Reconstitution and Characterization of the *Escherichia coli* Enterobactin Synthetase from EntB, EntE, and EntF†

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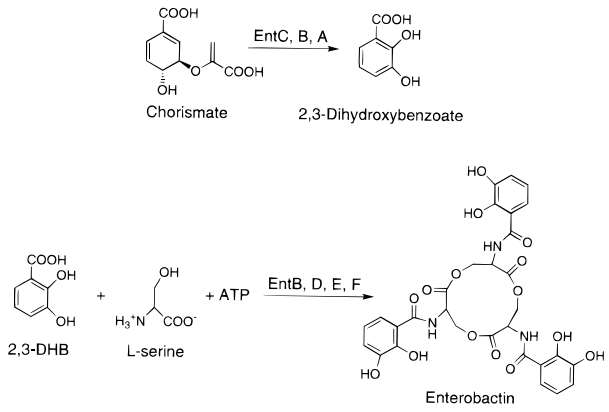
ABSTRACT: The siderophore molecule enterobactin, a cyclic trimeric lactone of *N*-(2,3-dihydroxybenzoyl)-serine, is synthesized and secreted by *Escherichia coli* in response to iron starvation. Here we report the first reconstitution of enterobactin synthetase activity from pure protein components: holo-EntB, EntE, and holo-EntF. Holo-EntB and holo-EntF were obtained by pretreatment of apo-EntB and apo-EntF with coenzyme A and EntD, thereby eliminating the requirement for EntD in the enterobactin synthetase. The holo-EntF monomer acts as the catalyst for the formation of the three amide and three ester bonds in enterobactin using ATP, L-serine, and acyl-holo-EntB, acylated with 2,3-dihydroxybenzoate by EntE, as substrates with a turnover rate of 120−140 min^{-1}. There is no evidence for a stable complex of the enterobactin synthetase components. Mutation of holo-EntF in the thioesterase domain at the putative active site serine residue (Ser1138 to Ala) eliminated enterobactin synthetase activity; however, the mutant holo-EntF retained the ability to adenylate serine and to autoacylate itself by thioester formation between serine and its attached phosphopantetheine cofactor. The mutant holo-EntF also appeared to slowly release *N*-(2,3-dihydroxybenzoyl)serine.

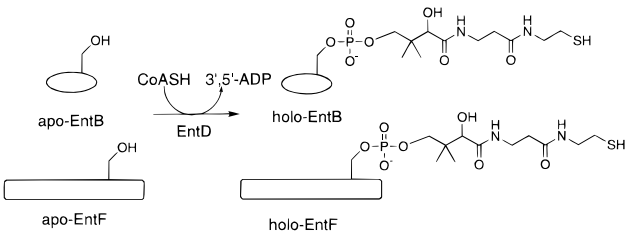
Bacteria starved for the essential nutrient iron respond by turning on the expression of genes that encode for the uptake of Fe^{III} complexed to siderophores, low molecular weight chelators with a high affinity for ferric iron (reviewed in *1*). Often the genes that encode siderophore biogenesis are likewise repressed when iron is plentiful and induced when iron is limiting (reviewed in *2*, *3*). The *Escherichia coli* enterobactin biosynthetic operons, encoding EntA−F, are one such paradigm (reviewed in *4*). EntA, -B, and -C divert the central metabolite chorismate to 2,3-dihydroxybenzoate (DHB),[1] and then EntB, -D, -E, and -F are required for the ATP-dependent conversion of three molecules each of the catecholic DHB and L-serine to the cyclic depsipeptide enterobactin which has a K_D of 10^{-52} M for Fe^{III} (*5*) (Scheme 1).

We have previously reported the purification and characterization of all six Ent proteins, EntA−F (*6*−*13*). Most recently we identified EntB as a bifunctional protein, containing both an N-terminal isochorismate lyase domain and a C-terminal aryl carrier protein (ArCP) domain (*14*).

