Addiction Toxin Fst Has Unique Effects on Chromosome Segregation and Cell Division in *Enterococcus faecalis* and *Bacillus subtilis*†

S. Patel and K. E. Weaver*

Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, South Dakota 57049

Received 10 April 2006/Accepted 22 May 2006

The Fst toxin of the *Enterococcus faecalis* pAD1-encoded *par* addiction module functions intracellularly to kill plasmid-free segregants. Previous results had shown that Fst induction results in membrane permeabilization and cessation of macromolecular synthesis, but only after 45 min. Electron micrographs of toxin-induced cells showed no obvious membrane abnormalities but did reveal defects in nucleoid segregation and cell division, begging the question of which is the primary effect of Fst. To distinguish the possibilities, division septae and nucleoids were visualized simultaneously with fluorescent vancomycin and a variety of DNA stains. Results showed that division and segregation defects occurred in some cells within 15 min after induction. At these early time points, affected cells remained resistant to membrane-impermeant DNA stains, suggesting that loss of membrane integrity is a secondary effect caused by ongoing division and/or segregation defects. Fst-resistant mutants showed greater variability in cell length and formed multiple septal rings even in the absence of Fst. Fst induction was also toxic to *Bacillus subtilis*. In this species, Fst induction caused only minor division abnormalities, but all cells showed a condensation of the nucleoid, suggesting that effects on the structure of the chromosomal DNA might be paramount.

Addiction modules or postsegregational killing (PSK) systems stabilize plasmids within host cell populations by programming for death any daughter cell that loses the plasmid. PSKs are ubiquitous on low-copy-number plasmids and have been identified in both gram-negative and gram-positive bacteria (for recent reviews see references 5, 14, and 20). PSKs encode at least two components, a stable toxin and its unstable antidote, the antitoxin. In most cases both toxin and antitoxin are proteins and toxin activity is regulated by direct interaction with its antitoxin. In a few cases the antitoxin is a regulatory RNA that binds to the mRNA for the toxin and inhibits translation. Proper segregation of plasmid DNA ensures continued production of the labile antitoxin and suppression of toxin activity or translation of the toxin. Plasmid loss leads to degradation of the antitoxin and activation or toxin activity or translation, leading to cell death. Similar modules have been found on the chromosomes of most bacterial species, where they are believed to play a role in stress response.

The targets of PSK toxins vary, and many are yet to be identified. The target of CcdB toxin of the *Escherichia coli* F-carried *ccd* locus was the first defined; CcdB was found to bind GyrA and poison the DNA-gyrase complex (1, 26). Recently the crystal structure of CcdB bound to the relevant GyrA fragment and a model of how it poisons gyrase were reported (9). The ParE toxin of the *parDE* system of the broad-host-range plasmid RK2 also targets DNA gyrase (24). The Kid toxin of the R1-carried Kis/Kid locus functions as an endoribonuclease, inhibiting protein synthesis by cleaving mRNA at 5'-UA(A/C)-3' sites (28, 33, 41). Toxins from a

number of chromosomally encoded toxin-antitoxin modules act in a similar manner (32, 42, 43) and appear to function as stress response loci (7). Finally, the Hok toxin of the R1-carried RNA-regulated *hok/sok* system leads to the formation of “ghost” cells and the collapse of membrane potential (13, 15), but the specific target and mechanism of action remain unknown.

Very few PSK systems have been identified on plasmids native to gram-positive bacteria, and they are less well described than their gram-negative bacterial counterparts. The *Axe-Txe* locus was identified on the pRUM plasmid in *Enterococcus faecium* (16). While the target of the Txe toxin is unknown, its sequence is similar to that of the YoeB protein encoded on the *E. coli* chromosome, which belongs to the family of endoribonucleases discussed above (6), and Txe is toxic to *E. coli*. The *Streptococcus pyogenes* plasmid pSM19035 encodes a unique three-component PSK consisting of the ω regulatory component, the ϵ antitoxin, and the ζ toxin (44). Induction of ζ in *Bacillus subtilis* leads to a variety of morphological defects, chromosome loss, and cell lysis. Induction in *E. coli* leads to filamentation without SOS induction and is bacteriostatic. Interestingly, ζ is also toxic to *Saccharomyces cerevisiae*, but the specific target is not known in any of these organisms.

The only RNA-regulated PSK system in gram-positive organisms is the *par* locus of *Enterococcus faecalis* plasmid pAD1. pAD1 is the prototype of a family of plasmids whose conjugative systems are induced by peptide sex pheromones secreted by potential recipients (10). *par* is a self-contained PSK locus less than 400 bp in size encoding two small RNAs, the $\sim$ 70-nucleotide regulatory RNA, RNA II, and the $\sim$ 215-nucleotide toxin-encoding RNA, RNA I (37, 38). Unlike most plasmid-encoded RNA-regulated systems, the two RNAs are transcribed convergently, overlapping only at a bidirectional intrinsic terminator. However, the RNAs are transcribed in opposite

* Corresponding author. Mailing address: Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD 57049. Phone: (605) 677-5169. Fax: (605) 677-6381.

