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# Characterization of EntF as a serine-activating enzyme

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**Abstract**

EntF is the enzyme responsible for serine activation during the biosynthesis of enterobactin (a cyclic trimer of *N*-dihydroxybenzoyl serine) in *Escherichia coli*. EntF has been overexpressed and purified to >90% homogeneity. The enzyme has been shown to complement the *entF*$^-$ MK1 strain in the synthesis of 2,3-dihydroxybenzoylserine derivatives and exhibits L-serine-dependent ATP[$^{32}$P] pyrophosphate exchange activity with a $K_m$ for serine of 260 mM and a turnover number of 760 min$^{-1}$. Release of PPi during incubation of EntF with serine and ATP was observed, but with a low turnover number of 1.0 min$^{-1}$. These results suggested the presence of an enzyme-bound intermediate, which has been shown by gel filtration analysis to be (L-serine)adenylate.

**Keywords:** enterobactin; EntF; phosphopantetheine; serine activation

Under conditions of iron starvation, *Escherichia coli* and other enteric bacteria synthesize and secrete enterobactin, an iron-scavenging molecule that chelates $Fe^{3+}$. The $Fe^{3+}$-enterobactin complex is then specifically reabsorbed and broken down to release intracellular iron. Enterobactin is a macrocyclic trimer of *N*-2,3-dihydroxybenzoyl-L-serine where the serine trilactone serves as scaffolding for the three catechol functionalities that provide hexadentate coordination for the tightly ligated $Fe^{3+}$ atom (Harris et al., 1979).

We previously purified the enzymes encoded by the genes *entC*, *entB*, and *entA*, which produce 2,3-dihydroxybenzoate from the central dihydroaromatic metabolite chorismate (Liu et al., 1989, 1990; Rusnak et al., 1990) and have begun molecular analysis of the enzymes responsible for assembly of the three amide and three ester linkages in the enterobactin molecule by overproducing and purifying the EntE and EntF enzymes (Rusnak et al., 1989, 1991). EntE produces stoichiometrically bound 2,3-dihydroxybenzoyl-AMP (DHB-AMP) activated for subsequent amide bond formation. The large, 140-kDa EntF polypeptide was expected to activate L-serine analogously and perhaps to catalyze additional reactions as well. Indeed our recent cloning, sequencing, and initial characterization of EntF revealed covalently attached phosphopantetheine, but we were unable to reliably demonstrate substantial catalytic activity in the EntF expressed from the pDD4 overexpressing construct (Rusnak et al., 1991). We report here a modified construct that produces EntF at levels up to 15-20% of soluble protein and leads to ready purification of enzyme active for reversible formation of bound seryl-AMP as assessed by L-serine-dependent exchange of radioactivity from [$^{32}$P]PPi into ATP.

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## Results

### *Subcloning and overproduction of EntF*

The EntF-producing plasmid pDD4 had a 4-bp spacer between the ATG start and the Shine-Dalgarno ribosome-binding site sequence (Shine & Dalgarno, 1974) and gave variable yields of EntF. The enzyme preparations generally had low enzyme activity in either a serine-dependent [$^{32}$P]PPi-ATP exchange assay or in a complementation assay using crude extract of an *entF*$^-$ strain followed by analysis for dihydroxybenzoylserine-containing products. To optimize the distance between the Shine-Dalgarno sequence and starting methionine codon, pMS14 was constructed as noted with a 6-bp spacing. Expression from pMS14 afforded EntF as ~5-10% of the soluble protein in crude extract; enzyme activity was detected in the two catalytic assays noted above, but for unknown reasons overproduction levels were variable.

At this juncture further attention was focused on the DNA sequence upstream of EntF as a 216-bp open reading frame (*orf1*) had been noted (Pettis & McIntosh,

