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Correlation of Host Range Expansion of Therapeutic Bacteriophage Sb-1 with Allele State at a Hypervariable Repeat Locus

Kirill V. Sergueev,[a] Andrey A. Filippov,[a] Jason Farlow,[a,b] Wanwen Su,[a] Leila Kvachadze,[c] Nana Balarjishvili,[c] Mzia Kutateladze,[c] Mikeljon P. Nikolich[a]

[a]Department of Bacteriophage Therapeutics, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA
[b]Farlow Scientific Consulting, LLC, Lewiston, Utah, USA
[c]G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia

ABSTRACT Staphylococci are frequent agents of health care-associated infections and include methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to first-line antibiotic treatments. Bacteriophage (phage) therapy is a promising alternative antibacterial option to treat MRSA infections. *S. aureus*-specific phage Sb-1 has been widely used in Georgia to treat a variety of human *S. aureus* infections. Sb-1 has a broad host range within *S. aureus*, including MRSA strains, and its host range can be further expanded by adaptation to previously resistant clinical isolates. The susceptibilities of a panel of 25 genetically diverse clinical MRSA isolates to Sb-1 phage were tested, and the phage had lytic activity against 23 strains (92%). The adapted phage stock (designated Sb-1A) was tested in comparison with the parental phage (designated Sb-1P). Sb-1P had lytic activity against 78/90 strains (87%) in an expanded panel of diverse global *S. aureus* isolates, while eight additional strains in this panel were susceptible to Sb-1A (lytic against 86/90 strains [96%]). The Sb-1A stock was shown to be a mixed population of phage clones, including approximately 4% expanded host range mutants, designated Sb-1M. In an effort to better understand the genetic basis for this host range expansion, we sequenced the complete genomes of the parental Sb-1P and two Sb-1M mutants. Comparative genomic analysis revealed a hypervariable complex repeat structure in the Sb-1 genome that had a distinct allele that correlated with the host range expansion. This hypervariable region was previously uncharacterized in Twort-like phages and represents a novel putative host range determinant.

IMPORTANCE Because of limited therapeutic options, infections caused by methicillin-resistant *Staphylococcus aureus* represent a serious problem in both civilian and military health care settings. Phages have potential as alternative antibacterial agents that can be used in combination with antibiotic drugs. For decades, phage Sb-1 has been used in former Soviet Union countries for antistaphylococcal treatment in humans. The therapeutic spectrum of activity of Sb-1 can be increased by selecting mutants of the phage with expanded host ranges. In this work, the host range of phage Sb-1 was expanded in the laboratory, and a hypervariable region in its genome was identified with a distinct allele state that correlated with this host range expansion. These results provide a genetic basis for better understanding the mechanisms of phage host range expansion.

KEYWORDS *Staphylococcus aureus*, phage Sb-1, adaptation, host range expansion, hypervariable DNA region, bacteriophage adaptation, genome analysis, putative host range determinant, therapeutic bacteriophage

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Address correspondence to Andrey A. Filippov, andrey.a.filippov.ctr@mail.mil.

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The increasing prevalence of multidrug-resistant (MDR) bacterial pathogens, including that of methicillin-resistant *Staphylococcus aureus* (MRSA), is a dangerous trend that limits options for treatment and hinders the ability of physicians to treat infections (1–3). The estimated incidence of health care-associated MRSA infection in the United States in 2014 was more than 77,000 cases (4). This situation, combined with the scarcity of new antibiotics currently in the pharmaceutical development pipeline, necessitates the implementation of alternative therapeutics, including bacteriophages (phages), for patients infected with MDR bacteria. Recently, phages and their lysins were identified by the National Institute of Allergy and Infectious Diseases (NIAID) as one of the viable alternatives to antibiotic treatment (5). Phages specifically infect and kill target bacteria but leave mammalian cells intact and do not have a detrimental effect on normal microflora; thus, they are considered nontoxic and safe for clinical use (6–8). Phages have been shown to disperse *S. aureus* biofilms (9, 10) and to provide successful treatment against various experimental and veterinary *S. aureus* infections, including septicemia (11–13) and skin infection (14) in mice, tibial implant infection in rats (15), and cutaneous abscesses (16) and biofilm-infected wounds in rabbits (17). Phage therapy of *S. aureus* infections in silkworm larvae and in mice showed that the larval model allows for adequate evaluation of therapeutic efficacy (18). Several case reports demonstrated successful phage treatment of refractory staphylococcal infections in humans, including diabetic ulcers (19–21), otitis media (22), and infected radiation injuries (23). Despite this progress, no FDA-approved phage therapeutics are currently available for treatment of infections caused by MRSA or any other MDR bacterium. A phage cocktail was applied against *Pseudomonas aeruginosa* and *S. aureus* burn wound infections in a small clinical trial (nine patients) in Belgium and showed no adverse effects (24). Recently, AmpliPhi Biosciences Corporation reported a successful phase I clinical trial in the United States to evaluate the safety of *S. aureus* phage cocktail AB-SA01 applied topically to the intact skin of healthy adults (https://www.reuters.com/article/idUSFWN1BP07D).

S. aureus phages identified to date are tailed double-stranded DNA phages that belong to the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families, characterized respectively by their long contractile, long noncontractile, or short tails (25–32). The broad-host-range *S. aureus* phage Sb-1 has been used extensively in former Soviet Union (FSU) countries and has shown efficacy in the treatment of various *S. aureus* infections, including those caused by MRSA (33–35). Phage Sb-1 is a member of the *Twortlikevirus* genus within the *Spounavirinae* subfamily of the *Myoviridae* family (27, 36–38) and is a close relative of phages K, G1, and Twort (31), as well as of *Bacillus subtilis* phage SPO1, from which the taxonomy of the subfamily is derived (39). The close genetic relationship between phages Sb-1, K, and SPO1 has been described previously, and genome architecture appears well conserved within the staphylococcal Twort-like phages (34, 38).

Phage Sb-1, produced by Eliava BioPreparations, Ltd., and referred to as "Staphylococcal Bacteriophage," is commercially available in the country of Georgia. This phage exhibits favorable safety and efficacy profiles (35). Originally, Sb-1 was isolated in 1977 from an already available antistaphylococcal phage cocktail (40). The biology and genome of Sb-1 have been studied in detail (34, 41); it is a strictly lytic phage (it cannot become a prophage) whose genome lacks any bacterial virulence or drug resistance genes. Phage Sb-1 has a broad host range that can be further expanded by passages on phage-resistant strains (34). The broad host range has been partially explained by the lack of GATC sequences in the Sb-1 genome, which precludes the phage DNA from digestion by major *S. aureus* restriction endonuclease Sau3AI (42, 43). However, the molecular basis for host range expansions of both Sb-1 (34) and closely related phage K (44) have remained unknown, although it was speculated that host range expansion of phage K was due to an unidentified antirestriction property (45).

Here, we describe the isolation and characterization of Sb-1 phage mutants with expanded host ranges. Our analysis revealed the presence of a novel hypervariable repeat region in the genomes of Sb-1, K, and other Twort-like phages. This repeat

region stabilized to a dominant allele state when Sb-1 was adapted to a previously phage-resistant bacterial host.

RESULTS AND DISCUSSION

Sb-1 testing on 25 military MRSA isolates and expansion of its host range. We tested the efficiency of plating (EOP) of a Sb-1 therapeutic preparation manufactured by Eliava BioPreparations, Ltd. (production stock designated Sb-1P, with "P" for parental), on an initial panel of 25 representative MRSA strains isolated from U.S. military personnel, including wound infection isolates (Table 1, strains 1 to 25). This Sb-1P phage preparation was active against 23/25 (92%) of the strains in this initial diversity panel. In order to expand the host range of Sb-1P, a phage adaptation procedure was performed as described earlier (34; see Materials and Methods) that resulted in a phage lysate with an expanded host range, designated Sb-1A ("A" for adapted). The Sb-1A lysate was able to lyse *S. aureus* strain MRSN9832, which was originally resistant to the parental Sb-1 phage (Sb-1P). The efficiencies of plating of Sb-1A and Sb-1P were then tested on an expanded panel of 90 global *S. aureus* isolates (see Table 1). Sb-1P had lytic activity against 78/90 strains (87%), while the Sb-1A phage preparation had lytic activity against eight additional strains (86/90 [96%]). Thus, the laboratory adaptation of Sb-1 phage to infect and lyse a single previously resistant MRSA strain (MRSN9832) resulted in a significant more generalized host range expansion. Although the process of adaptation enabled the phage to infect previously resistant strains, the efficiency of plating on those strains was approximately 20 times lower than those of the previously phage-susceptible strains (data not shown). We speculated that the Sb-1A lysate was a mixed population of phage clones, of which approximately 5% were expanded-host-range mutants. To confirm this, the Sb-1A phage lysate was plated on strain MRSN8383, and 46 random single plaques were picked, treated with chloroform, and spotted both on phage-susceptible strain MRSN8383 and on previously phage-resistant strain MRSN9832. Only 2/46 (4.3%) of these subclones were able to produce plaques on both strains and thus were considered to be expanded-host-range mutants. We designated these two host range mutants derived from single plaques Sb-1M ("M" for mutant).