Scheme 1



Scheme 2



EntD is a posttranslational modification catalyst, transferring the phosphopantetheinyl (Ppant) moiety of CoASH onto a serine side chain in both the ArCP domain of EntB and a homologous 80−100 aa peptidyl carrier protein (PCP) domain of EntF (Scheme 2) (*10*, *14*). The EntD-mediated conversion of apo-forms of EntB and EntF to phosphopantetheinylated holo-forms primes them for their acyl activation and transfer roles in enterobactin assembly. EntE is a

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[1] Abbreviations: ACP, acyl carrier protein; ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; ArCP, aryl carrier protein; BSA, bovine serum albumin; CoASH, coenzyme A; DHB, 2,3-dihydroxybenzoate; DHB-AMP, 2,3-dihydroxybenzoyladenylate; DHB-ser, *N*-(2,3-dihydroxybenzoyl)serine; DTT, dithiothreitol; ent, enterobactin; FAS, fatty acid synthase; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; PCP, peptidyl carrier protein; Ppant, 4′-phosphopantetheine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOE, splicing by overlap extension; TCA, trichloroacetic acid; TE, thioesterase; TLC; thin-layer chromatography.

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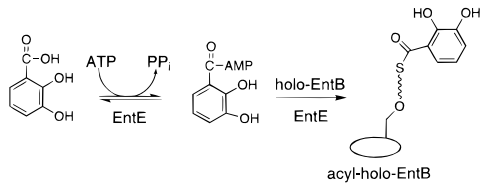
Scheme 3



dihydroxybenzoate-AMP ligase (*11*) that specifically transfers the DHB acyl group onto holo-EntB (Scheme 3), yielding the covalently arylated EntB to serve as the aryl donor for the amide bond formation in enterobactin assembly (*14*).

EntF, the sixth Ent protein, is an 142 kDa enzyme (*12*) with four domains recognizable by sequence similarity to other nonribosomal peptide synthetases: an N-terminal elongation/condensation domain, residues 1−475 (*15*), an adenylation domain (*16, 17*), residues 476−960, a PCP domain (*18*) with Ser1006 as the putative phosphopantetheinylation site, residues 961−1049, and a C-terminal 30 kDa thioesterase domain, residues 1050−1293 (*19*). The prior characterization of EntF was by L-serine-dependent ATP-PP_i exchange assay, demonstrating reversible serine-AMP formation as expected for the adenylation domain (*12, 13*). However, while we detected Ppant in EntF, we could not quantitate posttranslational stoichiometry or obtain fully active holo-EntF when this protein was overproduced in *E. coli* (*13*). The recent discovery that EntD is a PPTase (*10, 14*) that will specifically produce holo-forms of both EntB and EntF sets the stage to assess the role of EntB, -D, -E, and -F in enterobactin assembly. In this work we report on the robust reconstitution of enterobactin synthetase activity. EntE acylates holo-EntB via DHB-AMP and the DHB-S-Ppant-EntB formed then serves as an acyl donor substrate for holo-EntF. Holo-EntF is the catalyst, using L-serine, ATP, and DHB-EntB to make the three amide and three ester linkages in enterobactin assembly.

MATERIALS AND METHODS

Overproduction and Purification of EntB, -D, -E, and -F. EntD (*10*) and EntB (*14*) were overproduced and purified as previously described. EntE was overproduced and purified as described (*14*) but with gel filtration chromatography on a Sephacryl S-300 column (2.5 × 115 cm). The previously described plasmid pMS22 (*13*) was transformed into BL21(DE3) *E. coli* cells, and this strain was used for the overproduction of EntF (predominantly in the apo-form). Cultures of BL21(DE3)/pMS22 (2 L, 2 × YT media, 50 μg/mL ampicillin) were grown at 37 °C to an optical density of 0.9 and then induced with 1 mM IPTG. Cells were harvested after 3 h, yielding 6.6 g of wet cell paste which was resuspended in 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM (phenylmethyl)sulfonyl fluoride to inhibit proteolysis. Cells were lysed by two passages through a French pressure cell at 15 000 psi, and the lysate was clarified by centrifugation (27 000*g*). Purification of EntF from the cell lysate was similar to that described previously (*13*). The 0−50% ammonium sulfate fractionation pellet was dissolved in 20 mL of 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 5 mM DTT (buffer A) and dialyzed overnight against this same buffer. After dialysis, this material was applied to a Q-Sepharose column (2.5 × 10 cm) at a flow rate of 2 mL/min. The column was washed with 100 mL of buffer A, and EntF was eluted with a 500 mL gradient of 0−1.0 M KCl in buffer A. Fractions containing EntF (eluting at 0.45 M KCl) as judged by 8% SDS−PAGE were pooled and dialyzed against 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 10% glycerol and stored at −80 °C. Previous reports indicated that purified EntF retained activity for up to 14 days following purification (*13*); however, we observed no loss of activity even after several months of storage.

