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Isolation and Characterization of *Bacillus subtilis* Genes Involved in Siderophore Biosynthesis: Relationship between *B. subtilis sfp*^{0} and *Escherichia coli entD* Genes

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In response to iron deprivation, *Bacillus subtilis* secretes a catecholic siderophore, 2,3-dihydroxybenzoyl glycine, which is similar to the precursor of the *Escherichia coli* siderophore enterobactin. We isolated two sets of *B. subtilis* DNA sequences that complemented the mutations of several *E. coli* siderophore-deficient (*ent*) mutants with defective enterobactin biosynthesis enzymes. One set contained DNA sequences that complemented only an *entD* mutation. The second set contained DNA sequences that complemented various combinations of *entB*, *entE*, *entC*, and *entA* mutations. The two sets of DNA sequences did not appear to overlap. A *B. subtilis* mutant containing an insertion in the region of the *entD* homolog grew much more poorly in low-iron medium and with markedly different kinetics. These data indicate that (i) at least five of the siderophore biosynthesis genes of *B. subtilis* can function in *E. coli*, (ii) the genetic organization of these siderophore genes in *B. subtilis* is similar to that in *E. coli*, and (iii) the *B. subtilis entD* homolog is required for efficient growth in low-iron medium. The nucleotide sequence of the *B. subtilis* DNA contained in plasmid pENTA22, a clone expressing the *B. subtilis entD* homolog, revealed the presence of at least two genes. One gene was identified as *sfp*^{0}, a previously reported gene involved in the production of surfactin in *B. subtilis* and which is highly homologous to the *E. coli entD* gene. We present evidence that the *E. coli entD* and *B. subtilis sfp*^{0} genes are interchangeable and that their products are members of a new family of proteins which function in the secretion of peptide molecules.

Iron is essential to the growth of virtually all organisms (for reviews, see references 9 and 20). However, iron is not readily available in most biological systems. At neutral pH, iron forms insoluble salts, and in serum and secretory fluids, iron is tightly sequestered by host carrier proteins. Microorganisms have evolved various mechanisms to acquire iron when confronted with iron-limiting conditions. Many microorganisms are capable of synthesizing and secreting siderophore molecules in response to iron deprivation. Siderophores bind to ferric ions with high affinity and are then reinternalized via specific receptor molecules found at the cell surface. Ferric iron is reduced to the ferrous form in the cell.

Much of what is known about iron transport in bacteria has been derived from studies with gram-negative bacteria, especially *Escherichia coli* (20). In contrast, little is known about the mechanism(s) of iron acquisition in gram-positive bacteria. Because of the relative ease of genetic manipulation, we have chosen to use *Bacillus subtilis* as a model system to study gram-positive bacterial iron transport.

In response to iron deprivation, *E. coli* and *B. subtilis* both produce catecholic siderophores which are very similar in structure (Fig. 1) (4, 7). *E. coli* produces the siderophore enterobactin (Fig. 1A). The products of the *entC*, *entB*, and *entA* genes enzymatically convert chorismate to dihydroxybenzoate. Dihydroxybenzoate is then converted to a cyclic trimer of 2,3-dihydroxybenzoyl serine, i.e., enterobactin, by an enterobactin synthetase complex which is composed of the *entD*, *entE*, and *entF* gene products and the EntG activity encoded by the *entB* gene (14, 23). At present, this last stage of enterobactin biosynthesis is poorly understood. The genes responsible for enterobactin biosynthesis and utilization are clustered in several transcriptional units present on a 22-kb segment near min 13 of the *E. coli* chromosome. These genes are coordinately regulated in response to iron availability.

Because of the biochemical similarities of 2,3-dihydroxybenzoyl serine in *E. coli* and 2,3-dihydroxybenzoyl glycine in *B. subtilis* (Fig. 1B), we attempted to isolate *B. subtilis ent* genes by complementing *E. coli* enterobactin biosynthesis mutations with a plasmid library containing *B. subtilis* chromosomal DNA. We isolated two sets of *B. subtilis* DNA sequences with Ent$^+$ complementing activity. Here we describe the cloning of these genes, their genetic organization, and the nucleotide sequence of a DNA fragment containing the *B. subtilis entD* homolog. We also describe the construction and phenotype of a *B. subtilis* mutant containing an insertion in the region of the *entD* homolog. Finally, we demonstrate the structural similarity and functional interchangeability of gene products involved in the production of siderophore from *E. coli* and siderophore and surfactin from *B. subtilis*. On the basis of these findings and the observed similarities in their predicted amino acid sequences, we suggest that the *E. coli entD* gene, the *B. subtilis sfp* and *sfp*^{0} genes, and an uncharacterized open reading frame in a *Bacillus brevis* gramicidin S biosynthesis operon, *orfX*, belong to a family of genes that perform analogous functions in the secretion of short peptide molecules.