Analysis of *S. aureus* diversity. The genetic diversity of U.S. military MRSA isolates selected for this study, and also that of the expanded 90-strain global *S. aureus* panel used here, was analyzed using pulsed-field gel electrophoresis (PFGE) genotyping. Computer analysis of PFGE profiles identified 76 distinct restriction patterns in the expanded 90-strain diversity panel (Fig. 1). Using the Dice coefficient with BioNumerics software, isolates with $\geq$80% similarity were assigned to the same pulsotype. The isolates were distributed in 32 pulsotypes. Eight of the 12 strains that were resistant to Sb-1P (67%) were isolated on three different continents but were grouped into two closely related PFGE clusters (Fig. 1) suggesting some correlation between PFGE subtype and Sb-1 resistance and indicating partial association of resistance with phylogenetic grouping. Based on the extant multilocus sequence typing information available for the strains in this panel, some patterns of phage susceptibility could be observed in the *S. aureus* clonal complexes (CCs): strains representing CC1 (sequence type 1 [ST1]), CC5 (ST5 and ST225), CC8 (ST8 and ST250), CC22 (ST22), CC30 (ST30 and ST36), and CC45 (ST45) were susceptible. Strains in ST239 were also susceptible, with the notable exception of strain DBT12, which was resistant to both Sb-1P and Sb-1A. Clonal complexes with strains that were resistant to Sb-1P included CC59 (ST59 and ST87) and CC88 (ST78 and ST88), with CC88 strains DBT15 (ST78) and NAJAF22 (ST88) also retaining resistance to Sb-1A.

Parental Sb-1 and Sb-1M adsorb to phage-resistant and phage-susceptible *S. aureus* strains with similar efficiency. The first step of phage infection is adsorption of the virus to receptors on the host cell. In order to investigate why Sb-1P could not propagate on resistant strains, we sought to determine whether the phage was able to adsorb to the surface of phage-resistant *S. aureus* cells. The results of a phage adsorption assay presented in Fig. 2 suggest that both Sb-1P and Sb-1M variants were able to efficiently bind to the surface of both phage-resistant (MRSN9832) and phage-

TABLE 1 *S. aureus* strains used in this work and results of phage susceptibility testing

Strain	Geographic location	Source	Antibiotic resistance[a]	Susceptibility of phage[b]:	
				Sb-1	Sb-1A
MRSN18	Fort Bragg, NC	Wound	MRSA	S	S
MRSN30	Fort Bragg, NC	Wound	MRSA	S	S
MRSN42	Fort Bragg, NC	Wound	MRSA	S	S
MRSN214	Bethesda, MD	Wound	MRSA	S	S
MRSN219	Bethesda, MD	Urine	MRSA	S	S
MRSN250	Bethesda, MD	Urine	MRSA	S	S
MRSN352	Bethesda, MD	Wound	MRSA	S	S
MRSN549	Fort Bragg, NC	Wound	MRSA	S	S
MRSN563	Fort Bragg, NC	Wound	MRSA	S	S
MRSN1722	Bethesda, MD	Sputum	MRSA	S	S
MRSN1732	Bethesda, MD	Tissue	MRSA	S	S
MRSN1952	Bethesda, MD	Wound	MRSA	S	S
MRSN2339	Bethesda, MD	Wound	MRSA	S	S
MRSN2763	Bethesda, MD	Wound	MRSA	S	S
MRSN3573	Bethesda, MD	Blood	MRSA	S	S
MRSN3643	Bethesda, MD	Tissue	MRSA	S	S
MRSN3710	Bagram, Afghanistan	Wound	MRSA	S	S
MRSN3966	Tbilisi, Georgia	Blood	MRSA	S	S
MRSN4109	Bethesda, MD	Sputum	MRSA	S	S
MRSN4531	Bethesda, MD	Wound	MRSA	S	S
MRSN4535	El Paso, TX	Wound	MRSA	S	S
MRSN9832	El Paso, TX	Nasal swab	MRSA	R	S
MRSN9834	El Paso, TX	Nasal swab	MRSA	S	S
NAJAF22	Najaf, Iraq	Wound	MRSA	R	R
NAJAF33	Najaf, Iraq	Wound	MRSA	S	S
MRSN4344	Bethesda, MD	Wound	MSSA	S	S
MRSN5079	Bethesda, MD	Wound	MSSA	S	S
MRSN6168	Bethesda, MD	Blood	MSSA	R	S
MRSN7983	Bethesda, MD	Wound	MSSA	S	S
MRSN8383	Bethesda, MD	Wound	MSSA	S	S
MRSN9127	Bethesda, MD	Respiratory	MSSA	S	S
MRSN9287	Bethesda, MD	Wound	MSSA	S	S
MRSN10110	Bethesda, MD	Wound	MSSA	S	S
MRSN10185	Bethesda, MD	Blood	MSSA	S	S
MRSN12239	Bethesda, MD	Eye	MSSA	S	S
IS-3	Baghdad, Iraq	Wound	MRSA	S	S
IS-24	Baghdad, Iraq	Wound	MRSA	S	S
IS-55	Baghdad, Iraq	Wound	MRSA	S	S
IS-88	Baghdad, Iraq	Wound	MRSA	S	S
IS-91	Baghdad, Iraq	Respiratory	MRSA	S	S
IS-99	Baghdad, Iraq	Wound	MRSA	S	S
IS-105	Baghdad, Iraq	Wound	MRSA	S	S
IS-111	Baghdad, Iraq	Wound	MRSA	S	S
IS-122	Baghdad, Iraq	Wound	MRSA	R	S
IS-125	Baghdad, Iraq	Wound	MRSA	S	S
IS-157	Baghdad, Iraq	Wound	MRSA	S	S
IS-160	Baghdad, Iraq	Wound	MRSA	R	S
IS-189	Baghdad, Iraq	Respiratory	MRSA	S	S
IS-195	Baghdad, Iraq	Blood	MRSA	S	S
IS-278	Baghdad, Iraq	Respiratory	MRSA	R	R
IS-M	Baghdad, Iraq	Blood	MRSA	S	S
CO-06	Camp Cropper, Iraq	Abscess	MRSA	S	S
CO-08	Camp Cropper, Iraq	Abscess	MRSA	S	S
CO-23	Camp Cropper, Iraq	Abscess	MRSA	S	S
CO-30	Camp Cropper, Iraq	Wound	MRSA	S	S
CO-32	Camp Cropper, Iraq	Abscess	MRSA	R	S
CO-41	Camp Cropper, Iraq	Wound	MRSA	S	S
CO-49	Camp Cropper, Iraq	Abscess	MRSA	S	S

(Continued on next page)

TABLE 1 (Continued)

