Surprising Dependence on Postsegregational Killing of Host Cells for Maintenance of the Large Virulence Plasmid of *Shigella flexneri*

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Low-copy-number plasmids all encode multiple systems to ensure their propagation, including replication, partition (active segregation), and postsegregational killing (PSK) systems. PSK systems kill those rare cells that lose the plasmid due to replication or segregation errors. PSK systems should not be used as the principle means of maintaining the plasmid. The metabolic cost of killing the many cured cells that would arise from random plasmid segregation is far too high. Here we describe an interesting exception to this rule. Maintenance of the large virulence plasmid of *Shigella flexneri* is highly dependent on one of its PSK systems, *mvp*, at 37°C, the temperature experienced during pathogenesis. At 37°C, the plasmid is very unstable and *mvp* efficiently kills the resulting cured bacterial cells. This imposes a major growth disadvantage on the virulent bacterial population. The systems that normally ensure accurate plasmid replication and segregation are attenuated or overridden at 37°C. At 30°C, a temperature encountered by *Shigella* in the outside environment, the maintenance systems function normally and the plasmid is no longer dependent on *mvp*. We discuss why the virulent pathogen tolerates this self-destructive method of propagation at the temperature of infection.

*Shigella flexneri* is an etiologic agent of bacillary dysentery, a serious invasive disease of the human colonic mucosa. Virulence is dependent, in large part, on the products of a 230-kb virulence plasmid referred to by a number of names, including pMYSH6000 and pWR100, according to the particular isolate and form red colonies on Congo Red agar (the Crb correlated with the ability of the cells to bind the dye Congo Red pMYSH6000 and pWR100, according to the particular isolate virulence plasmid referred to by a number of names, including

**MATERIALS AND METHODS**

**Media, chemicals, and DNA manipulations.** Media, reagents, enzymes, buffers, and chemicals were as previously described (1). Cells were grown in Luria-Bertani (LB) broth supplemented when needed with kanamycin (12.5 µg/ml), chloramphenicol (25 µg/ml), or ampicillin (50 µg/ml). Strains were tested for Congo Red binding on tryptic soy broth (TSB) agar plates (1.5% agar) containing 0.025% Congo Red (Sigma Chemical Co., St. Louis, Mo.). Plasmid DNA was prepared using the Wizard Plus Miniprep/Maxiprep DNA purification system during cell division and also one or more PSK systems (10). PSK systems consist of genes for the production of a toxin and its specific antidote. Should a cell lose the plasmid, the antidote rapidly decays but the toxin persists and kills the cell or inhibits its growth. In this way, cured cells are effectively eliminated from the population, thus ensuring the continued maintenance of the plasmid (20). When the core plasmid maintenance systems, including the partition system, function efficiently, the PSK system should rarely come into play, as very few cured cells are produced (10). Thus, PSK systems can be regarded as back-up plasmid maintenance systems that deal with occasional errors. This makes sense, because frequent use of the system would impose a major disadvantage to the host due to frequent cell killing.

This functional relationship between PSK and the plasmid partition system has recently been demonstrated directly in *Escherichia coli* (3). A model plasmid was constructed with just a low-copy-number replicon and the PSK system *mvp*. It was accurately maintained, but at a cost to the host growth rate and viability. Its maintenance was highly dependent on *mvp* function. When a partition system (*P*tpur) was added, accurate maintenance was retained but the normal cell growth rate was restored. The plasmid was no longer dependent on *mvp* function for maintenance (3). Thus, plasmids with both PSK and partition systems are stably maintained and do not measurably disadvantage the host, because the PSK system rarely comes into play.

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overnight growth. The broth cultures were grown for 8 h at 37°C, diluted nies at 37°C, and the proportion of red to white colonies was determined after
were immediately plated on TSB-Congo Red plates (16) to give separate colo-
pALA136 or its
chloramphenicol resis-
tate, and the equivalent sites in the vector pGB2.

BamHI-SalI fragment of pALA2529 containing the
resistance cassette in place of the ampicillin resistance gene, as described else-
pALA136 was derived from pALA33 by introduction of a chloramphenicol
region, and a gene for ampicillin resistance, as described previously (12). Plasmid
is required for the assembly of the type III secretory apparatus that is essential
for measuring virulence plasmid loss. Aliquots were removed periodically and
was assayed by PCR amplification of the
PCR tests for loss of the virulence plasmid.

