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Virulence Plasmid Instability in *Shigella flexneri* 2a Is Induced by Virulence Gene Expression

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Expression of the predominantly plasmid-encoded virulence regulon of *Shigella flexneri* 2a is induced by growth at 37°C and repressed by growth at 30°C. During growth at 37°C, spontaneous *S. flexneri* mutants arise which have undergone virulence plasmid curing or rearrangement and no longer display the virulent phenotype. In the laboratory, the unstable nature of the virulence plasmid causes complete loss of virulence in a growing population. We have undertaken an analysis of virulence plasmid instability, classifying events which produced individual avirulent derivatives within a virulent population and identifying the factor(s) which controlled conversion. Multiplex PCR analysis of DNA obtained from spontaneous avirulent derivatives indicated that *virF* and *virB* were deleted or otherwise inactivated in over 97% of the isolates. The *virF* and *virB* loci encode regulatory proteins required for transcriptional activation of the virulence regulon. Inactivation of these key regulatory loci in the vast majority of avirulent derivatives which arose during growth at 37°C suggested that virulence gene expression induced virulence plasmid instability. Consistent with this hypothesis, we observed stable virulence plasmid maintenance during growth of a wild-type strain at 30°C where virulence gene expression was repressed. The virulence plasmid was also stably maintained in *virF* and *virB* mutants grown at 37°C. Conversely, virulence plasmid destabilization was induced at 30°C and accelerated at 37°C through expression of VirF or VirB from multicopy plasmids. These results indicate that exposure of *S. flexneri* to conditions favoring induction of the virulent phenotype also favor its loss. The significance of this paradox of *Shigella* pathogenicity is discussed.

Ingestion of *Shigella flexneri* initiates a series of host-parasite interactions, the ultimate outcome of which is bacillary dysentery. Central to these interactions is bacterial invasion of the colonic mucosa, a multistep process which involves internalization of shigellae by epithelial cells followed by intracellular growth, intra- and intercellular spread, and finally host cell death (25). The ability of *S. flexneri* to productively infect humans is in part conferred by products of loci encoded on a 220-kb virulence plasmid (29). Many of the plasmid-encoded genetic determinants of virulence are localized within a 31-kb "invasion region," which encompasses over 30 genes (22, 31). Among the products of these loci are secreted effectors of the invasion process (Ipa proteins) as well as proteins dedicated to their secretion (Mxi and Spa proteins) (2, 31). An interesting aspect of the regulation of *ipa*, *mxi*, and *spa* expression is a dependence on temperature; growth at 37°C induces the virulence regulon (the bacteria are phenotypically invasive), while growth at 30°C represses it (rendering the bacteria phenotypically noninvasive) (24). Temperature regulation is exerted through transcriptional activation of the *virB* locus, which encodes a positive regulatory element that directs invasion gene expression (1, 38). Expression of *virB* is itself controlled by an additional positive regulatory element encoded by *virF* (37, 38). Together, VirF and VirB form a regulatory cascade responsible for induction of the invasive phenotype.

Laboratory assessment of the virulent phenotype of *S. flexneri* relies on such features as (i) the ability to provoke a positive Sereny reaction (Ser) (33), (ii) the ability to invade (15) and proliferate (27) in cultured epithelial cell lines (Inv), and (iii) the ability to bind Congo red dye (Crb) (23, 36). The red staining of *Shigella* colonies grown on agar plates containing Congo red is the most easily studied of these phenotypes and is often used to distinguish between invasive (colonies that bind Congo red or Crb^+ colonies) and noninvasive (colonies unable to bind Congo red or Crb^- colonies) strains. While the Congo red binding phenotype is not a virulence factor, it is a very reliable marker for virulence (23) as loci required for its expression are most likely members of the VirB regulon. Spontaneous loss of the ability to bind Congo red by virulent, wild-type *S. flexneri* occurs at a frequency between 5×10^{-3} to 1.2×10^{-4} per CFU (23, 35). Loss of Congo red binding is invariably associated with loss of additional virulence phenotypes and is usually accompanied by molecular alterations in the large virulence plasmid (7, 23, 32). Virulence plasmid instability in the laboratory is also observed in strains of *Shigella sonnei* (30) and *Shigella dysenteriae* (14), clinical isolates of *S. flexneri* (12), and pathogenic strains of *Escherichia coli* (6, 16).

It has been proposed that instability of the virulent phenotype may be beneficial to *Shigella* for survival outside of the host, by allowing conservation of energy that would otherwise be directed towards virulence plasmid maintenance (26). That the Crb^- phenotype may confer a selective growth advantage is supported by the observation that spontaneous avirulent Crb^- derivatives of *S. flexneri* grow faster than their Crb^+ parental strain in vitro (32). By virtue of this selective advantage a predominantly Crb^+ culture will eventually be overgrown by its Crb^- derivatives. Reversible inactivation of the virulent phenotype may then enable *Shigella* to optimally exploit the environment inside of a host as well as outside (26).