Strain	Geographic location	Source	Antibiotic resistance[a]	Susceptibility of phage[b]:	
				Sb-1	Sb-1A
CO-85	Camp Cropper, Iraq	Wound	MRSA	S	S
CO-86	Camp Cropper, Iraq	Wound	MRSA	S	S
CO-99	Camp Cropper, Iraq	Wound	MRSA	S	S
DBT1	Japan	NA[c]	MSSA	S	S
DBT2	USA	NA	MRSA	S	S
DBT3	USA	NA	MRSA	S	S
DBT4	USA	NA	MRSA	S	S
DBT5	NA	NA	MRSA	R	S
DBT6	Sydney, Australia	NA	MRSA	S	S
DBT7	Sydney, Australia	NA	MRSA	S	S
DBT8	Sydney, Australia	NA	MRSA	S	S
DBT9	Sydney, Australia	NA	MRSA	S	S
DBT10	Sydney, Australia	NA	MRSA	S	S
DBT11	Sydney, Australia	NA	MRSA	S	S
DBT12	Sydney, Australia	NA	MRSA	R	R
DBT13	Sydney, Australia	NA	MRSA	S	S
DBT14	Sydney, Australia	NA	MRSA	R	S
DBT15	Sydney, Australia	NA	MRSA	R	R
DBT16	Sydney, Australia	NA	MRSA	S	S
DBT17	Sydney, Australia	NA	MRSA	S	S
DBT18	Sydney, Australia	NA	MRSA	S	S
DBT19	Sydney, Australia	NA	MRSA	R	S
DBT20	Sydney, Australia	NA	MRSA	S	S
DBT21	Sydney, Australia	NA	MRSA	S	S
DBT22	St Leonards, Australia	Wound	MSSA	S	S
DBT23	St Leonards, Australia	Wound	MSSA	S	S
DBT24	St Leonards, Australia	Wound	MSSA	S	S
DBT25	St Leonards, Australia	Wound	MSSA	S	S
DBT26	St Leonards, Australia	Wound	MSSA	S	S
DBT27	St Leonards, Australia	Wound	MSSA	S	S
DBT28	St Leonards, Australia	Wound	MSSA	S	S
DBT29	St Leonards, Australia	Wound	MSSA	S	S

[a]MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.
[b]S, susceptible; R, resistant.
[c]NA, data are not available.

susceptible (MRSN8383) host bacteria. In all cases, more than 99% of phage particles were bound to *S. aureus* cells and thus lost from the supernatant after centrifugation. Therefore, phage resistance in this case is likely to be independent of phage interaction with receptors on the bacterial cell surface.

Genome comparisons of Sb-1 and its host range variants. To explore the molecular basis of the phage Sb-1 host range expansion, we performed whole-genome sequencing of the Sb-1P phage (production stock), picked from a single plaque and propagated on phage-susceptible strain MRSN8383 (Sb-1_{8383}), and of two expanded host range mutants (Sb-1M), picked from single plaques and propagated on previously Sb-1-resistant strains MRSN6168 (Sb-$1M_{6168}$) and MRSN9832 (Sb-$1M_{9832}$). These whole genomes were compared to each other and to the reference genome of Sb-1 sequenced in 2010 (GenBank accession number HQ163896). The comparisons revealed the presence of multiple single-nucleotide differences, two insertion/deletions, and select unresolved regions corresponding to the positions of tandem and complex repeat structures in the genomes analyzed (see Table 2 and Table S1). Single mutations in two hypothetical proteins were observed, including an arginine-to-isoleucine mutation in open reading frame 147 (ORF147) and a lysine-to-glutamic acid mutation in ORF149, both of which were uniquely shared by both Sb1M phages (Table 2 and Table S1). We noted that the Sb-1_{8383} phage genome encodes six unique mutations in the

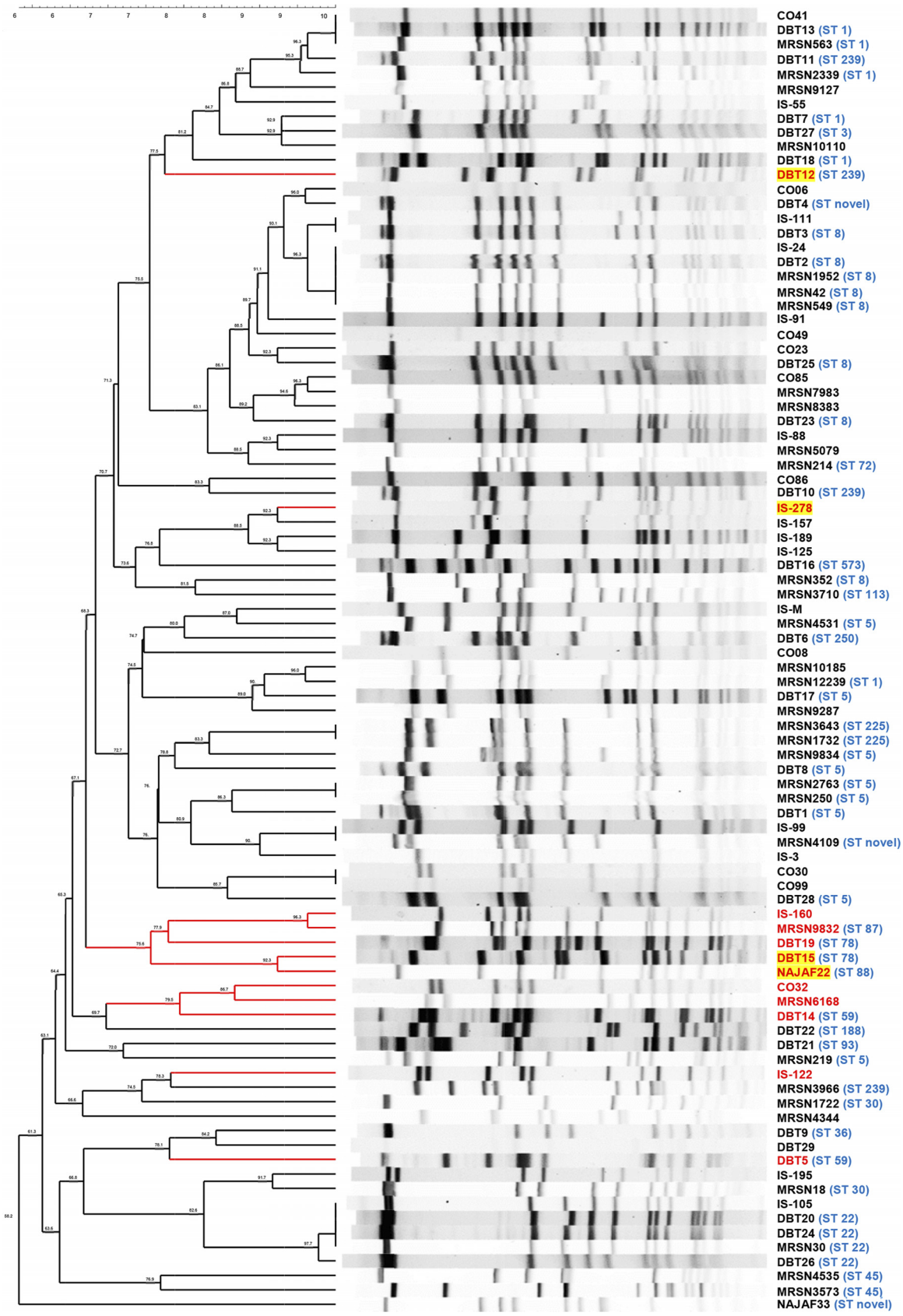

FIG 1 Diversity of *S. aureus* strains according to PFGE. Strains resistant to the parental Sb-1 therapeutic preparation (Sb-1P) manufactured by Eliava Biopreparations, Ltd., are marked in red. Strains that were also resistant to the adapted Sb-1 (Sb-1A) are marked with yellow highlighting. Sequence type (ST) based on multiple locus sequence typing is also provided in blue type, if available.

