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BRIEF REPORT

# A Novel Shiga Toxin 1a-Converting Bacteriophage of *Shigella sonnei* With Close Relationship to Shiga Toxin 2-Converting Pages of *Escherichia coli*

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In recent studies, strains of non-*dysenteriae* 1 *Shigella* (NDS) expressing Shiga toxin have been reported. In this study, we report a novel *stx1a*-converting bacteriophage of *Shigella sonnei* associated with travel to Mexico. Phylogenetic comparison between this and other *stx*-converting phages suggests that toxigenic NDS strains have arisen through separate horizontal transfer events from toxigenic *Escherichia coli*.

**Keywords.** bacteriophages; *Shigella sonnei*; Shiga toxin.

Shiga toxin-producing *Escherichia coli* (STEC) has been associated with adverse clinical outcomes including hemolytic uremic syndrome (HUS) [1]. Within STEC strains, the *stx* gene operon is phage-encoded, and it exists as one of several *stx* subtypes (*Stx1a*, *Stx1c*, *Stx1d*, and *Stx2a-f*) [2]. These *stx*-converting phages vary considerably in genomic size, morphology, and integration site preference (Supplementary Table 1) [2]. The *stx*-converting phage lytic cycle and toxin expression is induced in response to deoxyribonucleic acid (DNA)-damaging agents including fluoroquinolone antibiotics [3].

Previously, the expression of Stx toxins in *Shigella* species was believed to be unique to *Shigella dysenteriae* type 1. More recently, *stx*-encoding isolates of *Shigella sonnei* [4–6], *S dysenteriae* type 4 [7], and *Shigella flexneri* [8] have been reported. These isolates were associated with travel to Eastern Europe [4, 5], Morocco [6], and Hispaniola [7, 8]. The details of *stx* acquisition by these non-*dysenteriae* 1 *Shigella* (NDS) isolates remain unclear, including the chronology of toxin acquisition (ie, recent vs remote), the origin of the converting phages, and their global distribution.

## METHODS

Stool samples were screened for Stx by immunoassay according to the manufacturer's instructions (ImmunoSTAT! EHEC assay; Meridian Biosciences, Cincinnati, OH). Stool cultures were performed using Hektoen enteric and XLD selective agars, and automated biochemical speciation of suspicious colonies was performed (Vitek2, Durham, NC). A toxin-negative clinical isolate of *S sonnei* was isolated by similar means and used as a control. Identifications were confirmed by the San Diego County Public Health Laboratory.

Phage lysis was induced by treating *Shigella* cultures with 0.5 μg/mL mitomycin C (MMC) as previously described [8]. After MMC addition, culture aliquots were removed hourly for optical density ($OD_{600}$) reading. Large-scale phage genomic DNA isolation was performed after the QIAGEN Lambda kit protocol (QIAGEN, Valencia, CA).

Restriction analysis was performed by digesting purified phage genomic DNA with *Eco*RI and *Hind*III according to the manufacturer's recommended conditions (New England BioLabs, Ipswich, MA), and restriction products were resolved on 0.7% agarose. Invitrogen 1 kb plus ladder was used as a size standard (Invitrogen, Carlsbad, CA).

Sequencing library preparation was performed from genomic bacterial DNA and purified phage genomic DNA. Deoxyribonucleic acid was fragmented to 350 base pairs (bp) using the Covaris M220 (Covaris, Woburn, MA). The Illumina TruSeq Nano Kit (Illumina, San Diego, CA) was used to create barcoded libraries according to the manufacturer's protocol. Equimolar amounts of each library were pooled, and bidirectional sequencing was performed using the Illumina MiSeq platform for 300 cycles, using v2 reagents. The phage genomic sequence was determined by de novo assembly using the SPAdes 3.5.0 algorithm. To identify the integration site, bacterial genomic sequencing reads containing the phage terminal sequences were extracted, aligned, and subjected to Basic Local Alignment Search Tool (BLAST) search. The complete phage genome sequence is available through GenBank under accession number KR781488.

For phylogenetic analysis, phage sequences were obtained from National Center for Biotechnology Information using accession numbers found in Supplementary Table 1. Multiple sequence alignment was performed using MAFFT version 7, and maximum likelihood trees were inferred using RAxML v7.0.3 with general time reversible + gamma nucleotide substitution model [9]. Branch support was performed using 100 bootstrap iterations; all branches had bootstrap scores of >90, except the HUN/2013 – Stx1phi branch, which had a score of 58.