Spontaneous alterations of the *S. flexneri* virulence plasmid

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TABLE 1. *S. flexneri* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or property	Source or reference
Strains		
2457T	Wild type	21
2457O	2457T *virF*::IS*1*SFO	26
BS200	2457T *recA56 srl1300*::Tn*10*, *recA56* derivative of 2457T	Laboratory strain
BS527	2457T *virB*::Tn*5* (Km^r), Km^r transductant of 2457T	This study
BS528	2457O *yap*::Cm^r, Cm^r transductant of 2457O	Laboratory strain
BS555	2457T/pATM323	This study
BS557	2457T/pBR322	This study
BS558	2457T/pBAD18	This study
BS559	2457T/pATM104	This study
Plasmids		
pATM323	pBAD18::*virF*$^+$	This study
pBAD18	Arabinose-inducible expression vector	13
pATM011	pUC19::*virB*$^+$, 3.9-kb *Pst*I fragment from pHS5102 cloned into the *Pst*I site of pUC19	4
pATM104	pBR322::*virB*$^+$, *virB* open reading frame cloned into pBR322	11
pGEX-3X::*virF*	GST-VirF protein fusion vector,[a] 786-bp *virF* open reading frame cloned into *Sma*I site of pGEX-3X	Pharmacia Biotech, Inc.

[a] GST, glutathione *S*-transferase.

occur by complete plasmid curing, deletion of plasmid sequences, or insertion events. A common insertion event is the transposition of an IS*1*-like element into the *virF* locus (26). Chosa et al. (7) and Sasakawa et al. (32) found that many Crb^- derivatives suffered deletions within or near the invasion region; however, the exact targets of spontaneous deletion events in these derivatives are unknown. Mechanisms driving the appearance of virulence plasmid deletion derivatives remain unclear, although Daskaleros and Payne (10) indicate a role for IS*1*-mediated deletion events in a small number of altered plasmids.

In this paper we report observations in support of our hypothesis that (i) virulence plasmid deletion events target *vir* loci and (ii) *vir* gene expression induces virulence plasmid instability. In over 97% of the Crb^- derivatives of a Crb^+ wild-type strain, the *virF* and/or *virB* loci were either inactivated or deleted. These alterations appeared to be directly related to the expression of virulence loci. Conditions favoring induction of the virulence regulon, either growth at 37°C or expression of *virF* or *virB* at 30°C, resulted in loss of the Crb^+ phenotype and alterations of the virulence plasmid. Alternately, repression of virulence gene expression, either by growth at 30°C or by inactivation of *virF* or *virB*, allowed stable maintenance of the intact virulence plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids used. Bacterial strains and plasmids used in this study are described in Table 1. Plasmids were introduced into *S. flexneri* by electroporation. Complementation of the *virB*::Tn*5* allele (22) by pATM011 and pATM104 as well as that of the *virF*::IS*1*SFO allele (in 2457O) by pGEX-3X::*virF* was confirmed by analysis of Congo red binding (see below) and by the tissue culture invasion assay (19).

Plasmid pATM323 was constructed to place the expression of *virF* under the control of the arabinose-inducible and glucose-repressible promoter, P_{BAD} (13). The *virF* locus was initially amplified by PCR from the 2457T virulence plasmid with primers annealing to positions 179 to 199 and 997 to 977 from the published sequence (GenBank accession number M29172). The *virF* amplification product (5′ end cut with *Eco*RI and the 3′ end filled in with Klenow fragment) was ligated into the *Eco*RI-*Sma*I sites of pBAD18 to yield pATM323. To confirm that pATM323 encoded inducible, functional VirF, the plasmid was introduced into strain 2457O. In the presence of 0.2% arabinose, the resulting strain exhibited a Crb^+ phenotype and invaded tissue culture cells at a level nearly identical to that of BS558 and 2457T.

Media and chemicals used. *S. flexneri* strains were grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) with aeration at 37°C, unless otherwise indicated. Strains were tested for Congo red binding on TSB agar plates (1.5% agar) containing 0.025% Congo red (Sigma Chemical Co., St. Louis, Mo.). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 10 μg/ml; and kanamycin, 40 μg/ml. Cultures of BS555 and BS558 were additionally supplemented with 0.2% arabinose to induce the P_{BAD} promoter.

Growth conditions. Sequentially passaged subcultures of *S. flexneri* were initiated from a single fresh colony (Crb^+ for each strain except BS527 and BS528) inoculated into TSB and split into two separate 4.0-ml cultures. Each culture was then titered to determine the percentage of Crb^+ colonies at the start of the experiment. Duplicate cultures were grown at either 37 or 30°C (as indicated) with agitation into stationary phase (1×10^9 to 5×10^9 cells/ml). The cultures, at similar cell densities within each experiment, were then diluted 100-fold with fresh TSB and allowed to grow again to stationary phase under the appropriate conditions. Sequential subculture was repeated several times over the next 3 to 13 days depending on the experiment. The exact durations for each subculture varied between different experiments and are indicated in the figures. Prior to dilution, aliquots of each culture were plated and incubated overnight at 37°C. Roughly 0.5×10^4 to 2×10^4 colonies per subculture were screened at each time point to determine the percentage of Crb^+ colonies arising from each culture.

For analysis of specific inactivating events, 174 independently derived Crb^- mutants were obtained from separate overnight 2457T cultures. Single, fresh Crb^+ 2457T colonies were used to inoculate 4.0-ml cultures, which were incubated overnight (~16 h) at 37°C with agitation. From each overnight culture, aliquots containing 0.5×10^4 to 1×10^4 cells were plated and incubated overnight at 37°C. Two Crb^- colonies were chosen from each overnight culture plating for further study.

