Maintenance of the virulence plasmid in *Shigella flexneri* is influenced by Lon and two functional partitioning systems

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Summary

Members of the genus *Shigella* carry a large plasmid, pINV, which is essential for virulence. In *Shigella flexneri*, pINV harbours three toxin-antitoxin (TA) systems, CcdAB, GmvAT and VapBC that promote vertical transmission of the plasmid. Type II TA systems, such as those on pINV, consist of a toxic protein and protein antitoxin. Selective degradation of the antitoxin by proteases leads to the unopposed action of the toxin once genes encoding a TA system have been lost, such as following failure to inherit a plasmid harbouring a TA system. Here, we investigate the role of proteases in the function of the pINV TA systems and demonstrate that Lon, but not ClpP, is required for their activity during plasmid stability. This provides the first evidence that acetyltransferase family TA systems, such as GmvAT, can be regulated by Lon. Interestingly, *S. flexneri* pINV also harbours two putative partitioning systems, ParAB and StbAB. We show that both systems are functional for plasmid maintenance although their activity is masked by other systems on pINV. Using a model vector based on the pINV replicon, we observe temperature-dependent differences between the two partitioning systems that contribute to our understanding of the maintenance of virulence in *Shigella* species.

Introduction

Plasmids play a critical role in enabling bacteria to adapt to specific environments and stresses. Genes encoded on plasmids can confer resistance to antibiotics and other toxic compounds, while virulence plasmids in bacterial pathogens mediate invasion and survival within specific niches during the development of disease. For example, the four species of *Shigella* have emerged from commensal *Escherichia coli* following the acquisition of a large ~210 kb plasmid, pINV (Pupo *et al.*, 2000). pINV is critical for *Shigella* virulence as it carries a 30 kb pathogenicity island (PAI) that encodes a Type III Secretion System (T3SS), a molecular syringe that delivers effector proteins directly from the bacterium into human cells, mediating cell invasion (Sansonetti, 1991; Buchrieser *et al.*, 2000). In addition, pINV carries other virulence genes involved in adhesion and actin-mediated motility, such as *icsA* (Bernardini *et al.*, 1989; Brotcke Zumsteg *et al.*, 2014), as well as T3SS effector proteins encoded outside the PAI, including *Shigella* enterotoxin 2 (Farfan *et al.*, 2011) and the multicopy *ipaH* genes (reviewed by Ashida and Sasakawa, 2015).

Large virulence plasmids can impose a significant burden on the replication and fitness of their bacterial host, as in the case of *Shigella* (Sasakawa *et al.*, 1986). pINV is likely present in a single copy as suggested by its replicon (Buchrieser *et al.*, 2000) and next-generation sequencing read depth relative to the chromosome (unpublished data). During growth in the laboratory, plasmid instability can result in complete loss of pINV, internal plasmid deletions and chromosomal integration of pINV, leading to absent or reduced expression of the T3SS (Sasakawa *et al.*, 1986; McVicker and Tang, 2016; Pilla *et al.*, 2017). The plasmid has several mechanisms to ensure its retention in a growing bacterial population. These mechanisms include toxin-antitoxin (TA) modules that mediate post-segregational killing (PSK; Sayeed *et al.*, 2005; Winther and Gerdes, 2011; McVicker and Tang, 2016), as well as uncharacterised partitioning systems belonging to the ParAB and ParMR families (Buchrieser *et al.*, 2000).

TA systems employ a stable, toxic protein that interferes with a key aspect of bacterial viability, such as translation or DNA replication (Bernard and Couturier, 1992; Winther...
Plasmid partitioning systems are closely related to chromosomal partitioning systems and function by promoting the physical separation of newly replicated plasmids into the two daughter cells during replication. Partitioning occurs either by separating plasmid DNA bound by partitioning proteins along a localised concentration gradient (type I, ParAB systems), or by the assembly of dynamic, actin-like filaments that push each plasmid away from the mid-cell (type II, ParMR systems) (mechanisms reviewed by Ebersbach and Gerdes, 2005). Interestingly, VirB, which regulates genes both on the Shigella flexneri PAI (Watanabe et al., 1990) and elsewhere on the virulence plasmid (Wing et al., 2004; Le Gall et al., 2005; Weatherspoon-Griffin et al., 2018), has significant homology to proteins involved in partitioning (i.e. ParB and SopB encoded by plasmids P1 and F respectively) (Watanabe et al., 1990). Therefore, partitioning systems on virulence plasmids may have functions aside from their canonical role in plasmid segregation. S. flexneri pINV is predicted to encode two partitioning systems, ParAB and StbAB (Buchrieser et al., 2000), which are type I and type II partitioning systems respectively. Shigella ParAB shares 65.7% sequence identity with the well-characterised type I partitioning system on the E. coli P1 plasmid (Lobocka et al., 2004). StbAB is more distantly related to the ParMR system of the R1 plasmid (32.8% average amino acid identity to ParMR), yet retains a conserved aspartic acid at position 173 that is necessary for the ATPase activity and function of ParM (Jensen and Gerdes, 1997). To date, the activity of these partitioning systems on pINV has not been investigated, although the function of S. flexneri ParAB has been confirmed in E. coli using a mini-P1 vector (Sergueev et al., 2005).

