	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	599
600	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	660
661	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGCAACTATTGGAACAGCTACTGCAGAAGCCC	721
722	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGGC	783
784	GGGTTTATCCCCGCTGGCGCGGGGAACAC		812

BIM 39 CRISPR 2

- CRISPR start position : 58 ---------- CRISPR end position : 940 ---------- CRISPR length : 882
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

58	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	118
119	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGG	179
180	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACCTAACGAAAAAAACGATCATC	240
241	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	301
302	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	362
363	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	423
424	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	484
485	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	545
546	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	606
607	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	667
668	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCAACTATTGGAACAGCTACTGCAGAAGCCC	728
729	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	789
790	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTTCCACGTCGGCGCCGTGCC	850
851	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGACGCTTGATTTCGTTCACGCCTGTGACACG	911
912	CGGTTTATCCCCGCTGGCGCGGGGAACAC		940

BIM 3 CRISPR 2

- CRISPR start position : 56 ---------- CRISPR end position : 937 ---------- CRISPR length : 881
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

56	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	116
117	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGG	177
178	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACCTAACGAAAAAAACGATCATC	238
239	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	299
300	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	360
361	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	421
422	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	482
483	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	543
544	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	604
605	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	665
666	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCAACTATTGGAACAGCTACTGCAGAAGCCC	726
727	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	787
788	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTTCCACGTCGGCGCCGTGCC	848
849	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AGACGCTGATTTCGTTCACGCCTGTGACACG	908
909	CGGTTTATCCCCGCTGGCGCGGGGAACAC		937

BIM 6 CRISPR 2

- CRISPR start position : 49 ---------- CRISPR end position : 626 ---------- CRISPR length : 577
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 9

49	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	109
110	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	170
171	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	231
232	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGGTAGAGCC	292
293	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	353
354	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	414
415	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	475
476	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	536
537	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	597
598	CGGTTTATCCCCGCTGGCGCGGTGAACAC		626

BIM 7 CRISPR 2

- CRISPR start position : 62 ---------- CRISPR end position : 639 ---------- CRISPR length : 577
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 9

62	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	122
123	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	183
184	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	244
245	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGGTAGAGCC	305
306	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	366
367	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	427
428	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	488
489	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	549
550	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	610
611	CGGTTTATCCCCGCTGGCGCGGGGAACAC		639

BIM 10 CRISPR 2

- CRISPR start position : 43 ---------- CRISPR end position : 559 ---------- CRISPR length : 516
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 8

43	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	103
104	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	164
165	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	225
226	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGGTAGAGCC	286
287	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	347
348	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	408
409	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	469
470	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	530
531	CGGTTTATCCCCGCTGGCGCGGGGAACAC		559

BIM 15 CRISPR 2

- CRISPR start position : 44 ---------- CRISPR end position : 864 ---------- CRISPR length : 820
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

44	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	104
105	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGG	165
166	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	226
227	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGGCTTATCATTTTTGCTCCGTGCTAGACGC	287
288	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	348
349	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	409
410	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	470
471	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	531
532	CGGTTTATCCCCGCTGGCGCAGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	592
593	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	653
654	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGCAACAGCTACTGCNGAAGCCC	714
715	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	775
776	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTCCAGCGTCGGCGCGGGCGC	835
836	CGGTTTATCCCCGCTGGCGCGGGGAACAC		864

BIM 16 CRISPR 2

- CRISPR start position : 44 ---------- CRISPR end position : 865 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

44	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	104
105	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGG	165
166	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	226
227	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCGGCTTATCATTTTTGCTCCGTGCTAGACGC	287
288	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	348
349	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	409
410	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	470
471	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	531
532	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	592
593	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	653
654	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGCAACAGCTACTGCNGAAGCCC	714
715	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	775
776	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGCTGCAGCGTCGGCGCGGGCGC	836
837	CGGTTTATCCCCGCTGGCGCGGGGAACAC		865

BIM 40 CRISPR 2

- CRISPR start position : 59 ---------- CRISPR end position : 880 ---------- CRISPR length : 821
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 13

59	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	119
120	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGC	180
181	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	241
242	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCGCTTATCATTTTTCGTCCGTGGTAGACGC	302
303	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	363
364	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	424
425	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	485
486	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	546
547	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	607
608	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	668
669	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGGAACAGGTACTGCACAAGCCG	729
730	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	790
791	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTATTTACGGTGCCACGTGGGCGGGGCGC	851
852	CGGTTTATCCCCGCTGGCGCGGGGAACAC		880

BIM 42 CRISPR 2

- CRISPR start position : 57 ---------- CRISPR end position : 817 ---------- CRISPR length : 760
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

57	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	117
118	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGTTAGGATGAGTCCACAACCAAGCTACGC	178
179	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	239
240	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCGCTTATCATTTTTCGTCCGTGGTAGACGC	300
301	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	361
362	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	422
423	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	483
484	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	544
545	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	605
606	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	666
667	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGGAACAGGTACTGCACAAGCCG	727
728	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	788
789	CGGTTTATCCCCGCTGGCGCGGGGAACAC		817

BIM 43 CRISPR 2

- CRISPR start position : 43 ---------- CRISPR end position : 803 ---------- CRISPR length : 760
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

43	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	103
104	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	164
165	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	225
226	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGACGC	286
287	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	347
348	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	408
409	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	469
470	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	530
531	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	591
592	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	652
653	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTCGAACAGCTACTGCACAAGCCC	713
714	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCATCTGCAGGGGCAACAAAGGGGAAGACAGG	774
775	CGGTTTATCCCCGCTGGCGCGGGGAACAC		803

BIM 44 CRISPR 2

- CRISPR start position : 56 ---------- CRISPR end position : 818 ---------- CRISPR length : 762
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