Multiplex PCRs. The primers used to amplify *virF*, *virB*, *virR*, and *apy* were as follows (GenBank accession numbers in parentheses): *virF*, positions 1 to 26 and 906 to 885 (M29172); *virB*, positions 7 to 33 and 1227 to 1194 (X14340); *virR*, positions 5 to 25 and 530 to 509 (X66848); and *apy*, positions 12 to 36 and 1105 to 1086 (U04539). The *virF*, *virB*, *virR*, and *apy* primers yielded 906-, 1,221-, 526-, and 1,094-bp amplification products, respectively. Amplification of *virF*::IS*1*SFO with the same *virF* primers yielded a 1,690-bp product (26).

PCRs were performed with a DNA Thermal Cycler 480 and GeneAmp DNA amplification kit (Perkin-Elmer, Foster City, Calif.). DNA was prepared from *S. flexneri* by the following method: a single subcultured colony from a strain of interest was suspended in 25 μl of 0.5 mM NaOH and incubated for 30 min at room temperature. Twenty-five microliters of 1.0 M Tris, pH 8.0, was then added to the NaOH-treated cells, and the solution was promptly diluted with 450 μl of dH_2O. PCRs were carried out in 25-μl volumes containing ~100 ng of DNA, 10 to 15 pmol of *virF*, *virB*, *virR*, and *apy* primers, 1 U of Ampli*Taq* DNA polymerase, and 300 μM deoxynucleoside triphosphate. Amplification of target DNA occurred over 23 cycles, with each cycle consisting of 30 s at 94°C, 30 s at 48°C, and 4 min at 65°C. The PCR products were visualized on 1.0% agarose gels.

RESULTS

Targets of virulence plasmid deletion events. Insertional inactivation of *virF* in strain 2457O yields a virulence plasmid resistant to curing procedures (26). Spontaneous deletion derivatives of the virulence plasmid are also stable (32). Once rearranged, the virulence plasmid is no longer lost nor does it undergo any subsequent gross deletion. While exact targets of the deletions are unknown, evidence suggests that deletion events target virulence loci (7, 32). We undertook a series of experiments to determine if such specific *vir* deletion targets exist.

We propose that *virF* and *virB* are the most logical targets for virulence plasmid deletion events, owing to their central role in the induction of virulence gene expression and the highly pleiotropic nature of mutations in their open reading frames. To test this prediction, we isolated DNA from 174 independently derived Crb^- mutants and used it as template in multiplex PCRs containing primers specific to *virF*, *virB*, *virR*, and *apy*. The resulting amplification products differed in size such that they could each be distinguished in a single lane of an agarose gel. Primers specific for *apy* were included as controls

TABLE 2. Multiplex PCR analysis of spontaneous Crb$^-$ derivatives of 2457T[a]

Class	PCR products present[b]	% mutants analyzed
I	*virF virB virR apy*	5.2
II	*virF virR apy*	29.3
III	*virB virR apy*	8.0
IV	*virR apy*	1.7
V	*virF*::IS*1*SFO[c] *virB virR apy*	1.7
VI[d]	*virR*	54.1

[a] The procedure for generating the 174 derivatives examined is described in Materials and Methods.

[b] Gene designations listed represent loci identified by the multiplex reactions. Absence of any of the four loci indicates that the locus has been lost in the strains of that class.

[c] The *virF*-specific PCR product was of a size predicted for the *virF*::IS*1*SFO allele.

[d] These derivatives are presumed to have lost the entire virulence plasmid.

to confirm virulence plasmid curing in strains that lacked both *virF* and *virB*. The *apy* locus is encoded within the *Sal*I "A" fragment of the virulence plasmid (28) and may be maintained upon specific deletion of *virF* and *virB*. Primers for the chromosomal *virR* locus (encoding H-NS) (20) were included as a control for the PCR in the absence of virulence plasmid DNA.

Based on the banding patterns of multiplex PCR products, Crb$^-$ variants were assigned to one of six different classes (Table 2). Complete virulence plasmid loss (class VI) was the molecular alteration which occurred most often among the Crb$^-$ derivatives examined. Of the remaining plasmid alterations identified (40.7% of the total population analyzed), all involved loss of *virF* and/or *virB* or insertional inactivation of *virF* (classes II to V). The finding that *virF* and/or *virB* were absent in the majority of Crb$^-$ derivatives, coupled with the central regulatory functions identified for VirF and VirB, indicates that virulence gene expression may promote virulence plasmid instability.

The class I derivatives in Table 2 consist of nine independent mutants. This class may have arisen through mutations at virulence loci not examined or through mutations in or near *virF* and/or *virB* that could not be distinguished by the multiplex PCR technique. Small insertions, deletions or point mutations in *virF* or *virB* would not have been detected by our analysis, nor would we detect any type of mutation upstream of primer binding sites in regulatory regions. To determine whether such alterations occurred to prevent expression of *virF* or *virB*, we transformed plasmids expressing either VirF or VirB into each of the class I mutants and analyzed the transformants for complementation of the Crb and Inv phenotypes. Introduction of pATM011 (expressing *virB*) restored the Crb$^+$ and Inv$^+$ phenotypes to one of the class I mutants, while introduction of pGEX-3X::*virF* restored the Crb$^+$ and Inv$^+$ phenotypes to four (data not shown). These results strongly suggested that the class I mutants complemented by VirF or VirB expression had mutations in *virF* or *virB* (or in their regulatory regions), respectively. Therefore, of the spontaneous Crb$^-$ mutants derived from 2457T during growth at 37°C, 97.7% failed to express the *virF* and/or *virB* loci.

