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An Insight into CRISPR Regions of *Salmonella* Enteritidis Isolated from Poultry and its Bacteriophage Insensitive Mutants

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Abstract: Bacteria are continually exposed to foreign elements, such as bacteriophages and plasmids. It is very much archived that bacteria evolved defense systems against bacteriophages, which permit them to survive in an environment full of their predators. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR associated (Cas) genes adaptive immune systems provide heritable sequence-specific protection against these invaders. In this work the host Salmonella Enteritidis S49 was infected with its specific phage ΦSP-3 to isolate Bacteriophage Insensitive Mutants (BIMs). In order to understand the variation in the CRISPR regions of host and its mutants, they were amplified using specific primers of Salmonella. The amplicons obtained were sequenced and CRISPR identification was performed using CRISPRFinder online. Identities of CRISPR spacer regions were obtained using BLAST (Basic Local Alignment Search Tool). Host bacteria Salmonella Enteritidis S49 infected with its specific phage ΦSP-3 yielded BIMs. CRISPR regions were amplified and detected in CRISPRFinder online. A graphic representation of spacers based on its identity was prepared to analyse the spacer pattern in the BIMs and its host. Spacers were found deleted in certain BIMs and there was no novel addition of spacer in this case to infer CRISPR involvement in emergence of mutants. However, this study clearly shows that there were notable variations in spacer regions through phage challenges warranting further studies.
Keywords: CRISPR-Cas, Salmonella Enteritidis, BIMs, BLAST.

I. INTRODUCTION

Viruses of bacteria, bacteriophages represent the most abundant life forms on the planet. In addition, they are supposed to inhabit every niche in which potential hosts exist (1, 2). Hence, there is an adaptive pressure on bacteria to avoid phage infection in order to survive. As such, bacteria have evolved various phage resistance mechanisms including restriction/modification and abortive infection systems (3). Clustered regularly interspaced short palindromic repeats (CRISPRs) represent a component of a CRISPR/Cas system that confers adaptive immunity against viruses and plasmids (4, 5). 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 (6, 7, 8). The repeat sequence is highly conserved within a particular CRISPR locus. In contrast, the spacers differ greatly and their sequences have similarity to phages and plasmids and sometimes to host chromosomal sequences (9). Each CRISPR is commonly followed by a conserved AT-rich sequence known as a leader sequence. Adjacent to the CRISPR loci are located Cas genes, essential components of the system (10). Acquired immunity concerning CRISPR/Cas systems can be divided into two stages: the acquisition stage for uptake of the alien element as a spacer into the leader-proximal end of CRISPR, and the immunity stage involving interference with the focusing of DNA in a sequence-specific manner (10, 11). CRISPR interference is assisted by a set of Cas proteins that are encoded by the Cas genes usually found immediately adjacent to the repeats. Cas proteins can be organized into 45 different types but their precise biochemical functions are largely unknown. Only one protein, Cas1, has orthologs in all CRISPR loci (12). To provide immunity, the system follows three general steps: spacer acquisition, biogenesis of small RNAs and interference. Following a phage challenge in the acquisition step a bacterial cell (BIM) acquires a new repeat-spacer unit in its CRISPR locus. The new spacer in the CRISPR array is acquired from the invading DNA through the association of spacer acquisition motif located in the vicinity of a (proto) spacer in the phage genome. With the help of *trans*-acting RNA (tracrRNA) and the host RNase III the CRISPR locus is then transcribed and processed 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, by point mutation in the protospacer (PS) sequence or adjacent motif phage mutants can bypass the interference activity (CEM, CRISPR-Escape Mutants) (13).

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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.

II. MATERIALS AND METHODS

A. Bacterial cultures and Bacteriophages

Salmonella Enteritidis serotype strain S49 was selected to study CRISPRs in phage-host interaction. It was previously isolated from chicken gut. For that previously isolated, purified and characterized specific lytic phage, namely ΦSP-3 (14, 15) was used.

B. Isolation of Bacteriophage Insensitive Mutants (BIMs)

Salmonella Enteritidis serotype S49 was challenged with its lytic phage ΦSP-3. Briefly, 1 mL of overnight culture of *S*. Enteritidis strain S49 was mixed with 10 µL of phage lysate of ΦSP-3 (10^{12} pfu/mL) [15], followed by 1 mL of normal saline and incubated at 40˚C for one hour in a water bath (Scigenics, Chennai, India). After incubation, 3mL of sterile soft agar, (Nutrient broth (HiMedia, Mumbai, India) containing 0.8% agarose was added, mixed well and immediately overlaid on nutrient agar plates and plates incubated for 16 h at 37˚C. The colonies that appear in the top agar layer are the bacteriophage insensitive mutants and were picked from the plate, inoculated in nutrient broth and stored on nutrient agar slants at 4˚C until use (16).