56	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	116
117	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGG	177
178	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	238
239	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGACGC	299
300	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	360
361	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	421
422	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	482
483	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	543
544	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	604
605	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	665
666	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCCAACTATTCGAAACAGCTACTGCACAAGCCCC	728
729	GGTTTATCCCCCGCTGGCGCGGGGAACAC	GCATCTGCAGGGGCAACAAAGGGGAAGACAGG	789
790	CGGTTTATCCCCGCTGACGCGGAGACACA		818

S37 CRISPR 2

- CRISPR start position : 56 ---------- CRISPR end position : 818 ---------- CRISPR length : 762
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

56	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	116
117	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCGACAACCAAGCTACGC	177
178	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	238
239	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	299
300	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	360
361	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	421
422	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	482
483	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	543
544	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	604
605	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	665
666	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCCAACTATTGAAACAGCTACTGCAGAAGCGCC	728
729	GGTTTATCCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	789
790	CGGTTTATCCCCGCTGACGCGGAGACACA		818

BIM 27 CRISPR 2

- CRISPR start position : 56 ---------- CRISPR end position : 818 ---------- CRISPR length : 762
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 12

56	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	116
117	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCGACAACCAAGCTACGC	177
178	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	238
239	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGCTAGAGCC	299
300	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	360
361	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	421
422	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	482
483	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	543
544	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	604
605	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	665
666	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCCAACTATTGAAACAGCTACTGCAGAAGCGCC	728
729	GGTTTATCCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	789
790	CGGTTTATCCCCGCTGACGCGGAGACACA		818

BIM 28 CRISPR 2

- CRISPR start position : 43 ---------- CRISPR end position : 621 ---------- CRISPR length : 578
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 9

43	ACGGCTATCCCCGCTGACGCGGGGAACAC	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	103
104	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CCCGTTAGGATGAGTCCACAACCAAGCTACGC	164
165	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	225
226	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGGTAGAGGC	286
287	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	347
348	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	408
409	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCGACACGGCAATAATTGAAGGGTCCCGGAT	469
470	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	530
531	CGGTTTATCCCCGCTGGCGCGGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTTC	592
593	GGGTTTATCCCCGCTGGCGCGGGGAACAC		621

BIM 31 CRISPR 2

- CRISPR start position : 35 ---------- CRISPR end position : 245 ---------- CRISPR length : 210
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 3

35	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	95
96	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGGAAACAGCTACTGCAGAAGCCC	155
156	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GGATCTGCAGGGGCAACAAAGGGGAAGACAGG	216
217	CGGTTTATCCCCGCTGGCGCGGGAACACA		245

BIM 32 CRISPR 2

- CRISPR start position : 9 ---------- CRISPR end position : 709 ---------- CRISPR length : 700
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 11

9	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTTGCGCGACGTAACGAAAAAAACGATCATC	69
70	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TGCGCTTATCATTTTTGCTCCGTGGTAGAGGC	130
131	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCGCGATCCCCATGGCGGAGGTGATACCTGCG	191
192	CGGTTTATCCCCGCTGGCGCGGGGAACAC	TCCTCTTCAGTACCGTATTTCTGTTCCCACGT	252
253	CGGTTTATCCCCGCTGGCGCGGGAAACAC	GCCGACACGGCAATAATTGAAGGGTGCCGGAT	313
314	CGGTTTATCCCCGCTGGCGCGGGGAACAC	ATTCTGATGAGGTCATTTTAATTACATCGGTT	374
375	CGGTTTATCCCCGCTGGAGCAGGGAACAC	CTATCTGCTCCGGTGAATGTGTGCGCGAGTTT	435
436	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCAAAGCTGCCGCTTCTTTTCTCTATACAGAT	496
497	CGGTTTATCCCCGCTGGCGCGGGGAACAC	GCCAACTATTGGAAACAGCTACTGCAGAAGCCC	558
559	CGGTTTATCCCCGCTGGCGCGGGAACACG	GATCTGCAGGGGCAACAAAGGGGAAGACAGG	618
619	CGGTTTATCCCCGCTGGCGCGGGGAACAC	AAATTAATTTACGCTGCACGTCCGCGCCGCGGCGCC	680
681	GGTTTTATCCCCGCTGGCGCGGGGAACAC		709

BIM 33 CRISPR 2

BIM 35 CRISPR 2

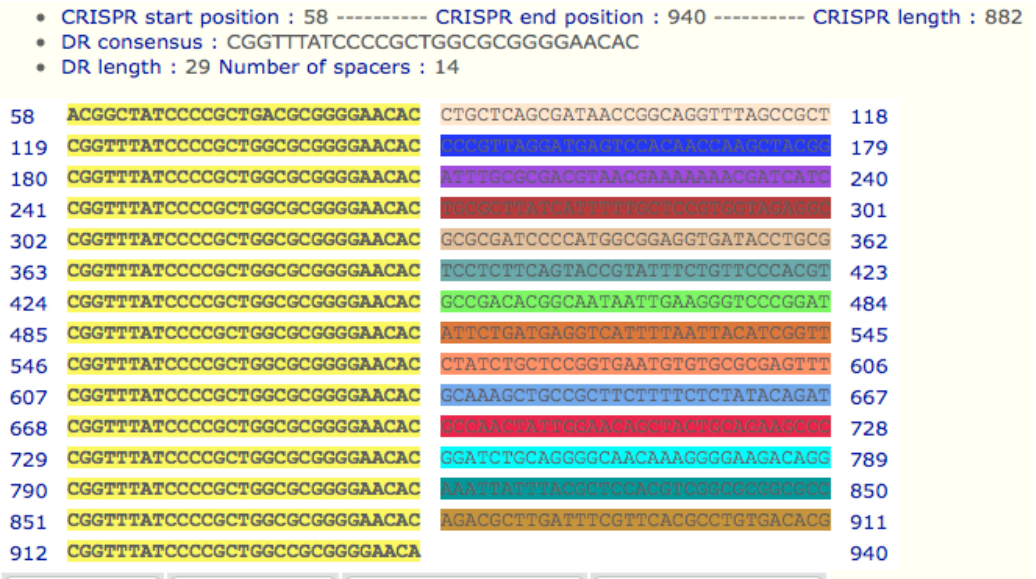

Fig 10.1 Representation of CRISPR 1 and CRISPR 2 regions of *Salmonella* Enteritidis S49, S37 and their BIMs obtained from CRISPRFinder online http://crispr.u psud.fr/Server/CRISPRfinder.php (Grissa *et al.*, 2007).