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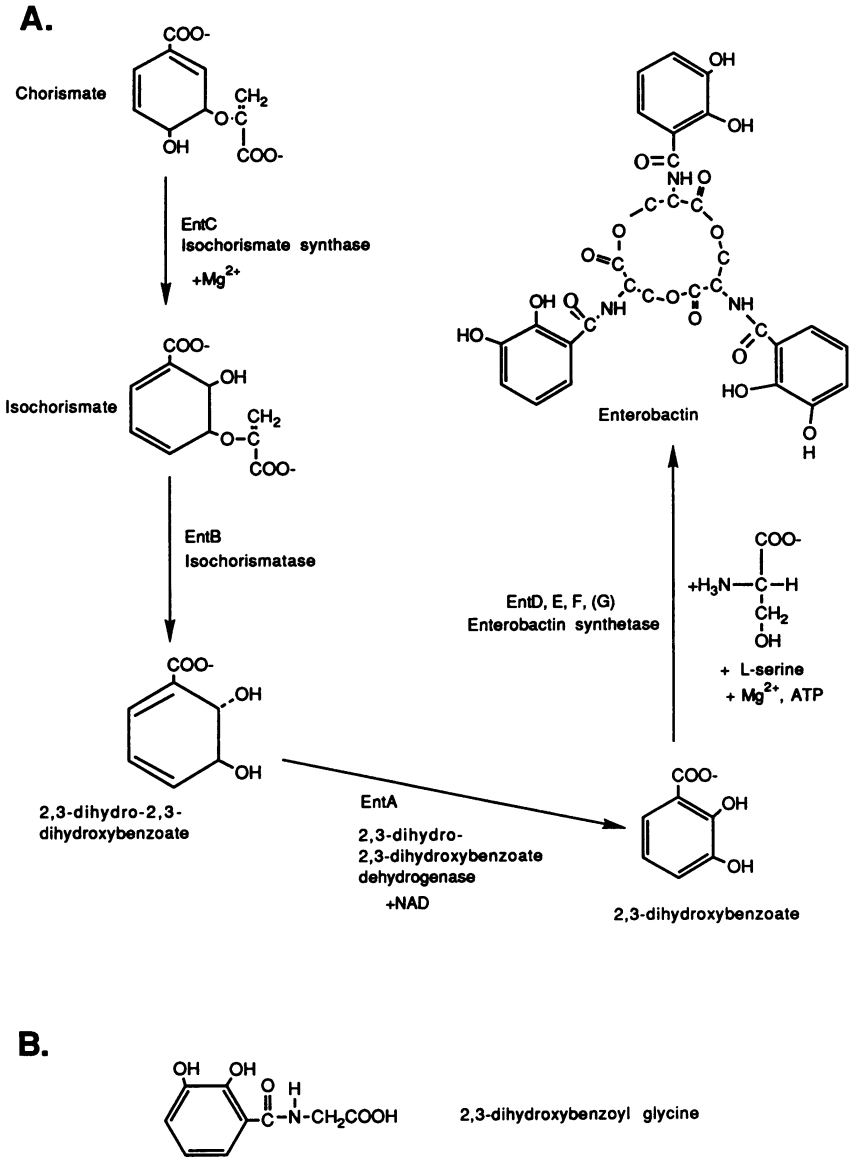

FIG. 1. (A) Biosynthesis of enterobactin in *E. coli*. The *ent* genes and corresponding enzymes which catalyze the steps of enterobactin biosynthesis are indicated. (B) Structure of 2,3-dihydroxybenzoyl glycine from *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. Bacterial strains, plasmids, bacteriophage, and their relevant properties are listed in Table 1. Our plasmid library contains *Sau*3AI fragments of *B. subtilis* 168 (*Bacillus* Genetic Stock Center strain 1A1) chromosomal DNA cloned into the *Bam*HI site of plasmid pBR322, inactivating the *tet* gene. Cells containing recombinant plasmids were selected by ampicillin resistance encoded by pBR322. The average insert size of the library is 6.9 kb.

Media, chemicals, and cell growth. Cells were routinely grown at 37°C with aeration or on agar plates. LB medium (13) was used as the standard iron-replete medium. LB medium treated either with Chelex 100 resin (25) or with ethylenediamine-N,N'-diacetic acid (EDDA) (17) was used as the iron-poor medium. Chrome azurol S (CAS) agar plates (21) were used to detect siderophore secretion by *E. coli*. When appropriate, media were supplemented with ampicillin or kanamycin (50 μg/ml), chloramphenicol (5 μg/ml for chromosomally encoded resistance and 10 mg/ml for episomally encoded resistance), or a combination of erythromycin (1 μg/ml) and lincomycin (25 μg/ml). Growth was estimated by measuring optical density at 600 nm or by using a Klett-Summerson colorimeter (green filter). Sporulation was in DSM medium (22). All chemicals were obtained from standard commercial sources.

Molecular biological methods. Standard recombinant DNA techniques were used to construct plasmids created in this study (12). Restriction enzymes and other reagents were obtained from standard commercial sources.

Transformation. *E. coli* was either transformed by electroporation, using a Bio-Rad unit according to the manufactur-

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or bacteriophage	Relevant genotype or phenotype	Source and/or reference
E. coli		
DH5α	*endA1 gyrA96 hsdR17 supE44 thi-1 recA1 relA1 ΔlacU169*(ϕ80*lacZ*ΔM15)	Laboratory stock
AN193-59	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Tn*10*::Δ*recA entA*	Mark McIntosh
AN192-60	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Δ*recA entB*	Mark McIntosh
MT147	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Δ*recA entC*::*kan*	Mark McIntosh
AN90-60	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Δ*recA entD*	Mark McIntosh
AN93-60	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Δ*recA entE*	Mark McIntosh
AN117-60	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA* Δ*recA entF*	Mark McIntosh
B. subtilis		
168	*trpC2*	Laboratory collection
168C	Chloramphenicol-resistant, Ent⁻	This study
168CTrp⁺	Spontaneous Trp⁺ revertant of 168C	This study
YB886 Din23	*metB5 trpC2 xin-1* SPβ⁻ *din*::Tn*917-lacZ*	R. Yasbin
Plasmids		
pHP13	Shuttle plasmid, *cat* and *ermC*	BGSC[a]
pENTA22	Contains *B. subtilis* DNA encoding an EntD-complementing activity	This study
pENT4	Contains *B. subtilis entA*, *entB*, *entE*, and *entC* homologs	This study
pENTA22H1	Same as pENTA22, except EntD-complementing activity is disrupted by mini-*kan* Tn*10* transposon.	This study
pENTA22HC1	Same as pENTA22H1 except *cat* from pHP13 inserted into *kan* gene	This study
pITS21	pBR328 vector containing *E. coli entD*, *fepA*, *fes*, and *entF* genes	M. McIntosh (19)
pITS21::pUB110	Shuttle plasmid, resistant to ampicillin in *E. coli* and neomycin in *B. subtilis*	This study[b]
pUB110	Encodes resistance to neomycin in *B. subtilis*	Laboratory collection (10)
Bacteriophage λ1105	λ phage containing mini-*kan* Tn*10* and Tn*10* transposase under p*tac* control	N. Kleckner (28)

[a] BGSC, *Bacillus* Genetic Stock Center.

