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Maintenance of the *Shigella sonnei* Virulence Plasmid Is Dependent on Its Repertoire and Amino Acid Sequence of Toxin-Antitoxin Systems

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**ABSTRACT** *Shigella sonnei* is a major cause of bacillary dysentery and an increasing concern due to the spread of multidrug resistance. *S. sonnei* harbors pINV, an ∼210 kb plasmid that encodes a type III secretion system (T3SS), which is essential for virulence. During growth in the laboratory, avirulence arises spontaneously in *S. sonnei* at high frequency, hampering studies on and vaccine development against this important pathogen. Here, we investigated the molecular basis for the emergence of avirulence in *S. sonnei* and showed that avirulence mainly results from pINV loss, which is consistent with previous findings. Ancestral deletions have led to the loss from *S. sonnei* pINV of two toxin-antitoxin (TA) systems involved in plasmid maintenance, CcdAB and GmvAT, which are found on pINV in *Shigella flexneri*. We showed that the introduction of these TA systems into *S. sonnei* pINV reduced but did not eliminate pINV loss, while the single amino acid polymorphisms found in the *S. sonnei* VapBC TA system compared with *S. flexneri* VapBC also contributed to pINV loss. Avirulence also resulted from deletions of T3SS-associated genes in pINV through recombination between insertion sequences (ISs) on the plasmid. These events differed from those observed in *S. flexneri* due to the different distribution and repertoire of ISs. Our findings demonstrated that TA systems and ISs influenced plasmid dynamics and loss in *S. sonnei* and could be exploited for the design and evaluation of vaccines.

**IMPORTANCE** *Shigella sonnei* is the major cause of shigellosis in high-income and industrializing countries and is an emerging, multidrug-resistant pathogen. A significant challenge when studying this bacterium is that it spontaneously becomes avirulent during growth in the laboratory through loss of its virulence plasmid (pINV). Here, we deciphered the mechanisms leading to avirulence in *S. sonnei* and how the limited repertoire and amino acid sequences of plasmid-encoded toxin-antitoxin (TA) systems make the maintenance of pINV in this bacterium less efficient compared with *Shigella flexneri*. Our findings highlighted how subtle differences in plasmids in closely related species have marked effects and could be exploited to reduce plasmid loss in *S. sonnei*. This should facilitate research on this bacterium and vaccine development.

**KEYWORDS** *Shigella sonnei*, virulence plasmid, TA systems, VapBC, insertion sequences, T3SS
Shigella spp. are important human pathogens and the leading cause of bacillary dysentery, resulting in over 160,000 deaths annually (1). The Global Enteric Multicenter Study demonstrated that shigellosis is a major cause of moderate to severe diarrheal disease among children under five, with Shigella flexneri and Shigella sonnei accounting for around 90% of cases of shigellosis worldwide (2, 3). While S. flexneri causes endemic disease in low- and middle-income countries, S. sonnei dominates in wealthier countries where the bacterium is largely transmitted from person to person (4). Infection with S. sonnei has become a particular concern because of the emergence of antimicrobial-resistant strains (5, 6). Currently, there are no licensed vaccines against any Shigella spp.

All species of Shigella arose from Escherichia coli following the acquisition of an ~210 kb plasmid, pINV (7), which encodes a type III secretion system (T3SS) on a 30 kb pathogenicity island (PAI) (8, 9). The T3SS delivers effector proteins, most of which are plasmid encoded, into host cells, leading to bacterial invasion and manipulation of host cell responses (10–13). Seminal studies demonstrated the contribution of pINV to Shigella virulence by showing that S. sonnei colonies, which lack pINV, cannot invade epithelial cells, a key step in Shigella virulence (14–16).

Avirulent S. sonnei cells arise at high frequency in the laboratory mostly due to pINV loss (14–17), posing a major obstacle for studying and developing vaccines against this species (18). For example, a significant proportion of strains in genome sequencing studies lack pINV (19), hampering phylogenetic analyses of plasmid-encoded virulence genes. In addition, pINV loss can confound studies of host-pathogen interactions and the development of animal models of infection (18). Furthermore, live attenuated and whole-cell S. sonnei vaccines can be noninvasive or lack pINV-encoded antigens if bacteria become avirulent (18, 20).

Here, we defined the molecular mechanisms responsible for the emergence of avirulence in S. sonnei. Consistent with previous work, we showed that loss of the S. sonnei virulence plasmid (pINVsonn) is the major event that results in avirulent S. sonnei (14, 15, 17). Previously, toxin-antitoxin (TA) systems were shown to be important for the maintenance of the S. flexneri virulence plasmid (pINVflex) by postsegregational killing (PSK) (17, 21). During PSK, if a daughter cell fails to inherit a plasmid, there is no de novo production of the antitoxin, which is preferentially degraded by cellular protease, allowing unopposed action of the toxin and resulting in growth arrest and/or death (22–24). In contrast to pINVflex, which has three functional TA systems (VapBC, GmvAT, and CcdAB) (17), we demonstrated that pINVsonn possessed two functional TA systems, RelBE and VapBC, which have the potential to promote pINVsonn maintenance through PSK. Our previous analysis revealed a 4.9 kb deletion that entirely encompasses gmvAT and a separate deletion that left a 23 bp fragment of ccdA, leading to the absence of both these TA systems from pINVsonn compared with pINVflex (17). Although introducing ccdA and gmvAT into pINVsonn at sites corresponding to their location in pINVflex significantly reduced the emergence of avirulence, we found that pINV loss remained the dominant genetic event leading to avirulence. Furthermore, we showed that RelBE from pINVsonn, despite being functional, had no discernible effect on pINVsonn maintenance during growth in the laboratory. In contrast, although it was not possible to delete vapBC presumably due to its essential role in plasmid maintenance, we demonstrated that single amino acid substitutions in VapBC also contributed to pINV loss.