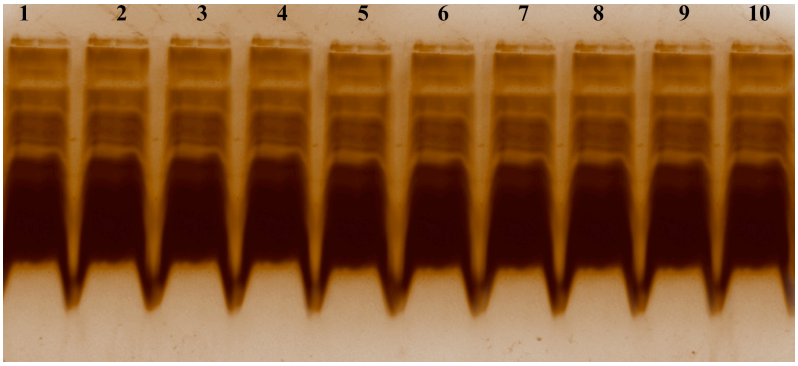

Fig 10.2 LPS profile of *Salmonella* Enteritidis S49 and their third set BIMs

Lane 1-S49, Lane 2- BIM 36, Lane 3-BIM 37, Lane 4-BIM 38, Lane 5- BIM 39, Lane 6-BIM 40, Lane 7-BIM 42, Lane 8-BIM 43, Lane 9- BIM 44

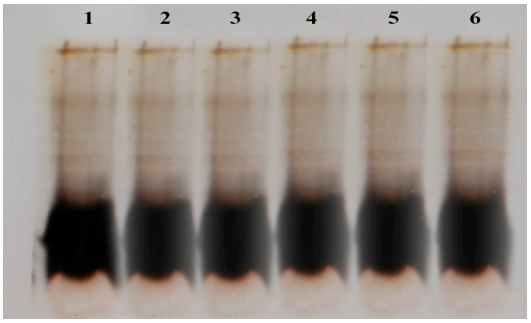

Fig 10.3 LPS profile of S*almonella* Enteritidis S37 and their third set BIMs.
Lane 1-S37, Lane 2- BIM 31, Lane 3- BIM 32, Lane 4- BIM 33, Lane 5-BIM 34, Lane 6-BIM 35.

LIST OF PUBLICATIONS

Publications in peer reviewed journals

Mridula V.G, Jeena Augustine and Sarita G. Bhat. (2015). Bacteriophage insensitive mutants of *Salmonella* Enteritidis. *Current Research in Microbiology and Biotechnology* Vol. 3, No. 1 (2015): 557‑560 ISSN: 2320-2246.

Jeena Augustine**, Mridula V. Gopalakrishnan** and Sarita G. Bhat. (2014). Application of ΦSP-1 and ΦSP-3 as a therapeutic strategy against *Salmonella* Enteritidis infection using *Caenorhabditis elegans* as model organism 2014b *FEMS Microbiology letters*, 356, 113-117.

Full paper in proceedings of National/ International symposium/ conferences/ seminars

Mridula V.G, Jeena Augustine and Sarita G. Bhat. (2014). Lytic Phage ΦSP-2 induced variability in CRISPR regions of Bacteriophage Insensitive Mutants (BIMs) of *Salmonella* Enteritidis S37. Proceedings of International Conference on Earth, Environment and Life sciences (EELS-2014) Dubai, UAE, December 23-24 2014. ISBN: 978-93-84422-02-8.

Mridula V.G, Jeena Augustine, Siju M Varghese and Sarita G. Bhat. (2012). Variations in CRISPRs in *Salmonella* Enteritidis and its Bacteriophage Insensitive Mutant (BIM): Indication of its role in phage host interaction. Proceedings of National Symposium on "Emerging trends in Biotechnology"

organized by Department of Biotechnology, CUSAT. December 12-13, 2012. ISBN: 978-93-80095-39-4.

Posters/Abstracts in National/International symposium

Mridula V.G, Jeena Augustine, Siju M Varghese and Sarita G Bhat (2012). Variations in CRISPRs in *Salmonella* Enteritidis and its Bacteriophage Insensitive Mutant (BIM): Indication of its role in phage host interaction. Proceedings of National Symposium on "Emerging trends in Biotechnology" organized by Department of Biotechnology, CUSAT. December 12-13, 2012.

Sarita G. Bhat, Jeena Augustine and **Mridula V.G.** (2015). Reaffirmming phage cocktail for biocontrol and to overcome BIM development in *Salmonella* Enteritidis. 1st International Conference in Antibiotic Resistance. Caparica, Portugal (26, 27, 28 January 2015).

Jeena Augustine, Linda Louis, **Mridula V.G.** and Sarita G. Bhat (2011). *Salmonella* phages as biocontrol agents to reduce *Salmonella* in experimentally contaminated chicken cuts, Proceedings of UGC sponsored two day national seminar on " Emerging trends in biopharmaceuticals" organized by St. Mary's College, Thrissur.

GenBank Submissions 165 (One sixty five) Nos

16S ribosomal RNA gene, partial sequences (36)

KF468776 - KF468777

KF527460 - KF527461

KP168667 - KP168670

KP226674 - KP226695.