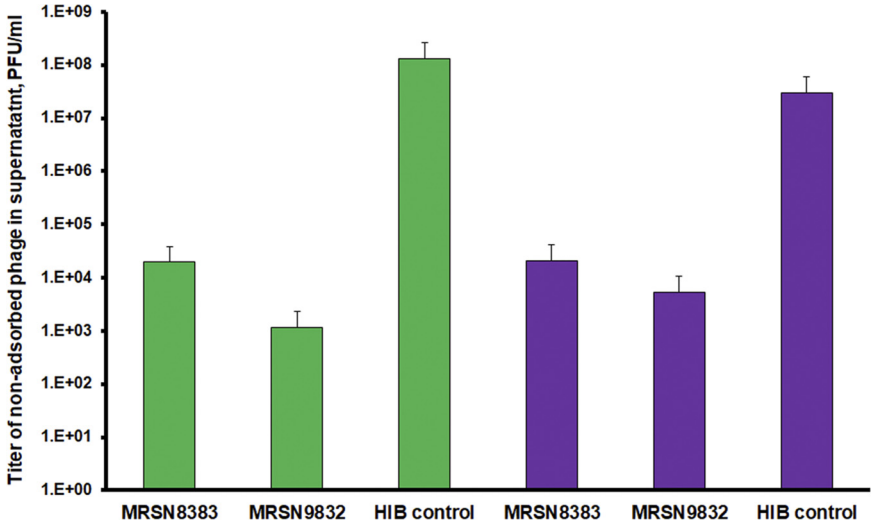

FIG 2 Phage adsorption assay. The bars represent the titers of nonadsorbed phage particles in supernatants after precipitation of bacterial cells and phage particles adsorbed to them. Green, Sb-1P; purple, Sb-1M.

terminal region of ORF82 relative to the Sb-1 reference genome. Although annotated as a hypothetical protein, ORF82 has >97% amino acid similarity to capsid proteins of *Staphylococcus* phages JD007, phiSA039, and pSco-10. The TAT to ATA mutation observed at positions 47966 to 47968 generates a 36-amino acid extension of the ORF82 homolog in Sb-1_{8383}.

In addition, unresolved repetitive sequences were also observed among the phage genomes. Errors in reconstructing repetitive DNA sequence loci by next-generation sequence assembly algorithms challenged the accurate determination of repeat copy number. Genomic microsatellite diversity has previously been hypothesized to impact *Staphylococcus* phage genome evolution and host adaptation, and the need to evaluate repeat composition in the context of host range was also noted (46). Our results indicated that such sequence repeat-mediated adaptation may have contributed to the phage host range phenotypes observed in our study. Subsequent bioinformatic analyses revealed the presence of three major intergenic repetitive sequence loci in the phage genomes reported here, which we arbitrarily designated regions 1, 2, and 3. The positions of these regions are illustrated in Fig. 3. We confirmed clone-specific allele states (Table 2) in the Sb-1/Sb-1M genome using PCR fragment sizing and Sanger sequencing. No sequence variation was observed between Sb-1P and Sb-1M at the

TABLE 2 Discriminating polymorphisms among Sb-1 phage genomes sequenced in this study[a]

Location	Polymorphism in phage:				Codon	ORF	Gene	GenBank protein accession no.
	Sb-1ref	Sb-1_{8383}	Sb-$1M_{9832}$	Sb-$1M_{6168}$				
47961	A[E]	C[D]	Adel	Adel	3rd	82	Conserved hypothetical protein	AEJ79720.1
47964	C[G]	T[G]	C[G]	C[G]	3rd	82	Conserved hypothetical protein	AEJ79720.1
47966	T	A	T	T	2nd	82	Conserved hypothetical protein	AEJ79720.1
47967	A	T	A	A	2nd	82	Conserved hypothetical protein	AEJ79720.1
47968	T	A	T	T	2nd	82	Conserved hypothetical protein	AEJ79720.1
47971	A	G	A	A	NA	NA	NA	NA
101833	C[A]	C[A]	C[A]	A[E]	2nd	NA	Hypothetical protein	AAX92102.1
108037	A	G	A	A	NA	NA	NA	NA
109146	G[R]	G[R]	T[I]	T[I]	2nd	147	Hypothetical protein	YP_008873656.1
109834	G[V]	G[V]	A[I]	G[V]	1st	149	Hypothetical protein	YP_008873658.1
109903	A[K]	A[K]	G[E]	G[E]	1st	149	Hypothetical protein	YP_008873658.1
123276–123740	Region 2 allele A	Region 2 allele B	Region 2 allele C	Region 2 allele C	NA	NA	NA	NA

[a]NA, data are not available.

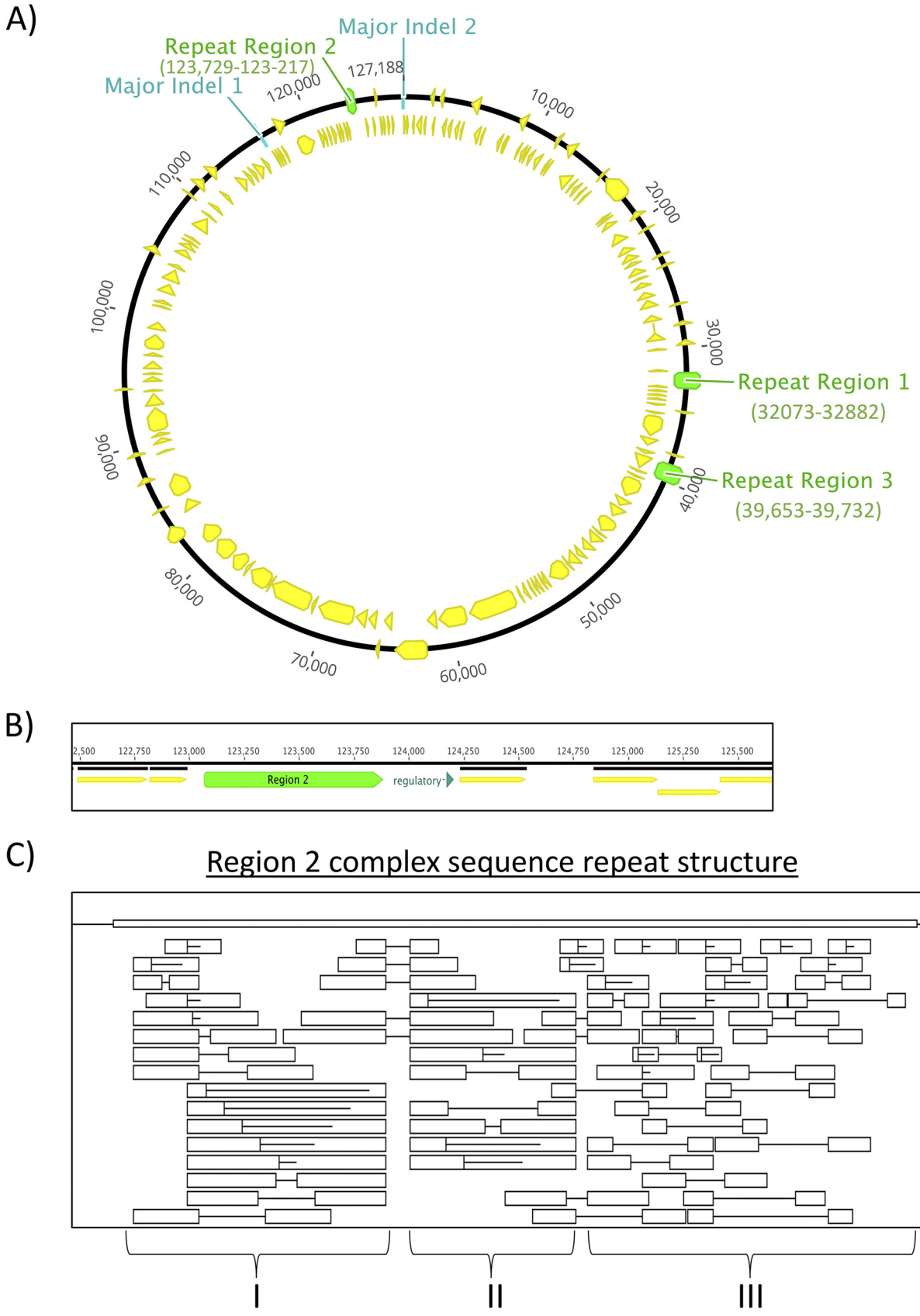

FIG 3 Repeat structure analysis of genomic sequence repeat regions. (A) Physical location of the three repeat regions and major indels in the Sb-1 reference genome; (B) annotation of the repeat region 2 locus; (C) complex repeat structure of the region 2 locus illustrating direct and indirect repeats. Boxed regions indicate repeated sequence units. Horizontal lines designate direct repeats.