Of the four Shigella species that cause bacillary dysentery, S. flexneri is the most prevalent, accounting for around 80% of all cases worldwide (Kotloff et al., 1999). S. flexneri pINV encodes three functional TA systems, MvpAT, CcdAB and GmvAT. MvpAT is a member of the VapBC family and contributes to plasmid maintenance at temperatures found in the environment and the human intestine (Sayeed et al., 2005; McVicker and Tang, 2016). Given the extensive studies in other bacteria on this and related TA systems (reviewed by Arcus et al., 2011), here we refer to MvpAT as VapBC, in which VapB is the antitoxin, and VapC is a toxic ribonuclease. CcdAB employs a DNA gyrase inhibitor, CcdB, as the toxin with CcdA acting as its antidote (Bahassi et al., 1999); this TA system does not appear to play a major role in plasmid stability in S. flexneri (McVicker and Tang, 2016). The third TA system, GmvAT, utilises an acetyltransferase toxin that prevents translation, and contributes significantly to pINV stability at 21°C (McVicker and Tang, 2016). Acetyltransferase TA systems are becoming more widely characterised (Jurenas et al., 2017; Qian et al., 2018; Rycroft et al., 2018; Wilcox et al., 2018). Interactions between TA and partitioning systems are poorly defined and the subject of speculation. For example, it has been proposed that S. flexneri VapBC and P1 ParAB act together to optimise plasmid stability whilst alleviating the growth deficit incurred by PSK (Brendler et al., 2004).

Here, we undertook a systematic analysis of TA and partitioning systems on S. flexneri pINV. We demonstrate that the TA systems responsible for pINV stability in S. flexneri are governed by the activity of Lon protease, while ClpP has no role in the function of these systems. To elucidate the contribution of individual PSK and partitioning systems to plasmid stability, we constructed a model vector harbouring the replicon from pINV so we could study the role of each TA module and partitioning system in isolation. Our results demonstrate that the partitioning systems ParAB and StbAB do not have a major impact on pINV stability in S. flexneri, irrespective of the presence of the TA systems, even though our model vector shows that both ParAB and StbAB are functional and influenced by the ambient temperature. Our findings further current understanding of the maintenance of virulence in important pathogens such as Shigella.

Results

pINV TA systems are dependent on the Lon protease for plasmid stability

Cellular proteases, such as Lon and ClpP, specifically degrade antitoxins from type II TA systems to effect PSK or bacterial entry into a persistent state (reviewed by Muthuramalingam et al., 2016). Therefore, we investigated the contribution of the Lon and ClpP proteases to pINV stability in S. flexneri using a dual marker system
as previously described (McVicker and Tang, 2016). Introduction of a sacB-neoR cassette into mxiH enables selection and enumeration of bacteria which have lost the T3SS PAI (through their ability to grow on media containing sucrose) and bacteria that retain this region (by their resistance against kanamycin); this allows quantification of the loss of the T3SS PAI with an error rate of <1% (McVicker and Tang, 2016). We deleted the chromosomal copies of lon or clpP in S. flexneri harbouring mxiH::sacB::neoR, and examined the stability of pINV during approximately 25 generations of growth. We found that pINV stability decreased by over two orders of magnitude at 37°C and at 21°C in a lon mutant compared to the parental strain (Fig. 1, p < 0.0001). In contrast, deletion of the gene encoding ClpP, which has been implicated in TA function previously (Lehnher and Yarmolinsky, 1995; Aizenman et al., 1996; Donegan et al., 2010), did not significantly alter the stability of pINV (Fig. 1). We measured plasmid loss over a fixed number of generations (cell doublings) to take into account any differential growth rate of the mutants.

Studies of the fate of Salmonella enterica VapB in E. coli indicate that this antitoxin is degraded by the Lon protease (Winther and Gerdes, 2012). Therefore, deletion of lon in S. flexneri should abrogate the contribution of VapBC to pINV stability. Consistent with this, in every pINV derivative containing VapBC at 37°C, plasmid stability was significantly reduced when lon was deleted (Fig. 2A, p < 0.0001). Furthermore, in the absence of VapBC, deletion of lon had no effect on pINV stability at 37°C (Fig. 2A, p = 0.5788), indicating that the effect of Lon at this temperature is due to its influence on VapBC.

VapBC is dispensable for pINV stability at 21°C (McVicker and Tang, 2016). However, deletion of lon also significantly reduced plasmid stability at this temperature, irrespective of the presence of VapBC (Fig. 2C, p < 0.0001), indicating that Lon acts through a different TA system at this temperature. Since GmvAT is sufficient to stabilise pINV at temperatures found outside the human host (McVicker and Tang, 2016), we next tested the impact of Lon on this TA system. We found that the stability of pINV containing gmvAT as the sole active TA system was reduced by the deletion of lon at 21°C (Fig. 2C, p < 0.0001), the temperature at which GmvAT functions, but not at 37°C (Fig. 2A, p = 0.6545). Crucially, plasmid stability in a lon-negative background was not significantly affected by the removal of all three TA systems (VapBC, GmvAT and CcdAB) at either temperature (Fig. 2A and C, p < 0.0001). Deletion of clpP did not affect pINV stability at 37 or 21°C (Fig. 2B and D, p > 0.23). Taken together, these results demonstrate that Lon affects the activity of both VapBC and GmvAT in S. flexneri; this is the first example of Lon affecting a TA system, such as GmvAT, containing a toxic acetyltransferase.