Although we were unable to identify the mechanism(s) of how these polymorphisms contributed to plasmid maintenance, we showed that modification of this TA system can stabilize pINVsonn. Finally, extended deletions encompassing T3SS-associated genes could also lead to avirulence in S. sonnei. These intramolecular events resulted from recombination between homologous insertion sequences (ISs), which are abundant in Shigella (25). Intriguingly, IS-mediated deletions have been also detected in S. flexneri pINVflex (26) but were different from those observed in pINVsonn due to the distinct profile of ISs and TA systems on these plasmids. Understanding the mechanisms of plasmid maintenance and loss in S. sonnei should enable research on the biology of
this important pathogen and be exploited for the development and evaluation of vaccines.

RESULTS

The absence of gmvAT and ccdAB from plINV{\text{SSon}} contributes to plasmid loss. Spontaneous avirulence occurs more frequently in {\text{S. sonnei}} than {\text{S. flexneri}} due to plINV loss (14, 15, 17, 26, 27). plINV{\text{SSon}} carries three functional plasmid maintenance TA systems, vapBC, gmvAT, and ccdAB, while gmvAT and ccdAB have been lost from plINV{\text{SSon}} through ancestral deletions (17). To determine whether the absence of ccdAB and gmvAT from plINV{\text{SSon}} is responsible for plINV loss, we analyzed a strain, ccdAB\textsuperscript{1}/gmvAT\textsuperscript{1}, with ccdAB and gmvAT and their promoters introduced into plINV{\text{SSon}} at sites corresponding to their positions in {\text{S. flexneri}} (17). The emergence of avirulent bacteria in ccdAB\textsuperscript{1}/gmvAT\textsuperscript{1} and wild-type (WT) {\text{S. sonnei}} was assessed in Congo red (CR) binding assays. Avirulent {\text{Shigella}} that lost T3SS expression do not bind CR when grown on CR-containing media and appear white (CR\textsuperscript{2}) while virulent T3SS-expressing bacteria bind CR and appear red (CR\textsuperscript{1}) (28–30). Therefore, we measured the emergence of avirulence as the emergence of CR\textsuperscript{2} colonies and then characterized these colonies by multiplex PCR. To prevent plINV{\text{SSon}} loss before the start of experiments, we introduced a chloramphenicol resistance cassette (\text{cat}) into plINV in the WT strain and ccdAB\textsuperscript{1}/gmvAT\textsuperscript{1} downstream of vapC. Bacteria were initially grown in the presence of the antibiotic then allowed to replicate in the absence of chloramphenicol for \textasciitilde25 generations by growth for 16 h at 37°C to allow for plINV loss during cell division. The emergence of avirulent colonies (CR\textsuperscript{2}) was then quantified by plating bacteria to media containing CR.

Consistent with previous work (17), the introduction of gmvAT and ccdAB into plINV{\text{SSon}} significantly reduced the emergence of avirulent CR\textsuperscript{2} colonies, by almost two orders of magnitude compared with the WT strain (\textit{P} = 0.0003 for \text{S. sonnei} \text{+/−} ccdAB/gmvAT) (Fig. 1A) (17). To determine the molecular events leading to avirulence, we used multiplex PCR to examine CR\textsuperscript{2} bacteria for the presence of (i) \text{virF}, which is present on plINV but not in the T3SS PAI and encodes a transcriptional regulator of T3SS genes, (ii) \text{virB}, which is in the PAI and encodes a transcriptional regulator, (iii) ori, the plINV origin of replication which was used as a proxy for the presence of plINV (26, 31), and (iv) hns as a chromosomal control (Fig. 1B and C). plINV loss was the dominant cause of avirulence in the WT strain, accounting for over 99% of CR\textsuperscript{2} bacteria (Fig. 1B).

FIG 1 CR\textsuperscript{2} colonies of \text{S. sonnei} mostly result from loss of plINV. (A) The percentage of CR\textsuperscript{2} colonies emerging from wild-type \text{S. sonnei} S33G (WT) and a strain with ccdAB and gmvAT introduced into plINV (ccdAB\textsuperscript{+}/gmvAT\textsuperscript{+}) after growth at 37°C for \textasciitilde25 generations. Solid line: mean (\textit{n} = 9 biological replicates). ***, \textit{P} \leq 0.001 by parametric Welch’s \textit{t}-test. (B and C) The number of CR\textsuperscript{2} colonies possessing plINV but lacking specified plasmid genes were assayed by multiplex PCR and shown as a percentage of all colonies. Loss of the entire plasmid is inferred by loss of the origin of replication (B). “Other” refers to CR\textsuperscript{2} colonies that contain \text{virB}, \text{virF} and the origin of replication (C). The value of each category is indicated inside the corresponding bar portion. Eight independent CR\textsuperscript{2} colonies were obtained from each of nine biological replicates (i.e., 72 colonies were analyzed per strain). ***, \textit{P} \leq 0.001; ****, \textit{P} \leq 0.0001; n.s., not significant; one-sample \textit{t}-test (parametric comparison of mean test/WT strain to 1) and Wilcoxon signed-rank test (nonparametric comparison of the median of strain/WT to 1, per specific event). l.o.d., limit of detection.