Protein concentrations for the purified enzyme preparations were determined using the calculated extinction coefficients (*20*) for the absorbance of these proteins at 280 nm: 51 710 M^{-1} cm^{-1} for EntB, 31 040 M^{-1} cm^{-1} for EntD, 55 250 M^{-1} cm^{-1} for EntE, and 180 360 M^{-1} cm^{-1} for EntF.

Preparation and Purification of Holo-EntB and Holo-EntF. Holo-EntB and holo-EntF were prepared by incubating the purified apo-forms of these proteins with purified EntD and CoASH. The following were incubated at 37 °C for 2 h to produce holo-EntB (4.5 mL final volume): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 1 mM CoASH, 125 μM apo-EntB, and 1 μM EntD. Holo-EntB was purified and stored as previously described (*14*) except a 0−0.7 M gradient of NaCl was used to elute holo-EntB from the POROS 20 HQ anion-exchange column.

The following were incubated at 37 °C for 45 min to prepare holo-EntF (5 mL final volume): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 500 μM CoASH, 30 μM apo-EntF, and 400 nM EntD. Holo-EntF was purified from the reaction mixture on a POROS 20 HQ anion-exchange column using the BioCAD SPRINT perfusion chromatography system (PerSeptive Biosystems, Inc.). The reaction mixture was applied to the column at a flow rate of 10 mL/min, and the column was washed with four column volumes of 10 mM Tris and 10 mM Bis−tris propane (pH 8.0). Holo-EntF was eluted with a 30 column volume gradient of 0−0.5 M NaCl in the wash buffer; fractions containing holo-EntF as judged by 8% SDS−PAGE were pooled, dialyzed against 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 10% glycerol, and stored at −80 °C.

Complete phosphopantetheinylation of the holo-EntB and holo-EntF preparations was confirmed using the radioassay for [^{3}H]phosphopantetheine incorporation described previously (*10, 14*). In these assays, 2.4 μM holo-EntB or holo-EntF were incubated with 150 μM [^{3}H]CoASH (192 Ci/mol) and 400 nM EntD at 37 °C for 30 min.

Construction and Purification of the EntF S1138A Mutant. The EntF site-directed mutant S1138A was constructed using the SOE method (*21*). The pMS22 plasmid (*13*) was used as the template for the first round of PCR amplification using (1) the forward primer 5′-GAATTCCATATGCAATTG-GTGGGCTATCTGG-3′ (904.for) and the reverse primer 5′-AGCGTACCGCCAAG*GGC*ATACCCCAGCAGG-3′ (mut.rev) and (2) the forward primer 5′-CCTGCTGGGG-TAT*GCC*CTTGGCGGTACG-3′ (mut.for) and the reverse primer 5′-CCGTTGAAGTACTTCC-3′ (1205.rev) (primers from Gene Link, Thornwood, NY). The base pair mismatch incorporated into the primers to give the TCC (serine) to GCC (alanine) mutation is italicized. The two PCR-amplified DNA fragments were gel-purified and used as the

template for a second round of PCR amplification using the 904.for forward primer and the 1205.rev reverse primer to give the 925 base pair DNA fragment containing the TCC → GCC mutation. This PCR fragment was digested with *Kpn*I and *Sty*I while pMS22 was digested with *Kpn*I, *Sty*I, and *Sal*I. Following gel purification, the *Kpn*I/*Sty*I-digested PCR fragment was ligated to the *Kpn*I/*Sal*I and *Sty*I/*Sal*I pieces derived from pMS22, and the ligation mixture was transformed into *E. coli* DH5α cells, yielding the plasmid pMS22−S1138A; the S1138A mutation was confirmed by DNA sequencing of the *Kpn*I/*Sty*I insert (Dana Farber Molecular Biology Core Facility, Boston, MA). pMS22−S1138A was transformed into BL21(DE3) *E. coli* cells, and this strain was used for the overproduction and purification of the mutant S1138A EntF.