C. Confirmation of BIMs as Salmonella

Confirmation of BIMs as *Salmonella* was according to the guidelines of the bacteriological analytical manual of the US Food and Drug Administration (17) and also by 16S rRNA gene sequence analysis using universal primers (18); with an initial denaturation at 94^0C for 1.5 min, 35 cycles of denaturation at 94^0C for 30s, annealing for 56^0C for 30s, extension at 72^0C for 2 min and final extension at 72^0C for 10min. The nucleotide sequences of the PCR amplicon were determined by the 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 (19).

D. PCR Amplification of CRISPR regions

The genomic DNA was isolated (20) and CRISPR regions were amplified.

Salmonella Enteritidis strain S49 and their BIMs were subjected to molecular characterization by PCR using specific primer pairs for Salmonella for CRISPR regions (21) (Table 1). After PCR amplification, the products were sequenced.

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

Sequences	Amplicon	Refrences
CRISPR 1 region CR1F-GCTGGTGAAACGTGTTTATCC CR2R-ATTCCGGTAGATYTKGATGGAC CRISPR 2 region CR2F-AACGCCATGGCCTTCTCCTG CR2RCAAAATCAGYAAATTAGCTGTTC	1300bp ,,	[21].

E. PCR Program

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

F. CRISPR Finder

CRISPR Finder 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. CRISPRFinder is freely accessible at http://crispr.u-psud.fr/Server/CRISPRfinder.php (22).

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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 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.

G. Analysis of CRISPR Spacer Sequences

The CRISPR identification application CRISPRFinder (22) 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 BLAST software (19) at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi. nlm.nih.gov). After doing the BLAST analysis of each 32-nucleotide spacer sequences, a graphic representation of spacers of CRISPR 1 and 2 regions in *Salmonella* Enteritidis S49 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 (23).

III. RESULTS

A. Isolation of Bacteriophage Insensitive Mutants and their confirmation as Salmonella

Infection of the host *Salmonella* Enteritidis serotype S49 individually by its lytic phage ΦSP-3 yielded 9 Bacteriophage Insensitive Mutants. These 9 mutants were confirmed as *Salmonella* Enteritidis and GenBank accession numbers obtained.

B. Analysis of CRISPR Regions

Following DNA isolation and amplification of the CRISPR 1 and CRISPR 2 regions in S49 and their BIMs, 1300bp-amplicons were obtained in all cases.

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

C. 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 1. The analysis of the sequences of both hosts and their BIMs from CRISPRFinder is shown in table 2. The sequenced products were deposited in the Genbank database and accession numbers were obtained (Table 2).

Table 2: GenBank accession numbers of CRISPR 1 and CRISPR 2 sequences of host *Salmonella* Enteritidis and their BIMs.

Organisms		GenBank accession numbers
CRISPR 1	S49	KT008939
	BIM 3	KT070140
	BIM 6	KT070142
	BIM 7	KT070143
	BIM 16	KT070149
	BIM 42	KT070151
	BIM 43	KT070152
	BIM 44	KT008940
CRISPR 2	S49	KT008941
	BIM 3	KT070173
	BIM 6	KT070177
	BIM 7	KT070176
	BIM 10	KT070180
	BIM 16	KT070167
	BIM 40	KT070163
	BIM 42	KT070164
	BIM 43	KT070165
	BIM 44	KT008942

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D. 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 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 had accession number KP184385.1.

Table 3: The features of the CRISPRs from *Salmonella* Enteritidis S49 and their BIMs obtained using the CRISPRFinder [22].