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S. flexneri
strain 2a 2457T was grown in LB broth, and plasmid
S. flexneri
lak
kanamycin at 30°C, and plasmid pALA33 or its derivative, plasmid pALA2515,
was introduced by electroporation. The resulting purified strains were grown at
kanamycin resistance. The cultures were subject to two further rounds of 5
106-fold dilution and incubation, and samples were plated after each round of
was introduced by comparison with the bands of the PCR size markers from Sigma-Aldrich, Inc. These appear correct
rpoS
of the chromosomal gene
rpoS
was assayed by PCR amplification of the
PCR tests for loss of the virulence plasmid.

Bacterial strains and plasmids. E. coli strain DH5α was used for plasmid
growth and DNA manipulations. Plasmid DNA was introduced into S. flexneri
strains by electroporation (18).

Competitive growth tests. S. flexneri strain BS547 was grown in LB broth plus kana-
mycin at 30°C, and plasmid pALA1056 or its derivative, plasmid pALA2515,
was introduced by electroporation. The resulting purified strains were grown at
30°C, mixed together in LB broth, diluted, and grown at 37°C using the protocol
for measuring virulence plasmid loss. Aliquots were removed periodically and
plated for viable counts on LB agar (total viable cells), LB agar with chloram-
phenicol (viable BS547 or pALA2515 cells), or LB agar with ampicillin (viable
BS547 pALA33 cells).

PCR tests for loss of the virulence plasmid. Presence of the virulence plasmid
was assayed by PCR amplification of the virF, virB, and rep4 genes. Amplification of
the chromosomal gene rpoS was used to control for the efficiency of the PCR.

RESULTS

Blocking the plasmid mvp function leads to rapid loss of a
virulence marker in the wild-type S. flexneri population. S. flexneri
strain 2a 2457T is a virulent, invasive strain and gives
the Crb+ (red colony) phenotype on Congo Red plates at 37°C. Any cells that lose the virulence plasmid are avirulent and give the Crb− (white colony) phenotype (9). The virulence plasmid
strain 2a 2457T appears to be essentially the same as
pWR100 from strain 2a M90T that was recently sequenced (4). It encodes an mvp PKS system.

The PSK function of mvp can be blocked by introducing an
extra source of the MvPA antitoxin on an additional plasmid (14). Now, if the mvp−containing plasmid is lost, MvPA
continues to be produced and the MvPT can no longer kill the cell.

We blocked mvp activity in strain 2457T in this way by intro-
ducing a pBR322-based plasmid carrying the entire mvp locus (pALA1196) into the strain by electroporation. We did not expect this to have any measurable effect on virulence plasmid
maintenance. The virulence plasmid encodes replication and
partition systems closely resembling those that promote faith-
ful plasmid replication and segregation to daughter cells in
other plasmid types (4). Thus, very few plasmid-free cells
should ever arise, and PSK by mvp should hardly ever come into play.

At 30°C, this expectation was justified; introduction of
pALA1196 had no measurable effect on virulence plasmid
stability as measured by retention of the Crb+ phenotype (Ta-
Materials and Methods.

Generations of unselected growth at the specified temperature, as described in chloramphenicol resistance.

However, at 37°C, 30% of cells containing pALA1196 formed Crb− (white) colonies after 20 generations of growth (Table 1). Virulent cells containing the vector without mvp (pALA136) produced no Crb− colonies under the same conditions. This suggested that the virulence plasmid is an exception to the rule and relies heavily on the action of the mvp PSK system for its continued maintenance at 37°C.