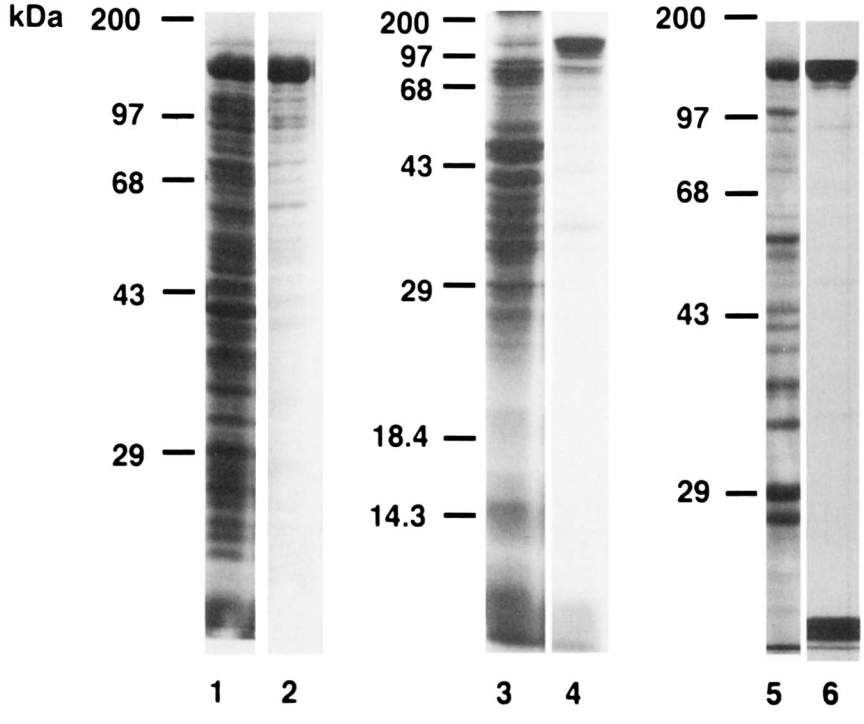


**Fig. 1.** SDS-PAGE analysis. Lane 1. Crude homogenate from JM109(DE3)/pMS22. Lane 2. After purification by chromatography on a Fast-Flow Q column as described in the Materials and methods section (10% gel, stained with Coomassie blue). Lane 3. Autoradiograph of $^{35}$S-labeled proteins from whole cells of induced JM109(DE3)/pMS22. Lane 4. Autoradiograph of $^{35}$S-labeled proteins from whole cells of induced JM109(DE3)/pMS22 treated with rifampicin as described in the Materials and methods section (15% gel, stained with Coomassie blue; Kodak X-OMAT AR film exposed for 0.5 h). Lane 5. Crude homogenate from SJ16/pGP1-2/pMS22 containing $\beta$-[$^3$H]alanine-labeled proteins (10% gel, stained with Coomassie blue). Lane 6. Autoradiograph of lane 5 (Kodak X-OMAT AR film exposed for 7.5 h).

1987; Rusnak et al., 1991) that, although out of the reading frame with EntF, overlapped it by 1 bp. Thus *orf1* could potentially encode a polypeptide of 72 amino acid residues with a predicted molecular weight of ~10,000. Although there was no obvious homology to acyl carrier protein sequences (Jackowski & Rock, 1987), those proteins are of a similar size (75–80 amino acids) and contain phosphopantetheine (Alberts & Vagelos, 1966; Jackowski & Rock, 1981). To determine whether the *orf1* upstream sequence was relevant to production of active EntF, the plasmid pMS22 containing the putative *orf1* and the *entF* gene with 6 bp between the Shine-Dalgarno sequence and the proposed start codon of *orf1* was engineered as described in the Materials and methods. pMS22 consistently yields overproduction of EntF as ~15% of soluble protein in *E. coli* crude extract and has been the construct of choice for reliable purification of active EntF.

*Purification and characterization of EntF*

EntF from pMS22 could be purified to >90% homogeneity by initial ammonium sulfate precipitation followed by a single Fast-Flow Q ion exchange column (Fig. 1, lane 2). A net fourfold increase in specific activity as monitored by the serine-dependent [$^{32}$P]PPi-ATP isotope exchange assay was observed (Table 1). A rather higher 35-fold purification is estimated using the more complex complementation assay. The apparent higher increase may be due to removal of an inhibitor in crude extract of pMS22.

Active enzyme could also be detected in the pMS14 crude extract, suggesting that *orf1* was not essential for active EntF per se. At this point, a search for a protein product from *orf1* was undertaken using selective $^{35}$S-labeling of the plasmid-encoded proteins (Tabor, 1990). A culture of JM109(DE3)/pMS22 was grown in M9 me-

**Table 1.** *Purification of EntF from Escherichia coli JM109 (DE3)/pMS22*

| | Volume (mL) | Concentration (mg/mL) | Total amount (mg) | DBS | | ATP-PPi | |
|---|---|---|---|---|---|---|---|
| | | | | Activity[a] | Purification | Activity[a] | Purification |
| Crude extract | 12.0 | 45.0 | 540 | 615 | 1 | $17.2 \times 10^6$ | 1 |
| $(NH_4)_2SO_4$, 0–50% supernatant | 10.0 | 9.0 | 90 | 2 | 0 | $0.2 \times 10^6$ | 0.01 |
| $(NH_4)_2SO_4$, 0–50% precipitate | 15.0 | 15.1 | 226 | 1,797 | 3 | $12.0 \times 10^6$ | 0.7 |
| Fast-Flow Q anion exchange | 5.5 | 1.3 | 7.2 | 21,736 | 35 | $67.5 \times 10^6$ | 3.9 |

[a] Activity is defined as $\Delta$DPM min$^{-1}$ (mg of protein)$^{-1}$.

dium supplemented with 18 amino acids (minus cysteine and methionine), then induced with isopropylthiogalactosidase (IPTG). Rifampicin was added to inhibit *E. coli* RNA polymerase. Addition of [$^{35}$S]methionine resulted in selective incorporation of $^{35}$S into the proteins produced by pMS22. Intense labeling of EntF was observed; however, no 10-kDa protein could be detected (Fig. 1, lane 4). This result suggests that EntF is the only protein overproduced by the plasmid pMS22. Furthermore, examination of the crude homogenate of induced cultures of JM109(DE3)/pMS22 by SDS–polyacrylamide gel electrophoresis (PAGE) revealed no 10-kDa protein (data not shown). Thus *orf1* does not appear to encode for a protein.