Organism		Length of CRISPR 1 (bp)	Length of CRISPR 2 (bp)	Repeat Length		Number of spacers	
Bacterio phage	Bacterium			CR1SPR 1	CRISPR 2	CRISP R 1	CRIS PR 2
	Salmonella Enteritidis S49	883	821	29	29	14	13
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 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

E. Indicates the Regions CRISPR 1 and 2 that were not Amplified by PCR.

- CRISPR start position : 24 ---------- CRISPR end position : 907 ---------- CRISPR length : 883
- DR consensus : CGGTTTATCCCCGCTGGCGCGGGGAACAC
- DR length : 29 Number of spacers : 14

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24   CGGTTTATCCCCGCTGGCGCGGGGAAGAC  TTTAAAACTCTTGCTGGAGACATGGGCGTCCC  84
85   CGGTTTATCCCCGCTGGCGCGGGGAACAC  ATTCGCACCTCCAGCCGTCTGGCGTATGCACT  145
146  CGGTTTATCCCCGCTGGCGCGGGGAACAC  [illegible]                        206
207  CGGTTTATCCCCGCTGGCGCGGGGAACAC  [illegible]                        267
268  CGGTTTATCCCCGCTGGCGCGGGGAACAC  TCGTGGTTGTCCTGCACCCGCTCGAATAAATC  328
329  CGGTTTATCCCCGCTGGCGCGGGGAACAC  TTGACCTGGAGCATCTGAAAAGTATTCACAAG  389
390  CGGTTTATCCCCGCTGGCGCGGGGAACAC  TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC  450
451  CGGTTTATCCCCGCTGGCGCGGGGAACAC  TTATTGGTATTGGGCGTTTCTTTTTTTAGCGG  511
512  CGGTTTATCCCCGCTGGCGCGGGGAACAC  ATTTTTGCGAACCAGATGTTATCGTCGGTGCG  572
573  CGGTTTATCCCCGCTGGCGCGGGGAACAC  TTGTGACGTCTGGCCGCCGAACGCCTCGGCAC  633
634  CGGTTTATCCCCGCTGGCGCGGGGAACAC  [illegible]                        694
695  CGGTTTATCCCCGCTGGCGCGGGGAACAC  ATTTTTGCGAACCAGATGTTATCGTCGGTGCG  755
756  CGGTTTATCCCCGCTGGCGCGGGGAACAC  CGAGTCTATGACATAAAAAGCACTATTGAAGT  816
817  CGGTTTATCCCCGCTGGCGCGGGGAACAC  GCGAACATTCGCCCACTCAATCGTAACGTGATC  878
879  GGGTTTATCCCCGCTGGCGCGGGGAACAC                                    907
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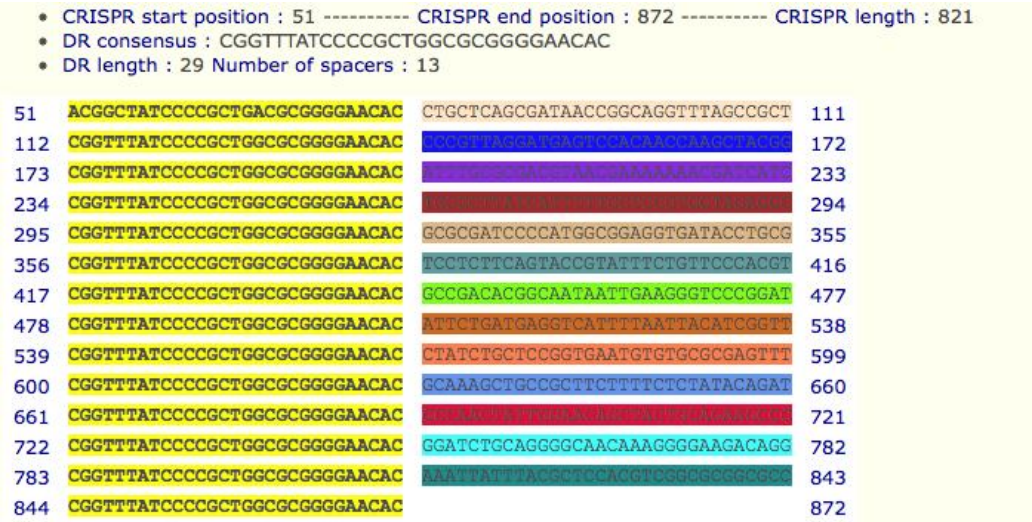

Figure 1: Representation of CRISPR 1 and CRISPR 2 regions of *Salmonella* Enteritidis S49 obtained from CRISPRFinder online http://crispr.u-psud.fr/Server/CRISPRfinder.php [22].

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

Table 4: Top hit annotations obtained for 32 nucleotide spacers of CRISPR 1 and 2 regions, for *Salmonella* Enteritidis S49 and their BIMs by BLAST in NCBI database.