The ParAB partitioning system contributes to pINV maintenance

Shigella flexneri pINV contains two potential partitioning systems, ParAB and StbAB. stbAB is predicted to encode an uncharacterised ParMR-related partitioning system (42.5% identity to ParM and 23.1% identity to ParR of the E. coli R1 plasmid, Supplementary Fig. 1; Gerdes and Molin, 1986). Nothing is known about the contribution of StbAB to pINV stability. Therefore, we assessed the impact of StbAB on plasmid stability by deleting stbAB from S. flexneri pINV. There was no difference in the stability of the plasmid with or without StbAB at 37 or 21°C, measured using the sacB-neoR assay (Fig. 3, p > 0.63). As there could be functional redundancy between StbAB and ParAB, we constructed a double parAB/stbAB mutant and examined its effect on plasmid stability at 37 and 21°C relative to the single mutants (Fig. 3). Removal of parAB or both partitioning systems did not significantly destabilise the plasmid irrespective of temperature (p > 0.74). However, at 21°C, pINV was significantly more stable (p = 0.0001) in the parAB mutant compared with the wild-type strain, and removal of stbAB in the parAB mutant restored wild-type levels of stability (Fig. 3B). Additionally, the T3SS regulator VirB has homology with the partitioning protein, ParB (Watanabe et al., 1990). Therefore, we analysed the effect of deleting virB in the presence and absence of the other putative partitioning systems at 37°C; the absence of VirB does not significantly decrease pINV stability under these conditions (Fig. 4, p < 0.85). Deletion of parAB does, however,
increase stability in the strain lacking VirB (Fig. 4, \( p = 0.0228 \)), consistent with its effect at 21°C (Fig. 3, discussed above), given that \( \text{virB} \) is not expressed at this temperature (Tobe et al., 1995).

It is possible that the effect of partitioning systems on plasmid maintenance is masked by the presence of the TA systems on \( \text{pINV} \), which could eliminate bacteria following defective plasmid segregation through PSK. Therefore, we also deleted \( \text{stbAB} \) and \( \text{parAB} \) from a plasmid lacking the TA systems, VapBC, CcdAB and GmvAT. Again, \( \text{stbAB} \) had no effect on plasmid stability even in the absence of these TA systems (Fig. 5, \( p > 0.74 \)). In contrast, while deletion of \( \text{parAB} \) did not reduce \( \text{pINV} \) stability in the presence of the TA systems, the stabilising effect of this partitioning system at 37 and 21°C became evident in the absence of the TA systems (Fig. 5, \( p \leq 0.0001 \)).

Taken together, these results indicate that ParAB is a functional partitioning system that operates at 37 and 21°C and that StbAB contributes to plasmid stability at 21°C in the absence of ParAB. However, we could not examine the contribution of \( \text{stbAB} \) in the absence of \( \text{parAB} \) and the three TA systems together, as \( \text{pINV} \) became increasingly unstable with each deletion, and we were not able to generate the necessary strain despite multiple attempts.

**Construction of a model vector to assess factors contributing to plasmid stability**

The impact of partitioning and PSK mechanisms on a large element such as \( \text{pINV} \) can be difficult to define given the multiplicity of maintenance systems, the likelihood of redundancies and interdependencies, and the presence of insertion sequences which allow for chromosomal integration (Pilla et al., 2017). Therefore, we generated a vector, pSTAB, to enable analysis of the effect of individual systems in isolation. The replicon of \( \text{Shigella} \ \text{pINV} \) is a \( \text{RepFIIA} \)-like element that is sufficient for plasmid propagation and incompatibility (Silva et al., 1988). pSTAB (Fig. 6A) contains the \( \text{S. flexneri} \ \text{pINV} \) replicon (nucleotides 202,317–204,916 of \( \text{pWR100} \); Buchrieser et al., 2000) with \( \text{sacB-neo} \) to allow positive selection for the presence or absence of the plasmid. When subjected to plasmid loss assays in \( \text{S. flexneri} \), the rate of pSTAB loss was similar to the rate of instability of \( \text{pINV} \) lacking the
three TA systems (Fig. 6B and C). Furthermore, we tested colonies at random from our loss assays with pSTAB and examined them by PCR for the presence of the replicon as a marker for plasmid loss; results demonstrate that selection on sucrose accurately measures the number of plasmid-free cells (data not shown).

To investigate the contribution each TA system has in plasmid maintenance, we inserted ccdAB, gmvAT and vapBC individually into pSTAB and monitored plasmid retention in S. flexneri lacking pINV (Fig. 6B and C). In comparison to pSTAB without any inserted sequence, we observed that ccdAB slightly increased maintenance of pSTAB in S. flexneri at 37°C but not 21°C (Fig. 6B and C, \(p = 0.0409\) and \(p = 0.1822\) respectively). As expected, VapBC had a dramatic effect on pSTAB, increasing stability by approximately two or three orders of magnitude at 37 and 21°C respectively (Fig. 6B and C, \(p < 0.0001\)). Insertion of gmvAT into pSTAB increased plasmid maintenance at

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21°C by over two orders of magnitude in comparison to the empty vector ($p < 0.0001$). However, at 37°C, the effect of GmvAT on plasmid stability was not significant ($p = 0.0888$).