† Supplemental material for this article may be found at <http://jb.asm.org/>.

directions across a pair of direct repeats, resulting in dispersed regions of complementarity more similar to chromosomally encoded RNA regulators than to traditional antisense-regulated systems (18, 19, 36). Binding of RNA II to RNA I suppresses the translation of a 33-amino-acid peptide designated Fst which functions as the toxin of the system (17). Overproduction of Fst results in a loss of cell viability, loss of membrane integrity, abnormalities in chromosomal segregation and cell division, and hypersensitivity to the lantibiotic nisin (39). While the small size and apparent hydrophobicity of Fst suggest that it could aggregate in the membrane and facilitate pore formation, the effects on membrane integrity and the collapse of macromolecular synthesis occurred relatively late, nearly 45 min, after induction. Furthermore, electron micrographs revealed no apparent membrane abnormalities and none of the leakage of cell contents that characterizes Hok toxicity in *E. coli*. These results suggest that the membrane effects could be secondary to division or segregation defects. To examine this possibility, we performed time course experiments using fluorescent vancomycin (Fl-Van), a dye that stains un-cross-linked and therefore recently incorporated peptidoglycan (8), and a variety of membrane-permeant and -impermeant DNA stains. Multiple cell division abnormalities and aberrant chromosomal distribution, including the segregation of chromosome-free cells, were observed as early as 15 min after Fst induction. At such early time points little DNA staining was observed with membrane-impermeant stains in unfixed cells, suggesting that loss of membrane integrity is a secondary effect of division and segregation defects. The conclusion that the primary Fst target is involved in chromosomal segregation and/or structure was further supported by studies of Fst toxicity in *Bacillus subtilis*, which showed a primary effect on nucleoid structure and only minor effects on peptidoglycan synthesis.

MATERIALS AND METHODS

Strains and growth conditions. Experiments with *E. faecalis* were performed using strain OG1X (21) containing the plasmid pAM2005K. pAM2005K is an erythromycin resistance-encoding pAD1 miniplasmid in which the *fst* gene is fused to the pheromone cAD1-inducible promoter of the *traE1* gene (35). The Fst-resistant mutant m7 was derived from OG1X(pAM2005K) by selection of spontaneous pheromone-resistant mutants as described in reference 39. Both pAM2005K and plasmid-free derivatives were used in this study. For studies with *B. subtilis* a promoterless version of the RNA I gene was constructed by PCR using primers DAS-5 (5' ACGCGTCGACGCGGCAGCTCGCCTCGATTGG 3') and DAS-3 (5' ACATGCGATGCCACAAAAGCAATCCTACGGCGA 3') under the following conditions: 94°C for 30 s, 94°C for 30 s, 44°C for 30 s, and 72°C for 60 s for 25 cycles with pDAK606 (36) as template DNA. Sites for restriction enzymes SalI and SphI were included in the primers (underlined) for cloning purposes. The 0.26-kb PCR fragment was cloned into pGEM-T-Easy (Promega, Madison, WI) and sequenced (Lone Star Labs, Texas) and transformed into competent DH5 α cells (Invitrogen, Carlsbad, CA). The PCR fragment was then removed from pGEM-T-Easy using the appropriate restriction enzymes and subcloned to similarly cut pDR66 (23) kindly provided by Alan Grossman, Department of Biology, Massachusetts Institute of Technology. The plasmid was then transformed into *B. subtilis* strain BG1 (30), kindly provided by D. Bechhofer, Mount Sinai School of Medicine. pDR66 does not encode a functional replicon for *B. subtilis* but contains the Pspac promoter, any cloned genes, and the chloramphenicol resistance gene between upstream and downstream segments of the *B. subtilis* chromosomal *amyE* gene. Selection for chloramphenicol resistance results in selection of transformants with the genes of interest inserted at the *amyE* chromosomal locus. This was confirmed by demonstrating loss of amylase activity by the starch test. Briefly, the culture was streaked on a 0.2% Luria-Bertani (LB) starch plate and grown overnight at 37°C.

The plate was flooded with Gram's iodine solution, and the absence of clearing around the streak demonstrated disruption of *amyE*. In addition, chromosomal DNA was purified from a selected transformant using the MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI), and the RNA I gene and flanking DNA were amplified by PCR using the primers lacI (GCCCCACT GACGCGTTGCGCG) and pDR66 reverse (GGATAACAATTAAGCTTG GGC) and sequenced to ensure proper sequence and fusion to the Pspac promoter. This strain was designated BG565. A control strain, BG1:pDR66, containing only the empty vector was also constructed.

E. faecalis strains were cultured in Todd-Hewitt broth (THB; Sigma-Aldrich) with erythromycin, 10 $\mu\text{g ml}^{-1}$. The strains were grown at 37°C in tubes with shaking at 250 rpm in an Innova 4230 incubator shaker (New Brunswick Scientific Co., Edison, NJ). For microscopy, 0.2 ml of overnight-grown cultures was used to inoculate 10 ml of THB and grown at 37°C to an optical density at 660 nm of ~ 0.1 for ca. 1 h. The pheromone cAD1 was added at this point, and the cultures were further grown for 1 h at 37°C before being processed for staining.

Growth was monitored by the change in optical density at 660 nm in a Milton Roy Spectronic 21D (Fisher Scientific) densitometer fitted for direct measurement of tubes with a 13-mm diameter.

The *fst* gene of OG1X(pAM2005K) was induced by addition of synthetic cAD1 (Sigma-Genosys) at 200 ng ml $^{-1}$ from a 200- $\mu\text{g ml}^{-1}$ stock in dimethyl sulfoxide.

Bacillus subtilis BG1 and its derivative strains were grown in standard LB medium for 18 to 20 h at 37°C in an incubator-shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm with chloramphenicol (10 $\mu\text{g ml}^{-1}$) before they were diluted 1:20 in fresh medium without the antibiotic. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the medium at a 1 mM concentration to induce the expression of RNA I under the control of the Pspac promoter after 30 min of growth.

RNA isolation from *B. subtilis* strains and Northern blot assays were done as described earlier with *E. faecalis*, with the only modification being that cultures were grown for 90 min in LB rather than 120 min in THB prior to harvest (39). The RNA I-specific probe used had the sequence 5'-ATAACCAACGACATT AAATCTTCAC-3'. It was used along with a standard probe for *B. subtilis* 5S rRNA with the sequence 5'-AACGGGTGTGACCTCTTCGCTAT-3'.