	Spacer No:	Spacer sequence	Target region	Accession No:
CRISPR	1	TTTAAAACTCTTGCTGGAGACATGGGCGTCCC	Predicted Vitis vinifera uncharacterizd 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

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	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_scaffold 0000048	LM583103.1
CRISP	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	Cylicostephans 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 anophageffers hypothetical protein partial mRNA	XM_009036681.1

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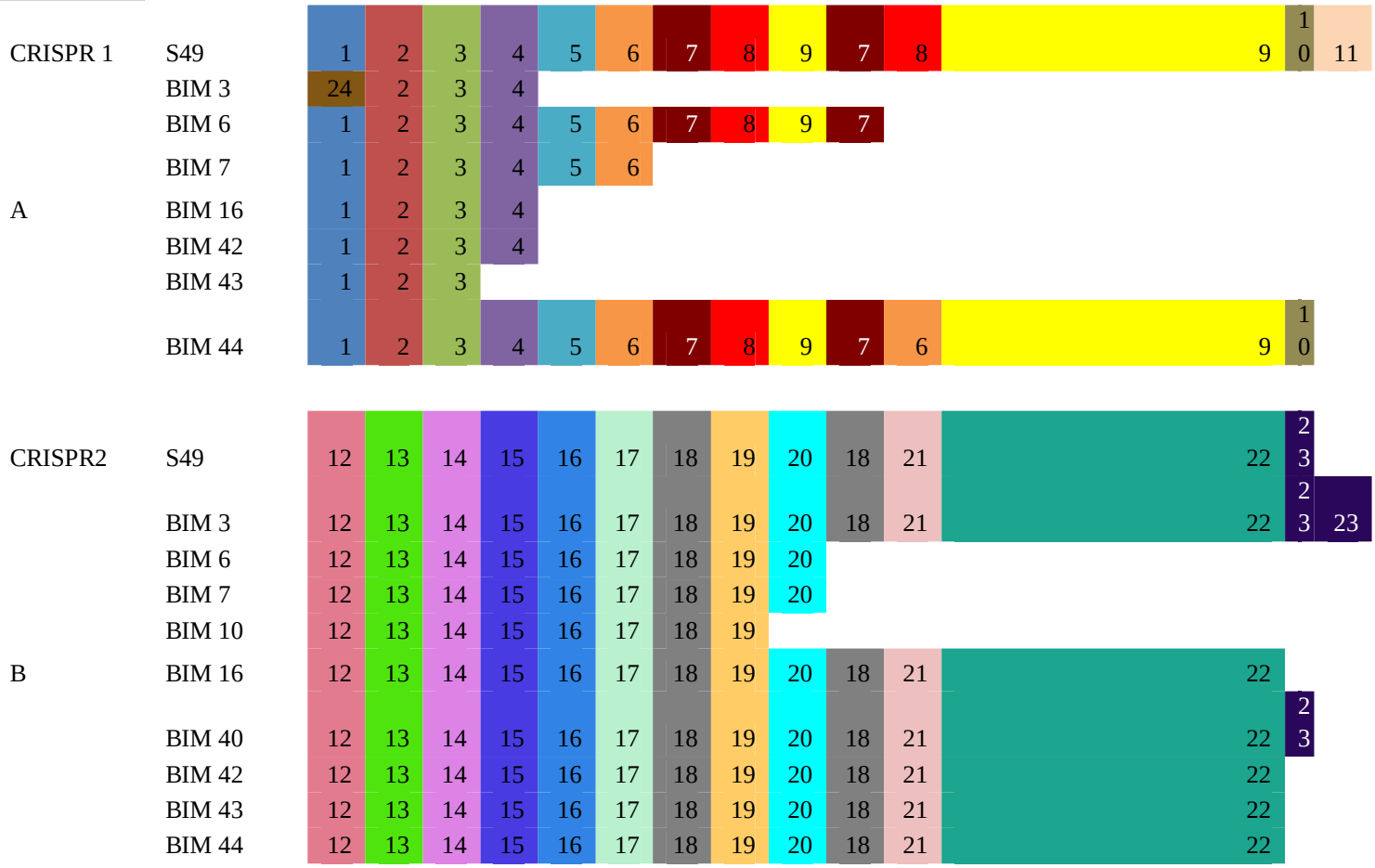

Figure 2: Graphic representation of spacers of CRISPR 1 (A) and 2 (B) regions in *Salmonella* Enteritidis S49 and their BIMs. Boxes represent spacers; repeats are not included. The 5' ends are oriented on the left of each array; the 3' end spacers are oriented on the right side of each array. The same number and color represent identical spacers [23].