We also conducted these experiments in a strain lacking the Lon protease, which would be expected to reduce the effectiveness of the TA systems. As predicted, the $\Delta$lon mutation completely abrogated the stabilising effect of the TA systems at both temperatures (Fig. 6B and C, $p > 0.31$) except for GmvAT, which still retained some, albeit greatly reduced, functionality in the $\Delta$lon strain at 21°C alone (Fig. 6C, $p = 0.0043$). Taken together, these results confirm that CcdAB, GmvAT and VapBC found on pINV in S. flexneri are functional TA systems, and that GmvAT is most effective at temperatures found outside the human host, in agreement with our previous work (McVicker and Tang, 2016).

Furthermore, the TA systems enhance the maintenance of pSTAB under the conditions tested, respond as expected to the absence of Lon and hence validate the use of pSTAB as a tool to measure plasmid stability.

**ParAB and StbAB are functional and influenced by temperature**

We next examined whether the partitioning systems parAB and stbAB promote the stability of pSTAB by introducing these constructs into S. flexneri lacking pINV (Fig. 7). StbA contains an aspartic acid residue at position 173 that is conserved in ParM at position 170 (Supplementary Fig. 1) and is essential for ATPase activity; ParM is rendered non-functional by D170E substitution (Jensen and Gerdes, 1997), therefore an inactive form of stbAB containing the equivalent D170E mutation was included as a control. At 37°C, results demonstrated that both parAB and stbAB stabilise pSTAB by approximately one order of magnitude over 25 generations of growth (Fig. 7A, $p < 0.0001$), whereas the inactive form of stbAB did not contribute to plasmid stability (Fig. 7A; $p = 0.66$ when compared with empty pSTAB). A version of pSTAB containing stbAB and parAB in tandem was also significantly stable relative to the empty vector (Fig. 7A, $p < 0.0001$).

We also examined the influence of parAB and stbAB on the stability of pSTAB in S. flexneri pINV at 21°C (Fig. 7B). Consistent with S. flexneri pINV lacking the TA systems (McVicker and Tang, 2016), the stability of unmodified pSTAB was dependent upon temperature, with a significantly lower level of plasmid loss after 25 generations at 21°C than at 37°C (Fig. 7, $p < 0.0001$). This is not unprecedented, as at least one other T3SS-encoding virulence plasmid replicon is controlled by temperature...
Interestingly, we observed that there is a significant correlation between the size of sequence inserted into pSTAB and plasmid loss (Supplementary Fig. 2, $r^2 = 0.9787$, $p = 0.0107$). The only operon insertion that does not fit this pattern is the wild-type version of stbAB, indicating that this system contributes to plasmid segregation at this temperature. The nonfunctional stbAB D173E allele serves as an effective size-matched control for this comparison.

Taken together, these results indicate that S. flexneri StbAB and ParAB are both active partitioning systems on pINV and that ambient temperature affects the function of pINV’s partitioning systems, similar to its TA systems (McVicker and Tang, 2016) and T3SS (Tobe et al., 1995).

**Discussion**

Large, low-copy plasmids encode important virulence determinants in many enteric pathogens, including the four species of *Shigella* (reviewed by Pilla and Tang, 2018). Whilst some plasmids are capable of moving horizontally from strain to strain, large, low-copy virulence plasmids must also encode systems to ensure their vertical transmission from parent to daughter cells upon division, otherwise important traits would be lost.

The *Shigella* invasion plasmid, pINV, carries several functional TA systems and putative partitioning systems. In *S. flexneri*, two type II TA systems, VapBC and GmvAT, are required to stabilise the plasmid across the distinct temperatures experienced by this important human pathogen, which has to survive in the external environment as well as at the higher temperatures found within the human intestine (McVicker and Tang, 2016). Type II TA systems require the action of a specific protease to degrade the antitoxin (Muthuramalingam et al., 2016). Many TA systems in enteric bacteria are governed by activity of the Lon protease (van Melderen et al., 1994; Christensen and Gerdes, 2004; Hansen et al., 2012; Winther and Gerdes, 2012). Therefore, we examined the impact of Lon and another antitoxin-targeting protease, ClpP, on plasmid stability in *S. flexneri*. We found that deletion of Lon, but not ClpP, destabilised the virulence plasmid and abrogated the effect of mutations in the TA system loci, demonstrating that the action of the main *S. flexneri* pINV TA systems, VapBC and GmvAT, relies upon Lon (Figs 1 and 2). The impact of Lon on an acetyltransferase-based TA system such as GmvAT has not been shown previously.

Lon is a housekeeping protease that has many distinct biological roles in prokaryotes and eukaryotes (van Melderen and Aertsen, 2009). Its N-terminal domain is responsible for substrate recognition (Li et al., 2005) and carries out ATP-dependent unfolding and sequestration
of substrates, activities that are divorced from its proteolytic activity (van Melderen and Gottesman, 1999). Of relevance, Lon substrates can be protected against degradation by binding to their ligands, as is the case for TA antitoxins like CcdA (van Melderen et al., 1996). Lon influences many virulence traits in bacteria, including quorum sensing (Bertani et al., 2007), motility, biofilm production (Claret and Hughes, 2000; Marr et al., 2007) and the activity of T3SSs (Jackson et al., 2004). Therefore, it is interesting that Lon is also necessary for enhanced stability of the Shigella invasion plasmid, with this housekeeping protease involved in the activity as well as retention of the T3SS.