Cultures of BL21(DE3)/pMS22−S1138A (2 L, 2 × YT media, 50 μg/mL ampicillin) were grown at 37 °C to an optical density of 0.6 and then induced with 1 mM IPTG; cells were harvested after 4 h, yielding 8.5 g of wet cell paste. Purification of the S1138A EntF mutant was as described for wild-type EntF but with the addition of a gel filtration chromatography step as well as the following alterations. Cells were resuspended in 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 5% glycerol (buffer B) prior to lysis by passage through the French pressure cell. A 25−50% ammonium sulfate pellet was dissolved in buffer B and applied to a Sephacryl S-300 column (2.5 × 115 cm) at 1 mL/min, and EntF was eluted with this same buffer. The EntF S1138A-containing fractions were then loaded on the Q-Sepharose column (2.5 × 11 cm).

The preparation of holo-EntF S1138A was also as described above for wild-type holo-EntF. The following were incubated at 37 °C for 45 min (5 mL final volume): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 500 μM CoASH, 18 μM apo-EntF, and 400 nM EntD. Holo-EntF S1138A was purified from the reaction mixture and stored as described for the wild-type enzyme.

Assays for Enterobactin Formation. (a) Ethyl Acetate Extraction Radioassay. The radioassay for the detection of enterobactin and its DHB-ser breakdown products was as described with minor modifications (*22*). Reaction mixtures (100 μL) contained 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 1.5 mM [^{3}H]-L-serine (16.6 Ci/mol, 2.5 μCi/reaction) (Dupont NEN), 500 μM 2,3-dihydroxybenzoate, 10 mM ATP, holo-EntB, EntE, and holo-EntF to initiate the reaction. Prior to the addition of holo-EntF, the reaction mixture was incubated for 10 min at room temperature to allow the acylation of holo-EntB with DHB by EntE. After the addition of holo-EntF, reactions were incubated at 37 °C for a specified time and then quenched with 150 μL of 1 N HCl. The acidified reaction mixture was extracted with 0.75 mL of ethyl acetate. A portion of the ethyl acetate layer was removed (0.5 mL) and added to 3.5 mL of scintillation fluid (Ultima Gold, Packard), and the radioactivity was quantified by liquid scintillation counting.

(b) HPLC Assay for the Identification and Quantitation of Enterobactin Synthetase Reaction Products. The enterobactin synthetase reaction mixtures were prepared as described above (except using unlabeled L-serine), acidified, and extracted into ethyl acetate (0.75 mL). A portion of the ethyl acetate layer (0.5 mL) was concentrated under reduced pressure, and the residue was dissolved in 0.25 mL of 30% acetonitrile/water. Samples of 50 μL were analyzed by HPLC (Waters) on a C18 reverse-phase column (VYDAC, 250 × 5 mm) using a gradient of 10−50% acetonitrile in water (with 0.1% TFA) over 30 min, a detector wavelength of 254 nm, and a flow rate of 1 mL/min (*23*).

To prepare large quantities of enterobactin for HPLC purification and mass spectrometry, the following were incubated at 37 °C for 1 h: 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 1.5 mM L-serine, 1.5 mM DHB, 10 mM ATP, 5 μM holo-EntB, 300 nM EntE, and 300 nM holo-EntF in a final volume of 1 mL. The reaction was quenched with 1.5 mL of 1 N HCl and extracted with 7.5 mL of ethyl acetate, and the ethyl acetate layer was concentrated under reduced pressure. The resulting residue was dissolved in 3 mL of 30% acetonitrile/water, and 2 mL of this was loaded on a C18 reverse-phase HPLC column (YMC−Pack ODS, 200 × 20 mm). Enterobactin was eluted with a 10−40% gradient of acetonitrile in water (with 0.1% TFA) at a flow rate of 10 mL/min and a detector wavelength of 254 nm. The fraction containing enterobactin (approximately 0.5 mg) was lyophilized and submitted for ion-spray mass spectrometry analysis (NuMega, California) on a PE-SCIEX API 100 instrument (Perkin-Elmer) in both positive and negative modes.