In the CRISPR 1 region of Salmonella Enteritidis S49 (Figure 2 and table 4) of the total 14 spacers present, spacer 7, 8 and 9 were tandemly repeated. These repeats 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.

Almost the same spacers were observed among the BIMs of S49. BIM 43 was different from others only three spacers (1,2 and 3) were detected. It seemed that after spacer 3 all were lost. The integration of novel spacers by CRISPR 1 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.

In the CRISPR 2 region of Salmonella Enteritidis S49 and its BIMs (Figure 2 and Table 4), the spacers were totally different from those present in CRISPR 1; only13 spacers were noted in host. In BIM 3 spacer 23 was repeated. In BIM 10 the spacers after 19 were absent from the 3' regions. In most BIMs studied the last spacer at the 3' regions were deleted. The analysis of the CRISPR region showed that the BIMs generated following a single infection were not only different from the host S49 they were derived from, but were also different from each other.

IV. DISCUSSION

Microbes, like bacteria and viruses, do not exist in isolation but shape intricate ecological interaction webs (24). 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 associates. Such co-evolutionary interactions create the natural cycle of adaptation and counter adaptation of ecologically interacting species, thereby driving rapid molecular evolution (25, 26). Nowhere is this dynamic arms race as prevalent as in

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microbe-phage interactions. The constant exposure to phage infection forces a strong selective pressure on bacteria to develop viral resistance strategies that promote prokaryotic survival. Consequently, this parasite-host relationship results in an evolutionary arms race of adaptation and counter adaptation between the associating partners. The evolutionary impact is a spectrum of remarkable strategies used by the bacteria and phages as they attempt to coexist. These techniques include adsorption inhibition, injection blocking, abortive infection, toxin-antitoxin, and CRISPR-Cas systems (27).

The CRISPR locus of the recently found CRISPR/ Cas defense system in prokaryotes is a map of the "immunological memory" of the microorganism as it protects against invading viruses and plasmids (23). The spacer sequences that are integrated into the CRISPR loci impart a historical view on the exposure of the bacteria to a variety of foreign genetic elements (11).

In the current study, the CRISPR arrays in *Salmonella* Enteritidis S49 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* (28). Within *Salmonella*, which contains the Type I-E CRISPR-Cas system (29), there are two CRISPR loci (CRISPR 1 and CRISPR 2) that differ in both the identity and number of spacers and repeats (30, 31).

The amplified size of CRISPR 1 and CRISPR 2 were 1300bp in both host S49 and their BIMs. The PCR products of CRISPR 1 and CRISPR 2 were in between 1000bp and 3000bp for *Salmonella* Typhimurium in a study on CRISPR typing and subtyping for improved laboratory surveillance of *Salmonella* Infections (32). 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 (33).

The repeat sequences with 29 nucleotides were analyzed in the present study. It was similar in host and BIMs, in both CRISPR 1 and CRISPR 2 regions, 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 two CRISPR loci in *Salmonella*; CRISPR 1 and CRISPR 2 separated by ~16 kb and sharing the same consensus direct repeat sequence (29 nucleotide) and the spacers 32nucleotide in length (32, 34, 35, 36).

When CRISPRs spacers of strain S49 and their BIMs 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 the highest was fifteen in this study. Shariat *et al*. (36, 33, 37) 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 disclosed different activities of the CRISPR/Cas loci across *S*. Enteritidis S49 and their BIMs (Table 4). Normally phage-host interactions may lead to insertions of phage nucleotides into the spacer region as protospacers (38). In an immune active system, array differences arise from spacer acquisition (39). 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 (Figure. 3).

Spacer acquisition itself differs drastically among different species, and endogenous acquisition has been observed in the laboratory in only a few bacteria (4, 40,41). Acquisition, along with spacer loss and duplication, makes CRISPR elements among the fastest evolving loci in bacteria (42, 43).

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 (44). The low number of arrays lacking 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 (45).

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 (46).

It is becoming apparent that CRISPR-Cas systems do have alternative functions (47,48). For example, these systems have been

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shown to be involved in biofilm formation (49), host infection in humans and amoeba, (50, 51) symbiotic colonization in nematodes (52) and DNA damage (53).