Fluorescence microscopy. Aliquots (500 μl) of bacterial cultures grown as described above were centrifuged at 10,000 rpm for 1 min at room temperature. The pellets were resuspended in 20 μl staining solution containing 2 M glucose, 1 M Tris-Cl, pH 8.0, and 0.5 M EDTA, pH 8.0. The cells were stained directly without fixation by mixing Fl-Van (a 1:1 mixture of vancomycin and BODIPY Fl-conjugated vancomycin [Molecular Probes]; final concentration, 2 $\mu\text{g ml}^{-1}$) and, where used as costain, propidium iodide (PI) (Molecular Probes; final concentration, 20 $\mu\text{g ml}^{-1}$) for 5 min at room temperature in the dark. Fixed cells were treated with an equal volume of ice-cold 100% methanol, vortexed, and then centrifuged. Stained bacterial cells were spread on microscopic slides coated with poly-L-lysine (Electron Microscopy Sciences, Hatfield, PA). DAPI (4',6'-diamidino-2-phenylindole; Molecular Probes) was used at a final concentration of 0.2 $\mu\text{g ml}^{-1}$.

BG565 and BG1:pDR66 cells were grown in LB at 37°C overnight and diluted (1:20) in fresh medium. IPTG (1 mM) was added after 30 min of growth, and cultures were grown for a further 30 min before the cells (500 μl) were harvested by centrifugation at 10,000 rpm for 1 min. The pellet was resuspended in 20 μl of staining solution. Cells were incubated with 200 ng ml $^{-1}$ FM4-64 and 0.1 μl of 5 mM Sytox Green (Molecular Probes) for 5 min at room temperature in the dark. The samples were then spread and dried on poly-L-lysine-coated slides.

Samples were viewed on an Olympus BX61 confocal laser scanning microscope utilizing argon, Helium Neon Red, and Helium Neon Green with a 60 $\times$ Plan Apo oil-immersion objective (numerical aperture, 1.40) with 6 $\times$ zoom using the generic green filter set for Fl-Van and the PI filter set for PI. DAPI staining was done on an Olympus AX70 upright compound microscope using an Olympus DP70 digital camera. The images were processed using Adobe PhotoDeluxe BE 1.0.

RESULTS

Fst causes early effects on cell division that do not correlate with loss of membrane integrity. Previous results showed that after 1 h of Fst induction cells became permeable to the membrane-impermeant DNA stain Sytox Green. In addition, after 40 to 45 min of induction RNA, DNA, and protein synthesis stopped simultaneously. These results suggested that exposure to Fst resulted in disruption of the membrane. However, scan-

FIG. 1. Effects of Fst induction on peptidoglycan synthesis and membrane permeability as determined by FI-Van and PI staining, respectively. All panels show, from left to right, FI-Van staining, PI staining, and an overlay of both. Uninduced cells are shown in the 0-min panel. Similar results were obtained with cells that were induced and immediately stained. Cells labeled 1, 2, and 3 show the normal progression through the division cycle as described in the text. The time after induction is shown to the left of the other panels. The 15-min panel shows two elongated cells at the right end of the chain. In the terminal cell the septal band is off center. In the penultimate cell a second, fainter septal band (marked by an arrow) is observed to the right of the main septal band, which is in turn off center in the direction opposite that in the terminal cell. In the top 30-min panel a chain of cells showing various abnormalities is shown. The leftmost "cell" shows multiple partially constricted bands, the second and fifth cells from the left show asymmetric secondary septal bands, and the third and fourth cells from the left each show two brightly staining septal bands. The bottom panel shows a chain of filamenting cells, and the arrows highlight segments of two filaments that stain with PI while the rest of the "cell" does not. Note that the two terminal cells on the right of the figure show filamentation but do not stain with PI. Bars = 2 μ m.

ning electron micrographs (SEMs) made of cells after 1 h of Fst exposure also showed cell division defects, predominantly premature separation of cell wall bands (the cell wall protrusions at the center of cells that mark the site of the next cell division) and cell chaining with multiple apparent incomplete and misoriented septae (39). Because samples were prepared at only a single time point, it was impossible to determine whether membrane disruption or aberrant cell division was the primary defect resulting from Fst exposure. To distinguish cell division and membrane permeability defects, cells were simultaneously stained with FI-Van to visualize cell division defects and the membrane-impermeant DNA stain PI to identify cells with membrane defects.

As shown in Fig. 1 (for a color version, see Fig. S1 posted at <http://www.usd.edu/biomed/biomedfaculty/weaver>) cells stained

prior to Fst induction show a pattern of FI-Van staining similar to that previously observed in other members of the streptococcal family (29). The majority of cells were in chains with multiple oval cells showing a bright spot of FI-Van staining between each cell and a centrally placed band of staining marking the position of the new, as yet unconstricted septum. In some cells septal constriction had begun, observed as a bright constricting belt at midcell (for example, the dividing pairs labeled 1, 2, and 3). In these cells, faintly staining secondary bands were symmetrically placed on each side of the constricting band. These faint bands got brighter as constriction of the central band progressed. As expected, no PI staining was detected in uninduced cells.