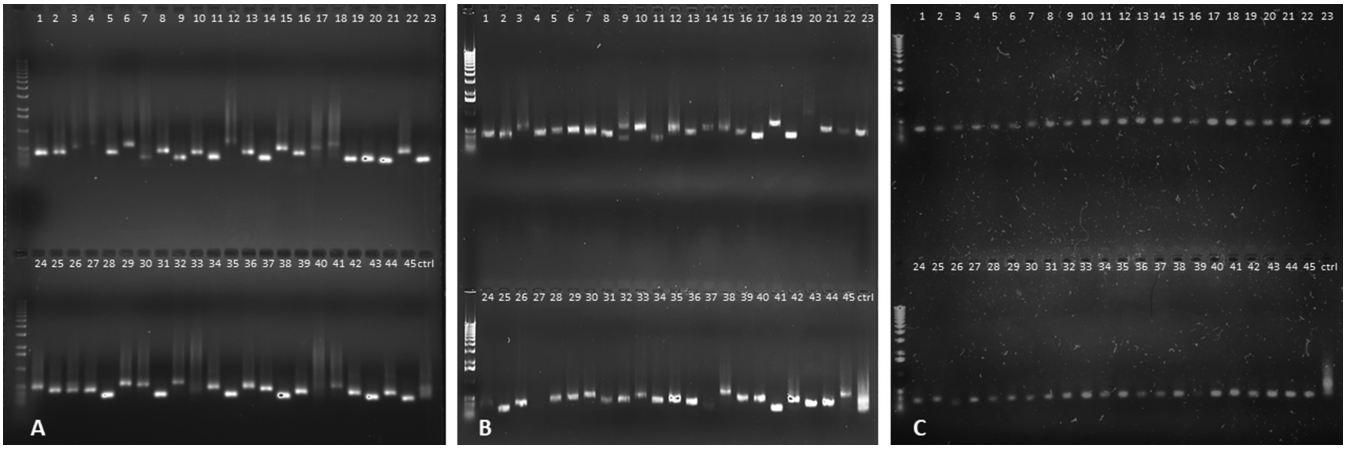

FIG 4 Region 2 allele diversity in Sb-1P and Sb-1A phage plaques. (A) PCR products of region 2 from Sb-1P clones plated on MRSN8383. (B) PCR products of region 2 from Sb-1A clones plated on MRSN8383. (C) PCR products of region 2 from Sb-1A clones plated on MRSN9832. The control lanes (ctrl) contain the PCR product of region 2 of the Sb-1 production stock (Sb-1P) without any plaque/clone purification.

region 1 locus. Region 1 is comprised of an iteron repeat structure in the left long terminal repeat (LTR) region. We observed that the genomes of the parental and expanded-host-range Sb-1 phage genomes sequenced in this work (Sb-1_{8383}, Sb-$1M_{6168}$, and Sb-$1M_{9832}$) differed from the Sb-1 reference genome at this locus, in that Sb-1_{8383}, Sb-$1M_{6168}$, and Sb-$1M_{9832}$ display a single-copy allele state, while the repeat locus in the Sb-1 reference genome contains four copies of the repeat (Fig. 3). Surprisingly, our analysis revealed the presence of a repeat-derived mutant spectrum arising from apparent hypermutability of the region 2 locus in Sb-1_{8383}. Region 2 is an intergenic repeat locus that exhibited a complex degenerate repetitive structure comprised of tandem and indirect repeats (Fig. 3A to C), including a core repeat unit sequence of "TACTACTATTAC." Region 2 is located upstream of the right terminus and immediately adjacent to a regulatory region that could control the transcription of the right long terminal repeat (R-LTR) genes (Fig. 3C). The region 3 locus exhibited no sequence polymorphism across host range phenotypes and the 2010 Sb-1 reference genome.

Diversity of genomic regions 1, 2, and 3. The sequence variability of Regions 1, 2 and 3 was compared in the therapeutic phage Sb-1 production stock (Sb-1P) and the Sb-1A adapted phage preparation. PCRs were performed on 45 randomly selected individual plaques from Sb-1P plated on MRSN8383 (phage-susceptible strain) and from Sb-1A plated on both MRSN8383 and on MRSN9832 (Sb-1P-resistant strain). Region 2 amplicons exhibited a high degree of length variability when Sb-1P or Sb-1A was plated on MRSN8383 (Fig. 4A and B), while Sb-1A plaques produced on previously phage-resistant strain MRSN9832 generated PCR amplicons of relatively uniform length (Fig. 4C). The control PCR product of region 2 of the Sb-1 production stock (Sb-1P) without isolation of individual plaques/clones appears as a highly diffused band that is consistent with variability in region 2 size in the Sb-1 genome. We observed no detectable length variability of PCR fragments in regions 1 and 3 in Sb-1P and Sb-1A clones plated on MRSN8383 or Sb-1A clones plated on MRSN9832 (data not shown). The results suggested that phage plaques on MRSN9832 represented Sb-1M host range mutants with a stabilized region 2 structure.

Sanger sequence analysis of regions 1, 2, and 3. The PCR products of regions 1, 2, and 3 for each individual clone of Sb-1P production stock and Sb-1A plated on MRSN8383 (see Fig. 4A and B), and Sb-1M clones picked from previously phage-resistant strain MRSN9832 (see Fig. 4C) were sequenced by the Sanger method.

Sequencing analysis and dot plot comparisons of sequence data confirmed that Sb-1P and Sb1A clones picked from phage-susceptible strain MRSN8383 had highly diverse region 2 genotypes (see Fig. 5A to C), while the Sb-1M host range mutants