In view of the restricted spectrum of catalytic activity of pure EntF (see below), the presence of covalently bound phosphopantetheine in EntF overproduced to a high level by pMS22 was assessed using the *panD*$^-$ *E. coli* SJ16 strain (Jackowski & Rock, 1981) and the incorporation of [$^3$H]β-alanine noted previously for chromosomally encoded EntF. As can be seen in Figure 1 (lane 6), intense radiolabeling of EntF on an SDS–polyacrylamide gel was observed.

### *Activity of EntF: Serine-dependent [$^{32}$P]PPi–ATP exchange*

The ability of pure EntF to carry out reversible formation of L-seryl-AMP was probed by the classic L-serine-dependent [$^{32}$P]PPi–ATP isotopic exchange assay, and by this criterion EntF is a substantial catalyst with $K_m$ of 0.26 mM and $k_{cat}$ of 760 min$^{-1}$ for L-serine and $K_m$ of 0.75 mM and $k_{cat}$ of 660 min$^{-1}$ for ATP (Table 2). The L-seryl-AMP is most likely bound tightly at the active site, then released free into solution in vivo. This could be assessed by measuring the catalytic production of PPi product by adding inorganic pyrophosphatase as a coupling enzyme to assay Pi generation colorimetrically. As a serine-dependent ATP-splitting enzyme, EntF shows a turnover number of 1.0 min$^{-1}$.

The exchange assay permits determination of EntF specificity for other amino acids. Glycine, alanine, cysteine, homoserine, and both L-threonine and L-allo-threonine were not detectably activated as the aminoacyl-AMP derivatives. Neither was *N*-2,3-dihydroxybenzoyl-L-serine (DBS). The only amino acids recognized were *S*-methyl-L-cysteine, L-β-chloroalanine, and *O*-acetyl-L-serine (Table 2). D-β-Chloroalanine was not activated. These three alternate substrates show $K_m$ values 12–30-fold higher than L-serine, although the L-β-chloroalanine and *O*-acetyl-L-serine $k_{cat}$ values are quite high. At high levels of the β-substituted L-chloroalanine and *O*-acetyl-L-serine (e.g., 3–4-fold $K_m$; ~15–20 mM) time-dependent inactivation ensued, and full loss of activity could be monitored in preincubation studies. The addition of ATP resulted in some protection, and the prospect was raised that these β-substituted alanines partitioned between carboxyl activation and enzymatic alkylation. However, D-β-chloroalanine caused similar time-dependent inactivation at 20 mM concentration, and as D-β-chloroalanine does not support [$^{32}$P]PPi–ATP exchange, it is likely that the inactivating modification is nonspecific and probably due to second-order collisional alkylation.

**Table 2.** *Measured values of $K_m$ and $k_{cat}$ for L-serine, ATP, and some alternate substrates of EntF in the ATP–PPi exchange reaction*[a]

| Substrate | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ |
|---|---|---|---|
| L-Serine[b] | 0.26 | 760 | 2,900 |
| ATP[c] | 0.75 | 660 | 880 |
| (*O*)Ac-L-serine[d] | 5.0 | 720 | 150 |
| β-Cl-L-alanine[d] | 8.0 | 870 | 110 |
| (*S*)methyl-L-cysteine | 3.1 | 38 | 12 |

[a] The following molecules failed to support the exchange reaction ($k_{cat}$ < 1): L-alanine, D-alanine, β-F-L-alanine, β-Cl-D-alanine, glycine, L-threonine, allo-L-threonine, L-cysteine, L-homoserine, (*O*)phospho-L-serine, (*O*)benzyl-L-serine, D,L-serinol, and dihydroxybenzoylserine.

[b] Concentration of ATP in the reaction mixture was 5 mM.

[c] Concentration of L-serine was 1 mM.

[d] Ent F shows ATP–PPi exchange activity dependent upon the substrate at the substrate concentrations <5 mM ($K_m$ and $k_{cat}$ are shown as above). However, inhibition was observed at concentrations of substrate >5 mM.

### *Search for enzyme-bound intermediates*

The serine-dependent [$^{32}$P]PPi–ATP exchange suggests initial formation of a seryl-AMP–EntF complex. The existence of such a species had previously been proposed by Bryce and Brot (1972) based on studies of slightly purified enzyme. As expected then, when ATP, [$^{14}$C]-L-serine, and pure EntF were incubated for 15 min and subjected to rapid gel filtration on a Sephadex G-25 column, a radioactive peak accompanied the enzyme and was dependent on the presence of ATP in the incubation (Fig. 2). Calculation of stoichiometry in this and an independent repeat of the experiment indicated that 10% of the EntF molecules contained a bound serine species. To determine if any of the enzyme-associated [$^{14}$C]L-serine label was covalently attached to EntF, the complex was precipitated with cold 10% trichloroacetic acid (TCA). In contrast to the earlier study of Bryce and Brot (1972) on impure EntF, no label was found associated with the protein.