CRISPR sequences (55)

KT008939- KT008942

KT070139- KT070189

MLST sequences (74)

KT855092 - KT855175

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Bacteriophage insensitive mutants of *Salmonella* Enteritidis

Mridula V.G, Jeena Augustine and Sarita G Bhat*

Cochin University of Science and Technology, Cochin 682022, India

* Corresponding author: Sarita G Bhat; e-mail: vg.mridula@gmail.com

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ABSTRACT

The battle between predator and prey is perhaps the second oldest conflict on earth, and phage represents one of oldest predators on the planet. All cellular systems evolve ways to combat predators and genomic parasites. Phages form a formidable force against bacteria and archaea, being responsible for 4%–50% of their destruction. To combat this, numerous resistance mechanisms have emerged against phage leading to emergence of bacteriophage insensitive mutants (BIMs). In this study, BIMs of *Salmonella* Enteritidis (S49), developed after phage host interaction with *Salmonella* specific lytic phage ΦSP-1(a candidate phage for phage therapy), were isolated and characterized by biochemical and molecular analysis, after reconfirmation as *Salmonella*. Antibiotic sensitivity by disk diffusion method was used to detect for variation in antibiotic sensitivity of host and its BIMs. Cefuroxime resistance observed in BIM 21 and 22, was an altered response from that in S49. Tetracycline resistance observed in S49, was changed to an intermediate response in BIM 19. The emergence of BIMs is a cause for concern as it challenges the use of the phage as an alternative to antibiotics in treating bacterial infections.

Keywords: *Salmonella* Enteritidis, phage host interaction, bacteriophage insensitive mutants (BIMs), lytic phage.

1.0 INTRODUCTION

Salmonella enterica serovar Enteritidis (SE) caused salmonellosis during the late 1970s and early 1980s and continues to be a principle reason for enteric disease in humans [1], in both Europe and North America [2-4]. Extensive varieties of food of animal origin, especially poultry, poultry products and raw eggs, are often implicated in sporadic cases, and outbreaks of human salmonellosis are attributable to *Salmonella enterica* [5-6]. Poultry is widely acknowledged as a reservoir of *Salmonella*, due to their ability to proliferate in the gastrointestinal tract of chicken [7], and subsequently survive on commercially processed broiler carcasses. The 1980s saw the emergence of resistance in *Salmonella* to an assortment of antimicrobials, including first-choice agents for the treatment in human, and today multi-drug resistance to "critically important antimicrobials" is further compounding problems. [8-9].

Investigations for new alternative anti-microbials, effective against bacterial pathogens have become increasingly relevant for both human and veterinary applications, with bacteriophage as potential candidates [10]. The first and foremost advantage of phage as an alternative biocontrol is their activity even against bacteria resistant to antibiotics [11-14]; second is their specificity, preventing secondary bacterial infection which is commonly observed in antibiotic therapy [16].

One of the major problems that can arise due to the use of phages as therapeutics is the development of phage-resistant mutants [17-19], also called as BIMs. Microbes devise various strategies allowing them to survive exposure to foreign genetic elements. Although out-populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments. The constant exposure to exogenous

DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements. These systems maintain genetic integrity, yet occasionally allow exogenous DNA uptake and conservation of genetic material advantageous for adaptation to the environment. Certain strategies, such as prevention of adsorption, blocking of injection, and abortive infection, are effective against viruses; other defense systems specifically target invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases.

Specific lytic phage ΦSP-1 [20] was previously isolated and characterized for potential use in phage therapy. In this study, we take a look at the bacteriophage insensitive mutants that emerged due to the interaction of *Salmonella* Enteritidis S49 and ΦSP-1. BIMs were characterized by biochemical and molecular testing. This information will aid in the development of phage cocktail to overcome phage resistance.

2.0 MATERIALS AND METHODS

2.1 Bacterial culture and Bacteriophage

Salmonella Enteritidis serotype S49 and its specific lytic phage, ΦSP-1 used in this study was previously isolated, purified and characterized [20].

2.2 Isolation of Bacteriophage Insensitive Mutants (BIMs)

Bacteriophage Insensitive Mutants were obtained when *Salmonella* Enteritidis serotype S49 was challenged by phage ΦSP-1. Briefly 1 mL of overnight culture of *S.* Enteritidis strain S 49 was mixed with 10 µL of phage lysate(10^{12} pfu/mL) [20], added1 mL of normal saline and incubated at 40°C for one hour in a water bath (Scigenics, Chennai, India). After incubation, 3mL of soft agar, (Nutrient broth (HiMedia, Mumbai, India) containing 0.8% agarose) was added, mixed well and immediately overlaid on nutrient agar plates [21], and plates incubated for 16 h at 37°C. The colonies that appeared in the top agar layer were picked from the plate as bacteriophage insensitive mutants inoculated in nutrient broth and stored on nutrient slants at 4°C until use.

2.3 Confirmation of BIMs as *Salmonella.*

The BIMs were confirmed as *Salmonella* following the guidelines of the bacteriological analytical manual of the US Food and Drug Administration [22], KBMOO2 HiMotilityTM Biochemical kit for *Salmonella* (HiMedia) was used for biochemical characterization. DNA was isolated from BIMs [23], 16SrRNA gene was amplified using universal primers [24]; with an initial denaturation at 94°C for 1.5 min, 35 cycles of denaturation at 94°C for 30 s, annealing for 56°C for 30s, extension at 72°C for 2 min and final extension at 72°C for 10min. Nucleotide sequences of the PCR amplicons were determined by ABI Prism 310 genetic analyzer, using big dye terminator kit. The identity of the sequence was determined by comparing the sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST) software [25].