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Only 0.3% of avirulent bacteria emerging from wild-type S. sonnei retained pINV but had lost virB and/or virF (Fig. 1C). Introduction of ccdAB and gmvAT into pINV^Ssonn significantly reduced pINV loss, which dropped from 2.5% to 0.02% of all colonies in the absence or presence of these TA systems, respectively (P < 0.0001) (Fig. 1B) and increased “other” events (P = 0.0070) (Fig. 1C), which lead to avirulence but not through the loss of virB, virF, and ori. This demonstrated that the introduction of ccdAB and gmvAT reduced pINV loss overall, making less frequent events more easily detectable. However, pINV loss was still the main genetic event leading to avirulence in ccdAB^-/gmvAT^- and occurred in almost 50% of CR^- bacteria (Fig. 1B). Loss of virB and/or virF, were also detected at low frequency in both strains (Fig. 1C). Therefore, the ancestral loss of ccdAB and gmvAT from pINV^Ssonn does not fully explain the high rate of pINV loss in S. sonnei.

Polymorphisms in VapBC contribute to pINV loss in S. sonnei. To further understand the factors responsible for the high rate of pINV loss in S. sonnei, we examined the sequence of the plasmid for TA systems. pINV^Ssonn is predicted to encode a RelBE which is absent in pINV^Sflex and a VapBC TA system (32). The RelBE TA system is encoded near the origin of replication and a closely related system stabilizes p307 in E. coli (see Fig. S1A and B in the supplemental material) (33). Analysis of whole-genome sequences from a global collection of 132 S. sonnei isolates (19) showed that pINV had been lost from most of the isolates; however, RelBE was found on pINV from all 43 S. sonnei isolates that contained the pINV sequence (Table S1). Although RelBE is a functional TA system, we found that it does not contribute to pINV maintenance under laboratory conditions (Fig. S1C and D).

Therefore, we focused our attention on the VapBC TA system, which is sufficient and necessary to maintain pINV^Sflex and the only TA system found on pINV from both S. sonnei and S. flexneri (17). The VapC toxin cleaves the initiator trNA, trNA^Met, preventing initiation of translation, while the VapB antitoxin binds to and blocks the activity of VapC (34, 35). VapBC expression is autoregulated by conditional cooperativity through binding of heterocytomeric VapBC complex to two sites in the vapBC promoter, known as operator sites (vapO), resulting in transcriptional repression when VapB is in excess (34, 36). When pINV is lost following cell division, PSK occurs as VapB is readily degraded by Lon and cannot be replaced, leaving VapC free to cleave trNA^Met (35–37).

The promoter region of vapBC, including the vapO sites, was identical in S. flexneri and S. sonnei (Fig. S2A). Comparison of the predicted amino acid sequences of this TA system from S. flexneri M90T (accession number AL391753) and S. sonnei 53G (accession number NC_016833) revealed single amino acid differences in VapB and VapC (Fig. S2B and C). The polymorphic residue in VapB (T58A, S. flexneri versus S. sonnei) is located toward the C terminus of the antitoxin, which is involved in neutralizing VapC, while the polymorphic site in VapC (K32R, S. flexneri versus S. sonnei) is not located near its active site, in the self-dimerization interface, or its interface with VapB (Fig. S2B and C) (34). To determine if the vapBC sequences in S. flexneri M90T and S. sonnei 53G are representative of these species, we examined available S. flexneri and S. sonnei genome sequences for vapBC. The predicted amino acid sequence of VapBC in S. flexneri M90T and S. sonnei 53G were found in most isolates belonging to these species (83% and 78%, respectively) (Fig. 2B and C). Therefore, we subsequently refer to VapB T58 as VapB^K58, VapB A58 as VapB^K58, VapC K32 as VapC^K32, and VapC R32 as VapC^K32.

We attempted to delete vapBC from pINV^Ssonn. However, despite multiple attempts, we failed to obtain CR^- vapBC mutants, which is consistent with this TA system having a critical role in pINV^Ssonn maintenance. Therefore, we analyzed the effect of the VapBC substitutions on plasmid maintenance using the model vector, pSTAB (37). pSTAB contains the origin of replication from pINV along with a sacB-neo cassette that allows detection of the presence or absence of the plasmid (37). We constructed pSTAB derivatives containing vapBC from S. sonnei (generating pSTAB::VapBC^K58), or from S. flexneri (pSTAB::VapBC^K58), or containing chimeric vapBCs (i.e., pSTAB::VapB^K58/C^K32 or pSTAB::VapB^K58/C^K32). The plasmids were introduced into S. sonnei 53G lacking...
and plasmid loss was determined after ~25 generations by growth for 16 h at 37°C. While pSTAB with \(\text{vapBC}^{\text{fl}}\) was lost at a substantially lower rate than empty pSTAB (\(P = 0.0039\)) (Fig. 2A), the introduction of \(\text{vapBC}^{\text{sonn}}\) into pSTAB had a more limited effect (\(P = 0.0195\), pSTAB versus pSTAB::\(\text{vapBC}^{\text{sonn}}\)) (Fig. 2A). pSTAB containing \(\text{vapBC}^{\text{sonn}}\) exhibited significantly reduced plasmid loss compared with empty pSTAB, similar to pSTAB::\(\text{vapBC}^{\text{fl}}\) (\(P = 0.0039\), pSTAB versus pSTAB::\(\text{vapBC}^{\text{fl}}\)) (Fig. 2A). In contrast, \(\text{vapBC}^{\text{fl}}\cdot\text{vapBC}^{\text{sonn}}\) did not have a significant effect on pSTAB loss (\(P = 0.0742\), pSTAB versus pSTAB::\(\text{vapBC}^{\text{fl}}\cdot\text{vapBC}^{\text{sonn}}\)) (Fig. 2A). These results indicate that the amino acid polymorphism in VapC (K32R) affects the function of the VapBC TA system on plasmid maintenance in pSTAB.