After 5 to 10 min of Fst induction, the presence of elongated cells could be detected. At 15 min, approximately 10% of cells

FIG. 2. Maximal effects of Fst on cell division and membrane permeability and comparison with SEMs. (A to C) The set of three micrographs shows, from left to right, FI-Van-stained cells, a phase-contrast image of the same field, and an analogous SEM. (A) Uninduced cells show the typical streptococcal cell morphology. Thick and thin arrows show the normal alternation of constricting primary bands and unconstricted secondary bands, respectively. In the SEM, thin arrows mark the cell wall bands that correspond to faintly staining FI-Van bands and the thick arrow marks a constricting septum that corresponds to the brightly staining FI-Van bands. (B) After 45 min of Fst induction bright and faint bands no longer alternate consistently as indicated by the arrows. The arrow in the bright-field image shows a visible cross band that corresponds to a faintly staining FI-Van band. (C) Normal-looking chains with regular constrictions show secondary bands that stain brightly and show visible cell separation in bright-phase images (arrows). These bands correspond to the prematurely separated cell wall bands observed in SEMs (thin arrows). (D) The images show, from left to right, FI-Van staining, PI staining, and an overlay after 45 min of induction. In the overlay, arrows mark two segments of a filament that stain with PI while others do not. SEMs were reproduced from reference 39 with permission. Bar = 2 μ m.

were longer than 2 standard deviations above the length of uninduced cells ($0.85 \pm 0.04 \mu\text{m}$). Frequently the new septae in these elongated cells were off center, and occasionally a second faint band could be observed on one side of the brighter band (see arrow). Other division defects could also be observed, such as cells with brightly staining secondary bands adjacent to incompletely constricted central bands and the beginnings of filaments. One to three percent of cells stained with PI after 15 min, but most cells showing division defects failed to stain with PI.

By 30 min, cell division defects became more prominent, more widespread, and more diverse. Virtually all cells were elongated or showed some other abnormality. Abnormalities included filaments containing multiple partially constricted or unconstricted bands, cells with asymmetrically placed bands, and cells with an even number of bands. Examples of all of these defects can be observed in the chain shown in the top 30-min panel of Fig. 1. Although at this point it became difficult to define where one cell ended and another began, approximately half of the cells showed PI staining. However, cells showing division defects were frequently not stained while ap-

parently normal-looking cells stained brightly. Also, filamented and segmented cells frequently showed staining in one segment but not others (arrows in bottom 30-min panel of Fig. 1).

At 45 min effects were maximal and multiply segmented filaments became the most frequent cell form. At this stage, effects of Fst on FI-Van staining could be compared to previous results obtained with SEM (Fig. 2; for a color version, please see Fig. S2 posted at <http://www.usd.edu/biomed/biomedfaculty/weaver>). Figure 2A compares uninduced FI-Van-stained cells with a SEM of uninduced cells, each showing a typical streptococcal pattern. The centrally located chain in the FI-Van image contains several cells at an early stage of cell division. In uninduced cells such chains invariably showed alternating brightly staining partially constricted bands and lightly staining unconstricted bands. The bright constricted bands correlate with clear separations between cells on the phase-contrast image while the faint bands do not. In the SEM, the bright bands correspond to the constrictions between cells while the faint bands correspond to the centrally located ridges that have been previously referred to as cell wall bands.

Figures 2B to D show typical late effects of Fst induction.

FIG. 4. Cell division and chromosome segregation in the Fst-resistant strain m7. (A) Two panels of typical m7 chains; FI-Van staining is on the left, and bright-field microscopy is on the right. The closed-headed arrows show cells with aberrant septal formation. The open-headed arrow shows a cell with a stretched central division site. (B) Fixed cells stained with FI-Van and PI. This particular chain showed a lot of variability in nucleoid staining and several apparently DNA-free cells. Bars = 2 μ m.

Figure 2B shows filamentation; note that, in contrast to the uninduced cells, bright and faint bands did not always alternate and that faint bands frequently corresponded to cell separations in the phase-contrast image. This pattern corresponds to the multiply partially constricted filaments observed in previous SEMs. Even in chains that looked relatively normal (Fig. 2C), the unconstricted bands were frequently unusually bright and showed separations in phase contrast. In the SEM, this is observed as a premature separation or collapse of cell wall bands. Figure 2D shows a typical field of cells stained with FI-Van and PI after 45 min of Fst expression. Approximately 75% of cells showed some PI staining, but frequently not all segments of a filament showed staining, suggesting either that intact membrane had closed between segments restricting diffusion of the dye or that segments of the same filament might contain differing amounts of DNA.

Fst causes aberrant nucleoid segregation. The unusual distribution of PI staining in the segments of cell filaments suggested that the segregation of chromosomal DNA might be affected by Fst exposure. This possibility was supported by previous transmission electron micrographs that showed cells with misplaced and apparently incomplete complements of chromosomal DNA (39). To better examine the effect of Fst on

DNA segregation, cells induced for Fst expression were simultaneously stained with FI-Van and DAPI, a membrane-permeant DNA stain.

Figure 3A shows the effects of Fst on DAPI staining over time. As expected, in uninduced cells, DAPI staining resolved into well-separated nearly equally staining spots that coincided with the dividing cells. Incompletely divided cells showed a bilobed staining that was constricted at the midpoint. In contrast, induced cells at all time points showed a highly irregular pattern of staining with bright streaks of staining extending over several cells and unequal amounts of DNA in adjacent cells. Even at 15 min, cells lacking apparent staining were observed, and this became more prominent at later time points with multiply segmented cells showing differential staining among the segments, as observed at later time points with PI staining. Frequently it appeared that all or most of the chromosome had segregated into one segment of a multiply segmented cell. To further examine this effect, cells induced for Fst expression for 15 min were fixed with methanol to permeabilize the membranes and then stained with FI-Van and PI (Fig. 3B). Although the fixing procedure decreased the resolution of the FI-Van staining, it was still clear that nucleoid-free cells were present as early as 15 min after Fst induction.

FIG. 5. Fst-dependent growth inhibition of *B. subtilis* cells. (A) Growth in the presence and absence of IPTG-induced Fst production. Fst was produced from an RNA I gene linked to the IPTG-inducible Pspac promoter that had been integrated into the chromosome at the *amyE* locus as described in Materials and Methods. IPTG was added to a final concentration of 1 mM after 30 min of culture. ■, uninduced control; ▲, IPTG induced. (B) Northern blot showing IPTG-dependent production of Fst-encoding RNA I. RNA was purified from uninduced and induced cultures, fractionated, transferred to a membrane, and probed with RNA I and 5S rRNA-specific probes as described in Materials and Methods. Lane 1, uninduced culture; lane 2, IPTG-induced culture.

