Genomic Characterization and Physical Mapping of *Shigella flexneri* Serotype-Specific Antigen V Temperate Bacteriophage

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Shigella flexneri hosts a temperate bacteriophage SfV responsible for the conversion of serotype Y strains (3,4) to serotype 5a (V; 3,4) through its glucosyl transferace gene. This enzyme mediated conversion effects a molecular antigenic conversion in glucosyl or glycosylic moiety attachment generating a corresponding immunogenic shift in the O-antigen component of the outer membrane lipopolysaccharide immune response among Shigellosis patients. With respect to vaccine development, further investigation into SfV's complete genome is crucial to explore and distinctly locate other genetic horizontal transfer contributing immunogenicity aside from the somatic antigen and proteins encoded by the invasion plasmid. SfV was placed in the group B of the Bradley classification scheme containing an approximate 39 kb intact DNA, double stranded, circularly permutted genome which appeared to be packaged by a headful mechanism at the Pac site. Bacteriophage SfV showed significant homology at the 2.5 kb fragment C serotype-conversion region of SfX but their morphology and protein profile were different. The construction of the physical map of SfV in this study gives a concrete blueprint stipulating the orientation and size of its genome. This information is vital for further studies involving library construction, cloning, sequencing, amino acid expression and functional evaluation and homology studies.

INTRODUCTION

Several serotypes of *Shigella flexneri*, the main cause of shigellosis in developing countries [1,2], is categorized based on the kind of antigenic determinant combined in the O-polysaccharide chain of the cell envelope lipopolysaccharide (LPS). The *S. flexneri* serotypes, except serotype 6, have a common tetrasaccharide repeating O-unit structure encoded by genes located in the *rfb* cluster adjacent to the his locus[3]. The various serotypes are expressed by the addition of glucosyl and/or O-acetyl residues to the individual monosaccharide in the

O-unit (Figure 1). A gene encoding the type-specific glucosyl transferase was mapped near the *pro-lac* region of an integrated prophage and the type-specific antigen expressed in *S. flexneri* Y converting it to either type V or group antigen 7,8 [4].

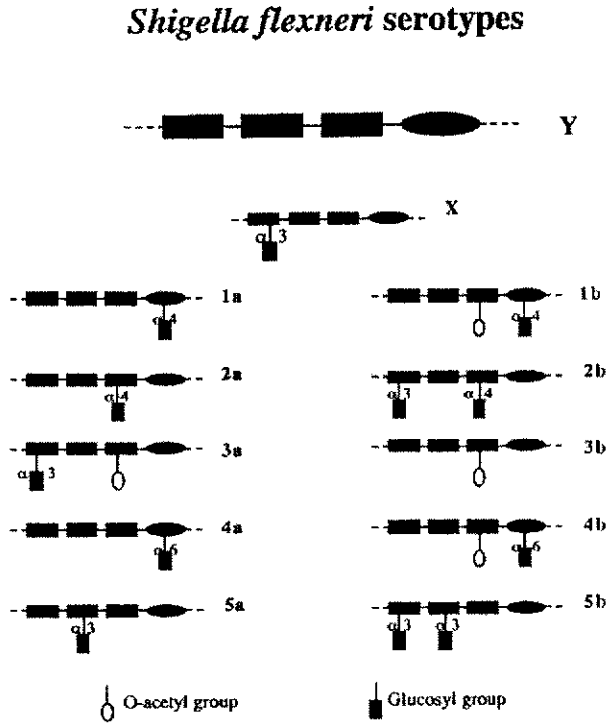

Figure 1. Biochemical Structure of *S. flexneri* Lipopolysaccharide

Site specific recombination is instigated by temperate phages to integrate their genome into the bacterial chromosome. The recombination event occurs between two segments at the specific attP site on the phage DNA and attB on the bacterial chromosome by a crossover event within a common locus core[5]. This process requires a phage-encoded integrase protein (Int) and a host-encoded integration host factor (IHF) [6,7,8]. The Int protein is necessary for both integration and excision activities, while the xis functions only for excision[9]. Int is a type-I topoisomerase, a site specific recombinase that can cut and rejoin the core sequence[10]. It is a specific DNA binding protein that recognizes the 'core type' and the 'arm type' two distinct classes of DNA sequences. IHF protects some regions of arm sequences from nuclease digestion[11]. In fact, attP arms are protected by both IHF and Int. The phage attachment, integration and excision genes form a cluster in the phage genome, and the serotype-conversion region is adjacent to the attP in the phages P22[12] and Sf6[13].