[b] Plasmid pITS21::pUB110 was created by digesting pITS21 with enzyme *Kpn*I within *entF*, removing the sticky ends with the Klenow fragment of DNA polymerase I and ligating the entire plasmid into plasmid pUB110 which was digested with enzyme *Pvu*II. The ligated plasmid was transformed into strain AN90-60. Ampicillin-resistant transformants surrounded by an orange halo on CAS agar plates (21) were selected, and plasmids were screened for the expected restriction pattern.

er's instructions, or made competent by standard calcium chloride methods (12). *B. subtilis* was transformed by growing cells to natural competence (22).

Genetic mapping in *B. subtilis*. Chromosomal mapping was performed both by PBS1 transduction and by transformation. PBS1 transduction was performed by the method of Takahashi (24), except that the recipient cells were grown in brain heart infusion medium obtained from Difco. The Tn*917* mapping kit and various auxotrophic strains were obtained from the *Bacillus* Genetic Stock Center. Chromosomal DNA isolated from strain 168CTrp⁺ was transformed into competent *B. subtilis* strains to determine closer genetic linkages.

Transposon mutagenesis of a plasmid containing *B. subtilis* EntD activity. *E. coli* DH5α(pENTA22) cells were transfected with bacteriophage λ1105 DNA by the method previously described (28). Transfected cells were spread onto LB plates containing ampicillin (to maintain the plasmid) and kanamycin (to select for transposon insertion). Ampicillin-and-kanamycin-resistant colonies were pooled, and plasmid DNA was isolated. The plasmid pool was then transformed into strain AN90-60. Transformants were selected on CAS agar containing ampicillin and kanamycin. Plasmids from ampicillin-and-kanamycin-resistant transformants which lacked orange halos on CAS agar (i.e., displayed an Ent⁻ phenotype) were examined by restriction enzyme analysis to confirm transposition events into the *B. subtilis* DNA of plasmid pENTA22.

Construction of a *B. subtilis* Ent⁻ mutant. A DNA fragment containing the *cat* gene of plasmid pHP13 was isolated following digestion of the plasmid with restriction enzymes *Dra*I and *Sma*I. This fragment was ligated into the *Xho*I site (within the Kan[r] gene) of the vector pENTA22H1. The recessed ends of the *Xho*I site were first filled in with the Klenow fragment of DNA polymerase I. Ligated DNA was transformed into *E. coli* DH5α, and transformants resistant to both ampicillin and chloramphenicol were selected. Transformants were then screened by restriction analysis, resulting in the identification of plasmid pENTA22HC1. This plasmid was transformed into competent *B. subtilis* 168, and chloramphenicol-resistant transformants were selected.

Nucleotide sequence determination and analysis. The *B. subtilis* DNA contained in plasmid pENTA22 was sequenced with Sequenase version 2.0 kit (U.S. Biochemicals) according to the manufacturer's recommendations. The template was denatured double-stranded DNA (12). Unidirectional exonuclease III deletions of plasmid pENTA22 were generated with the Promega Erase-a-Base system. All primers were synthesized in-house. Both strands of pENTA22 were sequenced to ensure accuracy. Nucleotide sequence compilation, analysis, and comparative searches were done by using the Genetics Computer Group (1992, version 7.2, Madison, Wisconsin) SEQED, MAP, GAP, and FASTA programs and GenBank and Swissprot data bases.

Detection of surfactin production. Surfactin production was detected by hemolysis of erythrocytes, using a modified version of the method reported by Nakano et al. (15). *B. subtilis* cells were streaked on thinly poured fresh LB plates containing neomycin (10 μg/ml) and defibrinated sheep blood (5% [vol/vol]) and incubated at 37°C for 24 to 48 h. Streaks of surfactin-producing cells were surrounded by a clear halo, indicating hemolysis.