For color versions of these figures, see Fig. S3 at <http://www.usd.edu/biomed/biomedfaculty/weaver>.

Fst-resistant mutants have altered division and DNA segregation in the absence of Fst. A spontaneous Fst-resistant mutant, designated m7, was previously described (39). The mutant shows a growth defect in the absence of Fst (generation time of 1.08 h as opposed to 0.85 h for the wild type) and is still partially affected by Fst induction (generation time of 1.43 h after induction). This suggests that if the mutation affects the target of Fst, it results in a moderate malfunction of the protein. Alternatively, a second site mutation could provide partial suppression of Fst toxicity.

Somewhat surprisingly, m7 mutants cultured in the absence of Fst (either uninduced or entirely lacking the Fst-encoding plasmid) showed division abnormalities that were similar to but less severe than those observed in the Fst-exposed wild-type strains (Fig. 4A; for a color version, please see Fig. S4A posted at <http://www.usd.edu/biomed/biomedfaculty/weaver>). Elongated cells with multiple brightly staining "septal" bands were frequently observed. However, the number of bands rarely exceeded three and filamentous cells with multiple partially invaginated septae were not observed. Dividing cells sometimes appeared to be stretched or twisted in m7 cells, a feature which was not observed in wild-type induced cells. Cells induced for Fst production for even prolonged periods were indistinguishable from uninduced cells, and neither induced nor uninduced cells showed detectable staining with PI without fixing (data not shown). DAPI staining showed much less variability in m7 whether induced or uninduced (see Fig. S4C in the supplemental material), but the higher resolution allowed by PI staining of fixed cells revealed that about 10% of cells in the presence or absence of Fst appeared to lack chromosomes. Figure 4B (for a color version, please see Fig. S4B posted at <http://www.usd.edu/biomed/biomedfaculty/weaver>)

shows a particularly variable chain. Therefore, it appears that the mutation(s) present in m7 that allows it to resist Fst results in alterations in septal development and chromosomal segregation. It appears that these two phenomena are linked in both Fst toxicity and resistance, suggesting that alteration of a single protein may affect both.

Effects of Fst on *Bacillus subtilis*. Attempts to introduce into *B. subtilis* the RNA I gene encoding Fst on a shuttle vector resulted in deletions of the inserted gene, suggesting that Fst was toxic to this host as well as its native host (data not shown). To test this, a promoterless version of the RNA I gene was cloned downstream of the IPTG-inducible Pspac promoter and inserted into the *B. subtilis* chromosome at the *amyE* locus. The resulting strain, BG565, showed IPTG-induced growth inhibition, and a transcript hybridizing with an RNA I-specific probe was produced in IPTG-induced cultures (Fig. 5). The toxicity of Fst was enhanced by exposure to subinhibitory levels of nisin (data not shown) as has been previously observed in *E. faecalis*, suggesting that the effects of the Fst toxin in *B. subtilis* were similar to those in the native host.

To examine whether the effects of Fst on cell wall growth and chromosome segregation were similar in *E. faecalis* and *B. subtilis*, IPTG-induced BG565 cells were stained with FI-Van and two DNA stains, DAPI and Sytox Green. FI-Van staining in uninduced cells (Fig. 6A) showed a regular pattern of either bipolar staining or septal staining, depending on the division state of the cell. Occasionally chains of cells were observed with multiple septa. The previously observed helical side wall staining (8) was not observed under our conditions, probably because the cells were growing relatively rapidly in rich medium. Growth in the defined medium used in previous studies was not possible here because induction of Fst had less of an effect on growth under these conditions (data not shown). Fst induction did not result in the aberrant division patterns ob-

FIG. 6. Effects of Fst on peptidoglycan synthesis in *B. subtilis*. (A) Typical patterns of FI-Van staining seen under the culture and staining conditions used in this experiment. Staining is predominantly polar and septal with some hazy staining on the side walls that may be indicative of the helices observed under other conditions. Such helices may not be observed here because of the more rapid growth of cells in rich medium. The last panel shows a cell chain which is occasionally observed. (B) Typical microscopic field of cells induced for Fst production 30 min before FI-Van staining. Although many cells appear unaffected by Fst production, some cells show an unusual accumulation of staining on the side walls that results in cell bending (arrows). Occasionally, cells show complete staining in limited regions of the cell (stars). Bars = 5 μ m.

served in *E. faecalis*, but more subtle effects on cell wall growth were observed (Fig. 6B). Frequently, brightly staining patches were observed at positions where the cells were kinked or curved, suggesting that excess new cell wall growth was affecting cell shape. These patches were sometimes but not always located near midcell, where the septum should be. Occasionally, large bright regions were observed within single cells, suggesting that excess side wall synthesis was occurring in these regions. However, many cells showed no obvious defects.

DAPI staining revealed an uneven distribution of DNA similar to what was observed in *E. faecalis* cells, with some cells staining brightly and others staining in relatively isolated spots (see Fig S5 in the supplemental material). To get a better idea

of the localization of DNA within cells, Sytox Green was used in combination with the membrane stain FM4-64. Although Sytox Green is supposed to be a membrane-impermeant stain, and indeed did not stain unfixed *E. faecalis* cells, enough penetration of the dye was observed in *B. subtilis* cells under the growth and staining conditions used here to allow visualization of the nucleoid in unfixed cells (Fig. 7; for a color version, see Fig. S6 posted at <http://www.usd.edu/biomed/biomedfaculty/weaver>). Uninduced cells showed a diffuse, even staining throughout individual cells, occasionally with some increased intensity at the poles as shown in Fig. 7A. In induced cells (Fig. 7B) the nucleoid was condensed either into a single point or into a short helical filament that extended about half the length