Next, we investigated whether the polymorphisms in \(S. \text{sonnei}\) VapBC affect pINV loss. We generated strains of \(S. \text{sonnei}\) containing pINV with \(S. \text{sonnei}\) \(\text{vapBC}\) (\(S. \text{sonnei} \text{VapBC}^{\text{sonn}}\)), or with the locus replaced with \(\text{vapBC}\) from \(S. \text{flexneri}\) (\(S. \text{sonnei} \text{VapBC}^{\text{flex}}\)), or a chimeric \(\text{vapBC}\) (i.e., \(S. \text{sonnei} \text{VapB}^{\text{flex}}\cdot\text{VapC}^{\text{sonn}}\) and \(S. \text{sonnei} \text{VapB}^{\text{sonn}}\cdot\text{VapC}^{\text{flex}}\)).
chloramphenicol resistance cassette (cat) was inserted downstream of vapBC in all strains to ensure retention of pINV before the start of experiments. As before, we quantified the emergence of CR– colonies after ~25 generations by growth for 16 h at 37°C in the absence of chloramphenicol. There was a significantly higher number of CR– bacteria arising from S. sonnei VapBCSV
ex and S. sonnei VapBC
exC
ex compared with wild-type S. flexneri (P = 0.0039) (Fig. 2B). In contrast, there was no difference in the number of CR– bacteria emerging from S. sonnei VapBC
ex or S. sonnei VapBC
exC
ex compared with wild-type S. flexneri (P = 0.8203 and P = 0.4961) (Fig. 2B).

We also used multiplex PCR to investigate whether the single amino acid substitutions in VapBC altered the molecular events that lead to avirulence. Of note, the presence of vapC from S. flexneri led to a significant reduction in pINV loss as a cause of avirulence compared with S. sonnei VapC (S. sonnei VapBC
ex versus VapBC
ex, P = 0.0039; S. sonnei VapBC
ex versus VapBC
exC
ex, P = 0.0039) (Fig. 2C). In contrast, replacement of vapB from S. sonnei with vapB from S. flexneri had less effect on pINV loss (S. sonnei VapBC
ex versus VapB
exC
ex, P = 0.0391) (Fig. 2C). Therefore, the presence of S. flexneri vapC significantly decreased pINV loss, which was consistent with the results obtained with pSTAB. Furthermore, results demonstrated that, in addition to pINV loss, loss of virB and/or virF could also lead to avirulence in S. sonnei (Fig. 2D).

Because the genetic background can influence TA function (38), we also examined the effect of the VapBC polymorphisms in S. flexneri. We transferred derivatives of pINV
ex into S. flexneri lacking pINV by triparental mating. CR-binding assays with pINV
ex derivatives in S. flexneri demonstrated that the presence of vapC
ex significantly reduced the emergence of CR– colonies compared with vapC
ex (pINV
ex VapBCSV
ex versus pINV
ex VapBC
ex, P = 0.0005) (Fig. 3A). Furthermore, multiplex PCR revealed that loss of the pINV was not detected among 72 CR– colonies analyzed when vapBC
ex was present on pINV
ex in S. flexneri (S. flexneri with pINV
ex VapBCSV
ex versus pINV
ex VapBC
ex, P < 0.0001) (Fig. 3B and C).

Taken together, these results demonstrated that single amino acid substitutions in the VapBC TA system influenced the frequency of pINV loss in S. sonnei with the substitution in VapC (K32R) having a major effect. However, replacement of the VapBC in S. sonnei with vapBC from S. flexneri made pINV loss undetectable in S. flexneri, suggesting that the genetic background also influenced VapBC function in S. sonnei in addition to single amino acid substitutions.

The effect of single amino acid substitutions on VapBC structure and function.

Next, we investigated the mechanism(s) by which the amino acid substitutions in
VapBC affect its function. VapBC forms a hetero-octameric complex that binds the operator sites in the promoter sequence (vapO) when VapB is in excess (34, 36). To establish whether the amino acid differences affect the overall architecture of the VapBC complex, we set up crystallization trials in the presence of vapO-containing DNA. However, the crystals obtained contained protein only, enabling us to determine the atomic structure of VapBC from \textit{S. sonnei} to establish whether the amino acid differences affect the overall architecture of the VapBC complex (Fig. 4A; Table S2). Alignment of the \textit{S. sonnei} and \textit{S. flexneri} (PDB accession number 3TND) VapBC hetero-octamers demonstrated that they are highly similar (root mean square deviation of 0.460 Å over

**FIG 4** Analysis of the function of VapC polymorphisms. (A) Atomic structure of the \textit{S. sonnei} 53G VapBC hetero-octamer (top) and the VapB\textit{sonn} dimer in complex with a VapC\textit{sonn} dimer (bottom). VapC\textit{sonn} is in light gray, and VapB\textit{sonn} is dark gray. The four residues comprising the VapC active site (D7, E42, D98, and E119) are highlighted in red. The location of the polymorphisms in VapB (T58A) and VapC (K32R) are highlighted in blue and orange, respectively. (B) Viability of \textit{S. sonnei} 53G lacking pINV following expression of VapC\textit{sonn} or VapC\textit{flex} or a nonfunctional version of VapC\textit{flex} (VapC\textit{flex}D7A) from pBAD33. Empty pBAD33, control. (C) Northern blotting of VapC-mediated cleavage of tRNA\textsubscript{Met} in \textit{E. coli} MG1655 at times (indicated) following expression of VapC\textit{sonn} or VapC\textit{flex} from an inducible promoter. (D) Viability of \textit{S. sonnei} 53G lacking pINV following expression of VapC\textit{sonn} or VapC\textit{flex} from pBAD33 with VapB\textit{sonn} or VapB\textit{flex} under the expression of their promoters on pGM101. Error bars and standard deviation (SD).
all Cα atoms) (Fig. 4A and Table S2), indicating that the substitutions did not significantly alter the complex.