To test whether a covalent seryl-X–enzyme might accumulate only on formation of an *N*-dihydroxybenzoyl-seryl species, EntF was mixed with [$^{14}$C]serine, ATP, dihydroxybenzoylserine, and pure EntE (Rusnak et al., 1989) under conditions known to produce DHB-AMP·EntE. No $^{14}$C-containing, TCA-precipitable covalent

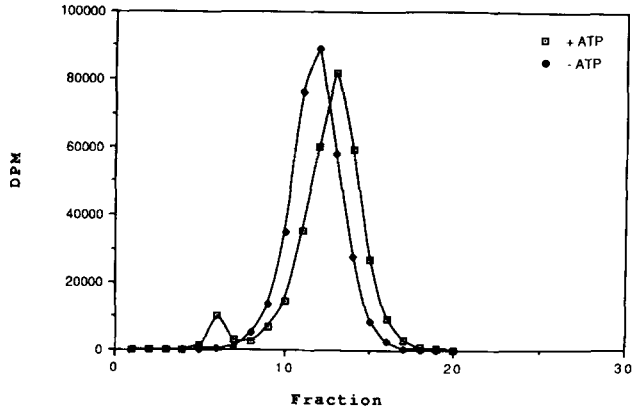


**Fig. 2.** Evidence of enzyme-bound intermediate. EntF was incubated with [$^{14}$C]serine in the presence (□) and absence (●) of ATP.

complex (bound to either EntE or EntF) was detected, nor was there discernible production of free dihydroxybenzoyl-[$^{14}$C]serine under such conditions, suggesting absence of covalent seryl-X-EntF and inability of pure EntE and EntF to make the amide linkage of dihydroxybenzoylserine.

## Discussion

In this work we report the purification to >90% homogeneity of the 140-kDa EntF enzyme in active form for the first time and the characterization of EntF as an L-serine-activating enzyme. The components of cyclic peptide and cyclic depsipeptide multienzyme synthases such as cyclosporin A synthetase and enniatin synthase have been notoriously labile (Zocher et al., 1982; Lawen & Zocher, 1990); activity of EntF enzyme preparations have previously been variable in our hands. The pMS22 construct reported here affords EntF as ~15% of soluble cell protein. A rapid one-step column purification then provides >90% pure enzyme, which remains active at 4 °C for 2 weeks. This has permitted the initial characterization of the catalytic activity of EntF. We were interested in determining whether the *orf1* upstream of *entF* produced a 72-amino acid polypeptide that might be a required subunit or a posttranslational modifying component, e.g., an acyl carrier protein-like subunit. We did not detect the expected 10-kDa protein product from *orf1* expressed in either $^{35}$S-Met-labeling studies or in SDS-PAGE analysis of induced cultures of JM109(DE3)/pMS22. This suggests that the presence of the 216-bp upstream sequence might only have a role in regulating translational efficiency.

Because EntF contains phosphopantetheine and sequence homology to gramicidin and tyrocidin synthase polypeptides as well as to thioesterase domains in triacylglycerol lipases (Wion et al., 1987; Brady et al., 1990; Winkler et al., 1990), it seemed likely that EntF would activate L-serine by the now classical L-seryl-AMP to L-seryl-*S*-enzyme route where the enzyme thiol could come from a cysteine or phosphopantetheine group. In fact, EntF as purified is highly active in L-serine-dependent [$^{32}$P]PPi-ATP exchange and the anticipated L-seryl-AMP·EntF species can be detected by gel filtration. Only 10% of the EntF sites show accumulated intermediate. This could arise from kinetics of buildup and decay, hydrolytic release, and/or having only a substoichiometric fraction of EntF molecules in active form. We noted the possibility of enzyme lability above. The enzyme releases PPi and seryl-AMP at a rate of 1.0 min$^{-1}$, some 760-fold slower than the rate of intermediate formation; this presumably reflects a "leak" rate to solution, as we have also noted with 2,3-DHB·AMP bound at the active site of EntE.

None of the [$^{14}$C]-L-seryl groups associated with EntF after gel filtration are covalently attached in a linkage stable to cold TCA (the typical protocol for isolating aminoacyl-*S*-enzyme species in this class of peptide and depsipeptide ligases). This could be an equilibrium issue between L-seryl-AMP and L-seryl-X-enzyme species; we could have detected 5% of covalent [$^{14}$C]serine. Attempts to accumulate a seryl-X-EntF species by adding DHB-AMP·EntE produced no labeled species attached to either protein nor was dihydroxybenzoylserine generated. We conclude at present that either EntF (the largest polypeptide by far in the EntD,E,F-containing enterobactin synthase complex) cannot react directly with DHB-AMP activated at the active site of EntE or perhaps some scaffolding component, e.g., EntD, is required before amide and ester bond assembly can proceed. One needs to keep in mind that the large 1,293-amino acid residue EntF enzyme, even though rapidly purified, might have lost one or more of its catalytic properties and retained only the partial reaction of L-seryl-AMP formation, as precedented in earlier work on cyclosporin A synthetase (Lawen & Zocher, 1990). The inability of EntF to make DBS-AMP also suggests that other components might be required for *N*-acylserine cyclic lactone assembly. At this point it is unclear what role the phosphopan tetheinyl group covalently tethered to EntF plays. Separate experiments with [$^{14}$C]salicyl-AMP·EntE, L-serine, ATP, and EntF did not produce detectable $^{14}$C-salicylated EntF. Thus EntE and EntF do not associate on gel filtration (Bryce & Brot, 1972). Finally, addition of fractions of ~10-fold purified EntD (data not shown) also did not yield detectable acylated EntE, F, or D in incubations containing ATP, serine, and dihydroxybenzoate. When all three EntD, E, and F enzymes are pure, they may serve to yield a complementation assay for detection of any missing components of the enterobactin synthase complex including, for example, the possible need for membrane components or an intact membrane system for vectorial assembly of the potent iron-chelating enterobactin.