In this study, we have induced phage SfV from EW 595/52 and enzyme restricted its genomic DNA. Afterwhich, a preliminary physical map was constructed, which will be valuable in further genetic and homology analysis. We have also generated its protein profile and compared it with a previously characterized serotype-converting phage SfX[14].

MATERIALS AND METHODS

Bacterial strains, Plasmids, Phage Isolation, Sensitivity. Bacteria were grown in Luria broth (LB) and on Luria agar (LA) supplemented with ampicillin, 50 ug/ml (Sigma). NZCYM broth, LB, LA and 0.7% LA were used for phage propagation and titration. Phage SfV was induced from the EW 595/52 *S. flexneri* strain stock by UV irradiation. A five ml overnight bacterial culture was spun down and resuspended in 10mM $MgSO_4$. The bacterial suspension was exposed under a germicidal lamp at 260 nm wavelength for 30 seconds and transferred to a tube containing 5 ml LB. The tube was protected from light by wrapping it with aluminum foil, then placed in a shaker for 5 h at 37°C. The lysate was collected by filtration through a 0.45 mm microfilter unit. *S. flexneri* serotype Y SFL124 strain was used for propagation as well as for checking serotype conversion. Serotype conversion was confirmed by agglutination tests against *S. flexneri* monoclonal antibodies MASF V and MASF Y-5. Susceptibility of *S. flexneri* strains to phage SfX and SfV were tested by applying a drop of phage containing 5.3×10^{10} pfu/ml on a lawn of test bacteria.

Propagation and Purification of Phage DNA[15]. An overnight culture of SFL 124 in five ml of LB broth

was incubated in shaker at 37°C. The culture was diluted 1:100 with NZCYM medium and incubated for another three hours. Cell suspension of density 10^9/ml was spun down and resuspended in 100 ul of SM buffer. Infected cell suspension with phage purified stock with $5.3x10^{10}$ titer using 0.001 multiplicity of infection value (MOI) was incubated for 20 minutes in 37°C. Note that MOI will vary depending on the bacteria and phage stock concentration. Phage titer was obtained through ten-fold serial dilution of phage stock followed by a 20 min incubation with the two hour log phase SFL 124. The bacteria and bacteriophage mixture was mixed with 3 ml melted 0.5% soft agar by swirlinbg over LB plates and overnight incubation. For the propagation step, the bacteria-phage mixture was transferred to 100 ml prewarmed NZCYM then incubated overnight. Two ml of chloroform was added to complete host lysis, then incubated for another 25 minutes. After spinning down in Sorvall centrifuge at 8000g (GSA rotor, 7000 rpm) at 4°C for 20 minutes, the supernate was treated with 1 ug/ml RNAse and DNAse in sterile flasks for 30 min at room temperature with continuous stirring. A 5.84g of NaCl was added, placed on ice for an hour, then transferred into centrifuge bottles and spun at 9000 rpm for 20 minutes in GSA rotor (13000g). Ten grams of PEG 6000 was added to the recovered supernate and ice incubated for another hour, the mixture was spun down again at 10,000 rpm (16,000g) for 20 minutes using Sorvall GSA rotor. As much supernatant was discarded and the sedimented phage particles were resuspended in one ml SM buffer, extracted three times with equal volume of chloroform, then aliquoted into 500 ul amounts. This was followed by addition of 0.5% SDS, 20mM EDTA, 10Mm Tris-HCl, pH8 and 150 ug from 20mg/ml stock of Proteinase K. The solution containing phage genomic DNA was incubated at 55°C water bath for 30 minutes. The aqueous phase was transferred into dialysis tubing after one time extraction with pure phenol, one time phenol-chloroform mixture and and chloroform. Dialysis at 4°C was performed overnight against three changes of 1x TE buffer to remove all phenol traces. DNA was extracted using 3M Sodium Acetate pH7, and 100% ethanol precipitation followed by a 30 min incubation at room temperature and a 10 min refrigerated microcentrifuge spin at 13000g. Two volumes of cold 70% ethanol was used to wash off Sodium acetate and concentration ethanol. After another 30 min spin, the pelleted DNA was placed at 40°C vacuum oven to dry before resuspension in water containing 20 ug/ml RNAse.