Effect of growth temperature on virulence plasmid stability. Stabilization of the virulence plasmid is achieved by spontaneous rearrangement events that target *virF* and *virB* (23, 26) (data from previous section). Because of a correlation between virulence plasmid instability and the presence of *virF* and *virB*, we investigated the effects of virulence regulon repression and induction on maintenance of the Crb$^+$ phenotype. If expression of virulence loci at 37°C has a destabilizing influence on the virulence plasmid, then growth at 30°C, which represses virulence gene expression, should allow stable maintenance of the virulence plasmid and the Crb$^+$ phenotype.

We subcultured 2457T at both 30 and 37°C for several days and monitored conversion to Crb$^-$ during this period (see Materials and Methods). Within 3 days of sequential subculture, more than 50% of cells in the 2457T culture grown at 37°C yielded Crb$^-$ colonies (Fig. 1). Similar results have been reported previously (32). In contrast, the Crb$^+$ phenotype of 30°C cultures was remarkably stable (Fig. 1). Throughout the time course, the proportion of Crb$^-$ derivatives in the 30°C cultures never rose above 0.09% of the total population, which was similar to the value found in the starting culture (0.06%). Examination of the 30°C cultures even as long as 7 days after inoculation also yielded similar results (i.e., low numbers of Crb$^-$ colonies) (data not shown). Therefore, an aspect of in vitro growth encountered at 37°C and absent at 30°C favors loss of the Crb$^+$ phenotype. The metabolic burden imposed by virulence gene expression at 37°C could be the driving force behind the instability. At 30°C the repression of virulence gene expression may prevent virulence plasmid rearrangements and, therefore, the generation of Crb$^-$ derivatives.

Virulence plasmid stability in *virF* and *virB* mutants. If virulence gene expression is responsible for destabilization of the virulence plasmid, then in strains unable to produce VirF or VirB and thus unable to express virulence genes, there should be no further plasmid alterations. At least in the case of the *virF* insertion mutant (2457O) the virulence plasmid does appear to be stably maintained (26). It may be, however, that *virF* and *virB* mutants are unstable and undergo further rearrangements (though not previously detected in 2457O) (23, 26). To determine if the virulence plasmids in *virF* and *virB* mutants were stable, we analyzed spontaneous plasmid curing and smaller-scale deletion events in sequentially subcultured populations of cells lacking either *virF* or *virB*.

Three strains (2457T, BS527, and BS528) were sequentially

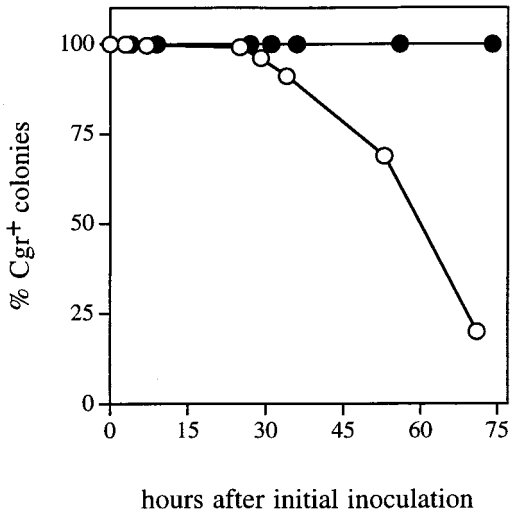

FIG. 1. Accumulation of Crb$^-$ mutants during growth. Loss of the Congo red binding (Crb$^+$) phenotype by 2457T grown at 37°C is shown by the open circles. 2457T grown at 30°C is represented by the solid circles. The bacteria were sequentially subcultured and monitored over a period of more than 70 h, and at the indicated time points the cultures were plated out at 37°C to enumerate the number of Crb$^+$ derivatives present per milliliter of culture. The percentages of Crb$^+$ colonies were then calculated by dividing the number of Crb$^+$ derivatives per milliliter by the total number of bacteria per milliliter at any given time point. The values shown are the averages obtained from two independent experiments performed in duplicate.