Extra copies of the mvp locus lead to complete loss of an mxiM mutant virulence plasmid at 37°C. We showed above that expression of additional copies of mvp leads to loss of the Crb+ phenotype in cells carrying the wild-type virulence plasmid. Is this due to loss of the entire plasmid, as was predicted, or to loss of a virulence function? This was investigated using S. flexneri strain BS547. This is an avirulent derivative of strain 2457T with an aphA-3 insertion mutation in the mxiM virulence gene of the virulence plasmid (15). Here, retention of the plasmid can be monitored by following the kanamycin resistance on LB plates at 30°C. As shown in Fig. 1C, the cells containing pALA2515 leads to the loss of the entire virulence plasmid kanamycin resistance marker throughout the experiment when grown at 30°C. After the initial lag, the slope of the response to pALA2515 at 37°C corresponded to a virulence plasmid loss rate of 2 to 3% per generation (2). (C) Viable counts of strain BS547 during competitive growth in LB broth at 37°C. Squares, BS547 cells containing the plasmid pALA2515, a derivative of pALA136 that contains the intact mvp locus. Both strains retained the virulence plasmid kanamycin resistance marker throughout the experiment when grown at 30°C. After the initial lag, the slope of the response to pALA2515 at 37°C corresponded to a virulence plasmid loss rate of 2 to 3% per generation (2). (C) Viable counts of strain BS547 during competitive growth in LB broth at 37°C. Squares, BS547 cells containing the plasmid pALA2515 containing the mvp locus; circles, BS547 cells containing the vector, pALA33. The two variants were grown together in competition and were distinguished by the different antibiotic resistance markers that they carry (see Materials and Methods).

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We blocked mvp function by introducing a multicopy, pBR322-based plasmid carrying an additional copy of the mvp genes (pALA2515). The kanamycin resistance (KmR) of the cells in the culture was progressively lost at 37°C. After an initial lag, the loss rate was 2 to 3% per generation. Virtually no KmR cells remained after 65 generations of unselected growth (Fig. 1A). Control strains containing no additional plasmid or containing the pALA136 vector produced no measurable loss.

The cells containing pALA2515 appeared to grow faster at 37°C than cells containing the vector in the above experiment (data not shown). To check this, we carried out a growth competition experiment (Fig. 1C). Cells containing pALA2515 (chloramphenicol resistance) were mixed in equal amounts with cells containing a variant of pALA136 (pALA33, ampicillin resistance). The mixture was grown at 37°C under the same conditions as the above experiment, and samples were plated periodically for chloramphenicol or ampicillin resistance. The mixture was grown at 37°C under the same conditions as the above experiment, and samples were plated periodically for chloramphenicol or ampicillin resistance. The mixture was grown at 37°C under the same conditions as the above experiment, and samples were plated periodically for chloramphenicol or ampicillin resistance. The mixture was grown at 37°C under the same conditions as the above experiment, and samples were plated periodically for chloramphenicol or ampicillin resistance.

The kanamycin-sensitive colonies produced in response to pALA2515 were screened for loss of other plasmid loci by PCR analysis. In addition to kanamycin resistance, the cells had lost sequences in the virulence plasmid replicon (repA) and the plasmid-encoded virulence genes virB and virF, while retaining the host chromosomal locus rpoS (Fig. 2). We conclude that introduction of the mvp region present in plasmid pALA2515 leads to the loss of the entire virulence plasmid at 37°C. No loss of the plasmid markers was seen in cells growing at 30°C (Fig. 1B).

Expression of the mvpA gene is sufficient to promote plasmid loss from the 37°C population. We reasoned that the virulence plasmid must be inherently unstable at 37°C and that killing of the cured cells by mvp must ensure that the cells retain the plasmid. This causes the growth of the population to slow down at 37°C. When killing is blocked by the MvpA

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<th>Incoming plasmida</th>
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<tr>
<td>pALA136</td>
<td>30</td>
<td>&lt;1</td>
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<tr>
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<td>pALA1196</td>
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a The relevant plasmid was introduced into S. flexneri 2a 2457T, selecting for chloramphenicol resistance.

b The rate of loss (as a percentage) of the Crb+ phenotype assayed during 20 generations of unselected growth at the specified temperature, as described in Materials and Methods.

c Loss was measured through 20 generations. This growth period was insufficient to detect the loss level of loss of the Crb+ phenotype normally seen when S. flexneri 2a 2457T cells are grown at 37°C (16).