DNA Labelling and Hybridization. The restriction fragments of SfV DNA on the agarose gel were blot transferred to Hybond N+ nylon membrane by alkaline transfer following instruction from supplier. Fragments used as probe were eluted from the gel using the BandPure DNA purification kit, then labelled with [$\propto^{32}$P]-dCTP by using the random primer method with the Gigaprime DNA labelling kit from Bresatec. Hybridization. The procedure was performed under high stringency conditions.

Physical Map Construction and Protein Fingerprinting. A preliminary restriction map SfV as constructed by 0.6% agarose gel analysis of single and double digests of SfV with EcoRI, BamHI and SacI. Care was taken to note submolar bands. Sizes of restriction fragments were determined by comparing relative mobilities on the gel with those of ul DNA molecular weight marker SPP1 cut with EcoRI. Alphabetical letters were used to designate the fragments and submolar bands were named as A' and D'. PEG 6000 precipitated and cesium chloride gradient purified phage particles were boiled for 10 min in SDS-PAGE loading buffer. Samples were run on 10% SDS-PAGE[16]. Protein bands were visualized by staining the gel with Coommassie brilliant blue.

RESULTS AND DISCUSSION

The morphology of bacteriophage SfV places it in Bradley's classification Group B because of its

isometric head and long non-contractile tail (Figure 2). As compared to the morphology of phage SfX and Sf6 of *S. flexneri*[17], both phages possess a short

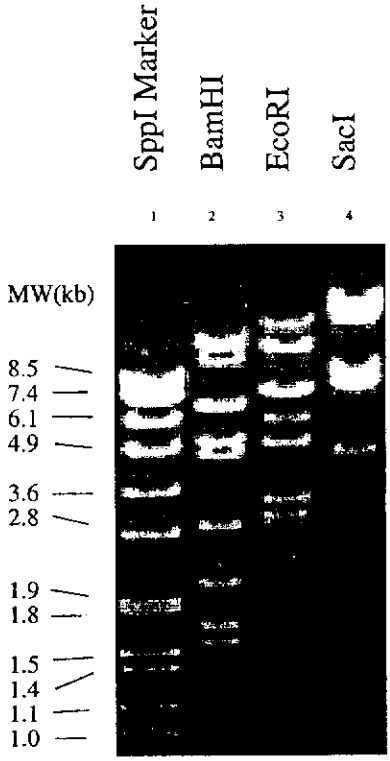

Figure 2. 0.7% Agarose Gel Electrophoresis of SfV DNA including submolar bands. Lane 1. SPPI marker cut with EcoRI; Lane 2. BamHI endonuclease cleavage products; Lane 3. Eco RI endonuclease cleavage products; Lane 4. SacI endonuclease cleavage products. Refer to Table 2 for the fragment moleculear weight.

Table 1. Phage Sensitivity

Strain (serotype)	SfV	SfX
Y411(x)	R	R
Y53(1a)	S	R
Y868(1b)	S	R
2457T(1a)	S	S
Y357(2b)	S	R
Y2417(3a)	S	R
Y1025(3b)	S	R
F2291(5a)	R	R
Sf6124(Y)	S	S

S - Sensitivity
R - Resistant

Table 2. Molecular Weights of SfV genome Restriction Fragments.

BamHI Fragments	EcoRI Fragments	SacI Fragments
A - 13.0 kb	A - 18.3 kb	A - 21.2 kb
B - 7.0	B - 7.2	B - 9.0
C - 5.0	C - 5.1	C - 5.0
D - 4.8	D - 3.4	
E - 3.1	E - 3.2	
F - 2.2	F - 1.1	
G 1.6		
H - 1.5		

tail and a narrow host range. Although, it has been well discussed that tail fibers are the most important substructures of phages involved in the process of host attachment, not to mention their role in molecular ligand recognition and binding to specific receptors on the cell surface [18,19], the host range for phage SfV appears to be wider than that of phage SfX (Table 1). It is probable that the sensitivity of *S. flexneri* serotypes Ia, 2b, 3a, 3b sharing the common group antigen 3,4, to phage SfV but resistant to SfX was brought about by the addition of glucosyl or acetyl residue to the rhamnose or N-acetyl D-glucosamine in the repeating unit other than the position for SfV, which do not interfere in the adsorption of the phage. The 3,4 receptor is probably more scattered on the cell surface of these *S. flexneri* serotypes compared to the serotype Y. Another explanation could be the presence of the SfV long tail which facilitates the attachment as opposed to the short tail of SfX or Sf6. An earlier experiment proved that phage Sf6 uses the group antigen as receptor and the addition of glucosyl or acetyl residues close to the linkage which is specific to Sf6 posed an interference on the adsorption of the phage[20]. The variability in host range and lytic

spectrum of these phages is still unclear and requires further investigation.