2.4 Multiple sequence alignment and phylogenetic tree construction

All nucleotide sequences were converted into FASTA format and multiple sequence alignment was done using the Clustal W program [26] in BioEdit software [27]. Aligned sequences were imported into MEGA 5 (Molecular Evolutionary Genetics Analysis version 5.0) [28]. The ends were trimmed to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method [29] using nucleotide based TN84evolutionary model for estimating genetic distances based on synonymous and non synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

2.5 Antibiotic susceptibility test

Antibiotic susceptibility test was done according to disc diffusion method [30]. To obtain a uniform bacterial lawn, the culture suspension was swabbed, on Mueller-Hinton agar (HiMedia) plate. Antibiotic discs were placed, and spaced to provide room for the zone of inhibition. The plates were incubated at 37°C for 24 h before the examination. The result was interpreted as resistant, intermediate or sensitive based on the zone diameter (mm) as per the manufacturer (HiMedia, India). The tested antibiotics included ampicillin (5 µg/disc), tetracycline (30 µg/disc), trimethoprim (5 µg/disc),azithromycin (15 µg/disc), ciprofloxacin (5 µg/disc), chloramphenicol (30µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc),nalidixic acid (30 µg/disc) and gentamicin (10 µg/disc). The experiment was independently repeated thrice and the interpretation was based on the average of the three measurements.

3.0 RESULTS AND DISCUSSION

The challenge of *Salmonella* Enteritidis S49 by its specific lytic phage ΦSP-1 led to generation of four bacteriophage insensitive mutants-namely BIM19, BIM20, BIM21 and BIM22. Repeated infection of these BIMs by ΦSP-1 did not lead to cell lysis, which confirmed their phage insensitivity. The biochemical characteristics of the four BIMs and their parent strain S49 were observed to be similar to each other and is represented in Table-1.

The PCR amplification of the 16S rDNA gene and subsequent sequence analysis using BLAST indicated the sequence similarity between the four BIMs and S49, and showed that they were all *Salmonella* Enteritidis. Accession numbers were obtained after submission of the sequence to GenBank (BIM19 KP168667, BIM20 KP168668, BIM21 KP168669, BIM22 KP168670). Based on the 16S rRNA gene sequences, the relatedness of the host bacteria S49 and its four BIMs were studied by constructing a phlyogenetic tree as depicted in

Figure 1. From the phylogenetic tree, it can be observed that the host bacteria and its four BIMs grouped together to form a single clade, pointing to the fact that they were related.

Table 1: Biochemical characteristics of Bacteriophage insensitive mutants and *Salmonella* Enteritidis S49 using KBMOO2 HiMotilityTM Biochemical kit for *Salmonella* (Hi Media).

No	Test	S 49	BIM 19	BIM 20	BIM 21	BIM 22
1	Motility	+	+	+	+	+
2	Citrate Utilization	+	+	+	+	+
3	Urease	-	-	-	-	-
4	Arginine Utilization	-	-	-	-	-
5	Lysine Utilization	+	+	+	+	+
6	H_2S production	+	+	+	+	+
7	ONPG	-	-	-	-	-
8	Arabinose	+	+	+	+	+
9	Lactose	+	+	+	+	+
10	Maltose	+	+	+	+	+
11	Trehalose	+	+	+	+	+

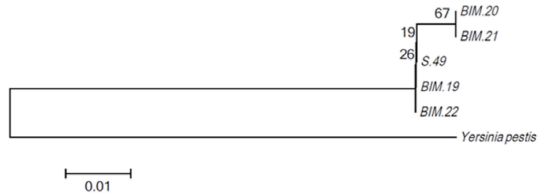

Figure 1: Phylogenetic tree indicating the relationship of S49 and its four BIMs based on 16SrDNA sequences. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on neighbour- joining analysis of 1000 resampled data set. *Yersinia pestis* was used as the out group.

The antibiotic sensitivity was interpreted as per the manufacturers instruction as sensitive, intermediate and resistant. The antibiogram of S49 and its BIMs is shown in Table-2. Strain S49 was resistant only to tetracycline and intermediate for azithromycin, cefuroxime, ciprofloxacin and nalidixic acid. The antibiotic sensitivity profile of the mutants was different from that of its host, with BIM 21 and BIM 22 showing resistance to cefuroxime while its host S49 indicated intermediate sensitivity. All exhibited resistance to tetracycline, but BIM 19 showed intermediate sensitivity. Many findings indicated that tetracycline resistance in *Salmonella* can be attributed to the production of an energy dependent efflux pump to remove the antibiotic from within the cell. The modification of the ribosomal target, enzymatic inactivation of tetracycline, and other mechanism of resistance, have been documented in other bacterial species but has yet to be reported in *Salmonella* [31]; [32].

It was observed that BIMs 21 and 22 were resistant to cefuroxime. Beta-lactams comprise of penicillin derivatives, cephalosporins, carbapenems and monobactams [34]; [35]. There is growing concern about more than 340 β- lactamases resistance genes, such as blaTEM, blaOXA, blaPER, blaPSE, blaSHV, blaCTX-M, and blaCMY, while some are more prevalent in *Salmonella* globally [36]. In *Salmonella*, the secretion of a β-lactamase is the common mechanism of resistance to beta-lactams. Moreover, in *Salmonella* the class Aβ lactamases are most commonly found, which provide resistance against penicillins, cephalosproins, and carbapenems, and are plasmid encoded. The class C β- lactamases providing resistance against cephalosporins such as cefoxitin and ceftiofur, are normally encoded by chromosomal ampC genes. However these genes are plasmid mediated in *Salmonella,* which have no chromosomal ampC gene [37].

Table 2: Antibiotic sensitivity profile of S49 and its BIMs.