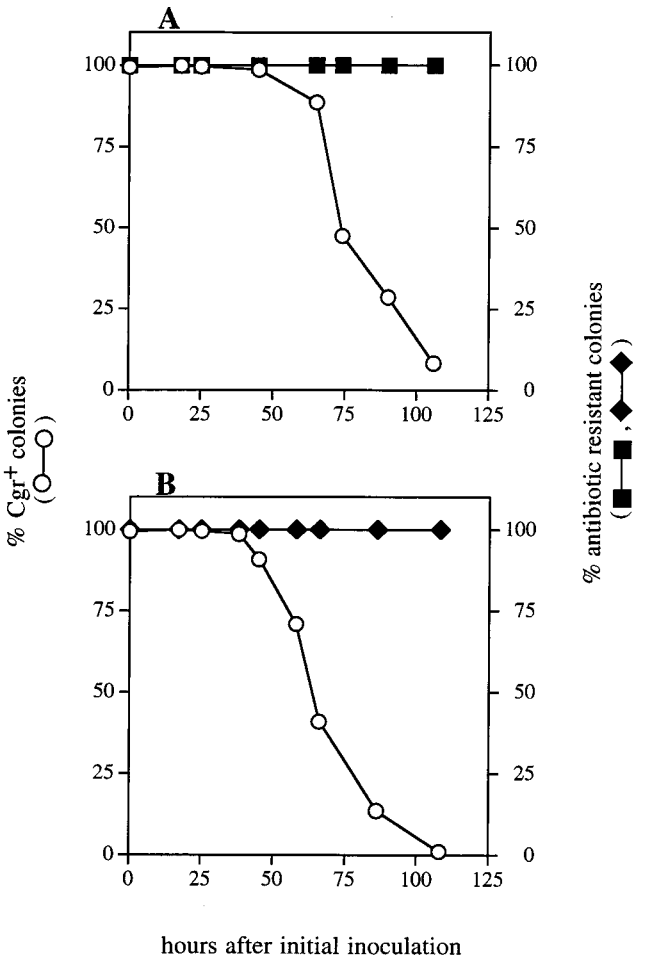

FIG. 2. Virulence plasmid loss in the absence of virulence gene expression. Sequentially passaged subcultures of the *virF* mutant BS528 (A) and the *virB* mutant BS527 (B) were analyzed for loss of chloramphenicol and kanamycin resistance markers (respectively) integrated into the virulence plasmid. The percentages of antibiotic-resistant colonies derived from cultures of BS527 and BS528 (diamonds and squares, respectively) are shown in comparison to the percentage of Crb^+ colonies derived from 2457T (circles). The values shown represent the averages of results obtained from two independent experiments performed in duplicate.

subcultured in duplicate over a 4-day period in the absence of antibiotics (see Materials and Methods). Strain BS527 is a derivative of 2457T containing a kanamycin-resistant transposon insertion in *virB* (22). Strain BS528 is a *virF* mutant bearing a chloramphenicol resistance gene inserted in the *yap* locus (encoding an acid phosphatase) on the virulence plasmid (28). Bacteria from each subcultured population were plated out at the time of each dilution on nonselective TSB agar plates. Since colonies of BS527 and BS528 are unable to bind Congo red, 100 colonies of each strain were picked at each plating and screened for either kanamycin resistance (BS527) or chloramphenicol resistance (BS528). As antibiotic selection was not applied prior to this screen, BS527 and BS528 derivatives that lost the virulence plasmid-encoded antibiotic resistance markers (by plasmid curing or rearrangement) would be identified. The results of this screen are shown in Fig. 2A and B. In the absence of any selective pressure to maintain the virulence plasmid, its spontaneous loss in either BS527 or BS528 was not observed. Maintenance of the Crb^+ phenotype in separate 2457T cultures grown alongside either the *virF* mutant (Fig. 2A) or the *virB* mutant (Fig. 2B) was assessed at various points throughout the time course. Results obtained from these analyses were similar to those described in Fig. 1 in that 50% of sequentially subcultured 2457T populations lost the ability to bind Congo red 60 to 75 h after the initial inoculation.

To determine if the virulence plasmids of BS527 and BS528 had suffered specific deletions of virulence loci, we prepared DNA from 30 colonies obtained from each culture throughout the experiment. The DNA was analyzed by multiplex PCR with primers specific to *virF*, *virB*, *virR*, and *apy*. In each case, the *virB* mutant derivatives retained *virF*, *virR*, and *apy*, while the *virF* mutant derivatives retained *virB*, *virR*, and *apy* (data not shown). The virulence plasmids in BS527 and BS528 were, therefore, stable and underwent no alterations at typically unstable virulence loci (*virB* or *virF*, respectively). These results are consistent with the hypothesis that expression of the VirF-VirB regulon is responsible for destabilization of the virulence plasmid.

VirF or VirB expression at 30°C destabilizes the Crb^+ phenotype. Results described above indicate that the capacity for the Crb^+ phenotype is stable during growth at 30°C. The stability can be attributed, presumably, to the absence of high-level VirF or VirB activity and/or to repression of virulence gene expression associated with growth at 30°C. If either of these assumptions are correct, then induction of *virF* or *virB* expression to levels sufficient for virulence gene expression during growth at 30°C should lead to destabilized maintenance of the intact virulence plasmid.

To determine whether virulence plasmid alterations can be induced at 30°C, we utilized strains capable of producing either VirF or VirB during growth at 30°C at levels sufficient to induce invasion gene expression. Induction of *virF* expression from the arabinose-inducible promoter of pBAD18 (in pATM323) may activate virulence gene expression through VirB (37). Transcription of *virB* initiates from its own promoter in pATM104 but is nonetheless expressed at high levels during growth at 30°C (data not shown). This high-level *virB* expression from pATM104, which was likely an effect of the elevated copy number of *virB* and its promoter in BS559, provided a means to directly induce virulence gene expression at 30°C. To determine if pATM323 and pATM104 could direct virulence gene expression at 30°C, we introduced each plasmid into *S. flexneri* strains carrying either an *ipaB-lacZ* or a *mxiC-lacZ* reporter fusion (19). Induction of *virF* expression from pATM323 during growth at 30°C in the presence of 0.2% arabinose resulted in high levels of both *ipaB-lacZ* and *mxiC-lacZ* expression (data not shown). There was no reporter gene induction in the presence of 0.2% glucose (data not shown). Expression of *virB* from pATM104 during growth at 30°C similarly induced high levels of both *ipaB-lacZ* and *mxiC-lacZ* expression. These results indicated that expression of either *virF* or *virB* under the conditions tested was capable of inducing virulence gene expression at 30°C.