Construction of plasmids lacking multiple maintenance systems is challenging as they become increasingly unstable, making it more difficult to perform each sequential genetic manipulation. Interpretation of the function of individual systems on a large plasmid is also complex as there are often multiple systems which might have redundant roles, and contain insertion sequences which can mediate localised deletions without plasmid loss (Pilla et al., 2017); furthermore, pINV can spontaneously integrate into the chromosome via insertion sequences, affecting expression of genes on the PAI (Pilla et al., 2017). To circumvent these problems, we constructed a test vector, pSTAB, which contains the origin of replication from pINV, as well as the same counter-selectable marker we used to assay pINV stability previously (McVicker and Tang, 2016); experiments with pSTAB allowed us to delineate the function of each individual maintenance system.

The impact of partitioning systems ParAB and StbAB on pINV stability has not previously been analysed. Deletion of either system did not affect the stability of pINV (Fig. 3), suggesting they are functionally redundant, except that StbAB seemed to have a subtle but significant stabilising effect at 21°C in the absence of ParAB. Deletion of the regulatory protein VirB, which shares homology with ParB (Watanabe et al., 1990), did not reduce pINV stability (Fig. 4).

To further evaluate the function of the partitioning systems required the use of pSTAB. We found that parAB and stbAB are capable of stabilising pSTAB by approximately one order of magnitude over 25 generations at 37°C (Fig. 7A), confirming ParAB function within a model system (Sergueev et al., 2005) and providing evidence that StbAB is active.

Similar to TA systems (McVicker and Tang, 2016), the stabilising effect of the partitioning systems is influenced by the ambient temperature. StbAB was most effective at 37°C and initially appeared dispensable at 21°C, while ParAB was functional at 37°C and actually destabilised the plasmid at 21°C (Fig. 7). The increased importance of these proteins at 37°C may reflect the higher growth rate, and hence the reliance on partitioning systems to ensure the rapid and faithful separation of plasmids into daughter cells. At lower temperatures, it is conceivable that cell division is slow enough to allow segregation, so partitioning systems are less important. The reason for the destabilising effect of ParAB at lower temperatures is not clear, although it is noteworthy that a non-functional version of stbAB allele (based on Jensen and Gerdes, 1997) was also detrimental to pSTAB retention at 21°C, consistent with the effect on plasmid instability at 21°C being due to the change in size of the plasmid. Indeed, the plasmid with the largest insertion (i.e. stbAB and parAB in tandem) was dramatically unstable at 21°C, with instability directly correlated with insert size (Supplementary Fig. 2, $r^2 = 0.9787$, $p = 0.0107$). This was not seen at 37°C, where instead, tandem insertion of the segregation systems stabilised pSTAB (Fig. 7A). Crucially, direct comparison of the effect of the functional and nonfunctional stbA alleles at 21°C indicates that StbAB is active at this temperature. This is consistent with the role of StbAB in pINV at 21°C requiring the absence of ParAB, as discussed above. Interestingly, an enteropathogenic E. coli virulence plasmid, pB171, also carries both type I and type II partitioning loci (Ebersbach and Gerdes, 2001), but these systems are adjacent and share a single regulatory region. In Shigella pINV, the two partitioning systems are encoded approximately 90 kb apart (Fig. 8) so there is unlikely to be direct crosstalk between ParAB and StbAB.

In this study, we provide evidence for the influence of global protease activity upon plasmid stability in S. flexneri and define further the maintenance systems encoded by pINV that operate at different temperatures (summarised with current knowledge in Fig. 8). We constructed a model vector using the pINV replicon that allows us to interrogate individual plasmid maintenance elements and have used it to confirm the temperature dependency of the TA systems, as shown previously (McVicker and Tang, 2016). Furthermore, we have demonstrated that while the partitioning systems StbAB and ParAB display redundancy, they are both functional at 37°C, with StbAB providing a degree of stabilisation at 21°C. This observation is important, since, like the TA system GmvAT that is solely functional at temperatures outside the human host, the stbAB operon is missing in Shigella sonnei (Supplementary Fig. 3). While the precise deletion has not been mapped, we could not detect stbAB in pINV of 132 sequenced S. sonnei isolates (Holt et al., 2012), providing further evidence that this species is adapted to retain pINV at temperatures found inside the human body (McVicker and Tang, 2016).
Plasmid stability mechanisms in Shigella flexneri

Experimental procedures

Strains and growth media

The bacteria and plasmids used in this study are shown in Supplementary Table 1. E. coli and Shigella were propagated in liquid Lysogeny broth (LB; Invitrogen, Waltham, MA), or on solid media containing 1.5% (w/v) agar (Oxoid, Basingstoke, UK). Antibiotics were used at the following concentrations: carbenicillin, 50 µg ml−1; chloramphenicol, 20 µg ml−1; kanamycin, 50 µg ml−1. For Shigella, Congo red (0.01% w/v, final concentration) was added to tryptic soy broth (Fluka, Buchs, Switzerland) for solid media. For selection on sucrose, 1% (w/v) tryptone (Fluka), 0.5% (w/v) yeast extract (Fluka) and agar as above were autoclaved in water, with sucrose (final concentration of 10% w/v) added prior to pouring plates.