Antibiotics	Range			S49	BIM 19	BIM 20	BIM 21	BIM 22
	R	I	S					
Ampicillin	≤ 11	12-14	≥ 15	S	S	I	S	S
Azithromycin	≤ 13	14 -17	≥ 18	I	I	I	I	I
Cefixime	≤ 15	15-17	≥18	S	S	I	I	I
Cefuroxime	≤14	15-17	≥18	I	I	I	R	R
Chloramphenicol	≤ 12	13-17	≥ 18	S	S	S	S	S
Ciprofloxacin	≤ 15	16-20	≥ 21	I	S	S	S	S
Tetracycline	≤ 14	15-18	≥ 19	R	I	R	R	R
Gentamicin	≤ 12	13-14	≥ 15	S	S	S	S	S
Nalidixic acid	≤ 13	14-18	≥ 19	I	S	I	S	S
Trimethoprim	≤10	11-15	≥16	S	S	S	S	S

R-resistant, I-intermediate, S-sensitive

4.0 CONCLUSION

The misuse of antimicrobial agents as chemotherapy in human and veterinary medicine or as growth promoter in food for animals can potentially lead to widespread dissemination of antimicrobial resistance to *Salmonella* and other pathogens via mobile genetic elements [33]. In bacteriophage therapy, the use of viruses that infect bacteria as antimicrobials has been championed as a promising alternative to conventional antibiotics. The long-term use of phages as antimicrobials may lead to phage resistance, emphasizing the need for phage cocktails for the effective phage therapy. Hence the search for more phages to add to this armoury against pathogenic bacteria is imminent.

5.0 Acknowledgements

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FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Application of ΦSP-1 and ΦSP-3 as a therapeutic strategy against *Salmonella* Enteritidis infection using *Caenorhabditis elegans* as model organism

Jeena Augustine, Mridula V. Gopalakrishnan & Sarita G. Bhat

Department of Biotechnology, Cochin University of Science and Technology, Cochin, India

Correspondence: Sarita G. Bhat, Department of Biotechnology, Cochin University of Science and Technology, Kalamassery, Cochin-22, Kerala, India. Tel.: 91 484 2576267; fax: 91 484 2577595; e-mail: saritagbhat@gmail.com

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Enteritidis; lytic phage; biocontrol agent; antibiotic resistance.

Abstract

The potential of *Salmonella*-specific phages ΦSP-1 and ΦSP-3 as biocontrol agents was studied in *vitro,* employing host cell lysis test and *in vivo*, using *Caenorhabditis elegans* as a model organism. For *in vivo* testing, stage 4 *C. elegans* larvae were experimentally infected with the pathogen *Salmonella.* Worm mortality was scored for 10 days. TD_{50} (the time required for 50% of the nematodes to die) of infected worms in the presence of bacteriophages was comparable to uninfected worms, and the two phages provided an increased protection than each one. This study in addition demonstrated the simplicity, elegance, and the cost effectiveness of the *C. elegans* model for *in vivo* validation.

Introduction

Foodborne pathogens are the principal cause of illness and death in less developed countries, killing *c.* 1.8 million people annually (Faruque, 2012). *Salmonella* is prominent among the bacterial pathogens. The incidence of Salmonellosis is higher in industrialized and developing countries (Majowicz *et al.*, 2010), and there have been periodic reports of *Salmonella* in a variety of sources from India, where it is endemic (Gupta & Verma, 1989; Hatha & Lakshmanaperumalsamy, 1997; Tankhiwale *et al.*, 2003; Suresh *et al.*, 2006; Singh *et al.*, 2010; Harsha *et al.*, 2011). The beginning of the 1980s saw the emergence of *Salmonella* strains resistant to a range of antimicrobials used for treatment in human. Multidrug resistance to 'critically important antimicrobials' are further compounding the problem (Ribot *et al.*, 2002; Faldynova *et al.*, 2003). Investigations for alternative antimicrobials, effective against bacterial pathogens including *Salmonella,* have become increasingly relevant for both human and veterinary applications. Bacteriophages, obligate intracellular parasites that multiply inside bacteria, are potential candidates (Boyle *et al.*, 2007). Newly isolated phages usually have to undergo thorough characterization, before being tested for its bacteriolysis capacity *in vitro* and *in vivo*. Here, *Caenorhabditis elegans,* a small free-living soil nematode that feeds primarily on bacteria, was selected as model organism to study the effectiveness of phages in combating the bacterial pathogen *in vivo.* The experimental advantages associated with *Caenorhabditis elegans* include simple growth conditions and a rapid generation time. The transparent body allows clear observation of internal organs. Different strains of *Salmonella* like *S.* Typhimurium as well as other *Salmonella enteric* serovars including *S.* Enteritidis and *S.* Dublin were found to be effective in killing *C. elegans* (Aballay *et al.*, 2000), and it was demonstrated that *S. enterica* could establish a persistent infection within the gut of the nematode to achieve full pathogenicity (Labrousse *et al.*, 2000). The ability of phages to protect *Salmonella* infection in *C. elegans* has been previously reported (Santander & Robeson, 2007).

Considering the above factors, this study sought to test the ability of two *Salmonella* lytic phages, previously isolated in the laboratory to control *Salmonella* infection in *C. elegans* as model organism.

Materials and methods

Salmonella host culture

Salmonella previously isolated and purified in the laboratory and designated as S49 was used for the present study. The host strain S49 was confirmed to be *Salmonella* Enteritidis 9, 12: g, m:- by serotyping at National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh, India. The 16S rRNA gene sequence analysis of S49 by BLAST showed maximum sequence similarity with *Salmonella enterica* ssp. *Enterica* and the sequence were submitted to GenBank (Accession Number: S49: HQ268500) (Augustine *et al.*, 2013a).

Bacteriophage isolation

Bacteriophages previously isolated, purified and characterized in the laboratory, and designated as ΦSP-1 (Augustine *et al.*, 2013a) and ΦSP-3 (Augustine *et al.*, 2013b) were used for the present study. ΦSP-1 is a T7-like phage (Accession Number JQ638925), and ΦSP-3 is a T5-like phage (Accession Number JQ638926) (Augustine *et al.*, 2013a, b). The phage lysate for the experiments were prepared using polyethylene glycol precipitation method as described by Sambrook *et al.* (2000).