FIG. 7. Fst-induced effects on nucleoid structure and distribution in *B. subtilis*. Uninduced (A) and 30-min IPTG-induced (B) cells were stained with the membrane stain FM4-64 and the DNA stain Sytox Green. The rows show FM4-64 staining on the left, Sytox Green staining in the center, and overlays on the right. Sytox Green staining produces a diffuse and even staining extending the length of the cell in uninduced cells. In the majority of induced cells the stain is localized near midcell and appears to be membrane associated. Occasionally, the brightest staining adopts a helical configuration (arrows). Cells show various amounts of background staining in addition to the condensed spots or helices. Bars = 5 μ m.

of the cell. Some additional DNA staining was observed in cells, suggesting that not all the DNA had condensed, and the amount of background staining varied from cell to cell, which probably accounts for the variability in DAPI staining. Cells lacking DNA were occasionally seen but were rare (data not shown). Importantly, in contrast to the Fl-Van staining, effects on DNA staining were universal. Images shown in Fig. 7 were obtained after 30 min of induction, but the effects were observed almost immediately after addition of IPTG. Unlike Sytox Green, PI did not penetrate uninduced unfixed cells, but stained approximately one-third to one-half of induced cells (see Fig. S7 in the supplemental material), indicating that, as in *E. faecalis*, membrane permeability effects were not as widespread as the alterations in DNA.

DISCUSSION

The results presented in this report show that the *E. faecalis* plasmid pAD1 addiction module toxin Fst was toxic to *B. subtilis* as well as its native host and that effects on nucleoid structure, segregation, and cell division were observed in both species. Also in both species, the division and segregation effects preceded effects on membrane permeability, suggesting that Fst does not function primarily as a pore-forming toxin in either host. The nucleoid effects appeared to be more consistent than the cell division defects, particularly in *B. subtilis*,

where virtually all cells showed effects on nucleoid structure but less than half of the cells showed relatively minor effects on peptidoglycan synthesis. In both species, distribution of chromosomal DNA to daughter cells appeared to be uneven. This was particularly apparent in *E. faecalis*, where it frequently appeared that nearly all chromosomal DNA was partitioned into one daughter cell with little or no DNA remaining in the other daughter cell. This was apparently not due to the presence of only a single chromosome in these dividing cells, since effects on partition were observed as soon as 15 min after Fst induction, whereas DNA replication continues for 40 to 45 min. Thus, it would appear either that the two chromosomes are incompletely separated (e.g., by failure of topoisomerases to unlink the copies or by failure to resolve dimers) prior to being partitioned or that the partitioning apparatus mistakenly partitions both chromosomes to a single daughter cell. Both the DAPI staining and previous electron micrographs indicated that some small fragments of DNA may be left behind in the less fortunate daughter cell. Whether this is a specific chromosomal locus that remains attached to some membrane structure or is just random DNA pinched off during aberrant division cycles is unclear.

While unequal DNA segregation was also observed in *B. subtilis*, the most distinctive characteristic of the effect of Fst on this species was an apparent condensation of most of the DNA

in the cell to a single focus. Most frequently these foci were single spots located near the midpoint of the cell, but short arcs and helices were also commonly observed. Such a condensation of the nucleoid was not observed in *E. faecalis* even after prolonged incubation, but this may simply reflect an inability to adequately resolve the nucleoid within the smaller spherical cells of this species. However, the possibility that differences in cell cytoskeleton between the two species might also affect differences in the effects of Fst on nucleoid structure should be considered. In particular, *B. subtilis* genes encode a number of actin homologs which form helical structures at the cell surface that may be involved in both peptidoglycan synthesis and chromosome partitioning (8, 34). *E. faecalis*, like other spherical cells, appears to lack such proteins, which might account for the difference in chromosomal appearance compared to *B. subtilis* cells. Of course, it is also possible that Fst targets different proteins or the same protein with different effects in the two hosts, but the fact that Fst induction in both hosts leads to hypersensitivity to nisin suggests that the effects are related.

Fst's effects on cell division in *E. faecalis* were quite dramatic, with the organism displaying cellular elongation, hyperseptation, and altered septal placement even at early time points. Morlot et al. previously proposed a model of cell wall growth and division for *Streptococcus pneumoniae* based on visual localization of penicillin binding and other cell division proteins (27) which probably applies at least in broad outline to *E. faecalis* as well. This model requires coordination of lateral cell wall growth, septal formation, and constriction. Fst exposure seemed to affect primarily the constriction phase and also appeared to uncouple lateral wall growth and septal placement, resulting in variability in the spacing of septal planes. It is possible that all of these effects are secondary effects of the improper segregation of chromosomal DNA. It is known that in both *E. coli* and *B. subtilis* the presence of DNA near the normal division site can suppress the earliest phases of cell division, a phenomenon known as nucleoid occlusion (2, 40). However, it should be noted that, if nucleoid occlusion does occur in the chain-forming streptococci, it must differ in its particulars from that in rod-shaped organisms. Thus, nucleoid occlusion in *B. subtilis* and *E. coli* occurs at the formation of the FtsZ ring before septal synthesis begins, but in *S. pneumoniae* and *E. faecalis* synthesis of the next septal peptidoglycan rings occurs early during the preceding cell division and presumably before DNA segregation. The presence of excess, improperly segregated DNA could, however, favor lateral growth over septal formation and/or inhibit septal in-growth, a feature apparent in Fst-exposed cells. Conversely, the absence of a complete DNA complement could release nucleoid occlusion, leading to increased lateral and septal wall formation, features also observed in Fst-exposed cells. Interestingly, preliminary results indicate that exposure to nalidixic acid results in cell division defects similar to, though less severe than, those caused by Fst (data not shown).