Nucleotide sequence accession number. The nucleotide

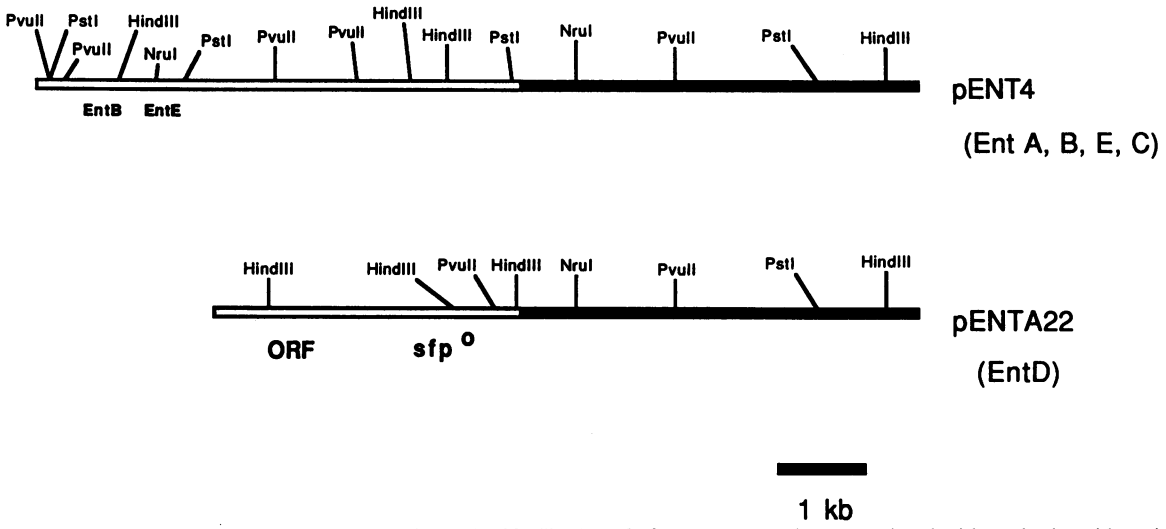

FIG. 2. Restriction maps of plasmids pENT4 and pENTA22. The *B. subtilis ent* gene analogs associated with each plasmid are indicated. The presence of *entA*, *-B*, *-E*, and *-C* analogs on plasmid pENT4 was inferred from the results of genetic complementation experiments, as described in the text. The *entB* and *entE* analogs have been precisely located, as indicated. The precise locations of *entA* and *entE* analogs have not been determined. The white portion of the plasmid represents *B. subtilis* chromosomal DNA, and the dark portion of the plasmid represents pBR322 DNA.

sequence reported here is available in the GenBank data base under accession number L17438.

RESULTS

Isolation of *B. subtilis* DNA sequences containing Ent$^+$ complementing activities. Six *E. coli ent* mutants (Table 1) were transformed with our *B. subtilis* plasmid library. Transformants were selected for ampicillin resistance on CAS agar plates. The normal blue color of CAS agar changes to orange when iron is removed from CAS dye by a chelator, such as a siderophore (21). *E. coli ent* mutants with defective enterobactin biosynthesis enzymes were unable to produce orange halos on CAS agar. However, approximately 1% of the transformants formed colonies that produced orange halos, indicating that siderophore was secreted as a result of genetic complementation.

Seven independent DNA sequences, each able to complement at least one *E. coli ent* mutation, were then transformed into all six *E. coli ent* mutants to determine, by genetic complementation, whether these DNA sequences encoded other *ent*-like genes. Complementation was defined as the ability of ent mutants to produce orange halos on CAS agar. The six DNA sequences fell into two sets; one set complemented only *entD* mutations, and the other set complemented either *entB* mutations only, *entB* and *entE* mutations, *entB*, *entE*, and *entC* mutations, or *entA*, *entB*, *entE*, and *entC* mutations. Restriction maps of a representative plasmid from each set, pENTA22 and pENT4, are shown in Fig. 2. Restriction patterns and Southern hybridization analyses indicate overlap among the cloned DNA sequences within each set but no overlap between the two sets.

Construction and characterization of a *B. subtilis* Ent$^-$ mutant. As a first step in determining the roles of the cloned genes in their native host, we inactivated the EntD activity encoded by plasmid pENTA22 and transformed the plasmid encoding the mutated gene back into *B. subtilis*. Plasmid pENTA22 was mutagenized as described in Materials and Methods. Two independent insertions which inactivated EntD activity were localized on the same 0.6-kb *Pvu*I restriction fragment located within the *B. subtilis* DNA of pENTA22. One plasmid containing an inactivating insertion, pENTA22H1, was chosen. Plasmid pENTA22H1 failed to complement an *E. coli entD* mutant gene *entD* cells containing this plasmid could not form halos on CAS agar.

The next step was to transform the interrupted gene(s) on plasmid pENTA22H1 into the *B. subtilis* chromosome. Because the *kan* marker in the transposon did not appear to be expressed in *B. subtilis*, we first inserted the *cat* gene from plasmid pHP13 into the transposon of pENTA22H1, forming plasmid pENTA22HC1 (see Materials and Methods). Plasmid pENTA22HC1 was transformed into competent *B. subtilis* 168. Since pENTA22HC1 cannot replicate in *B. subtilis*, it was expected that the plasmid would integrate into the chromosome by virtue of homologous recombination between *B. subtilis* DNA on the plasmid and on the chromosome. Southern hybridization analysis confirmed that one chloramphenicol-resistant transformant, 168C, contained the *cat* gene inserted by a double crossover event at the expected location in the cloned fragment (not shown).

We next measured the ability of *B. subtilis* 168C to grow in iron-poor medium. Strain 168C and its parent strain, 168, were grown overnight in LB medium, washed in saline, diluted into either LB medium (iron replete) or LB medium containing 120 μM EDDA (iron poor), and growth was monitored. Both strains grew equally well in LB medium. In the presence of EDDA, the growth curve of the parent strain, 168, was diauxic (Fig. 3), suggesting that the parent strain could produce and utilize siderophore once iron was depleted from the growth medium. Strain 168C, however, failed to grow once iron was depleted from the medium.

We also tested the abilities of strains 168 and 168C to germinate in low-iron medium. Spores of the parent strain were able to germinate, and the resulting cells grew to moderate density in LB medium containing 120 μM EDDA, whereas spores of the mutant strain did not germinate in this medium. The media were inoculated with 10^6 spores. Following incubation at 37°C with shaking for 24 h, growth was