## Materials and methods

### *Materials*

α-[$^{35}$S]thio-dATP (500 Ci/mmol), disodium hydrogen [$^{32}$P]pyrophosphate (60 mCi/mmol), and β-[3-$^{3}$H]alanine (87 Ci/mmol) were from NEN DuPont (Wilmington, Delaware). [3-$^{14}$C]serine (56 mCi/mmol) was from Amersham (Arlington Heights, Illinois). Adenosine 5′-triphosphate and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, Missouri). 2,3-Dihydroxybenzoate was from Aldrich (Milwaukee, Wisconsin). X-Gal and IPTG were from Boehringer-Mannheim Biochemicals. Restriction enzymes and the bacteriophage cloning vector pUC18 were from New England Biolabs (Beverly, Massachusetts), and were used according to the manufacturers' instructions. T4 DNA ligase and ligase 10× buffer were from IBI (New Haven, Connecticut). The oligonucleotide primers used for polymerase chain reaction (PCR) were synthesized by Dr. Alexander L. Nussbaum of Harvard Medical School. GeneAmp DNA amplification reagent kit was from Perkin Elmer Cetus (Norwalk, Connecticut). The gene amplification PCR was performed with oligonucleotide primers and pITS32 or pJS4700 as a template DNA according to the manufacturers' instructions.

### *Growth media and conditions*

LB broth (1% bactotryptone, 1% NaCl, 0.5% bacto yeast extract) and LB agar (LB broth plus 1.5% bactoagar) were used for culture growth and maintenance. When necessary, the antibiotics ampicillin, kanamycin, and tetracycline were supplied at 50 μg/mL, 10 μg/mL, and 12.5 μg/mL, respectively. In vivo labeling of pantetheine-containing proteins with β-[3-$^{3}$H]alanine was carried out by inoculating 5 mL of Dex-E-B1-met medium (Vogel & Bonner, 1956) containing 0.4% α-D-glucose, $10^{-4}$% thiamine, 0.002% methionine, 50 μg/mL ampicillin, and 50 μg/mL kanamycin with a culture of *E. coli* SJ16 harboring the plasmid of interest and incubating at 30 °C for 38–40 h. The faintly turbid β-alanine starved culture was diluted 25-fold into 1 mL Dex-E-B1-met medium containing 50 μg/mL ampicillin, 50 μg/mL kanamycin, and β-[3-$^{3}$H]alanine at a final concentration and specific activity of 0.55 μM and 87.2 Ci/mmol, respectively. The cells were grown for an additional 46 h at 30 °C ($OD_{595}$ = 1.1), then heat shocked at 42 °C for 3 min. The cells were harvested after an additional 4 h at 30 °C.

### *Bacterial strains and plasmids*

*Escherichia coli* strain XL1Blue [*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 ($r_k^-$, $m_k^-$), *sup*E44, *rel*A1, λ$^-$, (*lac*), {F′, *pro*AB, *lac*I$^q$, *lac*ZDM15, Tn10 (tet$^R$)}], from Stratagene was used to maintain all pUC18 recombinant clones. The *panD* mutant SJ16 (Jackowski & Rock, 1981) was a gift from Dr. Eugene Kennedy, Harvard Medical School. *Escherichia coli* MK1 (*entF, mini-kan*-1), a derivative of AB1515 (Pettis & McIntosh, 1987) and pITS32, a recombinant plasmid harboring the *entF* gene cloned into the *Nru*I/*Hin*dIII sites of pBR328, were provided by Dr. M.A. McIntosh of the University of Missouri–Columbia. The plasmid pJS4700 containing the *entF* gene under the control of the T7 promoter was provided by Drs. Janet Staab and Charles Earhart of the University of Texas at Austin. JM109 (DE3), a λ-lysogen containing the gene for bacteriophage T7 RNA polymerase under control of the *lac* promoter, was purchased from Promega (Madison, Wisconsin), and is similar to the strain BL21(DE3) as developed by Studier and Moffatt (1986).

### *DNA manipulation*

DNA manipulations were carried out as described by Maniatis et al. (1982).