Using the *virF* and *virB* expression vectors in 2457T (strains BS555 and BS559, respectively), we assessed the impact of virulence gene expression on virulence plasmid maintenance during growth at 30°C. Strains BS555 and BS559, as well as BS557 and BS558 (control strains bearing the vector plasmids pBR322 and pBAD18, respectively), were sequentially subcultured in duplicate at both 37 and 30°C. Growth was monitored for a period of at least 9 days in the presence of ampicillin. As expected, growth of BS557 and BS558 at 37°C led to a loss of the Crb^+ phenotype (Fig. 3A and C). Expression of either *virF* or *virB* in BS555 or BS559, respectively, at 37°C hastened this loss (Fig. 3A and C). At 30°C, the Crb^+ phenotypes of BS557 and BS558 were stable (Fig. 3B and D). This was, however, not

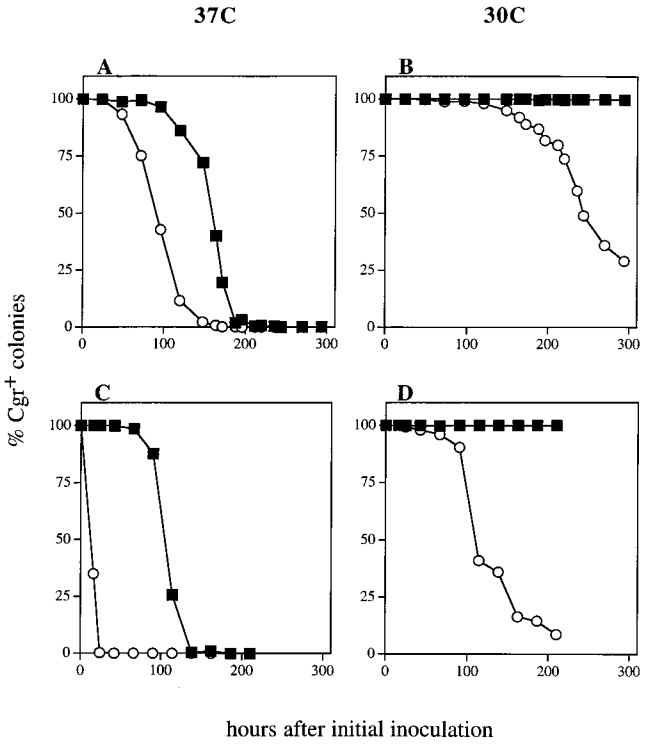

FIG. 3. Effects of *virF* and *virB* expression on the stability of the Crb^+ phenotype during growth at 30°C. Sequentially passaged subcultures of BS555 (A and B, circles) and BS559 (C and D, circles) were grown and monitored at either 37 or 30°C. As control strains, BS558 (A and B, squares) and BS557 (C and D, squares) were also analyzed. At the indicated time points after each initial culture inoculation, bacteria were diluted and plated out at 37°C, and the percentage of bacteria in each culture giving rise to Crb^+ colonies was determined. The values shown represent the averages of results obtained from two independent duplicate cultures.

the case for BS555 (induced *virF*), as loss of the Crb^+ phenotype was noticeable within 100 h of growth at 30°C and was greater than 50% by 250 h (Fig. 3B). Loss of the Crb^+ phenotype for BS559 (induced *virB*) at 30°C was even more rapid (Fig. 3D), reaching 50% within 100 h of the initial inoculation. These results confirmed that expression of either *virF* or *virB*, and/or subsequent expression of invasion loci, can destabilize the Crb^+ phenotype during growth at 30°C.

Molecular rearrangements in a *recA56* background. Recombination, as well as related processes such as transposition, mediate alterations in DNA structure and are likely responsible for rearrangements observed in the *S. flexneri* virulence plasmid. IS*1* and IS*1*-like elements are present in more than 40 copies in the *S. flexneri* genome (5), with 2 to 5 copies residing on the virulence plasmid (9, 10, 26, 32). However, because virulence plasmid deletions mediated by IS*1* replicative transposition occur in only a minority of events leading to the appearance of Crb^- derivatives (10), the general or homologous recombination reaction may be of central importance to the process of virulence plasmid rearrangement. Therefore, the possibility was considered that in the absence of the *recA* product (which is a crucial component of the homologous recombination reaction), molecular rearrangement of the virulence plasmid would be reduced significantly.

To assess the role of RecA in virulence plasmid instability, we monitored sequential subcultures of BS200 (a 2457T derivative bearing the *recA56* allele). Fresh Crb^+ BS200 colonies were inoculated in duplicate into TSB (as described in Materials and Methods) and sequentially subcultured for 6 days at 37°C. Control cultures of 2457T were also established. Culture aliquots were removed at various intervals throughout the time course and plated on TSB agar for the purpose of monitoring loss of the Crb^+ phenotype.