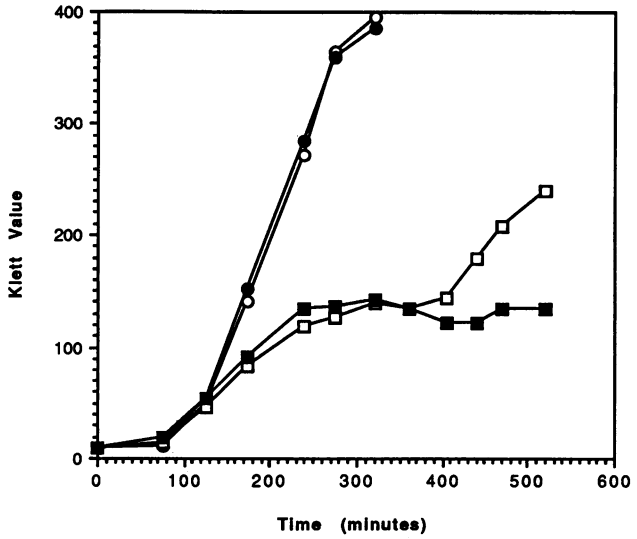

FIG. 3. Growth of *B. subtilis* Ent⁻ mutant 168C in low-iron medium. *B. subtilis* strains were grown overnight at 37°C in LB medium. The next day, cells were diluted into either LB medium or LB medium containing 120 μM EDDA (Klett value of 10), and allowed to grow. Symbols: ○, 168 grown in LB medium; □, 168 grown in LB medium plus EDDA; ●, 168C grown in LB medium; ■, 168C grown in LB medium plus EDDA.

determined by measuring the optical density at 600 nm of each culture. In LB medium, the optical densities were 1.783 for strain 168 and 1.772 for strain 168C. In LB medium containing 120 μM EDDA, the optical densities were 0.499 for strain 168 and 0.055 for strain 168C. Mutant and parent strains sporulated equally well in DSM medium (not shown), indicating that the mutation itself had no apparent effect on sporulation.

Similar results were obtained when Chelex-treated LB was used as the iron-poor medium. In these experiments, all cations removed by the resin were added back to the medium before use, except for iron.

These data strongly suggest that the product of the *entD* homolog is essential for iron transport by *B. subtilis* in iron-poor medium. However, it cannot be ruled out that the insertion in strain 168C may be a polar mutation affecting the function(s) of an element(s) downstream essential for iron transport.

We observed that the mutant strain formed filaments after several hours of incubation in iron-depleted medium. We therefore investigated the possibility that the *B. subtilis* SOS-like response was induced in the mutant upon iron deprivation. *B. subtilis* Din23, containing a *din*::Tn*917-lacZ* fusion (10), is known to produce an SOS-like response, reflected by the production of β-galactosidase, when treated with a variety of DNA-damaging agents and DNA synthesis inhibitors (10, 18). Strain Din23 was transformed with chromosomal DNA from strain 168C, and chloramphenicol-resistant transformants were selected and shown to have an iron-related phenotype identical to that of the original 168C mutant. Neither the parent strain Din23 nor strain Din23 encoding the iron mutation produced β-galactosidase when grown in high- or low-iron medium, whereas they both produced β-galactosidase when treated with novobiocin. This result suggests that the filamentation exhibited by strain 168C in low-iron medium is unrelated to the SOS-like response. It has been established previously that filamentation in *B. subtilis* is not always linked to induction of the SOS regulon (11).

Nucleotide sequence of pENTA22. The nucleotide sequence of the *B. subtilis* DNA contained in plasmid pENTA22 is shown in Fig. 4. The entire sequence was analyzed for homologies to previously reported DNA and/or protein sequences, using the Genetics Computer Group FASTA program. At least two open reading frames were identified. The predicted amino acid sequence of ORF, an open reading frame from bp 370 to 1106, was found to have significant homology to the sequences of a family of periplasmic permease ATP-binding proteins involved in the energy-dependent transport of numerous substrates (1, 27). The highest scoring homologies to ORF were with the predicted amino acid sequences of the *E. coli glnQ* (glutamine transport), *E. coli hisP* (histidine transport), *Salmonella typhimurium hisP* (histidine transport), and *E. coli cysA* (sulfate transport) genes. Extended regions of identity and similarity between ORF and the ATP-binding protein family included specific amino acid motifs which are thought to be involved in the binding of ATP (1, 27). Similar homologies to the predicted amino acid sequences of additional members of this family, including the *Serratia marcescens sfuC*, *E. coli fhuC*, *E. coli fepC*, and the *E. coli fecE* genes, all thought to be involved in energy-dependent transport of various siderophores, were observed. The function of ORF, and its possible involvement in the uptake of siderophore in *B. subtilis*, will be the subject of future investigation.

A second open reading frame, spanning bp 2168 to 2666, was found to be virtually identical to a previously reported *B. subtilis* gene, *sfp*⁰, shown to be involved in the secretion of surfactin, a lipopeptide biosurfactant (15). The full-length *sfp* gene encodes a 224-amino-acid polypeptide (15). The *sfp*⁰ gene is thought to encode a truncated 165-amino-acid polypeptide, which differs from the homologous portion of the full-length *sfp* gene by five base pair substitutions and one base pair insertion (15). Upon comparison of our sequence with the previously published *sfp*⁰ sequence, we observed differences in the flanking nucleotide sequence of the *sfp*⁰ gene and a single difference within the coding region (Fig. 4). The inferred amino acid sequence of *sfp*⁰ contains a threonine residue at position 22 (15), while the corresponding residue in the pENTA22-encoded *sfp*⁰ polypeptide is serine, a conservative amino acid change (Fig. 4).

The DNA sequence confirmed that the location of the chromosomal insertion in mutant 168C was either within or just upstream of the *sfp*⁰ gene. This data strongly suggests that the *sfp*⁰ gene encodes the pENTA22 EntD activity.

The *E. coli* Fur box has been shown to bind Fur repressor, a negative regulator which controls iron genes in response to iron availability (3). A GAP comparison search between the *B. subtilis* DNA sequence in plasmid pENTA22 and the 14-bp consensus sequence of the *E. coli* Fur box (3) was performed. We identified a 37-bp inverted repeat downstream of ORF, with a central portion that exhibited 71% identity to the *E. coli* Fur box when aligned along the axis of symmetry (Fig. 5). The function of this inverted repeat, and the possibility that its role is iron related, remains to be elucidated.