FIG. 1. Blocking effect of an additional cloned mvp region at 37°C. (A and B) Retention of the kanamycin resistance marker carried on the virulence plasmid in strain BS547 was assayed after the stated number of generations of growth without selection in LB broth at 37°C (A) or 30°C (B). Circles, BS547 cells containing the vector, pALA136; squares, BS547 cells containing the plasmid pALA2515, a derivative of pALA136 that contains the intact mvp locus. Both strains retained the virulence plasmid kanamycin resistance marker throughout the experiment when grown at 30°C. After the initial lag, the slope of the response to pALA2515 at 37°C corresponded to a virulence plasmid loss rate of 2 to 3% per generation (2). (C) Viable counts of strain BS547 during competitive growth in LB broth at 37°C. Squares, BS547 cells containing the plasmid pALA2515 containing the mvp locus; circles, BS547 cells containing the vector, pALA33. The two variants were grown together in competition and were distinguished by the different antibiotic resistance markers that they carry (see Materials and Methods).

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antidote encoded by the extra copies of mvp on pALA2515, the cured cells survive and rapidly accumulate in the population. In order to confirm that MvpA synthesis from pALA2515 was responsible for the effect, we carried out a deletion analysis of pALA2515. As predicted, the critical determinant for the accumulation of cells lacking the virulence plasmid at 37°C mapped to the mvpA gene (Fig. 3).

**Induction of the** virB **regulon is not the cause of rapid plasmid loss at 37°C.** We have shown that the maintenance of virulence plasmid is inherently unstable at 37°C but not at 30°C. This implies that some key plasmid maintenance function or functions are attenuated or overridden at the higher temperature. The plasmid has a known temperature-dependent regulatory system, the virB regulon. A number of virulence-associated plasmid genes are controlled by virB and become induced only at the higher temperature (19). It seemed possible that some key plasmid maintenance genes might be under negative regulation within the virB regulon. Alternatively, plasmid loss at 37°C might be due to some unknown regulon product, such as a site-specific endonuclease, that damages the plasmid. In either case, induction of virB at 30°C should also make the plasmid unstable. We expressed virB in strain BS547 at 30 or 37°C by using an arabinose-inducible expression system (see Materials and Methods). Despite the fact that the virB regulon was demonstrably induced at both temperatures (Fig. 4), maintenance of the virulence plasmid continued to be inherently unstable only at 37°C (Table 2). Thus, temperature regulation of plasmid maintenance is not part of the virB regulon but is due to an independent temperature effect on plasmid maintenance, either involving the replication and partition systems or some function that actively damages the plasmid at 37°C.

**DISCUSSION**

**Inherent instability of the virulence plasmid at 37°C.** Our interpretation of the data presented here is shown in Fig. 5. It is based on the principles that have been established experimentally using a model mvp-containing plasmid in *E. coli* (3). At 30°C, the core plasmid maintenance systems of the virulence plasmid are efficient. Very few plasmid-free cells are produced, and the mvp PSK system rarely comes into play (Fig. 5A). When the mvp system is effectively switched off by supplying an additional copy of the mvpA gene in *trans*, the maintenance of the virulence plasmid is not measurably compromised (Fig. 5B). At 37°C, the core maintenance systems of the virulence plasmid become ineffective, due to down-regulation or temperature sensitivity of some key component or to induction of some function that damages the plasmid. Many cured cells are produced. These are killed by the MvpT toxin, which persists after the plasmid is lost (Fig. 5C). This causes a considerable growth disadvantage to the population (Fig. 1C). When the mvp system is switched off by supplying an additional copy of mvpA, plasmid-free cells rapidly accumulate in the 37°C population because they are not killed (Fig. 5D). This population can grow normally (Fig. 1C).

The identity of the component or components that compromise plasmid maintenance at 37°C is unknown. However, they do not appear to involve the virB regulon or inherent temperature sensitivity of one of the two plasmid partition systems, pWR100par (K. Sergueev and S. Austin, unpublished observations).