Strain and plasmid construction

DNA manipulations were performed in E. coli DH5α and plasmids assembled using NEBuilder HiFi DNA Assembly master mix (New England Biolabs, Ipswich, MA). All primers (Supplementary Table 2) were purchased from Sigma. The sacB-neoR cassette was amplified from pIB279 (Blomfield et al., 1991). Electroporation was used to transform bacterial cells with plasmids or linear DNA. For insertion of mutations into the chromosome or pINV, λ Red recombination (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000) was performed using PCR products including ~1 kb of upstream and downstream flanking sequence. After λ Red recombination in Shigella, bacteriophage P1vir was used as previously described (McVicker and Tang, 2016) to transduce mutations into a clean genetic background to reduce the risk of off-site mutations created by the λ Red system. All mutations were verified by PCR and sequencing.

Virulence plasmid stability assays

Bacteria were grown on LB agar from frozen stocks, and incubated at different temperatures for ~25 generations (i.e. 25 doublings); after growth, whole colonies were re-suspended in PBS, diluted, then plated to measure the number of bacteria harbouring pSTAB or pINV mxiH::sacB-neoR, either on media containing kanamycin (pSTAB+ or PAI+, kanamycin resistant) or sucrose (pSTAB− or PAI−) to detect the presence/absence of the sacB-neoR cassette. The sum of these numbers was used to calculate the total number of CFUs to confirm the number of generations elapsed. Colonies were excluded from analysis if they had ~100% sucrose resistance (indicating a founder effect). Any difference in the growth rates of strains was accounted for by measuring the number of generations by assessing the number of CFU rather than using particular growth times.

Statistical and computational methods

Data were log-transformed (normally-distributed) and analysed using unpaired t-tests, linear regression, or by one-way or two-way ANOVA with appropriate multiple comparisons tests as indicated in figure legends. Statistical significance was assumed if p < 0.05.

Author contributions

GM, SH and GP performed experiments and analysed data. GM, SH, GP and CMT designed experiments, interpreted data and wrote the manuscript. CMT secured funding.

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Conflict of interest

The authors declare no conflicts of interest.

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References

Aizenman, E., Engelberg-Kulka, H. and Glaser, G. (1996) An Escherichia coli chromosomal “addiction module” regulated by guanosine [corrected] 3′,5′-bispyrophosphate: a model for programmed bacterial cell death. Proceedings of the National Academy of Sciences, 93(12), 6059–6063.

Arcus, V.L., Mckenzie, J.L., Robson, J. and Cook, G.M. (2011) The PIN-domain ribonucleases and the prokaryotic VapBC toxin-antitoxin array. Protein Engineering, Design & Selection, 24(1–2), 33–40.

Ashida, H. and Sasakiwa, C. (2015) Shigella IpaH family effectors as a versatile model for studying pathogenic bacteria. Frontiers in Cellular and Infection Microbiology, 5, 100.

Bahassi, E.M., O’Dea, M.H., Allali, N., Messens, J., Gellert, M. and Couturier, M. (1999) Interactions of CcdB with DNA Gyrase: inactivation of GyrA, poisoning of the gyrase-DNA complex, and the antidote action of CcdA. Journal of Biological Chemistry, 274(16), 10936–10944.

Bertani, P. and Couturier, M. (1992) Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. Journal of Molecular Biology, 226(3), 735–745.

Bernardini, M.L., Mounier, J., d’Hauteville, H., Coquis-Rondon, M. and Sansonetti, P.J. (1989) Identification of icsA, a plasmid locus of Shigella flexneri that governs bacterial intra- and intercellular spread through interaction with F-actin. Proceedings of the National Academy of Sciences, 86, 3867–3871.

Bertani, I., Rampioni, G., Leoni, L. and Venturi, V. (2007) The Pseudomonas putida Lon protease is involved in N-acetyl homoserine lactone quorum sensing regulation. BMC Microbiology, 7, 71.

Blomfield, I.C., Vaughan, V., Rest, R.F. and Eisenstein, B.I. (1991) Allelic exchange in Escherichia coli using the Bacillus subtilis sacB gene and a temperature-sensitive pSC101 replicon. Molecular Microbiology, 5(6), 1447–1457.

Brendler, T., Reaves, L. and Austin, S. (2004) Interplay between plasmid partition and postsegregational killing systems. Journal of Bacteriology, 186(8), 2504–2507.

Brotcke Zumsteg, A., Goossman, C., Brinkmann, V., Morona, R. and Zychlinsky, A. (2014) IcsA is a Shigella flexneri adhesin regulated by the type III secretion system and required for pathogenesis. Cell Host & Microbe, 15, 435–445.

Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., D’Hauteville, H., Kunst, F., et al. (2000) The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of Shigella flexneri. Molecular Microbiology, 38(4), 760–771.

Cherepanov, P.P. and Wackernagel, W. (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene, 158(1), 9–14.

Christensen, S.K. and Gerdes, K. (2004) Delayed-relaxed response explained by hyperactivation of RelE. Molecular Microbiology, 53(2), 587–597.

Claret, L. and Hughes, C. (2000) Rapid turnover of FliH and FliC, the flagellar regulon transcriptional activator proteins, during Proteus swarming. Journal of Bacteriology, 182(3), 833–836.

Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences, 97(12), 6640–6645.