It was also observed that phage SfV can lyse

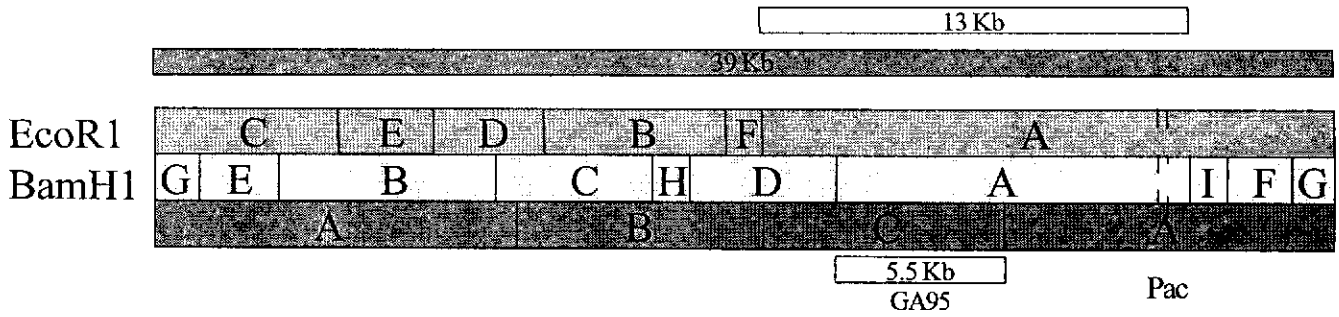

Figure 3. SfV Preliminary Physical map

partially sero-converted *S. flexneri 5a* strain, SfL1164, but not the fully sero-converted strain, SfL1168. The latter 5a strain was constructed by introducing a 5 kilobase BamHI fragment C of SfV into *S. flexneri* serotype Y strain SfL124, while SfL1164 was a clone with a 3.7 kilobase EcoRI/BamHI fragment of the aforementioned 5 kb BamHI. The expression of the group antigen 3,4 was very limited in the fully sero-converted SfL1168 strain. Since the SfL1164 but not SfL1168 was sensitive to the SfV phage, it can be deduce that this phage utilizes the group specific antigen 3,4 as receptor.

Upon induction of the SfV phage particle from its host and its subsequent purification and DNA extraction, SacI, EcoRI and BamHI enzymes were chosen to construct a physical map of SfV (Figure 3). The preliminary physical map was constructed in order to establish fragment location and sizes necessary for further library construction, cloning, sequencing and characterization of the SfV genome. Restriction enzyme digests with SacI, EcoRI and BamHI resulted in the generation of three, eight, and nine fragments respectively, including submolar bands produced by the headful packaging inherent in the viral mechanism.

The appearance of non-stoichiometric bands was explained by the headful packaging mechanism first proposed by Streisinger et al.[21], and modified by Tye et al.[22] on their experimental observation of *Salmonella* phage P22. The mechanism elucidates that mature DNA macromolecules inside a population of phage heads are a collection of linear DNA molecules with circularly permutted end and terminal repetition. These DNA molecules are nicked from long, concatemeric DNA precursors of several phage genomes in length, when the precursor DNA is encapsulated into the protein heads during the maturation process. The length of the DNA in the protein head is equal to the amount of DNA that fits into the head capacity or one "headful". Each DNA headful is slightly larger than one complete set of genes, thus resulting in terminal repetitious sequences. Since the phage DNA precursors are limited in length, the endpoints processed by this mechanism fall within a range on the physical map (20% of its genome for P22). The abnormal fragments shown as submolar bands on the agarose gel are believed to be generated between the package site (pac) and the first endonuclease cutting site[23,24]. Several bacteriophages like P22 and Streptomycete temperate phage FP43[25] were observed as having such submolar bands.