#### *Subcloning of the entF gene into pT7-7 alone or with orf1*

Subcloning of the *entF* gene was carried out in four steps (Fig. 3). The *Sal*I/*Hin*dIII fragment containing the 3′ end of *entF* was first cloned into the *Sal*I/*Hin*dIII sites of pT7-7, and the vector pDD2 was isolated (Rusnak et al., 1991). The 5′ portion of *entF* alone or with *orf1* was then amplified by PCR with AmpliTaq (recombinant Taq DNA polymerase). The PCR primers were constructed such that each fragment had a unique *Xba*I site just upstream of a Shine–Dalgarno sequence. The fragments were then cloned into pUC18 to afford pUMS1, which included the 5′ portion of *entF*, and pUMS2, which included *orf1* and the 5′ portion of *entF*. After digestion of pUMS1 and pUMS2 with *Xba*I/*Sal*I, the 0.85-kb fragment containing the 5′ portion of *entF* from pUMS1 and the 1.1-kb fragment containing *orf1* and the 5′ portion of *entF* from pUMS2 were each ligated into *Xba*I/*Sal*I-digested pDD2, to give the vector pMS1 containing the complete *entF* gene and the vector pMS2 containing *orf1* and the complete *entF* gene. The relevant steps in this process are as follows:

*PCR amplification of the 5′ end of entF alone or with orf1.* The reaction mixture (100 μL) containing 0.1–1.0 μg of pITS32, which harbors *orf1* and the *entF* gene, in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, each primer (oligo primer 1 and oligo primer 2 for the fragment MS1 or oligo primer 7 and oligo primer 2 for the fragment MS2) at 1 μM, each dNTP (dATP, dCTP,

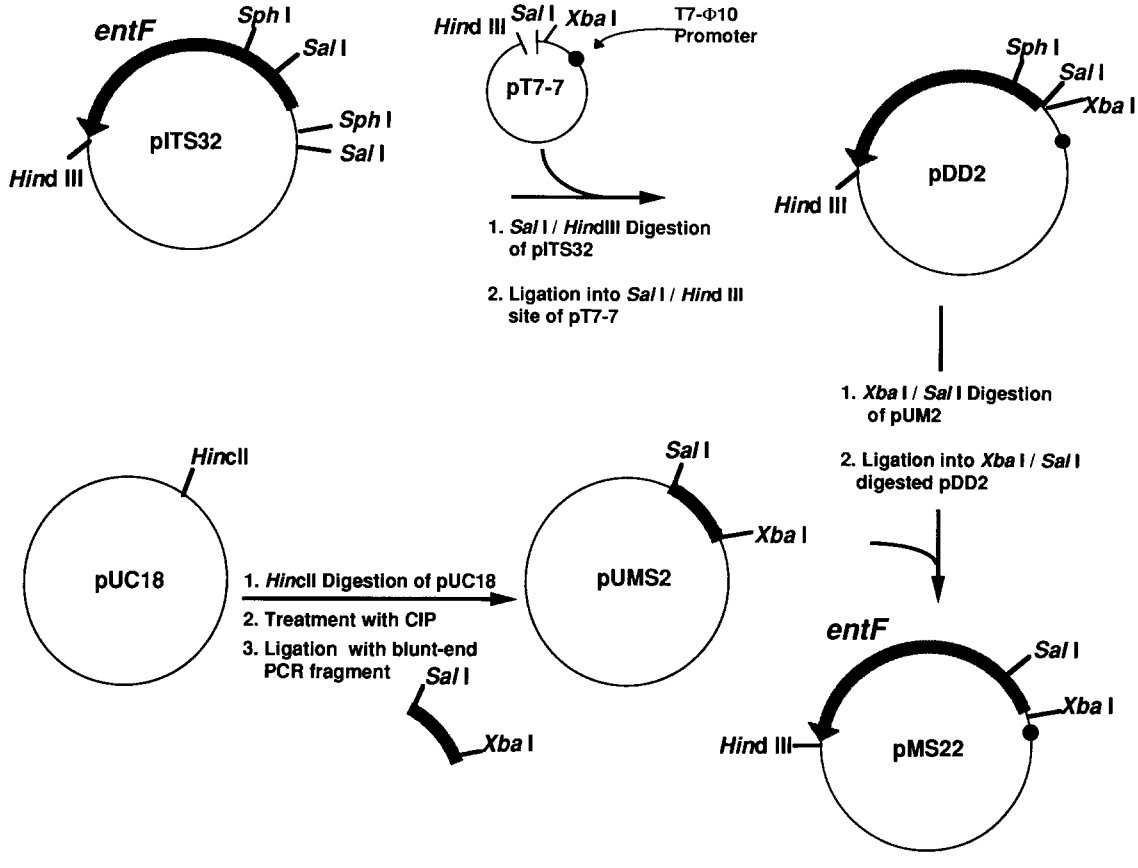


**Fig. 3.** Subcloning strategy used to generate the EntF overexpression vector pMS22. The 1.1-kb blunt-ended PCR fragment containing *orf1* and the 5′ region of *entF* was first cloned into the *Hin*cII site of pUC18. The *Xba*I/*Sal*I 5′ fragment of *entF* was then cloned into the *Xba*I/*Sal*I site of pDD2, which contains the 3′ end of *entF* cloned into pT7-7 behind the T7-Φ10 promoter.