The 2457T cultures yielded 50% Crb^+ colonies within ca. 68 h after the initial inoculation, while the BS200 cultures gave rise to 50% Crb^+ colonies only after ca. 135 h (Fig. 4). Therefore, while a mutation in *recA* did not stabilize the Crb^+ phenotype, it did delay its eventual loss. These results suggested that certain processes directing virulence plasmid destabilization in BS200 may be occurring slowly or not at all. This is supported by findings obtained by multiplex PCR analysis of Crb^- derivatives of the *recA56* strain (data not shown). Of 55 Crb^- BS200 colonies analyzed by PCR, 20 gave no amplification of a *virF* product (all other loci were amplified, however), 2 gave amplification of a *virF*-specific product corresponding to the expected size of *virF*::IS*1*SFO (again, all other loci were amplified), and 19 gave the expected amplification products for all primer pairs. Each of these classes (III, V, and I, respectively), which arose from RecA-independent events, was identified earlier (Table 2). An additional class was also identified, consisting of 14 Crb^- derivatives, in which we observed a previously unseen *virF*-specific band roughly 1,300 bp larger than that expected for *virF* (950 bp). This *virF* allele was named *virF*::1300 and was presumably created by the insertion of an IS element other than IS*1*SFO into *virF*. The 1,329-bp IS*10* element present in BS200 (and not in 2457T) may have been the source of the insert in *virF*. No examples of virulence plasmid curing or *virB* deletion were observed, indicating that the appearance of such derivatives occurs via a RecA-dependent mechanism.

DISCUSSION

Several enteric pathogens, including *S. flexneri*, possess large plasmids encoding a variety of virulence loci (16, 41). Loss of or rearrangement within these plasmids occurs spontaneously during growth and invariably results in the appearance of avirulent derivatives unable to infect host tissues (6, 14, 18, 23, 30,

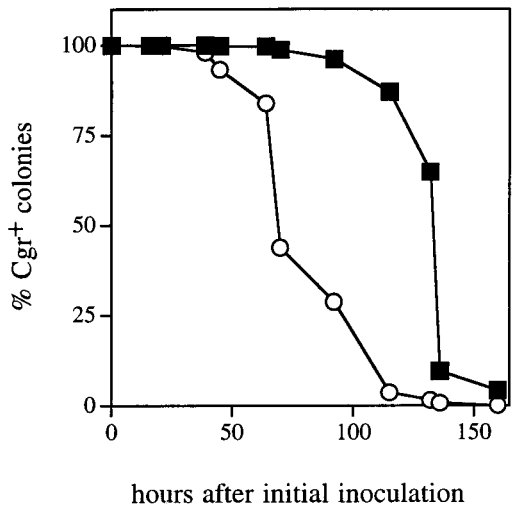

FIG. 4. Loss of the Crb phenotype in an *S. flexneri recA56* background. Sequentially passaged subcultures of 2457T (circles) and BS200 (squares) were analyzed for loss of the Crb^+ phenotype. Bacteria were subcultured and plated out at the indicated time points; the percentage of bacteria in each culture giving rise to Crb^+ colonies was then determined. Values shown represent the averages of two independent duplicate experiments.

42). The nature of the forces driving the plasmid alterations is unknown.

Once a spontaneously rearranged virulence plasmid arises in a population of *S. flexneri* it is stably maintained (23, 26) (see Results), indicating that specific genetic information which destabilizes the virulence plasmid is absent or nonfunctional in the rearranged derivative. Fine-detail analysis of *S. flexneri* 2457O, a spontaneous avirulent mutant bearing a stable virulence plasmid, indicated that stabilization was achieved through insertion of an IS*1*-like element in the virulence plasmid at the *virF* locus (26). Stable maintenance of an intact virulence plasmid by inactivation of a key positive regulatory protein (VirF) controlling virulence gene expression suggests that specific *vir* inactivation targets exist and that instability may be attributable to induction of the virulence regulon of *S. flexneri*. Results obtained in this study confirm these hypotheses.

Multiplex PCR analysis of Crb^- variants derived from a wild-type Crb^+ population of cells was used to identify spontaneous molecular alterations of the *S. flexneri* 2a virulence plasmid. In virtually all mutants analyzed (97.7%) the *virF* and/or *virB* loci were absent or otherwise inactivated. These findings were predominantly attributable to virulence plasmid curing and rearrangement (Table 2). Of those Crb^- derivatives isolated bearing some form of the virulence plasmid (45.9% of strains examined), 95.0% had sustained rearrangements which targeted and inactivated the *virF* and/or *virB* loci. Since *virF* and *virB* were primary targets for molecular rearrangement in the virulence plasmid, and because their loss correlated with stability of the altered plasmid, we hypothesized that *virF* and *virB* exert a destabilizing influence on intact plasmid maintenance.