To determine the influence of the VapC K32R polymorphism on its toxicity, we expressed each version of VapC under the control of an arabinose-inducible promoter in S. sonnei lacking pINV. An inactive version of the toxin, VapC D7A, was included as a negative-control (Fig. 4B) (35). Expression of vapCSVflex (encoding VapC K32) or vapCSSFsonn (encoding VapC R32) led to a similar reduction in bacterial viability from 1 h postinduction onwards (P = 0.6867, one-way ANOVA, pBAD33::VapCSSFsonn versus pBAD33::VapCSVflex) (Fig. 4B). Because the VapC K32R polymorphism was neither in the catalytic site nor at its interface with VapB, it could be involved in VapC cleavage of tRNAfMet. Therefore, to determine the influence of the VapC K32R polymorphism on VapC activity, we measured the cleavage of tRNAfMet, the target of the toxin, using an in vivo cleavage assay as previously described (35). VapC from S. sonnei or S. flexneri was expressed under an arabinose-inducible promoter in E. coli, and the cleavage of tRNAfMet was followed by Northern blotting. There was no detectable difference in the cleavage of tRNAfMet by VapCSSFsonn and VapCSVflex (Fig. 4C).

We also investigated whether the VapC K32R substitution altered the capacity of either VapBCSVflex or VapCSVflex to act as an antitoxin, although this residue was not located at the TA interface. VapC was expressed under the control of an arabinose-inducible promoter in S. sonnei lacking pINV with either homologous or heterologous vapB coexpressed under the control of its native promoter. There was no significant difference in the ability of either VapB to neutralize either VapC (P = >0.9999, one-way ANOVA) (Fig. 4D).

In conclusion, we found that the polymorphisms in VapBC that influence pINV maintenance did not affect the structure of the VapBC complex, VapC toxicity, or the ability of VapB to act as an antitoxin.

**IS-mediated deletions in pINVSSFsonn lead to avirulence.** pINV loss is not the only event that can result in avirulence in S. sonnei. Multiplex PCR of avirulent bacteria emerging from S. sonnei strains showed that some CR− colonies retained pINV but lost virB and/or virF (Fig. 1C and 2D), which is similar to S. flexneri (26). Of note, there were marked differences in the frequency of these events in S. flexneri strains compared to S. sonnei strains (Fig. 2D). Loss of virB alone dominated in wild-type S. flexneri as previously shown (26) but was infrequently detected in S. sonnei (S. flexneri versus S. sonnei VapBCCSVflex and S. sonnei VapBSSFsonnCSVflex, P = 0.0078; S. flexneri versus S. sonnei VapBCSSFflex and S. sonnei VapBSSFsonnCSVflex, P = 0.0039) (Fig. 2D). In contrast, loss of virF only and loss of virF with virB were rarely detected in S. flexneri but were relatively common in S. sonnei (Fig. 1C, 2D, and 3C).

Because the emergence of avirulence in S. flexneri usually occurs following recombination between homologous copies of ISs (26), we investigated whether the loss of virB and/or virF in S. sonnei resulted from IS-mediated deletions. We examined pINVSSFsonn for pairs of ISs that occur in the same orientation and flank the T3SS PAI and/or virF. Using primers binding external to selected ISs, we amplified and sequenced potential IS-mediated deletions. This revealed that three pairs of ISs could contribute to avirulence in S. sonnei. Copies of IS21 (positions 29959 to 32101 and 123799 to 125930 [blue arc in Fig. 5A]) mediated deletion of a 93.7 kb fragment and resulted in the loss of the T3SS PAI and virF. Copies of IS1 (positions 73733 to 74500 and 146228 to 146996 [gray arc in Fig. 5A]) led to the loss of the T3SS PAI. Copies of IS1294 (positions 33932 to 35621 and 68896 to 70585 [purple arc in Fig. 5A]) were involved in the deletion of virF alone.

To examine whether the deletions leading to avirulence in S. sonnei were due to the presence of distinct ISs on pINV compared with S. flexneri, we compared the content of ISs in pINV from these two species. In S. flexneri M90T pINV (pWR100), ISs accounted for 53% of open reading frames, and ISSfl4, IS1294, and IS600 were responsible for most T3SS PAI deletions (Fig. 5B) (26, 39). pINVSSFflex harbored a total of 29 different ISs, consisting of 12 ISs types. IS1294 was the most abundant IS (present in six copies) with five copies of IS629 and four of IS600 on the plasmid, respectively. Only four
ISs were present in a single copy (Fig. 5C and D). In contrast, only ~23% of open reading frames in pINVSon were intact ISs with 26 ISs belonging to 13 different types. IS600 was the most abundant IS in pINVSon with five copies on the plasmid. The remaining ISs were present in up to three copies per plasmid, and seven were only present in a single copy (Fig. 5C and D). Therefore, there was a substantial difference in the repertoire and organization of ISs on the two plasmids with pINVStex harboring multiple copies of more ISs than pINVSon.

Taken together, these results demonstrated that the number of ISs and their location influenced the different recombination events leading to avirulence in S. sonnei and S. flexneri.