![FIG. 2. The entire virulence plasmid is lost when mvp activity is blocked at 37°C. Lanes correspond to amplified products for the virulence plasmid genes *virB* (lane B), *virF* (lane F), or *repA* (lane A), the chromosomal gene *rpoS* (lane S), and size markers (lane M). The template DNA was derived from colonies of strain BS547 recovered from the experiments shown in Fig. 1 and 3. Panel 1, typical kanamycin-resistant line recovered from cells containing the plasmid vector pALA136; panel 2, kanamycin-sensitive line recovered from cells containing pALA2515, which has the entire mvp region; panel 3, kanamycin-sensitive line recovered from cells containing pALA2526 (mvpA gene only). The products of the *virF*, *virB*, *repA*, and *rpoS* primers were predicted to be 780, 725, 816, and 1,274 bp, respectively, and appear to be correct as estimated by comparison with the 1,636-, 1,018-, and 517-bp bands of the 1-kb DNA ladder (central three bands in the size marker lanes).](http://jb.asm.org/)

![FIG. 3. The determinant for blocking virulence plasmid stability at 37°C maps to the mvp4 gene. The extents of the mvp4 region carried by derivatives of the plasmid vector pALA136 are shown on the left. These plasmids were introduced into strain BS547 by electroporation, selecting for chloramphenicol resistance. After approximately 65 generations of unselected growth at 30 or 37°C, retention of the kanamycin resistance marker of the virulence plasmid was assayed as described in Materials and Methods. The intact mvp locus promoted loss of the virulence plasmid (pALA215). An in-frame deletion within mvp4 in an otherwise-intact 1,127-bp mvp region abolished the effect (pALA2546). Plasmid pALA2526 gave the full effect. It carries only the mvp4 gene and its upstream control region, showing that the mvp4 gene is necessary and sufficient to promote loss of the virulence plasmid at 37°C.](http://jb.asm.org/)
The virulence-dependent genetic instability previously described. Many of the virulence genes of the plasmid are located in a 31-kb invasion region containing the *ipa* and *spa* genes. These are positively regulated by the factor *virB*. Genes under the control of *virB* are expressed at 37°C but are repressed at 30°C (8). During growth at 37°C, some spontaneous variants arise on long-term culture that have lost the ability to express the virulence genes, either by plasmid mutation or by complete loss of the plasmid (16). This genetic instability reflects a selective disadvantage of virulence gene expression. We avoided this selective effect in our studies by limiting growth of cells with the wild-type plasmid to 20 generations at 37°C, or by using *mxiM* mutant plasmid that is not subject to selection because the cells are avirulent (see Materials and Methods). However, we can now extend the interpretation of this genetic instability effect, given our findings. The virulence plasmid is inherently unstable at 37°C. This effect is independent of the *virB* regulon. The cells lose the plasmid at a rate of 2 to 3% per generation. However, these cured cells are efficiently killed by the action of *mvp*, which remains active at 37°C. Very few cured cells survive. These remain virtually undetectable unless the cell population is virulent. In this case, the few cured cells have a growth advantage and eventually outgrow the population after extended growth (16). Because the original surviving cells are very rare, the outgrowing, avirulent population includes a high proportion of cells that retain the plasmid but...
have lost virulence by spontaneous mutation. Although the inherent instability of the plasmid is independent of virB control, the selection and eventual outgrowth of the rare cured cells that survive from the virulent population do depend on virB, because virB controls virulence.

Implications for the pathogenic lifestyle. The use of PSK as a major component for the maintenance of a naturally occurring plasmid is a completely unexpected phenomenon. Low-copy-number plasmids encode highly efficient replication and partition systems that ensure that PSK rarely comes into play. This is necessary because, although effective, PSK is an expensive strategy for preventing the accumulation of cured cells in the population. This is well illustrated in the growth competition experiment described in Fig. 1C: mvp is effective in preventing the accumulation of cured cells, but the cells are at a major growth disadvantage to cells in which mvp is disabled.

Virulent Shigella cells infect their human host at 37°C. If we assume that the properties of the plasmid are not grossly altered in the environment of the human host, virulence plasmid retention presumably depends on mvp killing during infection. Why would the virulence plasmid become unstable at the very temperature at which the functions that it carries are needed, and why should the bacterial cells tolerate a system that continuously kills a portion of the bacterial cell population during virulent infection? Perhaps the loss of the plasmid and death of part of the infecting bacterial population is not deleterious to the infection process or the transmission of the organism to other hosts. It may even be advantageous. Unrestricted exponential growth of the pathogen might kill the host prematurely and limit transmission. Also, it is possible that dead cells provide some advantage to the pathogen. For example, an accumulation of dead bacterial cells might help to encapsulate the infection, providing a barrier to infiltration by the immune system, or it might facilitate release of toxins into the host tissues that aid the infection process.

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REFERENCES