dTTP, and dGTP) at 200 μM, and 2.5 units of Amplitaq DNA polymerase. The samples were overlaid with 100 μL of mineral oil to prevent condensation and subjected to 30 cycles of 1 min at 94 °C, 1 min at 37 °C, and 2 min at 72 °C by using a programmable heat block (Perkin-Elmer-Cetus Thermocycler). After the last cycle, all samples were incubated for an additional 7 min at 72 °C to ensure that the final extension step was complete. The PCR fragments were purified by phenol-chloroform extraction followed by ethanol precipitation and resuspended in 100 μL of TE (10 mM Tris, 1 mM EDTA) buffer. The names and sequences of the synthetic oligonucleotides used in this report are oligo primer 1 (5′-GCATGCTCTAGAGGAGATATACATATGAGCCAG CATTTACCTTTG-3′), corresponding to the first 21 bp of the *entF* gene and 24 overhanging residues including an *Xba*I site inserted into the front of a Shine-Dalgarno sequence and 6 bp between the Shine-Dalgarno sequence and a start codon of *entF*; oligo primer 2 (5′-CCCAGT CGACGCATAAAGATAAAT-3′), corresponding to the 24 bp of the unique *Sal*I region of the *entF* gene; and oligo primer 7 (5′-GCATGCTCTAGAGGAGATATACA TATGGCATTCAGTAATCCCTTC-3′), corresponding to the first 21 bp of *orf1* and 24 overhanging residues including an *Xba*I site inserted into the front of a Shine-Dalgarno sequence and 6 bp between the Shine-Dalgarno sequence and a start codon of *orf1*.

*Subcloning into pUC18.* The 0.85-kb PCR fragment (MS1) containing the 5′ portion of *entF* and the 1.1-kb PCR fragment (MS2) containing *orf1* and the 5′ portion of *entF* were each converted to blunt-ended fragments as follows. A mixture (100 μL) containing 30 μg of the requisite PCR fragment, each dNTP at 25 μM, and 10 units of the Klenow fragment of *E. coli* DNA polymerase I in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl was incubated at 37 °C for 15 min and then heated at 75 °C for 10 min to inactivate the Klenow fragment. Blunt-ended fragments were purified by low melting point agarose gel electrophoresis, excised, and concentrated by electroelution. Those fragments were further purified by phenol-chloroform extraction followed by ethanol precipitation and resuspension in 100 μL of TE buffer. The fragments were subsequently ligated into the *Hin*cII-digested and dephosphorylated pUC18 vector with T4 DNA ligase at 16 °C for 20 h. The ligation mix-

tures were each transformed into *E. coli* XL1Blue, and white colonies were isolated. Plasmid DNA was isolated by miniprep; sequence determination, using the chain-termination method of Sanger (Sanger et al., 1977), showed the presence of the correct insert in the pUC18 vector.

*Cloning of the 5′ end of entF into pDD2.* Plasmids pUMS1 and pUMS2 were prepared from the *E. coli* XL1Blue cells and digested at 37 °C for 2 h with *Xba*I/*Sal*I in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl. The 0.85-kb fragment (MS1) containing the 5′ portion of *entF* and the 1.1-kb fragment (MS2) containing *orf1* and the 5′ portion of *entF* were each isolated on low-melting point agarose, excised from the gels, and concentrated by electroelution. The fragments were further purified by phenol-chloroform extraction followed by ethanol precipitation and resuspension in 50 μL of TE buffer. Plasmid pDD2 (Rusnak et al., 1991), which harbored the 3′ portion of *entF*, was linearized by *Xba*I/*Sal*I digest and purified in a similar fashion. Ligation of MS1 or MS2 and linearized pDD2 was performed as described above for 18 h at 16 °C. The ligation mixture was transformed into *E. coli* JM109(DE3), and ampicillin-resistant colonies were isolated and screened by plasmid preparation and restriction digest. The correct clone containing the entire *entF* gene with a new *Xba*I site inserted into the front of a Shine-Dalgarno sequence and 6 bp between the Shine-Dalgarno sequence and a start codon of *entF* cloned into the vector pT7-7 is called pMS14, whereas the correct clone containing *orf1* and the *entF* gene with a new *Xba*I site inserted into the front of a Shine-Dalgarno sequence and 6 bp between the Shine-Dalgarno sequence and a start codon of *orf1* cloned into the vector pT7-7 is called pMS22 (Fig. 3).