Alternatively, Fst could affect a protein involved in both chromosomal segregation and septal formation. For example, SpoIIIE/FtsK has been shown to be involved in septum formation; separation of chromosomal catenanes and dimers through interaction with topoisomerase IV and XerCD/dif, respectively; and translocation of chromosomal copies to daughter cells (3, 11, 22, 25). The genome sequence of *E.*

faecalis V583 contains five SpoIIIE homologs (31). The observation that the Fst-resistant mutant showed alterations in both septum formation and chromosome partitioning might support the hypothesis that Fst targets a protein common to both processes. The mutation leading to resistance could reduce the affinity of the target for Fst but also alter and/or compromise its function in both septation and partition. Of course, this assumes that resistance is due to a single mutation in the Fst target rather than to multiple mutations in genes other than the target, resulting in suppression of the toxin's effects, an assumption currently without experimental support.

Finally, any model of Fst toxicity and resistance must account for the fact that exposure to Fst sensitizes cells to nisin and Fst-resistant cells are cross-resistant to nisin (39). Nisin functions primarily as a pore-forming peptide (12), and it was originally proposed that nisin and Fst might act in concert to depolarize the bacterial membrane. The current results cast doubt on this proposal since Fst's primary effect did not appear to be on membrane integrity. However, nisin is also known to use lipid II as a docking molecule and to perturb peptidoglycan synthesis (4). Given the fact that Fst exposure altered the pattern of peptidoglycan synthesis in both *E. faecalis* and *B. subtilis*, it is possible that this is the source of the synergistic effect of the two toxins. Interestingly, even though the Fst-resistant mutant shows some features reminiscent of the Fst-induced wild-type strain, it is resistant to nisin, suggesting perhaps that a fundamental change in peptidoglycan synthesis has occurred.

In summary, Fst is a peptide toxin that simultaneously affects chromosomal segregation and cell division/peptidoglycan synthesis in both *E. faecalis* and *B. subtilis*. Since Fst contains a hydrophobic stretch of amino acids predicted to form a transmembrane domain, it seems likely that its target is located at or near the cell membrane. Whether this target affects only DNA segregation directly or affects both DNA segregation and cell division remains to be determined.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Francis Day and Volker Brozel, who provided essential assistance with the microscopy; David Bechhofer and Irina Oussenko for providing *B. subtilis* strains and expertise in dealing with them; and Chao Tang, Erik Ehli, Sonia Chahal, and Shirisha Reddy from our laboratory, who helped with the conduct of experiments.

This work was supported by Public Health Service grant GM55544.

REFERENCES

1. Bernard, P., and M. Couturier. 1992. Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J. Mol. Biol.* **226**:735–745.
2. Bernhardt, T. G., and P. A. J. de Boer. 2005. SImA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. *Mol. Cell* **18**:555–564.
3. Bigot, S., J. Corre, J.-M. Louarn, F. Cornet, and F.-X. Barre. 2004. FtsK activities in Xer recombination, DNA mobilization and cell division involve overlapping and separate domains of the protein. *Mol. Microbiol.* **54**:876–886.
4. Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H.-G. Sahl, and B. de Kruijff. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**:2361–2364.
5. Buts, L., J. Lah, M.-H. Dao-Thi, L. Wyns, and R. Loris. 2005. Toxin-antitoxin modules as bacterial metabolic stress managers. *Trends Biochem. Sci.* **30**:672–679.
6. Christensen, S. K., G. Maenhaut-Michel, N. Mine, S. Gottesman, K. Gerdes, and L. Van Melderen. 2004. Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the *yefM-yoeB* toxin-antitoxin system. *Mol. Microbiol.* **51**:1705–1717.