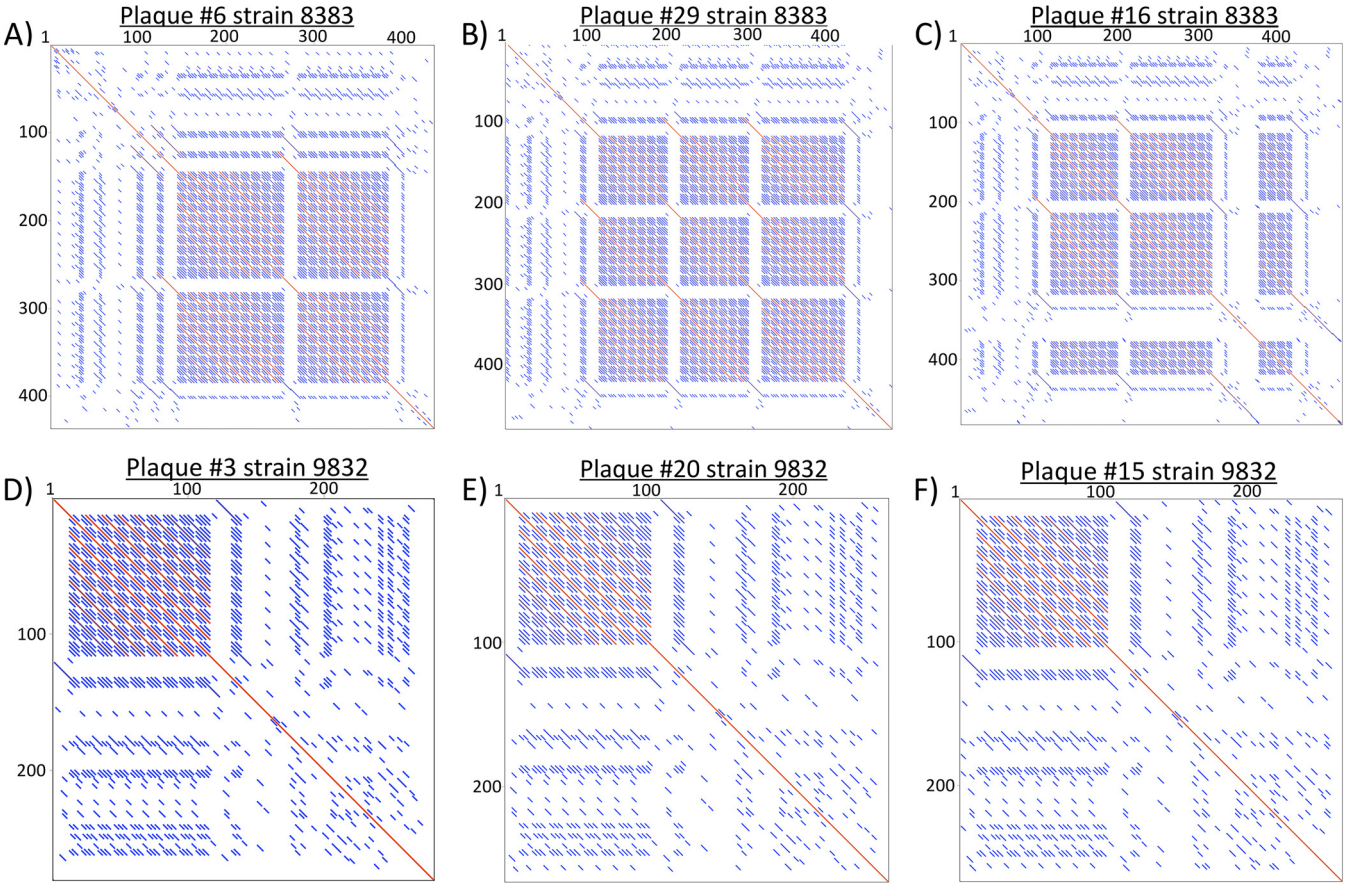

FIG 5 Dot plot analysis. Dot plots illustrating the region 2 repeat structure of individual clones derived from the dominant genotype of Sb-1A phage plated on the phage-susceptible strain MRSN8383 (A to C) and from the dominant genotype of Sb-1M plated on the previously phage-resistant strain MRSN9832 (D to F).

selected on the previously resistant strain show a single dominant genotype (clone) and roughly four minor genotype variants (Fig. 5D to F). The extent of allele diversity at the hypervariable locus correlated with host range. Given the stability observed in this complex repeat region when grown on the previously resistant host, it is feasible that selection may occur at this particular locus that impacts viral fitness on that specific host. Regions 1 and 3 yielded uniform DNA sequence in clones from Sb-1P and Sb-1A (data not shown).

Diversity generation during phage propagation from a single plaque. We determined whether progeny of individual clones of Sb-1P or Sb-1M generated diverse alleles in regions 1 to 3 during their propagation in broth culture, per the procedure described in Materials and Methods. For each stock, regions 1 to 3 from 45 randomly selected individual plaques were amplified by PCR, analyzed for fragment size variation, and sequenced. PCR banding patterns (Fig. 6) and dot plot analysis of sequenced amplicons (Fig. 7) consistently demonstrated that Sb-1P exhibited marked allelic diversity only in the region 2 locus and not in regions 1 or 3 (data not shown), while Sb-1M showed only a single allelic state at the region 2 repeat locus (Fig. 6B and 7B).

Thus, the only consistent genetic variation observed between the parental Sb-1P phage and the expanded host range Sb-1M phage were the host-expansion-specific allele states observed at the region 2 locus. Whether the observed variations in the complex repeat architecture at this locus alter viral fitness is unknown. An expanded annotation search for functionality of this specific sequence did not indicate significant homologies. Region 2 is positioned upstream of the right terminus and immediately adjacent to a regulatory region that we speculate could impact the transcription of select R-LTR genes. The presence of variable LTRs that exhibit multiple tandemly

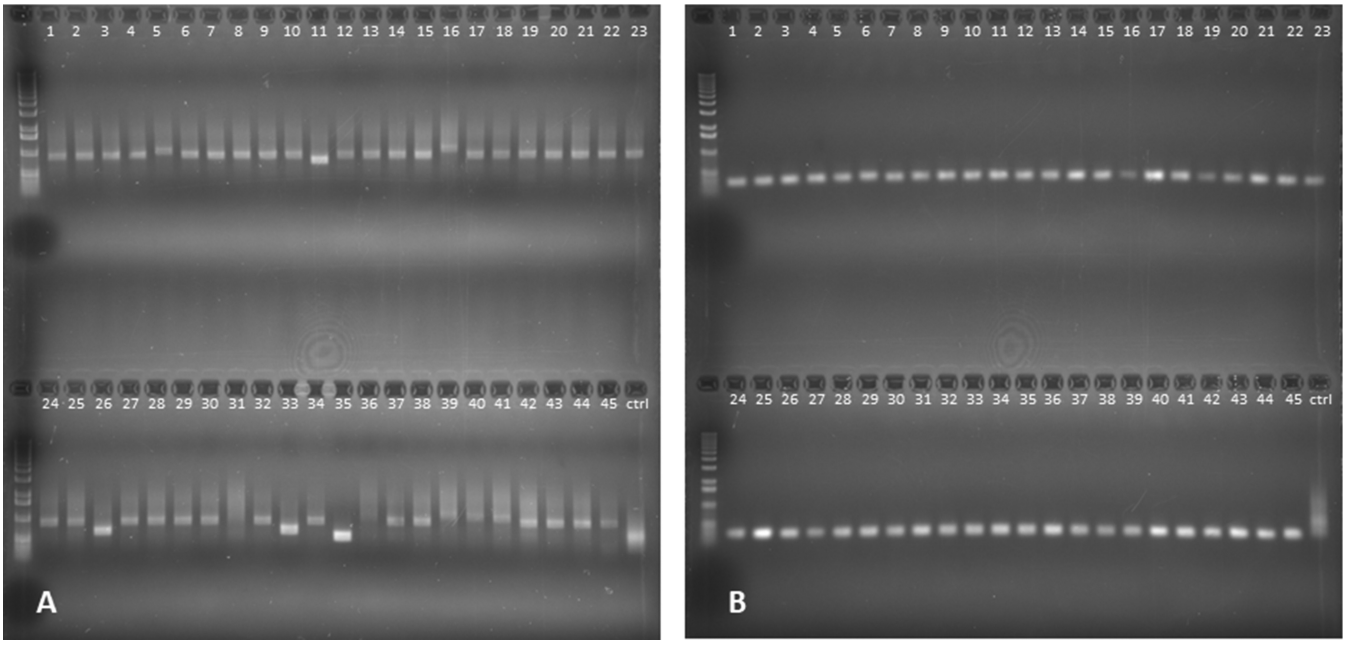

FIG 6 PCR fragment size variation at the region 2 locus for isolated plaque clones of Sb-1P (A) and of Sb-1M (B). Control lanes (ctrl) show the PCR product of region 2 of the Sb-1P production stock without plaque/clone purification.

repeated regions flanking the central nonrepetitive region of the genome is a shared feature of staphylococcal phages in the *Spounavirinae* subfamily (38). The region 2 locus is positioned at the border region of the right LTR and the nonrepetitive genome region adjacent to a palindromic sequence (36). The region 2 locus displays extensive variation among other representative *Staphylococcus* phage genomes characterized by the presence of repeat structure variation as well as by the presence of short hypothetical proteins (data not shown). The frequency of the singular Sb-1M region 2 allele state observed over subsequent host-specific passages argues against this being the result of neutral mutation. We speculate that the variation observed at the region 2 hypervariable repeat locus may provide an increase in fitness related to host range phenotype. The similar host adsorption efficiencies observed between Sb-1P and Sb-1M suggest a lack of differential receptor-mediated host range specificity, which would be consistent with this hypothesis. Alternatively, the observed variation in repeat structure may impart changes in gene expression of adjacent open reading frames that may participate in mediating host range. Such mechanisms are speculative, but the data reported here could guide future experimental efforts to dissect the basis of host range specificity. The basis for the novel allele variation observed in the Sb-1 phage genome that distinctly exhibits a unified allele state coinciding with host range expansion requires further investigation. Identifying potential functional consequences arising from mutations such as the ones described here that occur during host adaptation may enable the determination of the genetic requisites of host range expansion in Sb-1 and other Twort-like phages.