## RESULTS

The *stx1a*-converting phage described here was isolated from *S sonnei* that infected 2 epidemiologically unrelated subjects

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who presented to our hospital in 2014. Both subjects had recently returned from Baja California Norte, Mexico, and presented with diarrhea and abdominal discomfort. Both were treated with intravenous fluids and oral fluoroquinolone antibiotics, and both made uneventful recoveries. Stool samples from both individuals were positive for Stx by immunoassay, and, unexpectedly, stool culture on selective agar grew *S sonnei* from both subjects. *Shigella sonnei* species was confirmed by automated biochemical species determination and confirmed by polymerase chain reaction targeting a methylase gene sequence that is unique to *S sonnei* [10] (data not shown). The Stx-producing isolates were named Ss-VASD01 and Ss-VASD02.

Treatment of *S sonnei* cultures with the DNA-alkylating agent MMC induced lysis of both Stx-expressing isolates but not of an Stx-negative *S sonnei* isolate (Figure 1A), suggesting the presence of an inducible bacteriophage. Phage particles were purified from MMC-induced cultures by polyethylene glycol precipitation, and restriction analysis of purified phage genomic DNA revealed a large molecular weight genome from both isolates, which shared identical restriction patterns (Figure 1B).

To further characterize the phage (named ϕSs-VASD), deep sequencing of purified phage and the whole bacterial genome was performed. De novo assembly of sequencing reads resulted in a 62 851-bp genome with 712.5× mean coverage. A total of 189 sequencing reads were recovered that spanned the host-phage junction, and BLAST alignment of the non-phage portions revealed 100% identity to the *wrbA* gene of *S sonnei* reference strain Ss046 (host-phage junction at Ss046 position 1088102).

To assess the relationship of ϕSs-VASD to other stx-converting phages, we performed phylogenetic comparisons between the genomic sequences of ϕSs-VASD and 18 previously reported *stx*-converting phages (Supplementary Table 1) [2, 5, 8]. The

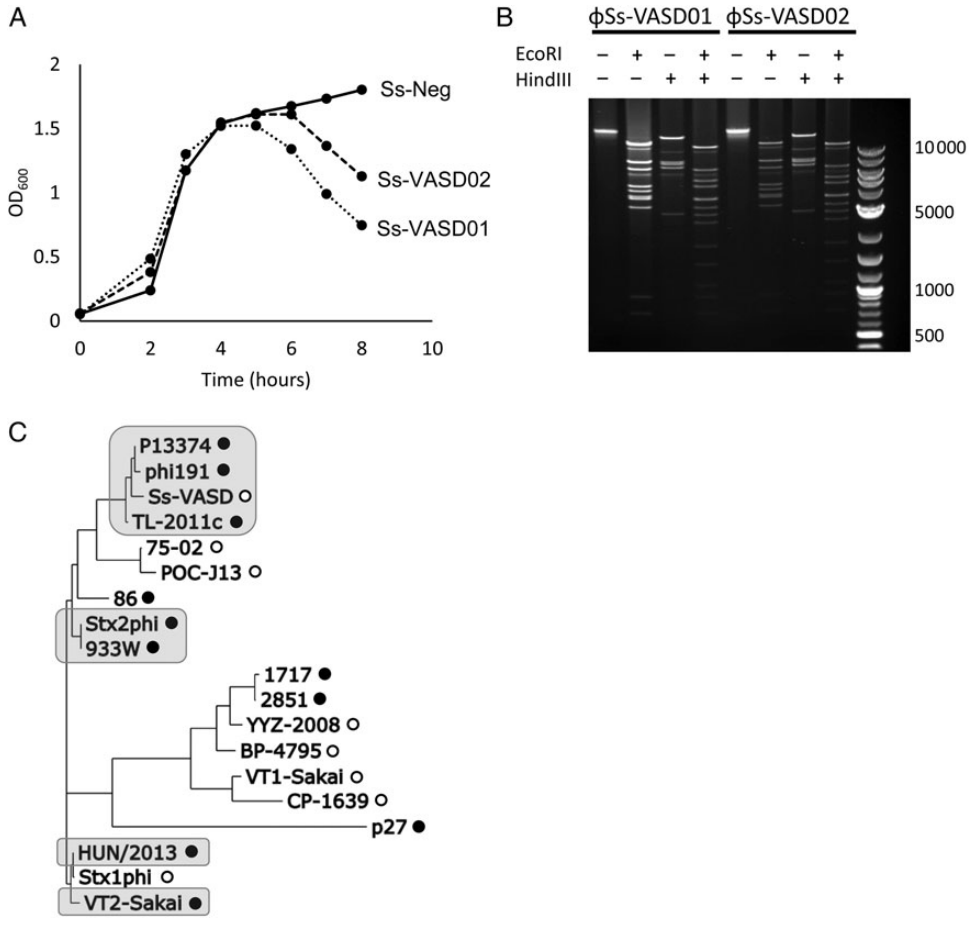


**Figure 1.** (A) Induction of bacterial lysis by mitomycin C (MMC). Culture optical density ($OD_{600}$) of Ss-VASD01, Ss-VASD02, and a nontoxigenic *Shigella sonnei* strain (Ss-Neg) was measured over time after addition of 0.5 µg/mL MMC (added at the 2-hour time point). (B) Restriction analysis of phage genomic deoxyribonucleic acid (DNA) obtained from purified phage particles after MMC induction. The DNA was digested with *Eco*RI, *Hind* III, or both. Invitrogen 1 kb plus ladder was run in the rightmost lane. (C) Whole genome phylogenetic analysis of ϕSs-VASD and other *stx*-converting bacteriophages. Phylogenetic tree of the complete ϕSs-VASD genome and other *stx*-converting phage genomes was inferred using the maximum likelihood method. Phages encoding *stx1* are denoted by open circles, and those encoding *stx2* are denoted by closed circles. Phages known to integrate into the *wrbA* gene are shaded in gray.