7. Christensen, S. K., K. Pedersen, F. G. Hansen, and K. Gerdes. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J. Mol. Biol.* **332**:809–819.
8. Daniel, R. A., and J. Errington. 2003. Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell* **113**:767–776.
9. Dao-Thi, M.-H., L. Van Melder, E. De Genst, H. Afif, L. Buts, L. Wyns, and R. Loris. 2005. Molecular basis of gyrase poisoning by the addiction toxin CcdB. *J. Mol. Biol.* **348**:1091–1102.
10. Dunny, G. M., B. A. Leonard, and P. J. Hedberg. 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication. *J. Bacteriol.* **177**:871–876.
11. Espeli, O., C. Lee, and K. J. Marians. 2003. A physical and functional interaction between *Escherichia coli* FtsK and topoisomerase IV. *J. Biol. Chem.* **278**:44639–44644.
12. Garcera, M. J., M. G. Elferink, A. J. Driessen, and W. N. Konings. 1993. In vitro pore-forming activity of the lantibiotic nisin. Role of protonmotive force and lipid composition. *Eur. J. Biochem.* **212**:417–422.
13. Gerdes, K., F. W. Bech, S. T. Jorgensen, A. Lobner-Olesen, P. B. Rasmussen, T. Atlung, L. Boe, O. Karlstrom, S. Molin, and K. von Meyenburg. 1986. Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli* *relB* operon. *EMBO J.* **5**:2023–2029.
14. Gerdes, K., S. K. Christensen, and A. Lobner-Olesen. 2005. Prokaryotic toxin-antitoxin stress response loci. *Nat. Rev. Microbiol.* **3**:371–382.
15. Gerdes, K., P. B. Rasmussen, and S. Molin. 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. *Proc. Natl. Acad. Sci. USA* **83**:3116–3120.
16. Grady, R., and F. Hayes. 2003. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. *Mol. Microbiol.* **47**:1419–1432.
17. Greenfield, T. J., E. Ehli, T. Kirshenmann, T. Franch, K. Gerdes, and K. E. Weaver. 2000. The antisense RNA of the *par* locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. *Mol. Microbiol.* **37**:652–660.
18. Greenfield, T. J., T. Franch, K. Gerdes, and K. E. Weaver. 2001. Antisense RNA regulation of the *par* post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNAII and its target, RNAI. *Mol. Microbiol.* **42**:527–537.
19. Greenfield, T. J., and K. E. Weaver. 2000. Antisense RNA regulation of the pAD1 *par* post-segregational killing system requires interaction at the 5' and 3' ends of the RNAs. *Mol. Microbiol.* **37**:661–670.
20. Hayes, F. 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* **301**:1496–1499.
21. Ike, Y., R. A. Craig, B. A. White, Y. Yagi, and D. B. Clewell. 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. *Proc. Natl. Acad. Sci. USA* **80**:5369–5373.
22. Ip, S. C. Y., M. Bregu, F.-X. Barre, and D. J. Sherratt. 2003. Decatenation of DNA circles by FtsK-dependent Xer site-specific recombination. *EMBO J.* **22**:6399–6407.
23. Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman. 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev.* **7**:283–294.
24. Jiang, Y., J. Pogliano, D. R. Helinski, and I. Konieczny. 2002. ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. *Mol. Microbiol.* **44**:971–979.
25. Levy, O., J. L. Ptacin, P. J. Pease, J. Gore, M. B. Eisen, C. Bustamante, and N. R. Cozzarelli. 2005. Identification of oligonucleotide sequences that direct the movement of the *Escherichia coli* FtsK translocase. *Proc. Natl. Acad. Sci. USA* **102**:17618–17623.
26. Miki, T., J. A. Park, K. Nagao, N. Murayama, and T. Horiuchi. 1992. Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*: mutants of DNA gyrase subunit A suppress *letD* (*ccdB*) product growth inhibition. *J. Mol. Biol.* **225**:39–52.
27. Morlot, C., M. Noirclerc-Savoye, A. Zapun, O. Dideberg, and T. Vernet. 2004. The d,d-carboxypeptidase PBP3 organizes the division process of *Streptococcus pneumoniae*. *Mol. Microbiol.* **51**:1641–1648.
28. Munoz-Gomez, A. J., M. Lemonnier, S. Santos-Sierra, A. Berzal-Herranz, and R. Diaz-Orejas. 2005. RNase/anti-RNase activities of the bacterial *parD* toxin-antitoxin system. *J. Bacteriol.* **187**:3151–3157.
29. Ng, W.-L., K. M. Kazmierczak, and M. E. Winkler. 2004. Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. *Mol. Microbiol.* **53**:1161–1175.
30. Oussenko, I. A., T. Abe, H. Ujiie, A. Muto, and D. H. Bechhofer. 2005. Participation of 3'-to-5' exonucleases in the turnover of *Bacillus subtilis* mRNA. *J. Bacteriol.* **187**:2758–2767.
31. Paulsen, I. T., L. Banerjee, G. S. A. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299**:2071–2074.
32. Pedersen, K., A. V. Zavialov, M. Y. Pavlov, J. Elf, K. Gerdes, and M. Ehrenberg. 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**:131–140.
33. Pimentel, B., M. A. Madine, and G. de la Cueva-Mendez. 2005. Kid cleaves specific mRNAs at UUACU sites to rescue the copy number of plasmid R1. *EMBO J.* **24**:3459–3469.
34. Soufo, H. J. D., and P. L. Graumann. 2003. Actin-like proteins MreB and Mbl from *Bacillus subtilis* are required for bipolar positioning of replication origins. *Curr. Biol.* **13**:1916–1920.
35. Weaver, K. E., and D. B. Clewell. 1989. Construction of *Enterococcus faecalis* pAD1 miniplasmids: identification of a minimal pheromone response regulatory region and evaluation of a novel pheromone-dependent growth inhibition. *Plasmid* **22**:106–119.
36. Weaver, K. E., E. A. Ehli, J. S. Nelson, and S. Patel. 2004. Antisense RNA regulation by stable complex formation in the *Enterococcus faecalis* plasmid pAD1 *par* addition system. *J. Bacteriol.* **186**:6400–6408.
37. Weaver, K. E., K. D. Jensen, A. Colwell, and S. I. Sriram. 1996. Functional analysis of the *Enterococcus faecalis* plasmid pAD1-encoded stability determinant *par*. *Mol. Microbiol.* **20**:53–63.
38. Weaver, K. E., K. D. Walz, and M. S. Heine. 1998. Isolation of a derivative of *Escherichia coli*-*Enterococcus faecalis* shuttle vector pAM401 temperature sensitive for maintenance in *E. faecalis* and its use in evaluating the mechanism of pAD1 *par*-dependent plasmid stabilization. *Plasmid* **40**:225–232.
39. Weaver, K. E., D. M. Weaver, C. L. Wells, C. M. Waters, M. E. Gardner, and E. A. Ehli. 2003. *Enterococcus faecalis* plasmid pAD1-encoded Fst toxin affects membrane permeability and alters cellular responses to lantibiotics. *J. Bacteriol.* **185**:2169–2177.
40. Wu, L. J., and J. Errington. 2004. Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. *Cell* **117**:915–925.
41. Zhang, J., Y. Zhang, L. Zhu, M. Suzuki, and M. Inouye. 2004. Interference of mRNA function by sequence-specific endoribonuclease PemK. *J. Biol. Chem.* **279**:20678–20684.
42. Zhang, Y., J. Zhang, K. P. Hoeflich, M. Ikura, G. Qing, and M. Inouye. 2003. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Mol. Cell* **12**:913–923.
43. Zhang, Y., L. Zhu, J. Zhang, and M. Inouye. 2005. Characterization of ChpBK, an mRNA interferase from *Escherichia coli*. *J. Biol. Chem.* **280**:26080–26088.
44. Zielenkiewicz, U., and P. Ceglowski. 2005. The toxin-antitoxin system of the streptococcal plasmid pSM19035. *J. Bacteriol.* **187**:6094–6105.