Phage Sb-1 has been used for decades for therapy of multiple types of *S. aureus* infections in Georgia and other FSU countries. The host range of Sb-1 was known to be expandable in the laboratory using a so-called adaptation procedure, but the genetic basis of this host range expansion was previously unknown. In this work, a hypervariable region (region 2) that is also present in other Twort-like phages was found in the genome of Sb-1. We showed that laboratory adaption of Sb-1 to expand host range to include previously resistant *S. aureus* strains produced a mixture of clones, including host range mutants (Sb-1M). The region 2 locus was variable in sequence in the parental phage preparation (Sb-1P) but exhibited a stable distinct allele in host range mutants (Sb-1M) that correlated with phenotypic host range expansion. The results

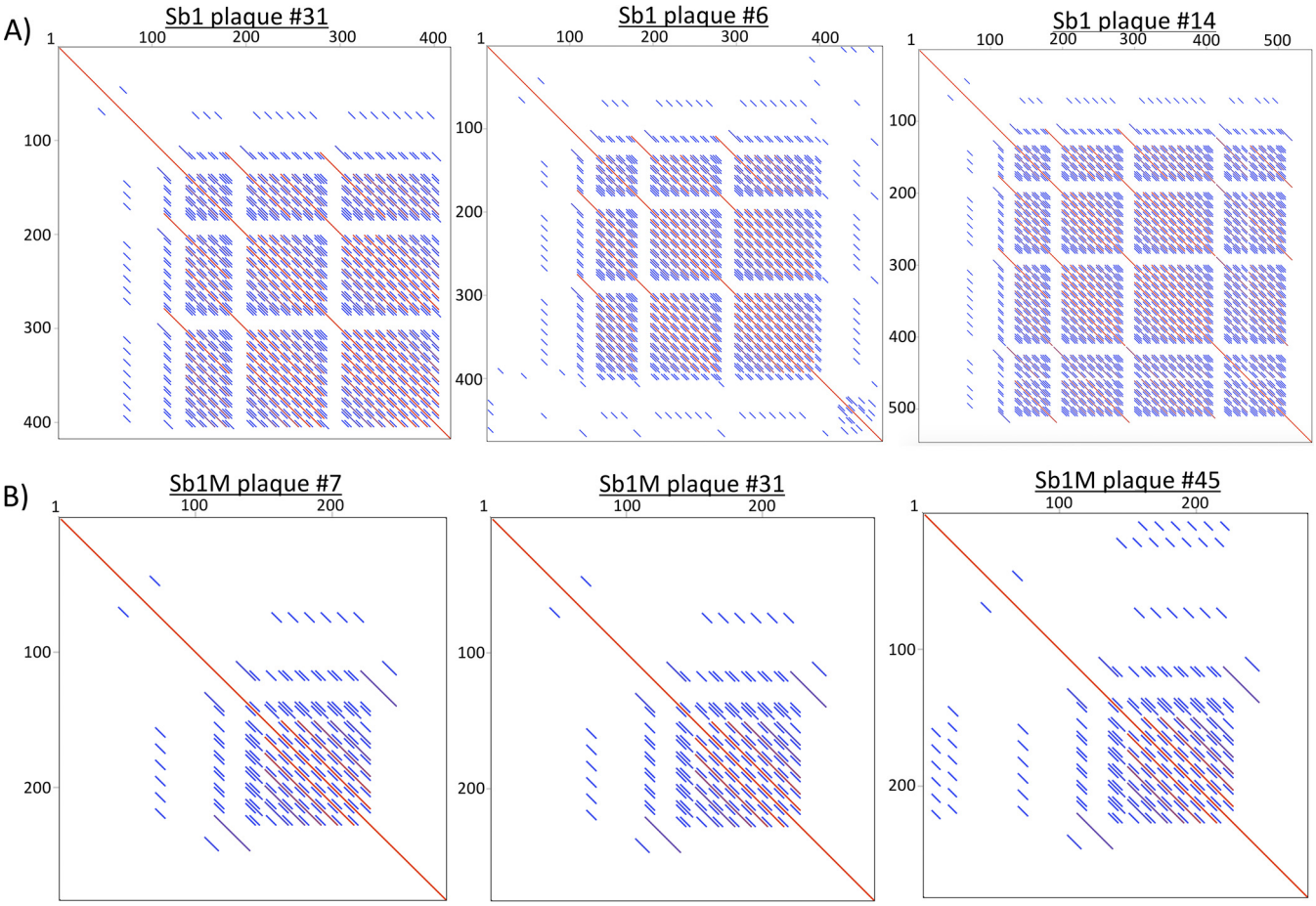

FIG 7 Dot plot comparisons of region 2 locus sequences among Sb-1P (A) and Sb-1M (B) plaques.

reported here can inform future studies to better understand the mechanisms of host range expansion in Twort-like phages such as Sb-1.

MATERIALS AND METHODS

Bacterial strains, phage, and growth media. *S. aureus* strains used in this work are presented in Table 1. The first 35 strains listed in Table 1 were received from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN; Bacterial Diseases Branch, Walter Reed Army Institute of Research), and the rest belong to the culture collection of the Department of Bacteriophage Therapeutics (in the same branch). The cultures were grown at 37°C in Bacto heart infusion broth (HIB; Becton, Dickinson and Co., Franklin Lakes, NJ). Solid medium contained 1.5% (wt/vol) Bacto agar (Becton, Dickinson). Semisolid agar overlay contained 0.7% (wt/vol) of Bacto agar (Becton, Dickinson). All *S. aureus* cultures were stored at −80°C in HIB containing 10% glycerol. Phage Sb-1 and its derivatives were filter sterilized through a 0.22-μm membrane and stored at 4°C in HIB or sodium chloride-magnesium sulfate (SM) buffer (Teknova, Hollister, CA).

Bacteriophage titer determination, plaque assays, and propagation. Phage dilution was done in SM buffer. Phage titer determination and plaque assays were performed by the double-layer agar method as described earlier (47) with overnight incubation at 37°C. Phages were propagated on methicillin-susceptible *S. aureus* (MSSA) strain MRSN8383 as follows. Phage stock lysate was added to 150 ml of an early-exponential-phase bacterial culture grown in HIB at a multiplicity of infection (MOI) of 0.01 and incubated in a 250-ml plastic Erlenmeyer flask (catalog no. 07-200-668; Fisher Scientific, Hampton, NH) at 37°C at 200 rpm until visible lysis occurred (for 3 to 5 h). Phage lysate was treated with chloroform added to a final concentration of 5% (vol/vol) in order to destroy all remaining live bacteria. Bacterial debris was removed by 15-min centrifugation at 5,000 × *g*. The supernatant was filtered through a sterile 0.22-μm membrane.

Phage host range test. Phage host ranges were determined using a micro-spot modification of an efficiency of plating assay. Briefly, 10-fold serial dilutions of phage were prepared in a sterile flat-bottomed 96-well plate. An aliquot (2 μl) of each phage dilution, ranging from 10^{-1} to 10^{-8}, was spotted using a multichannel pipette on semisolid HIB agar overlay infused with *S. aureus* culture and incubated overnight at 37°C. The results were scored, and the morphology of individual plaques was evaluated. The

efficiency of plating (EOP) of phage on each bacterial strain was calculated as phage titer on the test strain divided into the titer of the same phage on MSSA strain MRSN8383, which is consistently susceptible to phage Sb-1.

Host range expansion (adaptation procedure). Phage adaptation was performed by several rounds of coincubation of phage Sb-1P with initially phage-resistant strain *S. aureus* MRSN9832 in liquid medium (34). Briefly, 10^9 PFU of phage in 0.1 ml of SM buffer and 0.1 ml of overnight culture of MRSN9832 were added to 5 ml of HIB in a 50-ml conical centrifuge tube (catalog no. 06-443-18; Fisher Scientific) and incubated for 24 h at 37°C with shaking at 100 rpm. Bacterial cells were pelleted by 15-min centrifugation at 5,000 × *g*, 1 ml of supernatant and 0.1 ml of overnight culture were added to 5 ml of HIB, and the second round of the procedure was performed. Slight lysis of the culture was observed after the fifth round of coincubation, and the procedure was finished after six rounds. Bacterial debris was pelleted and supernatant was filter sterilized and considered Sb-1A. This preparation contained about 4×10^9 PFU/ml of phage capable of plating on MRSN9832.