## *Purification of EntF from E. coli JM109(DE3)/pMS22*

### *Crude cell extract*

Two 2-L cultures of *E. coli* JM109(DE3)/pMS22 were grown in LB broth containing ampicillin (100 μg/mL) at 37 °C until the $OD_{600}$ reached 0.5, at which point IPTG was added to a final concentration of 1 mM. Three hours later, the cells (2 g) were harvested by centrifugation and washed with 100 mM Tris-HCl, pH 8.0, resuspended in 6 mL of buffer containing 10 mM $MgCl_2$, 5.0 mM dithiothreitol, and 100 mM Tris-HCl, pH 8.0, and lysed by two passages through a French press operating at 12,000 psi at the orifice. Cell debris was removed by centrifugation (20 min, 10,000 × *g*), and nucleic acids were precipitated by the addition of one-fifth volume of 2% protamine sulfate to yield crude extract.

### *Ammonium sulfate fractionation*

Ammonium sulfate fractionation of EntF was performed as previously described (Rusnak et al., 1991).

### *Anion exchange chromatography*

The protein fraction remaining after ammonium sulfate fractionation was dialyzed against 1 L of 25 mM Tris, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol (buffer A) and applied to a 20 × 2.5-cm column of Fast-Flow Q (Pharmacia) at 4 °C and washed with 100 mL of buffer A. EntF eluted from the column with a linear 200-mL gradient of 0.2–0.6 M NaCl in buffer A at about 0.37 M NaCl. The flow rate under these conditions was 3.0 mL/min. Six-milliliter fractions were taken and assayed for protein by measuring the absorbance at 280 nm. Fractions of the major peaks that eluted were assayed for the formation of DBS-containing compounds (vide infra). A single peak that eluted after the void volume was found to synthesize DBS derivatives in the presence of 2,3-dihydroxybenzoate.

### *Analysis of proteins*

The analysis of proteins was performed as previously described (Rusnak et al., 1991).

### *Storage of active enzyme*

After anion exchange chromatography, the enzyme samples were pooled and concentrated in a 50-mL Amicon filtration cell with a PM-30 membrane. The resulting enzyme solution retained L-serine-dependent ATP-PPi exchange activity for 14 days.

## *Assays for EntF*

### *ATP-[$^{32}$P]pyrophosphate exchange assay*

The exchange of [$^{32}$P]pyrophosphate into ATP was carried out as previously described (Rusnak et al., 1991). $K_m$ and $k_{cat}$ values were usually calculated from six different substrate concentrations. The values for these kinetic constants were obtained from the program EnzymeKinetics (Trinity Software).

### *Synthesis of (dihydroxybenzoyl) serine derivatives*

Synthesis of (dihydroxybenzoyl) serine derivatives was carried out as previously described (Rusnak et al., 1991) with minor modifications. The differences between the present assay and that presented previously are the procedure for the preparation of MK1 crude extract and the amount of the crude extract of MK1 used in the assay. The procedure currently used is as follows: MK1 (*entF*$^-$ strain, a derivative of AB1515 [Pettis & McIntosh, 1987]) was grown in LB media in the presence of 2,2-dipyridyl (0.2 mM) and kanamycin (10 μg/mL) at 37 °C for 20 h. The cells (1 g) were harvested by centrifugation, washed in 100 mM Tris-HCl, pH 8.0, resuspended in 4 mL of buffer containing 10 mM $MgCl_2$, 5.0 mM dithiothreitol, and 100 mM Tris-HCl, pH 8.0, and lysed by two passages through a French pressure cell operating at 12,000 psi at the orifice. Cell debris was removed by centrifugation

(20 min, 10,000 × *g*) to give a crude extract. The resulting solution (30 μL per assay) was used to assay for the synthesis of DBS derivatives.

*Detection of enzyme-substrate complex*

The methods for observing substrate complexes with EntF were identical to those described previously for monitoring the formation of analogous complexes with EntE (Rusnak et al., 1989), the activating enzyme for 2,3-dihydroxybenzoate. Acid precipitations of enzyme reaction mixtures were performed as described previously (Rusnak et al., 1989).

*Pyrophosphate release assay*

The release of pyrophosphate was monitored as previously described (Rusnak et al., 1991).

*Selective labeling of EntF*

The procedure for selective labeling of plasmid-encoded proteins as described by Tabor (1990) was used with the following modifications. A colony of JM109 (DE3)/pMS22 was used to inoculate 5 mL LB medium containing 50 μg/mL ampicillin. After 16 h at 37 °C, the culture was diluted 1:40 into 20 mL LB medium containing 50 μg/mL ampicillin and grown until the $OD_{595}$ reached 0.8, at which point 1.0 mL of the culture was removed. The cells were washed with 1.0 mL M9 medium and then resuspended in 1.0 mL M9 medium containing 20 μg/mL thiamine and 0.01% each of 18 amino acids (minus cysteine and methionine). The cells were grown at 37 °C for 60 min; then IPTG was added to a final concentration of 1 mM. After an additional 15 min at 37 °C, 0.5 mL of the culture was removed and the remaining 0.5 mL of culture was warmed to 42 °C. Rifampicin was added to a final concentration of 200 μg/mL. The culture was kept at 42 °C for 10 min, returned to 37 °C for 20 min, then pulsed with 10 μCi of [$^{35}$S]methionine (>800 Ci/mmol) for 5 min. The cells were harvested, washed with water, and prepared for SDS-PAGE as described by Tabor (1990).

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