Hybridization experiment involving a gel-purified smaller Bg1II fragment probe strongly hybridized with a larger Bg1II fragment on the same locus. This indicates the existence of substantial terminal repetition sequences in the mature genome of phage SfV. The homology in DNA but heterology in size between bands A and A' from the enzyme digests indicated

that these fragments were a collection of the molecular gene ends of the phage population which have one enzyme cutting site while the other end was the consequence of a headful cutting mechanism. The SfV DNA is evidently permutted. P22 insert its DNA into *Salmonella typhimurium* at pro-lac region[26] and SfV also inserts its DNA into the same region of *S. flexneri* chromosome[27]. Since the attP-int-xis region of P22 and SfV is identical and P22 DNA is double circularly permuted with its DNA packed by headful mechanism, it is reasonable to suggest SfV's utilization of the same DNA packing mechanism. SacI fragment A probe hybridised with EcoRI band A, A', C, D, E and band A, A', B, C, D, E, F and I of BamHI indicating that SfV DNA is circular. The intact DNA size is approximately 39 kb.

An apparent doublet appearance was evident among the slowest migrating bands of BamHI, EcoRI and SacI digestion of SfV in 0.7% agarose gel. These doublets were visualized better into two distinct bands named A and A' when run in a lower agarose concentration of 0.5%. Bands A' of BamHI and EcoRi digests migrated faster than the bands and exhibited fainter fluorescence upon UV exposure providing further evidence of its submolarity. The size of EcoRI submolar B' is about 6 kb which also gave less intense band signal.

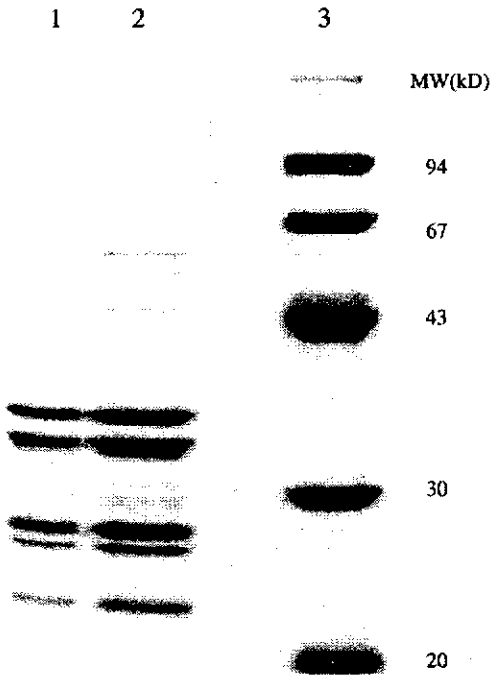

Figure 4. 12% SDS-PAGE gel showing SfV protein profile. Lane 1. contains 5 ul whole phage particle stock preparation; Lane 2. contains 10 ul whole phage stock preparation. Stock titer is 5.3x10 to the tenth. Lane 3. Low Molecular Weight protein marker.

Phage proteins were resolved by SDS-polyacrylamide gel electrophoresis. At least seven distinct protein bands for SfV can be visualized on the gel (Figure 4). The gels were run at different concentration to clearly distinguish the bands. Different concentrations of polyethylene glycol purified phage particles were also tested at various amounts to determine the optimum inoculum for sequencing. No common protein bands were observed between SfV and SfX despite sharing high degree of homology in some parts of their genome[28]. The majority of protein bands for SfV were over 30 kDa in contrast with that of SfX. SfV showed four major protein bands with molecular masses of 39, 35, 28 and 27 kDa; and four minor bands of lower yet significant concentrations (molecular masses of 65, 63, 50, and 47 kDa). Only one major band was observed in the SfX protein profiles. Although SfX and SfV share glucosylation function in *S. flexneri* their morphology is different and the DNA showed low homology except for the 5.0 kb DNA which is just an eight of SfV genome.

CONCLUSION

The SfV was place in the Group B of the Bradley classification scheme on the basis of its morphology. The 39 kb SfV genome was observed to be double stranded, circularly permuted and packaged by a headful mechanism. Compared with SfX, the two phages showed low DNA homology except for the serotype-conversion and putative attP-int regions, which predictably resulted in the considerable protein band difference. Further

sequencing work to supplement the results of the study is recommended followed by functional characterization of the phage genes and their protein products.

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