(c) TLC of Enterobactin Synthetase Reaction Products. Thin-layer chromatography was carried out on cellulose (Kodak Chromatogram Sheet 13255) using 5% (w/v) ammonium formate in 0.5% (v/v) aqueous formic acid. Compounds were detected by applying a spray of ninhydrin for L-serine detection followed by aqueous $FeCl_3$ (1% treated with saturated $NaHCO_3$) for detection of DHB-ser compounds (*24*).

Synthesis of Enterobactin and DHB-ser Standard Compounds. Enterobactin was synthesized according to a procedure reported by Ramirez (*25*). Dimer and trimer of DHB-ser were obtained by a partial hydrolysis of enterobactin under basic conditions (2 equiv of LiOH in THF/water at room temperature overnight) (*26*) followed by HPLC separation under the same conditions as used for the purification of enzymatically synthesized enterobactin. DHB-ser monomer was prepared by the method reported by Rastetter (*27*). Structures of all compounds were confirmed by ^{1}H NMR spectroscopy and mass spectrometry (NuMega, California).

Assays for the Partial Reactions Catalyzed by EntF. (a) ATP-[^{32}P]PP$_i$ Exchange Activity. ATP−pyrophosphate exchange was assayed as described previously (*12*) with minor modifications. Reaction mixtures contained (final volume 100 μL): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 1.5 mM L-serine, 10 mM ATP, and 10 nM holo-EntF (wild-type or S1138A mutant). The assay was initiated by the addition of 1 mM sodium [^{32}P]pyrophosphate (4.6 Ci/mol in wild-type assay, 3.8 Ci/mol in S1138A assay) and incubated at 37 °C for 0, 2, 4, 6, 8, and 10 min, respectively (each time point was assayed in triplicate). Reactions were quenched by the addition of 0.5 mL of a charcoal suspension (1.6% (w/v) activated charcoal, 0.1 M tetrasodium pyrophosphate, 0.35 M perchloric acid), and the charcoal was pelleted by centrifugation. The liquid was removed, and the charcoal pellet was washed twice with 0.8 mL of water. The pellet was then suspended in 0.5 mL of water and added to 3.5 mL of scintillation fluid (Ultima Gold, Packard); [^{32}P]

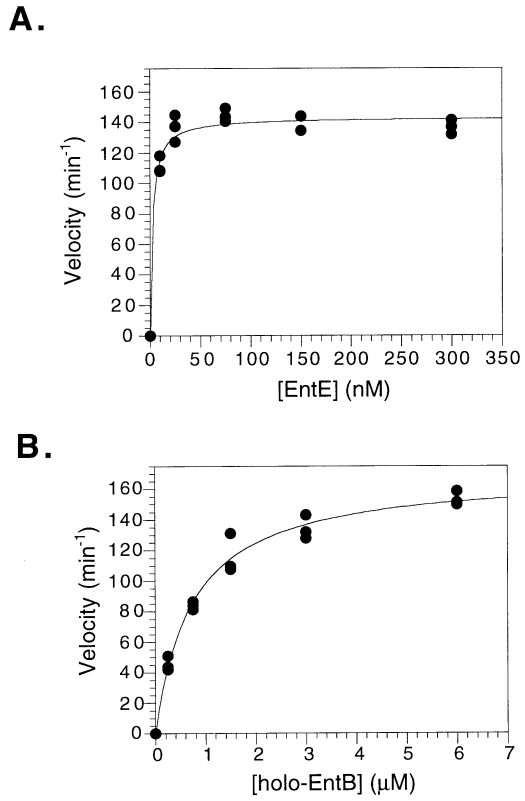


FIGURE 1: Saturation of enterobactin synthetase activity with EntE and holo-EntB. Holo-EntF (10 nM) was incubated for 10 min with DHB (0.5 mM), [^{3}H]serine (1.5 mM, 16.6 Ci/mol), ATP (10 mM), and increasing concentrations of (A) EntE (with holo-EntB at 6 μM) and (B) holo-EntB (with EntE at 200 nM). Enterobactin was quantitated by determining the amount of [^{3}H]serine extracted into ethyl acetate. Half-maximal activity is observed at 2.4 nM EntE and 0.73 μM holo-EntB, respectively. Velocity was calculated with respect to the amount of holo-EntF present in each assay (1 pmol).

incorporation into ATP was quantitated by liquid scintillation counting.