Host cell lysis test

The competency of phages in *Salmonella*-specific bacteriolysis was studied by the host cell lysis test (Hsieh *et al.*, 2011). The experimental set up consisted of four groups (group A. S 49, group B. S49 + ΦSP-1, group C. S49 + ΦSP-3 and group D. S49 + ΦSP-1 + ΦSP-3). Overnight cultures of S49 were diluted 1 : 100 in fresh nutrient broth and grown until log phase. One milliliter of this culture (*c.* 10^8 CFU mL^{-1}) was then transferred to fresh nutrient broth and infected with respective phage lysate. In case of group 2 and group 3, 10^{10} PFU mL^{-1} of lysate was added, and in case of group 4, each phage was added at 5×10^9 PFU mL^{-1}. Final volume was adjusted with nutrient broth. All mixtures were incubated at 37 °C at 120 r.p.m. (Scigenics, India). Readings were taken at definite intervals from all the sets and was read at $OD_{600\ nm}$ (Shimadzu, Japan) to check for cell lysis. The experiments were conducted in triplicate.

Caenorhabditis elegans strain and maintenance

The nematode *C. elegans* Bristol N2 wild type was used for the study. It was propagated and maintained in modified Nematode Growth Medium (NGM) agar (0.35% instead of 0.25% peptone) (Stiernagle, 2006) at 20 °C. Medium size plates (60 mm diameter) were used for general strain maintenance, and small plates of 35 mm diameter were used for the bioassay. *Escherichia coli* strain OP50, used as the food source (Brenner, 1974), is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is desired as it allows for easier observation of the worms. A starter culture of *E. coli* OP50, prepared by aseptic inoculation of Luria–Bertani broth and incubation overnight at 37 °C, was used to seed NGM plates. The worms were observed under a simple dissecting stereomicroscope (Labomed, CZM6) equipped with a transmitted light source.

Bacterial infection and phage treatment

The experimental design to study the ability of phage in controlling the infection consisted of six groups. *Caenorhabditis elegans* killing assays were conducted as per Aballay *et al.* (2000). *Salmonella* strains and *E. coli* OP 50 were grown overnight in Luria–Bertani broth and spread on NG agar medium in 3.5-cm-diameter plates. The plates were incubated at 37 °C for 2–12 h and allowed to equilibrate to room temperature. 15–20 worms (Larval-4 stage) were placed on each seeded plate and incubated at 25 °C for 24 h. Thereafter, the nematodes from plates of group 1, 2, and 3 were transferred to NGM plates seeded with *E. coli* OP 50. Nematodes from plates of group 4 and 5 are transferred to plate containing *E. coli* OP 50 lawn in which ΦSP-1 and ΦSP-3 are incorporated (1×10^{10} PFU), respectively. Worms from group 6 plates are transferred onto plates with *E. coli* OP 50 lawn incorporated with 5×10^9 PFU of ΦSP-1 and ΦSP-3 each. All the plates were incubated at 25 °C for 24 h, and the worms were transferred to each group's respective NGM for the next 4 days of the experiment or until no more progeny were evident. This was performed in order to avoid losing track of the original worms due to crowding by reproduction. Worm mortality was scored for 10 days, and a worm was considered dead when it failed to respond to touch. Worms that stuck to the wall and died were excluded from the analysis. The three independent experiments were conducted.

Statistical analysis

Statistical evaluations were performed by ANOVA followed by Student–Newman–Keul's test using GRAPHPAD INSTAT (version 2.04a, San Diego) computer program for host cell lysis test.

PRISM (version 5.04) computer program was utilized to calculate the time taken for 50% of the nematodes to die (time to death 50, TD_{50}) using the equation: Y = Bottom + (Top−Bottom)/(1 + 10^ ((LogEC50−X) × Hill

Slope)), where X is the logarithm of days and Y is the average of dead worms. The data represent the mean ± SE.

Results

Host cell lysis test

The ability of ΦSP-1 and ΦSP-3 individually and in combination to lyse *Salmonella* S49 was tested. At 0 h, the $OD_{600\ nm}$ reading for all the groups was 0.15. $OD_{600\ nm}$ increased in group A, as there was no phage infection and therefore no lysis of S49, and at the end of the experiment it was 0.25. On the contrary in group B, C and D where the phages effectively lysed the bacterial cells (Fig. 1), at the end of 7th hour, $OD_{600\ nm}$ was 0.05, 0.08, and 0.06, respectively. Values represent mean ± SD, and in all the cases, $P < 0.001$ when compared to group A, indicating high significance.

Phage treatment

The nematode *C. elegans* was used as a model system not only to study the bacterial pathogenesis of *Salmonella* strain S49 identified as *Salmonella* Enteritidis 9, 12: g, m:- by serotyping, but also to test the ability of the bacteriophages ΦSP-1 and ΦSP-3 to confer protection against the bacterial infection. The ability of these phages to combat *Salmonella* infection in the model organism is represented in Fig. 2, which shows the outcome of the phage treatment. The figure represents the percentage mortality of *C. elegans* over a span of 10 days when exposed to the pathogen S49 and the influence of the bacteriophages individually and in combination, in maintaining the lifespan of the worms to near normal levels when compared to the untreated (control) worms fed on *E. coli* OP50. It was observed that both the phages ΦSP-1 and ΦSP-3 had a positive influence in increasing the lifespan of the infected worm.