Donegan, N.P., Thompson, E.T., Fu, Z. and Cheung, A.L. (2010) Proteolytic regulation of toxin-antitoxin systems by ClpPC in Staphylococcus aureus. Journal of Bacteriology, 192(5), 1416–1422.

Ebersbach, G. and Gerdes, K. (2001) The double par locus of virulence factor pb171: DNA segregation is correlated with oscillation of ParA. Proceedings of the National Academy of Sciences, 98(26), 15078–15083.

Ebersbach, G. and Gerdes, K. (2005) Plasmid segregation mechanisms. Annual Review of Genetics, 39, 453–479.

Farfan, M.J., Toro, C.S., Barry, E.M. and Nataro, J.P. (2011) Shigella enterotoxin-2 is a type III effector that participates in Shigella-induced interleukin 8 secretion by epithelial cells. FEMS Immunology and Medical Microbiology, 61, 332–339.

Le Gall, T., Mavris, M., Martino, M.C., Bernardini, M.L., Denamur, E. and Parsot, C. (2005) Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system of Shigella flexneri. Microbiology, 151, 951–962.

Gerdes, K. and Molin, S. (1986) Partitioning of plasmid R1. Structural and functional analysis of the parA locus. Journal of Molecular Biology, 190(3), 269–279.

Hansen, S., Vulic, M., Min, J., Yen, T.J., Schumacher, M.A., Brennan, R.G. and Lewis, K. (2012) Regulation of the Escherichia coli HipBA toxin-antitoxin system by proteolysis. PLoS ONE, 7(6), e39185.

Harms, A., Brodersen, D.E., Mitarai, N. and Gerdes, K. (2018) Toxins, targets, and triggers: an overview of toxin-antitoxin biology. Molecular Cell, 70(5), 768–784.

Holt, K.E., Baker, S., Weill, F.X., Holmes, E.C., Kitchen, A., Yu, J., et al. (2012) Shigella sonnei genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. Nature Genetics, 44(9), 1056–1059.

Jackson, M.W., Silva-Herzog, E. and Plano, G.V. (2004) The ATP-dependent ClpXP and Lon proteases regulate expression of the Yersinia pestis type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. Molecular Microbiology, 54(5), 1364–1378.

Jensen, R.B. and Gerdes, K. (1997) Partitioning of plasmid R1. The ParM protein exhibits ATPase activity and interacts with the centromere-like ParR-parC complex. Journal of Molecular Biology, 269(4), 505–513.

Jurenas, D., Chatterjee, S., Konijnenberg, A., Sobott, F., Droogmans, L., Garcia-Pino, A. and van Melder, L.
(2017) AtaT blocks translation initiation by N-acetylation of the initiator tRNA(fMet). *Nature Chemical Biology*, **13**, 640–646.

Kotloff, K.L., Winickoff, J.P., Ivanoff, B., Clemens, J.D., Swerdlow, D.L., Sansonetti, P.J., *et al.* (1999) Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization*, **77**(8), 651–666.

Lehnerr, H. and Yarmolinsky, M.B. (1995) Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. *Proceedings of the National Academy of Sciences*, **92**(8), 3274–3277.

Li, M., Rasulova, F., Melnikov, E.E., Rotanova, T.V., Gustchina, A., Maurizi, M.R. and Wodawer, A. (2005) Crystal structure of the N-terminal domain of *E. coli* Lon protease. *Protein Science*, **4**(11), 2895–2900.

Lobocka, M.B., Rose, D.J., Plunkett, G. 3rd, Rusin, M., Li, M., Rasulova, F., Melnikov, E.E., Rotanova, T.V., Liu, H., Nataf, F., Xie, Y., Carninci, P., Fujita, T., Gough, J., Billington, P., Davis, R.W., Grishin, N.V., Spellman, P.T., Weng, Z., Zhu, X., Eppig, J.T., Shahab, S., Morris, J.B., Zheng, L., Wang, L.S., Han, W., Sato, T., Takashima, S., Watanabe, H., Arakawa, E., Ito, K.I., Kato, J.I. and Nakamura, Y. (2013) The genome of *Salmonella typhimurium* strain LT2. *Nature*, **499**(7457), 149–155.

Marr, A.K., Overhage, J., Bains, M. and Hancock, R.E.W. (2001) Lon-dependent proteolysis of CcdA is the key control for quality control by Lon-dependent proteolysis. *Proceedings of the National Academy of Sciences*, **98**(17), 9304–9309.

McVicker, G. and Tang, C.M. (2016) Deletion of toxin–antitoxin systems in the evolution of *Shigella sonnei* as a host-adapted pathogen. *Nature Microbiology*, **1**(1), 6204–6204.

van Melderen, L. and Aertsen, A. (2009) Regulation and quality control by Lon-dependent proteolysis. *Research in Microbiology*, **160**(9), 645–651.

van Melderen, L. and Gottesman, S. (1999) Substrate sequestration by a proteolytically inactive Lon mutant. *Proceedings of the National Academy of Sciences*, **96**(11), 6064–6071.

van Melderen, L., Bernard, P. and Couturier, M. (1994) Lon-dependent proteolysis of CcdB is the key control for activation of CcdB in plasmid-free segregant bacteria. *Molecular Microbiology*, **11**(6), 1151–1157.

van Melderen, L., Thi, M.H.D., Vecchi, P., Gottesman, S., Couturier, M. and Maurizi, M.R. (1996) ATP-dependent degradation of CcdB by Lon protease. Effects of secondary structure and heterologous subunit interactions. *Journal of Biological Chemistry*, **271**(44), 27730–27738.