**DISCUSSION**

Here, we investigated the molecular mechanisms of pINV maintenance in S. sonnei and the reasons for the frequent emergence of avirulence (17). While three functional TA systems (VapBC, GmvAT, and CcdAB) are found on pINVStex (17, 21, 40, 41), pINVSon only has two TA systems, VapBC and RelBE, both of which are close to the origin of replication. While a RelBE homolog can maintain plasmids in E. coli (33, 42), this system does not contribute to the maintenance of pINVSon under the conditions tested (see Fig. S1 in the supplemental material). Aside from the repertoire of TA systems, we showed that polymorphisms in VapBC and the number, type, and organization of ISs on pINV determine plasmid loss and plasticity in S. sonnei.

We confirmed that ancestral deletions affecting two TA systems, GmvAT and CcdAB, increase the loss of pINVSon (17). However, even when GmvAT and CcdAB
were introduced into plNV<sub>sonn</sub>, ~50% of avirulent bacteria still resulted from plNV loss (Fig. 1B). VapBC (also referred to as MvpAT) is the only TA system present in plNV from both <i>S. flexneri</i> and <i>S. sonnei</i> and plays a critical role in maintaining plNV<sub>flex</sub> (17, 21, 40, 41). Given this, we hypothesized that differences in VapBC could also contribute to the high plNV loss in <i>S. sonnei</i>. We found that single amino acid polymorphisms in VapBC, particularly the substitution in VapC, have a marked effect on the function of this TA system in the model plasmid pSTAB and plNV.

The K<sup>R</sup> substitution in VapC could affect plNV loss by several mechanisms during PSK. Of note, this change in VapC is not in the active site of the toxin, which is required for tRNA<sub>fMet</sub> cleavage (35) or in the region that interacts with VapB or in the toxin dimerization region (Fig. 4A) (34, 36). To investigate how the VapC polymorphism influences plNV loss, we determined the atomic structure of the <i>S. sonnei</i> VapBC complex and found that the amino acid substitutions do not affect the overall structure of VapBC (Fig. 4A). Furthermore, there was no detectable change in the toxicity of VapC, its ability to cleave tRNA<sub>fMet</sub>, or its susceptibility to being neutralized by VapB (Fig. 4B to D). However, these assays might not be sufficiently sensitive to detect subtle yet important differences in VapC activity and/or VapB antitoxicity or interactions of VapBC with its operator DNA (Fig. S2A) (36). For PSK, VapC must be liberated from VapB. Therefore, it is also possible that the VapC K<sup>R</sup> polymorphism reduces its affinity for VapB, increasing levels of free toxin in bacteria and enhancing PSK. Alternatively, the change in VapC might affect the degradation of VapB by the cellular proteases. Further studies are underway to examine these possibilities and the impact of the other VapBC sequence variants we identified (Fig. S2B and C) on the activity of this TA system.

ISs are critical for the architecture and evolution of plasmids in <i>Shigella</i> (39, 43). Indeed, recombination between ISs is likely to have resulted in the acquisition of the T3SS PAI (43). The spontaneous emergence of avirulence in <i>S. flexneri</i> is largely mediated by deletions of the T3SS PAI following intramolecular recombination between homologous copies of ISs (26, 31, 44). We found that some avirulent <i>S. sonnei</i> also arise following deletions of PAI-associated genes, but these events involve different ISs than <i>S. flexneri</i> because plNV from these two species harbor distinct IS profiles (Fig. 5C and D). Additionally, deletions involving the T3SS and its regulators may go undetected in <i>S. sonnei</i> because of the high level of plNV loss (Fig. 2C). As ISs can act as the substrate for horizontal gene flow, differences in ISs in plNV<sub>sonn</sub> and plNV<sub>flex</sub> might also affect the repertoire of T3SS effectors in the two species.

TA systems can stabilize local sequences by preventing IS-mediated deletions (26). Our results suggested that GmvAT might reduce IS1294-mediated loss of <i>virF</i> in <i>S. flexneri</i> because this system is located 7.4 kb from <i>virF</i> on plNV<sub>flex</sub> within a region bounded by two copies of IS1294 (39). The absence of <i>gmvAT</i> from plNV<sub>sonn</sub> might explain our finding that avirulent strains lacking <i>virF</i> only were more common in <i>S. sonnei</i> than <i>S. flexneri</i> (Fig. 2D). This was further supported by the decrease in <i>virF</i> loss observed when we introduced <i>gmvAT</i> into its native site in plNV<sub>sonn</sub> (Fig. 1C).

<i>S. sonnei</i> is a highly successful pathogen despite having an unstable virulence plasmid when grown in the laboratory (14–16). plNV may be highly stable in <i>S. sonnei</i> in vivo, with environmental signals in the intestinal tract enhancing plNV maintenance. However, the emergence of avirulent bacteria does generate phenotypic heterogeneity, which might be beneficial for the survival of virulent and/or avirulent <i>S. sonnei</i> in certain circumstances. For example, rapidly replicating avirulent bacteria could act as an immune decoy in the intestinal tract or provoke local inflammatory responses and modify the local microenvironment in favor of virulent bacteria (45). Of note, plNV from <i>S. sonnei</i> harbors genes responsible for the biosynthesis of the O antigen which is incorporated into the lipopolysaccharide (LPS) and capsule of the bacterium (14, 15, 46, 47). Therefore, loss of plNV in <i>S. sonnei</i> also results in loss of the O antigen and capsule, which could enhance inflammatory responses to infection (47, 48).