VirF and VirB participate in a regulatory cascade responsible for initiating and maintaining virulence gene expression (1, 37, 38). Based on their functions, VirF and VirB most likely alter virulence plasmid stability through induction of the virulence regulon (including, at least, the genes encoded within the *ipa*, *mxi*, and *spa* operons). Consistent with this idea, growth of *S. flexneri* at 37°C and the subsequent induction of virulence gene expression resulted in spontaneous alterations of the virulence plasmid and conversion of a predominantly Crb^+ culture to Crb^- (Fig. 1). This instability was not observed in the same growth conditions upon inactivation of *virF* or *virB*. In the absence of functional VirF or VirB and, consequently, no virulence gene expression, plasmid curing or rearrangement could not be detected (Fig. 2 and data not shown). Similarly, repression of virulence gene expression achieved by growth at 30°C stabilized the virulence plasmid (and, therefore, the potential for the Crb^+ phenotype) of a population of growing cells (Fig. 1). As our model predicted, it was the absence of virulence gene expression at 30°C that allowed stable virulence plasmid maintenance. When *virF* or *virB* was expressed from multicopy plasmids at 30°C at levels sufficient to induce the expression of *ipaB-lacZ* and *mxiC-lacZ* reporter fusions, the virulence plasmid was destabilized and Crb^- derivatives accumulated in an initially Crb^+ culture (Fig. 3).

Spontaneous alteration of virulence plasmid structure in vitro increases bacterial fitness for growth, as evidenced by the improved growth rate of Crb^- variants and their ability to overgrow wild-type Crb^+ populations (32). Release of *S. flexneri* from the metabolic load of virulence plasmid maintenance may explain the selective growth advantage. However, the stability of the intact virulence plasmid at 30°C or of deletion derivatives at 37°C, combined with additional findings in this study, suggest that it is the burden of expressing virulence loci and not the maintenance of a large plasmid that is causing the instability. How virulence gene expression destabilizes the virulence plasmid is unclear, although the process of transcription has been demonstrated to affect several bacterial recombination events including homologous and illegitimate recombination and could possibly play a role (40). Indeed, results presented here suggest that both RecA-mediated and RecA-independent events are responsible for directing virulence plasmid alterations (Fig. 4), although RecA-mediated rearrangements appear to be of primary importance. Of the 174 independent Crb^- derivatives of 2457T analyzed by multiplex PCR, 83.4% had cured the plasmid or generated a *virB* deletion derivative (Table 2); these alterations were not observed in the *recA56* background.

The unstable nature of the *S. flexneri* virulence plasmid in vitro presents a paradox in that induction of the virulence regulon in a host will impair its ability (through plasmid loss) to colonize that host and cause disease. The likelihood of this phenomenon occurring within the human gastrointestinal tract is, however, unknown, since laboratory growth conditions used in our study are artificial and do not completely mimic host conditions. It remains possible that an unknown host factor modulates virulence gene expression in such a manner that allows stable plasmid maintenance or that the virulence plasmid has stabilization systems to ensure its maintenance and that growth in the laboratory perturbs these systems.

If the virulence plasmid is unstable in vivo, how can *S. flexneri* maintain a virulence plasmid at all and why hasn't the plasmid been completely eliminated from the species? It is possible that Crb^- derivatives are constantly being generated in the infected host but that they are subsequently killed. It also remains possible that Crb^- derivatives generated in the host are shed and that some bear reversible virulence plasmid alterations. As indicated in this study, 1.7% of Crb^- derivatives analyzed carried the *virF*::IS*1*SFO allele, which has been shown to revert to the wild-type *virF* allele (26). Mills et al. (26) have suggested that IS*1*SFO insertion into *virF* could stabilize the virulence plasmid outside the host, and its excision from *virF* could allow reversion to full virulence inside the host. Additionally, integration of the entire virulence plasmid into the chromosome and then subsequent excision has been proposed as another mechanism for ensuring virulence plasmid maintenance (42). Evidence suggests that the virulence plasmid can integrate into and excise from the chromosome and that integration can prevent virulence gene expression and possibly stabilize the virulence plasmid (8, 42). The Crb^-, $virF^+$, and $virB^+$ nature of the class I mutants identified in this study (Table 2) suggests that they may have arisen by such a mechanism. The capacity of Crb^- derivatives bearing reversible virulence plasmid alterations to optimally exploit both host and nonhost environments could, therefore, provide it with a selective advantage over those bacteria bearing irreversible alterations. In the manners described, *S. flexneri* populations could maintain an intact virulence plasmid.

Attenuated strains of *Shigella* spp. have been proposed as live oral vaccines against shigellosis (17, 39) and as carriers for the delivery of foreign protein antigens (3) and DNA vaccines (34) into the human colonic mucosa. Large-scale production of vaccine strains can be confounded by virulence plasmid instability (17). Virulence plasmid rearrangements can remove virulence genes relevant for inducing immunity or can ablate the ability to invade epithelial cells and thus deliver antigen. A better understanding of the molecular basis for plasmid instability would lead to better vaccine strains which minimize or preclude loss of the plasmid. As has been demonstrated here, manipulation of *S. flexneri* under conditions which induce virulence gene expression will cause problems with virulence plasmid loss. Cultivation of *Shigella* vaccine strains will require

strict adherence to incubation temperatures which block induction of the virulence regulon and, thus, plasmid alterations.

In addition to problems associated with the growth of vaccine strains, *Shigella* virulence plasmid instability also poses problems for the use of diagnostic kits to detect *Shigella* infections. Many such kits depend on the identification of plasmid-borne virulence genes by PCR and are vulnerable to false negative results if the maintenance of virulence loci is unstable in the laboratory. Better understanding of growth (or transport) conditions which favor repression of virulence gene expression and maintenance of the intact virulence plasmid will preserve the effectiveness of these detection strategies.

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