Comparison of the *B. subtilis sfp*⁰, *E. coli entD*, and *B. brevis grs orfX* gene products. We performed a data base search using the FASTA program, looking for other DNA and protein sequences with homology to the *B. subtilis sfp*⁰ and *E. coli entD* genes and polypeptides. Several groups had previously identified homologies between *B. subtilis sfp* and *orfX* from the *B. brevis* gramicidin (*grs*) operon (8, 15). In

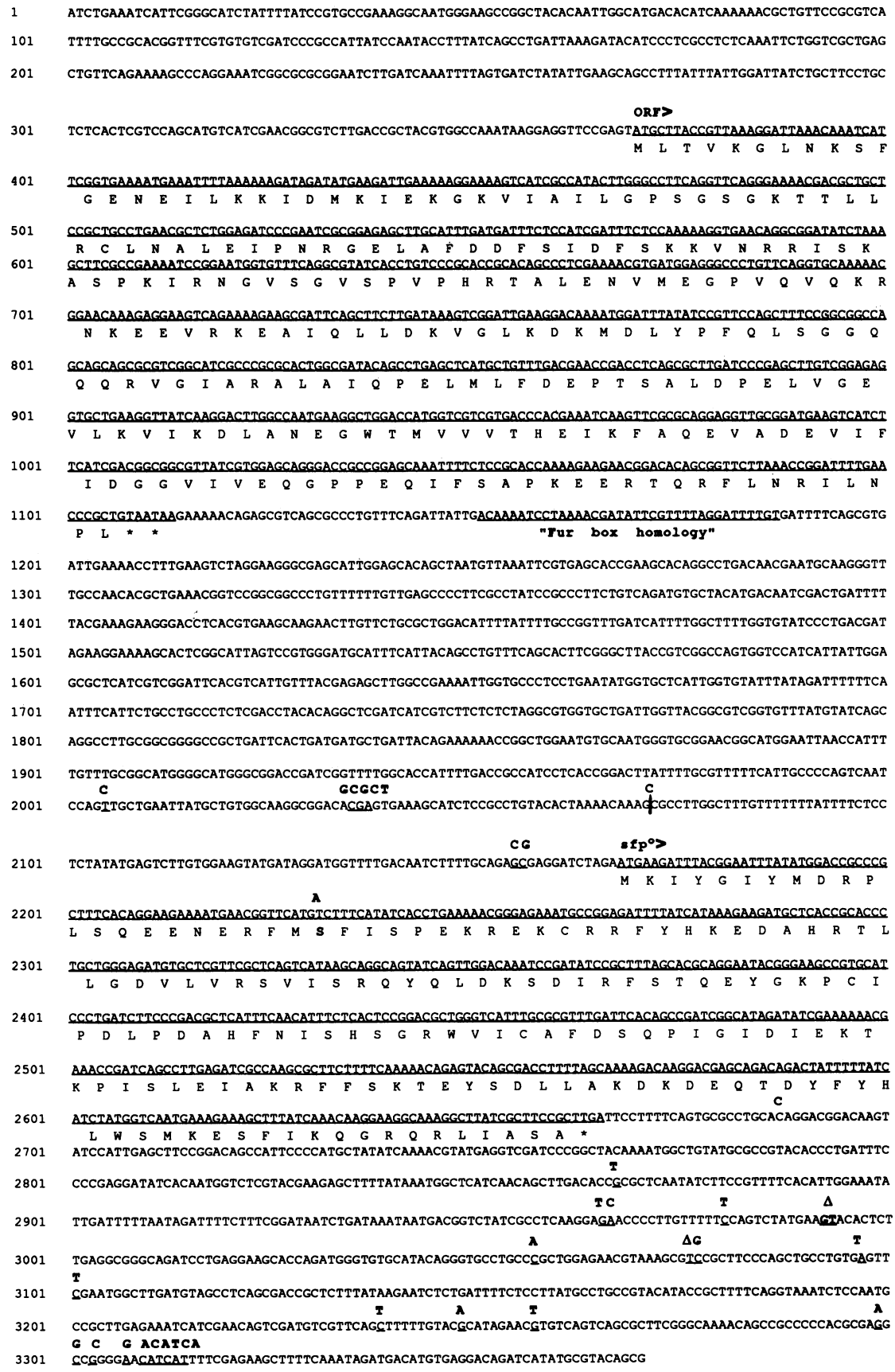
ORF>
"Fur box homology"
sfp°>

FIG. 4. Nucleotide sequence of the *B. subtilis* DNA in plasmid pENTA22. The location and orientation of the two open reading frames, ORF and *sfp*^{0}, are indicated. The location of a 37-bp inverted repeat which bears homology to the *E. coli* Fur box is also indicated. The predicted translation products of ORF and *sfp*^{0} are shown below the DNA sequence in single-letter amino acid symbols. For the *sfp*^{0} gene and the DNA sequence immediately flanking it, underlined nucleotides indicate deviations from the published sequence. The published sequence is noted in bold type above the underlined sequence. Insertions (I) and deletions (Δ) are indicated (15). The serine in bold type at amino acid position 22 indicates a deviation from threonine, the published predicted amino acid sequence (15).

our search, no other homologous DNA sequences were identified other than *orfX*. Using the GAP program, a comparison of the predicted amino acid sequences of *B. subtilis sfp*^{0}, *E. coli entD* (2), and the partially sequenced *B. brevis grs orfX* (8) revealed 23% identity between Sfp^0 and EntD and 44% overall similarity. Sfp^0 and *grs* OrfX proteins were 38% identical and showed an overall similarity of 57%. A comparison of EntD and *grs* OrfX revealed 21% identity and an overall similarity of 46%. Extensive regions of identity common to all three proteins were observed (Fig. 6).