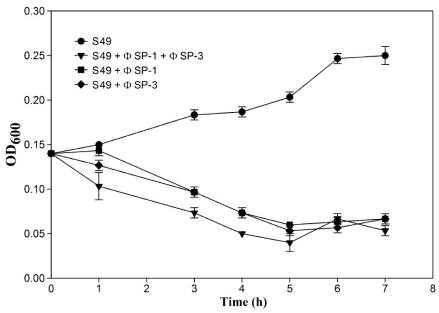

Fig. 1. Time course of host cell lysis by Φ SP-1 and Φ SP-3. Cultures of S49 were infected with Φ SP-1 and Φ SP-3 individually or in combination. $OD_{600\ nm}$ was measured following bacterial challenges at regular time intervals. The results shown are means ± SD s from three independent experiments. In all cases, $P < 0.001$ when compared to control with no phages.

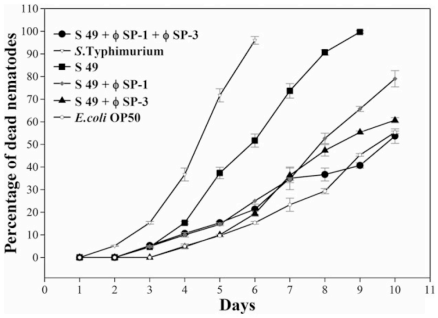

Fig. 2. Phage treatment *in Caenorhabditis elegans* infected by *Salmonella* strain S49. (The experiment was set up as per Aballay *et al.* (2000). Values represent mean ± SD from three independent experiments.

The time required for the mortality of 50% of the nematodes (TD_{50}) was also calculated. The TD 50 of S49 infected *C. elegans* was calculated as 5.85 ± 0.20 days. This indicates its pathogenic nature comparable to that of the known pathogen *Salmonella* Typhimurium's TD 50 which was calculated as 4.33 ± 0.12 days. The outcome of the phage treatment was found to be promising. When applied, the phages displayed their ability to control the *Salmonella* infection by cell lysis *in vivo* also thereby helping in reducing the mortality rate of infected worms. TD 50 was calculated as 7.91 ± 0.26, 8.29 ± 0.33, and 9.29 ± 0.26 days when infected worms were treated with ΦSP-1, ΦSP-3, and ΦSP-1 + ΦSP-3, respectively. Maximum protection was conferred by phages when they were applied together, when the TD_{50} reached to near control levels TD 50 of healthy *C. elegans* (9.48 ± 0.34 days).

Discussion

The prospects of lytic phages as biocontrol agents against pathogenic bacteria are being reconsidered worldwide with the surfacing of multiple antibiotic resistances (Sulakvelidze *et al.*, 2001). Thus, in the present study, the potential of ΦSP-1 and ΦSP-3 as a biocontrol agent was tested employing both *in vitro* and *in vivo* assays. The outcome of the host cell lysis test was promising.

*Salmonell*a-specific lytic phages ΦSP-1 and ΦSP-3 were effective in lysing the experimentally infected S49 *Salmonella* strain used in the study, thereby leading to a dip in the $OD_{600\ nm}$. Compared with the control, phages proved to be effective control agents in terms of their lethality, when applied individually or in combination.

The nematode *C. elegans* is long known for its use as an animal model system in the study of bacterial pathogenesis. Pathogens reported to be relatively virulent to *C. elegans* include certain strains of *Pseudomonas aeruginosa* (Tan & Ausubel, 2000), *Serratia marcescens* (Pujol *et al.*, 2001), and *Burkholderia paseudomallei* (O'Quinn *et al.*, 2001). It establishes intestinal infections and provokes obvious signs of sickness: locomotory problems, distension of the intestine, and cell lysis, followed by the precocious death of the worms (Ewbank, 2002).

Caenorhabditis elegans is reportedly also infected by *S.* Enteritidis and *S.* Pullorum (Aballay *et al.*, 2000; Aballay & Ausubel, 2001) and has been used to test for protection against *Salmonella* Enteritidis and *S. Pullorum* infection using bacteriophage prophylaxis assay (Santander & Robeson, 2007), where pretreatment with bacteriophage resulted in enhanced survival of *the* nematodes challenged with the bacterial pathogens.

In the present study, *Salmonella* strain S49 identified as *Salmonella* Enteritidis was also seen to infect and reduce the longevity of the worm. The results obtained from experiment revealed that pathogenesis of S49 in *C. elegans* was comparable with that of Aballay *et al.*, 2000, who had reported a TD50 of 5.3 ± 0.8 for *S.* Enteritidis and a TD50 of 9.9 ± 0.9 days when fed with *E. coli* OP50.

When the nematodes were exposed to lawns of *E. coli* OP50 containing the phages after their exposure to the bacterial pathogens, there was a significant drop in mortality. The ability of the bacteriophages ΦSP-1 and ΦSP-3 to confer protection against the bacterial infection by S49 was thus proved by this study. With the phage cocktail treatment, the TD_{50} of infected worms attained near control levels, the phage cocktail exhibiting maximum impact. The effectiveness of phage cocktail is due to the fact that bacteria which become resistant to one phage remain susceptible to the other (Gu *et al.*, 2012). In addition to that even when resistant forms appear, longer time is needed for mutations to develop resistance against multiple phages are involved (Tanji *et al.*, 2005). Recently, a group of workers also proved the effectiveness of bacteriophage cocktail as a biocontrol agent of *Salmonella* in several food matrices including pig skin, chicken breasts, fresh eggs, and packaged lettuce (Spricigo *et al.*, 2013).

The present study validated the hypothesis proposed regarding the application of phages in biocontrol and the animal model used. The simplicity, elegance, and the cost effectiveness of the *C. elegans* model for the *in vivo* validation were displayed by this study, taking us a step toward trails in other animal models. Future work in this area will also involve prescreening and electing other potential phages using the *C. elegan* model before extending the *in vivo* experiments in chicken following the methodologies of O'Flynn *et al.,* (2006) and Fiorentin *et al.* (2004).

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