Our findings could facilitate molecular studies of <i>S. sonnei</i> and aid the development of vaccines and animal/human challenge models (49). Vaccines based on live attenuated
S. sonnei strains can be adversely influenced by plINV loss (18, 49–52), which might affect other vaccine strategies, including those based on outer membrane vesicles by reducing levels of T3SS effectors and O antigen, which are both critical immunogens (20). However, stabilization of plINV in live vaccine candidates would require attenuation of virulence through additional genetic changes (49). Finally, detailed knowledge of plasmid dynamics in S. sonnei might offer novel approaches to target the plasmid maintenance systems to combat the threat posed by this important human pathogen.

**MATERIALS AND METHODS**

**Bacterial strains and growth.** Bacterial strains and plasmids used in this study are shown in Tables S3 and S4 in the supplemental material, respectively. Shigella spp. and E. coli were grown in tryptic soy broth (TSB; Sigma) and lysogeny broth (LB; Invitrogen), respectively, and 1.5% (wt/vol) agar (Oxoid) was added for solid media. Antibiotics were used at the following concentrations: carbenicillin, 100 μg/mL; chloramphenicol, 5 μg/mL (for S. flexneri) or 20 μg/mL (for S. sonnei); kanamycin, 50 μg/mL; streptomycin, 100 μg/mL. CR was added to TSB agar at a final concentration of 0.01% wt/vol (CR− TSA). For sucrose selection, 1% (wt/vol) bacto-tryptone (Sigma), 0.5% (wt/vol), yeast extract (Sigma), and agar were autoclaved in water, and sucrose (WVR, a final concentration of 10%, wt/vol) added before pouring plates.

**Construction of strains.** Lambda Red recombination was employed to construct mutants (53). Approximately 1 kb of sequence upstream and downstream of the gene interest was used to flank an antibiotic resistance cassette. Fragments were amplified by PCR (primers used in this study are shown in Table S5) then ligated into pUC19 (54) using NEBuilder HiFi master mix (New England Biolabs). The resulting plasmids were transformed into E. coli DH5α and used as the template to generate approximately 1 μg of linear DNA by PCR, which was transformed by electroporation into S. sonnei 53G expressing the recombinase from pKD46 (53). Bacteria were plated onto CR-TSA plates containing appropriate antibiotics and incubated overnight at 37°C. Strains were checked by PCR and sequencing. For construction of S. sonnei ccdAB+/gmvAT−, VapBCtrans/WT, VapBCtranscat, VapBflextrans, VapBsonntrans and S. flexneri, the cat cassette was introduced at nucleotide 204,038 in S. sonnei 53G plINV (available at https://www.ncbi.nlm.nih.gov/nuccore/NC_016833.1?report=genbank) and at site 191,776 bp in S. flexneri M90T (accession number AL391753), respectively (26). For S. sonnei ccdAB+/gmvAT−, ccdAB and gmvAT were introduced into plINVtrans at sites corresponding to their positions in S. flexneri (17).

**For the construction of pET28a-VapBCtrans, vapB and vapC were amplified from S. sonnei genomic DNA using the primers described in Table S5 and then inserted into pET28a digested with NdeI and Xhol using NEBuilder HiFi master mix.**

**Triparental mating.** To transfer plINVtrans into S. flexneri, tri-parental mating was performed using S. sonnei 53G with a cat cassette downstream of vapBC (conferring chloramphenicol resistance) as the donor strain, S. flexneri BS176 (streptomycin resistant) as the recipient, and E. coli containing pRK2013 (conferring kanamycin resistance) as the helper strain (55, 56). Strains were grown separately overnight at 37°C in 10 mL LB liquid medium with appropriate antibiotics. Cultures were resuspended in 10 mL PBS after washing and then mixed at a ratio of 1:1.5 of donor, helper, and recipient, respectively, in 100 μL. Bacteria were spotted onto LB agar without antibiotics and incubated overnight at 37°C. Bacteria were harvested from spots and resuspended in 1 mL TSB and plated onto CR-TSA containing chloramphenicol and streptomycin to select for transconjugants.

**sacB-neo assays.** S. sonnei strains possessing plINV with the sacB-neo cassette in mxhI or pSTAB derivatives (17, 37) were grown from frozen stocks at 37°C for 16 h on solid LB medium to reach ~25 generations. On three separate occasions, three colonies were resuspended in 100 μL PBS and serial dilutions plated on solid media with sucrose or with kanamycin only. PAI loss was calculated as the ratio of CFU on plates with sucrose and on plates without sucrose/sucrose with kanamycin and shown as a percentage.

**CR-binding assays.** Shigella spp. were grown at 37°C on CR-TSA plates containing chloramphenicol overnight to obtain single colonies. On three separate occasions, three independent CR− colonies were resuspended in a 5 mL TSB liquid medium and incubated at 37°C with shaking at 180 rpm for 16 h (~25 generations). Samples were diluted in PBS and plated onto CR-TSA and incubated overnight at 37°C before counting the number of CR− and CR+ colonies. The proportion of CR− colonies was quantified by dividing the number of emerging CR− colonies by the total number of colonies (CR+ and CR−) and expressed as a percentage. Colonies were assessed by visual inspection.

**Multiplex PCR.** Multiplex PCR was used to detect virF, virB, and ori (the plINV origin of replication). hns was included as a chromosomal control (26). Reactions included Taq polymerase (Sigma-Aldrich) with an annealing temperature of 51.2°C and an extension time of 1.5 min. For each strain, eight CR− colonies emerged from each biological repeat following the CR-binding assay were analyzed. For each strain, the percentage of all colonies showing a given gene loss was calculated by first quantifying the percentage of CR− colonies with a particular gene loss and then adjusting the result using the percentage of CR− colonies in the total bacterial population as described in the CR-binding assay.