Genetic mapping of the *sfp*^{0} gene from pENTA22. PBS1 lysates were prepared in each of the Tn*917* mapping kit strains. Each lysate was used to transduce strain 168CTrp$^+$ to erythromycin and lincomycin resistance. The recipient strain contained an insertion encoding the *cat* (chloramphenicol acetyltransferase) gene integrated into the chromosome at a location closely linked to the *sfp*^{0} gene derived from pENTA22. To determine linkage of the *sfp*^{0} gene to a particular Tn*917* insertion, erythromycin-and-lincomycin-resistant transductants were screened for sensitivity to chloramphenicol. The *cat* gene was linked to insertion *zfg-83*::Tn*917*, which maps at 216° on the *B. subtilis* chromosome (26); 29% of the erythromycin-and-lincomycin-resistant transductants were chloramphenicol sensitive. By genetic transformation with limiting DNA, we then showed that the *sfp*^{0} gene was 6 to 25% linked to the *trpC2* marker, near 199° on the chromosome. Our mapping results differ from the previously reported location of *sfp* at 40° (15). The reason for this difference is unclear.

The *E. coli entD* gene restored surfactin production to *B. subtilis* 168. We have shown that plasmid pENTA22, encoding the *B. subtilis sfp*^{0} gene, was able to restore enterobactin production to an *E. coli entD* mutant (discussed above), demonstrating that *sfp*^{0} can function in the production of siderophore in *E. coli*. On the basis of the observation that primary sequence similarities are shared by the *entD* and *sfp* gene products, we tested whether the *E. coli entD* gene in shuttle plasmid pITS21::pUB110 could restore surfactin production to the non-surfactin-producing strain *B. subtilis* 168. Neomycin-resistant transformants were assayed for surfactin production (Srf$^+$) on LB plates containing 5% defibrinated sheep blood and 10 μg of neomycin per ml (Materials and Methods). A Srf$^+$ phenotype was detected as a clear zone of hemolysis surrounding bacterial growth (Fig. 7). The zone of hemolysis surrounding 168(pITS21::pUB110) transformants indicated that the *E. coli entD* gene was expressed in *B. subtilis* 168 and was capable of complementing the chromosomal *sfp*^{0} allele to restore surfactin production. The control strain 168(pUB110) was surrounded by only a weak, narrow zone of hemolysis, indicating little or no surfactin production (Fig. 7).

DISCUSSION

***B. subtilis* siderophore biosynthesis genes.** The isolation of five *ent* homologs from a plasmid library containing *B. subtilis* DNA strongly suggests that there is a *B. subtilis* pathway analogous to the enteric pathway for the production of catecholic siderophore. As far as we are aware, this is the first report of the cloning and characterization of genes specifically involved in siderophore biosynthesis in a gram-positive organism. *B. subtilis* mutant 168C, containing an insertion in the region of the *entD* gene homolog *sfp*^{0}, grew poorly in low iron-medium. These data strongly suggest that DHBG is a major high-affinity siderophore of *B. subtilis*, if not the only siderophore produced under the growth conditions tested.

The linkage order of the *B. subtilis entC*, *entE*, *entB*, and *entA* homologs is the same as the order of the corresponding genes in *E. coli* (13, 19). The observation that all cloned DNA fragments containing the *B. subtilis entD* homolog were unlinked to the other *ent* genes is also consistent with the organization of the *ent* genes in *E. coli* (5). Restriction enzyme analysis suggests that the two sets of DNA sequences in Fig. 2 do not overlap. However, a DNA sequence that links the two sets may be missing from our collection. The fact that we did not identify a *B. subtilis entF* homolog may indicate that either an EntF-like activity does not exist in *B. subtilis* or it is not detectable using our method. We do not yet know whether gene expression from our DNA clones originates from native *B. subtilis* expression sequences or from other promoters present in the pBR322 vector. Transcription studies using these DNA fragments should help to answer these important questions, as well as other questions concerning the regulation of expression of genes involved in iron transport.

Interchangeability of *B. subtilis sfp*^{0} and *E. coli entD* genes. The role of the *B. subtilis sfp* gene has not yet been elucidated, although it has been suggested that the 224-amino-acid gene product is involved in surfactin secretion and regulatory functions (15). *B. subtilis* 168 does not secrete surfactin, although it possesses the *srfA* and *srfB* loci (15). Nakano et al. hypothesized that the deficiency was due to the presence of a defective form of *sfp*, termed *sfp*^{0}. This gene was thought to encode a truncated gene product of 165 amino acids whose function was unknown (15). *B. subtilis* mutant 168C, which could not grow to high density in low-iron medium, contains an insertion within or just up-

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Fur box consensus          ATAATGATTATCATTAT
                      1151                    1188
pENTA22 sequence      ACAAAATCCTAAAACGAATATCGTTTTAGGATTTTGT
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FIG. 5. Alignment of the *E. coli* Fur box consensus sequence and the pENTA22 37-bp inverted repeat. Nucleotides in bold type indicate identical base pairs. The nucleotide position of the pENTA22 inverted repeat is indicated at each end of the sequence.