phage genome sequences were noted to cluster by integration site preference, with distinct clusters containing *wrbA*-integrating isolates (Figure 1C). The closest genetic relationships were found between ϕSs-VASD and *stx2*-converting phages of *E coli*.

Analysis of the *stx* coding sequence of ϕSs-VASD revealed 100% amino acid identity to 7/8 *stx1*-converting phages, with only POC-J13 varying from the consensus sequence at 2 positions ($P_{284}Q$ in *stxA* and $A_{11}T$ in *stxB*). ϕSs-VASD was found to contain the *stx* operon promoter $P_R$, which drives fluoroquinolone-mediated toxin production from *stx2*-encoding phage and contributes to toxin expression in *stx1*-encoding phages [3, 11, 12].

## DISCUSSION

In this study, we report a novel *stx1a*-converting phage of *S sonnei* associated with travel to Baja California, Mexico. Prior reports of Stx-expressing NDS have been associated with travel (Hispaniola for *S dysenteriae* serotype 4 and *S flexneri*, and to the Eastern Europe and Morocco for *S sonnei*). To our knowledge, these are the first such isolates originating from North America. Furthermore, it appears that the subjects in this study may be part of a larger outbreak of Stx-toxigenic *S sonnei*, presently under investigation by the California Department of Public Health [13]. These findings suggest an expanding global emergence of Stx-toxigenic NDS. Alternatively, the presence of Stx-toxigenic phage in NDS may have been overlooked because, until recently, it was not common practice to screen diarrheal stools for Shiga-like toxins.

One concern surrounding Shiga toxin-producing bacterial strains is the potential for toxin induction by antibiotics. Fluoroquinolone antibiotics are of particular concern, because they can induce toxin production by inducing bacterial DNA damage responses. Even though ϕSs-VASD is inducible by DNA damage and encodes a fully intact *stx* operon, treatment with fluoroquinolone antibiotics in both subjects reported here did not appear to trigger any clinical worsening or complications. In fact, to our knowledge, there have been no cases of complications associated with Stx-NDS infection, an observation that could be explained by several theories including the predominance of the less-pathogenic Stx1 variant [2] or variability in toxin induction by antibiotics. Alternatively, there may simply be an insufficient numbers of cases detected thus far (even in outbreaks involving high-risk STEC isolates, HUS occurs in the minority of cases).

It is interesting to note that phylogenetic analysis of ϕSs-VASD reveals a relationship to other *wrbA*-integrating phages. This relationship seems to be irrespective of *stx* type, because the closest relationship was to ϕ191 and p13374, 2 *stx2*-converting phages of STEC associated with outbreaks of enteroaggregative hemorrhagic *E coli*. Given the overall similarity of these phages apart from the *stx* operon, the findings implicate a recombination event leading to similar phages with differing toxin operons, a phenomenon that has been observed in STEC phages [14]. A weaker phylogenetic relationship was observed between ϕSs-VASD and the *S flexneri* phage POC-J13, and the *S sonnei*-converting phage 75-02.

The origin of *stx*-converting phages in NDS stains is uncertain, as is the chronology of their existence. Possible theories include (1) recent horizontal transfer of phage from STEC and (2) longstanding presence of *stx*-converting phages in NDS that was previously undetected. The high degree of genetic similarity between ϕSs-VASD and previously reported *stx*-converting phages of STEC supports the theory that Ss-VASD arose from horizontal transfer between STEC and NDS strains, rather than by parallel evolution within NDS. We are not able to perform precise molecular clock analysis; however, the absence of major sequence divergence between ϕSs-VASD and STEC phages is suggestive of a recent transfer event. The occurrence of horizontal transfer events such as this seems plausible, given the discovery of large amounts of *stx*-converting phage found in human and agricultural wastewater [15].

## CONCLUSIONS

Important future directions will include characterization of additional Stx-NDS phages, which will create better understanding of their diversity and may allow more precise inference of their origins. These findings suggest the need for more careful clinical and epidemiological monitoring for Shiga toxigenic *S sonnei*, because these strains seem to be broadly distributed globally, and infections therewith have the potential to cause complications not previously associated with *S sonnei*.


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