**Toxicity, anti-toxicity, and VapC-meditated cleavage assays.** To assess the toxicity of VapC, the protein was expressed using pBAD33 (57) in S. sonnei 53G lacking plINV. Initially, bacteria were grown at 37°C with shaking at 180 rpm in LB liquid medium with 0.2% glucose (wt/vol) to repress toxin expression. At an optical density (OD) OD600 ~0.1 cultures were pelleted by centrifugation at 3000 × g then resuspended in LB liquid medium with 0.2% arabinose (wt/vol) to induce toxin expression and grown at 37°C with shaking at 180 rpm.
37°C with shaking at 180 rpm. Aliquots of cultures were taken at time points following induction then serially diluted in PBS and plated onto LB solid media containing 0.2% glucose (wt/vol) to measure bacterial viability.

To assess the ability of VapB to prevent VapC toxicity, VapB from S. flexneri or S. sonnei was expressed from their native promoter on pGM101 in S. sonnei 53G lacking pINV with or without pBAD33::vapC (17). Expression of VapC was induced as above in bacteria with or without a plasmid containing vapB, and viability was assessed by plating aliquots of cultures to solid media with 0.2% glucose (wt/vol).

To assess the ability of VapC to cleave tRNA^{met}, E. coli MG1655 containing pBAD33::vapC or pBAD33::VapBC were grown exponentially in LB liquid medium at 37°C with shaking at 180 rpm. At an OD_{600} of ~0.4, toxin expression was induced by the addition of 0.2% arabinose (wt/vol). Samples (1 mL) were collected before (0 min) and 2, 4, and 6 min after addition of arabinose then immediately mixed with 125 µL of 5% phenol in ethanol on ice to prevent further RNA degradation. Samples were harvested by centrifugation at 3000 × g for 5 min at 4°C, and RNA was extracted using the hotphenol method as previously (58). Total RNA (2.5 µg) was denatured in formamide and separated on a denaturing 8% polyacrylamide gel (19:1) containing 8 M urea buffered in TBE (19). The RNA was transferred to a Zeta-Probe membrane (Bio-Rad) by electroblothing. Membranes were prehybridized in hybridization buffer (0.9 M NaCl, 0.05 M NaH₂PO₄, 0.05 M EDTA, 5% SDS) at 30°C overnight. Hybridization was continued overnight at 42°C. To reduce nonspecific hybridization, membranes were washed in 2× SSC (0.3 M NaCl, 0.03 M Na₃C₆H₅O₇) at 50°C followed by 0.1% SDS at room temperature. Cleavage of tRNA^{met} was visualized by phosphorimaging.

**Protein purification and crystallography.** pET28a-VapBC (19) was transformed into E. coli C41. Following growth at 37°C to an OD_{600} of ~0.8, expression was induced with 1 mM IPTG and cultures incubated for a further 3 h before harvesting by centrifugation at 5000 × g for 10 min. VapBC was purified as described previously (34). In brief, 150 mM VapBC supernatant octamer was combined with double-stranded DNA (5′-CTTCCGGTTATGAGCCCGACGAGCTA) with 30 µl CIP (5′)-ATP using 1 µl T4 polynucleotide kinase (Thermo Fisher Scientific) in a total volume of 20 µL according to manufacturer’s instructions. Hybridization was continued overnight at 42°C. To reduce nonspecific hybridization, membranes were washed in 2× SSC (0.3 M NaCl, 0.03 M Na₃C₆H₅O₇) with 0.1% SDS at room temperature. Cleavage of tRNA^{met} was visualized by phosphorimaging.

**Bioinformatics.** Schematics of the position of RelBE or IS pairs on pINV from Shigella spp. were created from the sequence of S. flexneri M90T pINV (pWR100, accession number AL391753.1), and S. sonnei 53G pINV (accession number NC_016833.1). To determine the presence/absence of RelBE in a collection of S. sonnei from Holt et al. 2012 BLAST (BLASTn) v2.9.0 was used (19). Alignment of RelBE was performed using BLASTn and BLASTp v2.9.0 and subsequently Clustal O of sequence from RelBE from p307 from E. coli (accession number M26308) and S. sonnei 53G pINV (accession number NC_016833.1). Polymorphisms in VapB and VapC were identified in available S. flexneri or S. sonnei plasmid sequences using BLASTp v2.9.0 and alignment with Clustal O (v1.2.4). Statistical analyses. All data were analyzed using GraphPad Prism (version 7.4). The following statistical analyses were used as indicated in the legends of figures or the text: t test, one-sample t test/ Wilcoxon signed-rank test, one-way ANOVA, and two-way ANOVA. For t test, data were first analyzed for normal distribution using the Shapiro-Wilk test, and, depending on the result, parametric (Welch’s t test) or nonparametric t test (Mann-Whitney test) were performed. For one-sample t test/Wilcoxon signed-rank test analysis, data were log-transformed and divided by the respective control strain. The Shapiro-Wilk test was then used to test for normal distribution. If data were normally distributed, a parametric one-sample t test was performed to compare the mean of data of each strain to 1 because 1 is the result of the ratio control/control or to the limit of the detection (LOD = 0.00001, i.e., 0.001%), which was equal to ~5 log LOD in the absence of the control strain. If the data were not normally distributed, Wilcoxon signed-rank test was performed, comparing the median of data of each strain to 1 or the LOD. For one-way ANOVA, Kruskal-Wallis, Dunn’s multiple-comparison test was performed. For two-way ANOVA, Sidak’s or Tukey’s multiple-comparison test was performed.

**Data availability.** Coordinates and structure factors have been deposited in the Protein Data Bank (accession number 6SD6).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.9 MB**
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We comply with the Expects Data Sharing Policy.

We declare no conflicts of interest.

REFERENCES