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                    1                                                  49
E. coli EntD        MVDMKTTHTSLPFA.GHTLHFVEFDPANFCEQDLLWLPHYAQLQHAGRKR
                     1                                                 45
B. subtilis Sfp     .MKIYGIYMDRPLSQEENERFMTFISPEKREKC....RRFYHKEDAHRTL

B. brevis OrfX      ..........*IDRHVFNFLSSNVSKEKQQAFV....RYVNVKDAYRSL
                                      .
                                      .
                    50                                                 89
E. coli EntD        KTEHLA..........GRIAAVYALREYGYKCVPAIGDVRQPVWPAEVYG
                    46                                                 86
B. subtilis Sfp     LGDVLVRSVISRQYQLDKSDIRFSTQEYGKPCIPDLPDAH.........F

B. brevis OrfX      LGELLIRKYLIQVLNIPNENILFRKNEYGKPFV.DF.DIH.........F
                                      .
                                      .
                    90                                                137
E. coli EntD        SISHCGTTALAVVSRQPIGIDIEEI..FSVQTARELTDNIITPAEHERLA
                    87                                                136
B. subtilis Sfp     NISHSGRWVIGAFDSQPIGIDIEKTKPISLEIAKRFFSKTEYSDLLAKDK

B. brevis OrfX      NISHSDEWVVCAISNHPVGIDIERISEIDIKIAEQFFHENEYIWLQSKAQ
                                      .
                                      .
                    138                                               187
E. coli EntD        DCGLAFSLALTLAFSAKESAFKASEIQTDAGFLDYQIISWNKQQVIIHRE
                    137                                               183
B. subtilis Sfp     DEQTDYFYHL...WSMKESFIKQEGKGLSLPLDSFSVRLHQDGQVSIELP

B. brevis OrfX      NSQVSSFFEL...WTIKESYIKAIGKGMYIPINSFWIDKNQ.TQTVIYKQ
                                      .
                                      .
                    188                                 209
E. coli EntD        N.................EMFAVHWQIKE.KIVITLCQHD
                    184                                 225
B. subtilis Sfp     DSHSPCYIKTYEVDPGYKMAVCAAHPDFPE.DITMVSYEELL

B. brevis OrfX      NKKEPVTIYEPELFEGYKCSCCSLFSSVTNLSITKLQVQELCNLFLDSTFSENNNF
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FIG. 6. Comparison of the predicted amino acid sequences of the *E. coli entD* gene, the *B. subtilis sfp* gene, and the *B. brevis grs orfX* gene. Individual sequences were aligned, two at a time, using the Genetics Computer Group GAP program. Underlined amino acid residues indicate identical residues conserved within at least two of the proteins. The asterisk indicates the undetermined amino acid sequence of the amino terminus of *grs* OrfX (8). Amino acid residues are numbered as indicated.

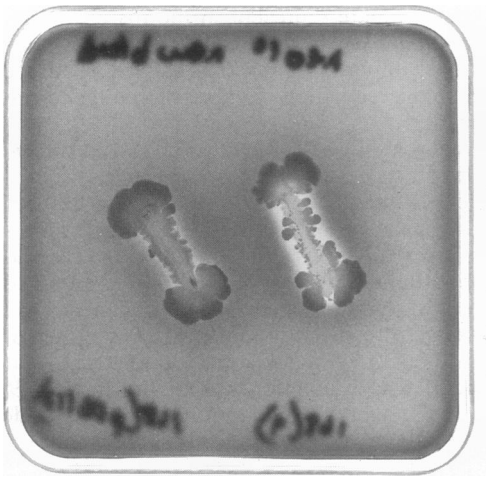

FIG. 7. Secretion of surfactin by *B. subtilis* 168 bearing a shuttle plasmid encoding the *E. coli entD* gene. *B. subtilis* 168 containing either plasmid pUB110 (left) or plasmid pITS21::pUB110 (right) were assayed for surfactin secretion on LB plates containing neomycin and 5% defibrinated sheep blood (see Materials and Methods). The more intense halo of hemolysis surrounding 168(pITS21::pUB110) cells indicates surfactin production.

stream of the chromosomal sfp^0 gene. Upon depletion of the residual free iron, cell growth stopped and no further siderophore was produced (6). In contrast, wild-type cells continued to produce siderophore in low-iron medium and were eventually able to grow to high density after a transient growth plateau. In addition, the DNA fragment encoding the sfp^0 gene restored enterobactin production to an *E. coli entD* mutant. These data support the idea that sfp^0 plays a role in iron transport in *B. subtilis* 168.

The fact that surfactin production can be restored to *B. subtilis* 168 by a high-copy-number plasmid encoding the *E. coli entD* gene and that a high-copy-number plasmid encoding the *B. subtilis* sfp^0 gene can complement an *E. coli entD* mutant for siderophore production demonstrate the functional interchangeability of the gram-positive bacterial sfp^0 gene and the gram-negative bacterial *entD* gene. This strongly supports the idea that their native functions may be very similar. Although the shuttle plasmid pITS21::pUB110 encodes the *E. coli fes* and *fepA* genes as well as the *entD* gene, it seems unlikely that the *fes* and *fepA* gene products play a role in surfactin production (4). We have observed conserved regions among the predicted gene products of *E. coli entD*, *B. subtilis sfp*, and the open reading frame *orfX* in the gramicidin operon of *B. brevis*. The high degree of conservation in discrete regions of all three proteins (Fig. 6) implies that these conserved regions are important for a function common to the three proteins. The data leads us to

suggest that the three gene products belong to a family of proteins that perform similar functions and that can be synthesized by either ribosomal or nonribosomal mechanisms (8, 16). On the basis of its association with the inner membrane and the gene products making up the enterobactin synthetase complex (1, 4, 5), it has been proposed that the *E. coli* EntD protein localizes the final stages of enterobactin biosynthesis near the cell surface and facilitates the secretion of enterobactin. Since enterobactin, surfactin, and gramicidin are all short, secreted peptide molecules, it seems reasonable that the *sfp* and *orfX* gene products have functions similar to that proposed for EntD. Functional specificity for these three proteins (i.e., association within a given synthetase complex) may reside within amino acid sequences that are unique to each protein.

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