Single-plaque diversity generation assay. A single isolated phage plaque was picked and suspended in 0.7 ml of SM buffer, filtered through a sterile 0.22-μm membrane, and diluted to a final concentration of 300 to 1,000 PFU/ml. The presence of viable phage particles was confirmed by plating on *S. aureus* MRSN8383. Phage suspension (100 μl) containing 30 to 100 viable phage particles was added to 10 ml of early mid-exponential-phase culture of MRSN8383 and incubated for 16 h at 37°C in a shaker incubator at 200 rpm. The resulting phage lysate was plated again on MRSN8383 to get isolated plaques. Sb-1 genome regions 1, 2, and 3 (see Results and Discussion) from 45 randomly selected individual plaques were analyzed by PCR and sequenced by the Sanger dideoxynucleotide chain termination method (48). The experiment is presented schematically in Fig. S1.

Phage adsorption assay. A phage adsorption assay was performed as described earlier (49–51), with some variations. Briefly, test strains were grown overnight in 5 ml of HIB supplemented with 5 mM $CaCl_2$. HIB without bacteria was used as a negative control. An aliquot (1 ml) of a tested culture was centrifuged, and the pellet was resuspended in 0.5 ml of HIB with 5 mM $CaCl_2$. Phage (50 μl, ~10^8 PFU) was added to each culture and control. Samples were incubated for 15 min at room temperature to allow phage to adsorb and then were centrifuged for 4 min at 13,000 rpm. The resulting supernatant was filter sterilized to eliminate the residual bacteria with adsorbed phage. The supernatant was diluted from 10^0 to 10^{-3}, and 10 μl of each dilution was spotted on a semisolid agar overlay infused with 0.1 ml of overnight culture of indicator strain MRSN8383. The negative control was treated the same way. The percentage of phage adsorbed by bacteria of each test strain was calculated by subtracting the phage titer in the test strain from the phage titer in the negative control and dividing the result by phage titer in the negative control. Adsorption assays were done in triplicate and averaged.

Isolation of phage genomic DNA for sequencing. Isolation of phage genomic DNA was performed as previously described (52), with minor modifications. Briefly, Sb-1P and Sb-1M phage lysates propagated from individual purified plaques were centrifuged at 13,000 × *g* for 4 h and resuspended in SM buffer in 1/100 of the original volume. The concentrated lysate was treated for 1 h at 37°C with RNase A (60 μg/ml) and DNase I (20 μg/ml) to remove residual *S. aureus* DNA and RNA. Proteinase K and SDS were added to final concentrations of 50 μg/ml and 0.5%, respectively, and the mix was incubated for 1 h at 56°C. DNA was purified by phenol-chloroform extraction followed by overnight DNA precipitation with ethanol at −20°C, centrifugation and resuspension in nuclease-free water. DNA purity was confirmed by restriction analysis with BamHI, EcoRI, and XbaI endonucleases (New England BioLabs Inc., Ipswich, MA) using horizontal agarose gel electrophoresis (47).

Whole-genome sequencing and genome assembly. Whole-genome sequences were determined for the Sb-1P phage picked from a single plaque and propagated on strain MRSN8383 ($Sb\text{-}1_{8383}$) as well as for the two Sb-1M host range mutants ($Sb\text{-}1M_{6168}$ and $Sb\text{-}1M_{9832}$) picked from single plaques and propagated on previously resistant strains MRSN6168 and MRSN9832, respectively. DNA sequencing was performed using the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA) with 600-cycle v3 reagents. Sequencing libraries were prepared with the HyperPlus PCR-free next-generation sequencing (NGS) kit (Kapa Biosystems, Wilmington, MA) containing 550 bp inserts. Paired-end sequencing reads were assembled using Velvet (https://www.ebi.ac.uk/~zerbino/velvet), CLC Bio (Qiagen, Germantown, MD), and Geneious 8.0.2 (Newark, NJ). Reference mapping and variant calling was performed using the 2010 Sb-1 reference genomic sequence (accession number HQ163896) using Geneious (51) with medium sensitivity and fine tuning at 5× iterations using trimmed sequences (yielding >100-fold coverage of each genome). Annotations were performed using Geneious and Sequin and using the 2010 Sb-1 genome as the reference. Geneious software was used for partial genome alignments and to analyze the physical organization of reference genomic sequences, including ORF content and synteny. Sequence repeat structures were analyzed using DNASTAR GeneQuest v14.1.0.

Analysis of staphylococcal DNA by pulsed-field gel electrophoresis. A single colony of each *S. aureus* strain was inoculated in 5 ml of BHI and grown overnight. The culture was diluted with BHI to an optical density at 600 nm (OD_{600}) of 0.9 to 1.1. The adjusted cell suspension (200 μl) was centrifuged at 13,000 rpm on an Eppendorf 5415C microcentrifuge for 4 min, and the pellet was resuspended in 300 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cell suspension was equilibrated at 37°C for 10 min and treated with 4 μg of lysostaphin (Sigma-Aldrich Corp., St. Louis, MO). To make plugs, 500 μl of 1.8% SeaKem Gold agarose (Lonza Inc., Allendale, NJ) in TE buffer equilibrated to 55°C was added to each cell suspension, gently mixed, and dispensed into the well of plug mold. The solid plugs were incubated at 37°C for at least 4 h with 3 ml of EC lysis buffer (6 mM Tris HCl [pH 7.6], 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% sodium deoxycholate, and 0.5% sodium lauroylsarcosine) in a shaker incubator. After incubation, EC lysis buffer was discarded, and the plugs were rinsed 4 times in 4 ml of

TABLE 3 Primers used in this work

Primer	Target region	Primer sequence
R1-F	1	CTGCTTCACTACGATTATCTTC
R1-R	1	GGAATGGATGAAACCTCTATA
R2-F	2	GAGGAAGATATAGAATGAGAACC
R2-R	2	GCTTCAGCATAGATTAACAGTAG
R3-R	3	CACAAGGCAATGAGGTAATC
R3-F	3	CAGGTAAATACTACTGATAGTGACG

TE buffer for 30 min at 37°C. After washing, the plugs were stored in 4 ml of TE buffer. DNA was digested with SmaI enzyme (New England Biolabs [NEB]) in NEB 4 buffer (30 units per tube) at 25°C for at least 3 h. DNA fragments were resolved using 1% SeaKem Gold agarose in 0.5× Tris-borate-EDTA (TBE) buffer (45 mM Tris-borate; 1 mM EDTA) using a Chef Mapper apparatus (Bio-Rad, Hercules, CA) for 21 h at 200 V and 14°C, with a 5 to 40 sec linear ramp. After electrophoresis, gels were stained with EnviroSafe DNA/RNA stain (Helixx Technologies, Inc., Scarborough, ON, Canada) per the manufacturer's protocol, rinsed, and photographed using a ChemiDoc XRS+ gel documentation system (Bio-Rad). Macrorestriction patterns were analyzed using BioNumerics version 7.6 (Applied Maths, Austin, TX) software based on the Dice similarity index (optimization, 5%; band matching tolerance, 0.7%). Cluster analysis was performed using unweighted pair group method with arithmetic mean (UPGMA) with 101 total entries (taxa) and normalized to *Salmonella enterica* serovar Braenderup H9812 strain standards.

Repeat region PCR. Primers used for amplification of repeat regions of Sb-1 and Sb-1M are shown in Table 3. The primers were designed using Beacon Designer program (Premier Biosoft Int.) within unique flanking sequences for each repeat region. PCR was performed using Invitrogen Platinum PCR supermix (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. PCR fragments were resolved using a standard horizontal electrophoresis in 0.8% Tris-acetate-EDTA (TAE) agarose gel. Sb-1P and Sb-1M phages were plated on strains MRSN8383 and MRSN9832, respectively. In each case, 45 well-separated single plaques were picked up using sterile tips. Each plaque was resuspended in 1 ml of SM buffer and filter sterilized, and 1 μl of the suspension (ca. 100 PFU) was used as a template for each of three separate PCRs targeting three genome regions.

Statistical analysis. Results of all EOP assays are presented as mean values of three independent biological replicas. Statistical significance was determined by one-way analysis of variance (ANOVA) analysis using a free online program at the website of Vassar College (53). P values of <0.05 were considered significant.

Data availability. The nucleotide sequences of the phages reported here were submitted to GenBank under the accession numbers MN336261 (Sb-1_{8383}), MN336262 (Sb-$1M_{6168}$), and MN336263 (Sb-$1M_{9832}$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01209-19.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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