This iterative phage challenges have typically added to CRISPR1 and CRISPR 2 locus. It helps in the separation of CRISPR intervened phage resistance from other natural antiphage defense systems. The other four systems like restriction modification systems, DNA ejection inhibition, adsorption inhibition, 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 (54).

V. CONCLUSION

This study design was to elucidate the CRISPR regions of the host bacteria and bacteriophage insensitive mutants and understand their role in the phage insensitivity. There were observable changes in the CRISPR regions not 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. However, this enables a touch of comprehension 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.

VI. ACKNOWLEDGEMENTS

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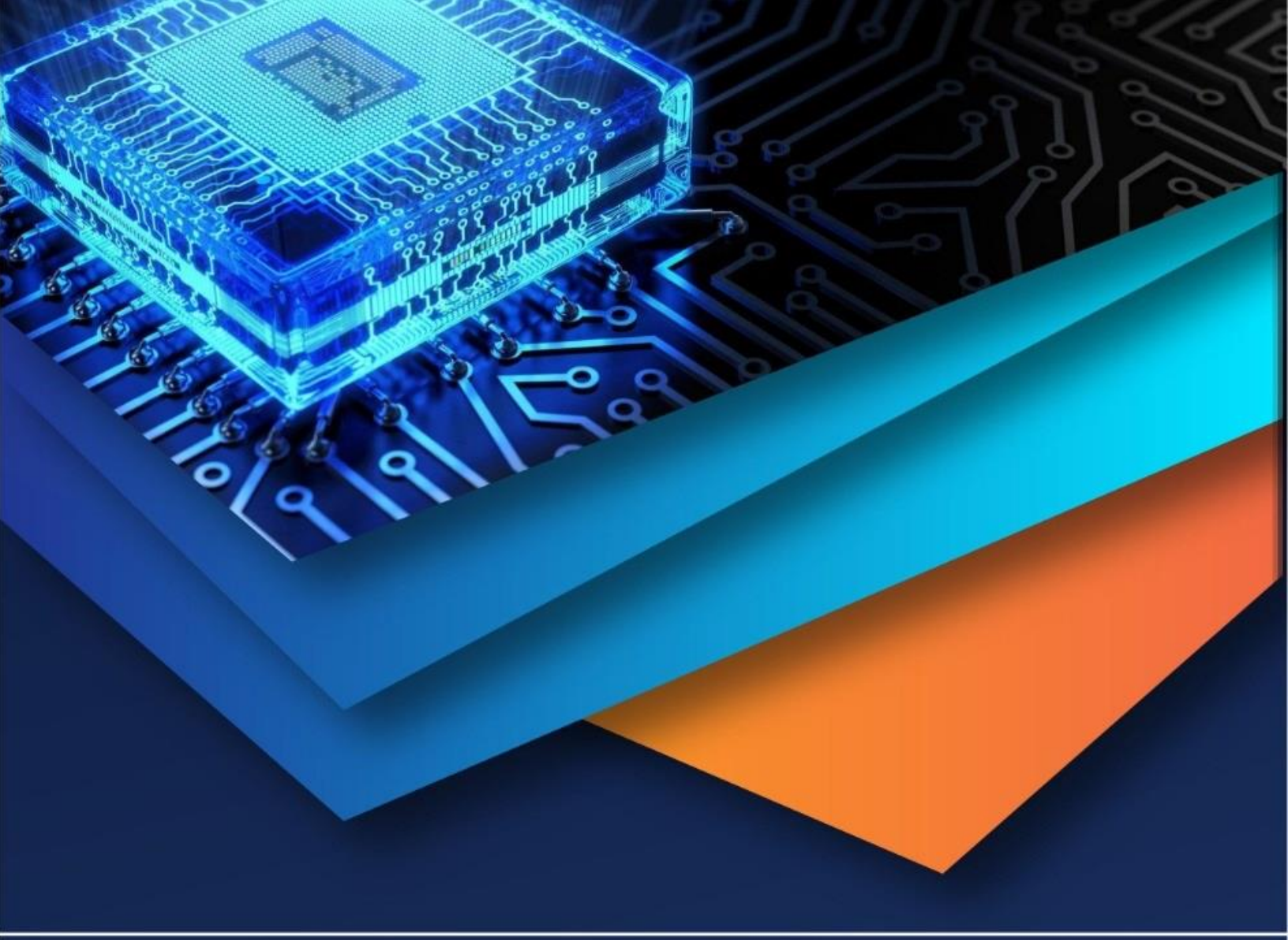

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