Muthuramalingam, M., White, J.C. and Bourne, C.R. (2016) Toxin-antitoxin modules are pliable switches activated by multiple protease pathways. *Toxins (Basel)*, **8**(7), pii:E214.

Pilla, G. and Tang, C.M. (2018) Going around in circles: virulence plasmids in enteric pathogens. *Nature Reviews Microbiology*, **16**(8), 484–495.

Pilla, G., McVicker, G. and Tang, C.M. (2017) Genetic plasticity of the *Shigella* virulence plasmid is mediated by intra- and inter-molecular events between insertion sequences. *PLoS Genetics*, **13**(9), e1007014.

Pupo, G.M., Lan, R. and Reeves, P.R. (2000) Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. *Proceedings of the National Academy of Sciences*, **97**(19), 10567–10572.

Qian, H., Yao, Q., Tai, C., Deng, Z., Gan, J. and Ou, H.Y. (2018) Identification and characterization of acetyltransferase-type toxin-antitoxin locus in *Klebsiella pneumoniae*. *Molecular Microbiology*, **108**(4), 336–349.

Rycroft, J.A., Gollan, B., Grabe, G.J., Hall, A., Cheverton, A.M., Larrouy-Maumus, G., *et al.* (2018) Activity of acetyltransferase toxins involved in *Salmonella* persistor formation during macrophage infection. *Nature Communications*, **9**(1), 1993.

Sansonetti, P.J. (1991) Genetic and molecular basis of epithelial cell invasion by *Shigella* species. *Reviews of Infectious Diseases*, **13**(Suppl 4), S285–S292.

Sasakawa, C., Kamata, K., Sakai, T., Murayama, S.Y., Makino, S. and Yoshikawa, M. (1986) Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infection and Immunity*, **51**(2), 470–475.

Sayeed, S., Brendler, T., Davis, M., Reaves, L. and Austin, S. (2005) Surprising dependence on postsegregational killing of host cells for maintenance of the large virulence plasmid of *Shigella flexneri*. *Journal of Bacteriology*, **187**(8), 2768–2773.

Sergueev, K., Dabrashynetskaya, A. and Austin, S. (2005) Plasmid partition system of the P1par family from the pWR100 virulence plasmid of *Shigella flexneri*. *Journal of Bacteriology*, **187**(10), 3369–3373.

Silva, R.M., Saadi, S. and Maas, W.K. (1988) Improved detection of virulence-associated plasmids of *Shigella* spp. and enteroinvasive *Escherichia coli* is homologous with a basic replicon in plasmids of IncF groups. *Infection and Immunity*, **56**(4), 836–842.

Tobe, T., Yoshikawa, M. and Sasakawa, C. (1995) Thermoregulation of virB transcription in *Shigella flexneri* by sensing of changes in local DNA superhelicity. *Journal of Bacteriology*, **177**(4), 1094–1097.

Wang, H., Avican, K., Fahlgren, A., Erttmann, S.F., Nuss, A.M., Dersch, P., *et al.* (2016) Increased plasmid copy number is essential for *Yersinia T3SS* function and virulence. *Science*, **353**(6298), 492–495.

Watanabe, H., Arakawa, E., Ito, K.I., Kato, J.I. and Nakamura, A. (1990) Genetic analysis of an invasion region by use of a Tn3-lac transposon and identification of a second positive regulator gene, *invE*, for cell invasion of *Shigella sonnei*: significant homology of *invE* with ParB of plasmid P1. *Journal of Bacteriology*, **172**(2), 619–629.

Weatherspoon-Griffin, N., Picker, M.A., Pew, K.L., Park, H.S., Ginete, D.R., Karney, M.M., *et al.* (2018) Insights into transcriptional silencing and anti-silencing in *Shigella flexneri*: a detailed molecular analysis of the icsP virulence locus. *Molecular Microbiology*, **108**, 505–518.

Wilcox, B., Osterman, I., Sererbyakovka, M., Lukyanov, D., Komarova, E., Gollan, B., *et al.* (2018) *Escherichia coli* IItA is a type II toxin that inhibits translation by acetylating isoleucyl-tRNA Ile. *Nucleic Acids Research*, **46**(15), 7873–7885. https://doi.org/10.1093/nar/gky560

Wing, H.J., Yan, A.W., Goldman, S.R. and Goldberg, M.B. (2004) Regulation of IcsP, the outer membrane protease of...
the *Shigella* actin tail assembly protein IcsA, by virulence plasmid regulators VirF and VirB. *Journal of Bacteriology*, **186**, 699–705.

Winther, K.S. and Gerdes, K. (2011) Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. *Proceedings of the National Academy of Sciences*, **108**(18), 7403–7407.

Winther, K.S. and Gerdes, K. (2012) Regulation of enteric *vapBC* transcription: induction by VapC toxin dimer-breaking. *Nucleic Acids Research*, **40**(10), 4347–4357.

Wozniak, R.A.F., Fouts, D.E., Spagnoletti, M., Colombo, M.M., Ceccarelli, D., Garriss, G., *et al.* (2009) Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. *PLoS Genetics*, **5**(12), e1000786.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.