(b) Radioassay for the Detection of Covalent Incorporation of Serine into EntF. A trichloroacetic acid (TCA) precipitation assay was used to detect and quantitate Ser-S-Ppant-EntF species. The reaction mixture (100 μL final volume) included 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 1.5 mM [^{3}H]-L-serine (66.5 Ci/mol, 10 μCi/reaction) (Dupont NEN), and 2.4 μM EntF (apo- or holo-wild-type or holo-S1138A mutant); ATP (10 mM) was added last to initiate the reaction. Reactions were quenched with 0.8 mL of 10% TCA, 375 μg of BSA was added as a carrier, and the precipitated proteins were pelleted by centrifugation. The supernatant was removed, and the pellet was washed three times with 0.8 mL of 10% TCA. The protein pellet was then solubilized with 0.12 mL of 1 M Tris base and 0.03 mL of 10% SDS; the solubilized protein was added to 3.5 mL of scintillation fluid, and covalently incorporated radioactivity was detected by liquid scintillation counting.

Data shown in Figure 3 are an average of at least four assays; incubation was either at 37 °C or room temperature (23 °C) for 1−10 min; all conditions give complete loading of EntF with serine. In some cases, 8 μM holo-EntB (6 μM in S1138A reaction), 400 nM EntE (200 nM in S1138A reaction), and 500 μM salicylate (DHB in S1138A reaction) were added to the reaction mixture as noted in the figure legend. For the $t_{1/2}$ determination of the autoacylation of

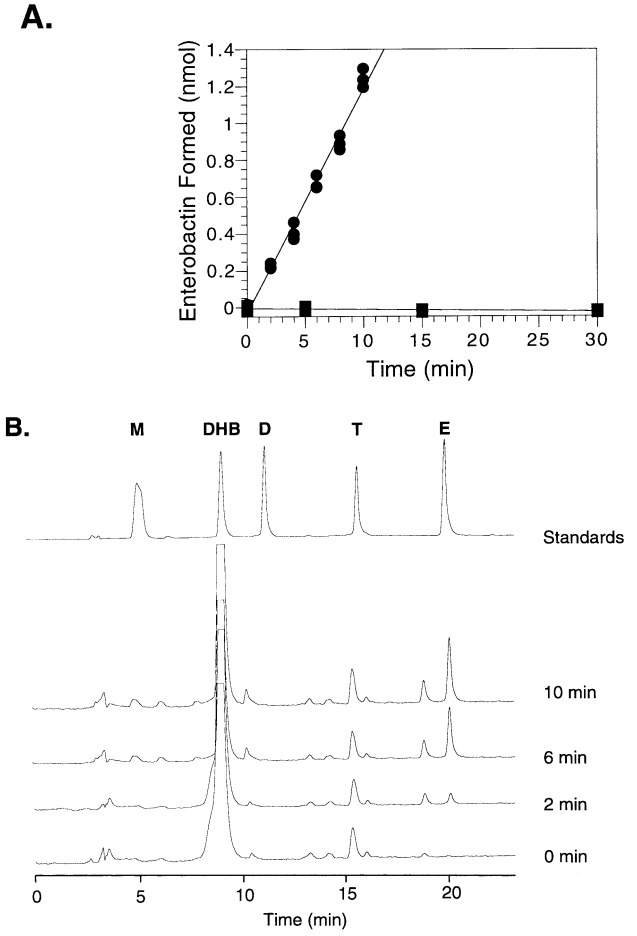


FIGURE 2: Time course for the formation of enterobactin by the ent synthetase components. Reaction mixtures (pH 7.5) included 6 μM holo-EntB, 200 nM EntE, and holo-EntF. (A) Ethyl acetate extraction radioassay for the detection of enterobactin. Circles show the progress of the reaction with 10 nM wild-type EntF and squares the reaction with 500 nM S1138A EntF. A rate of 121 min^{-1} is calculated for catalysis by wild-type EntF, while no catalysis by the EntF thioesterase mutant is observed. (B) HPLC analysis of time points as prepared in A using wild-type EntF. Included above is an HPLC trace of standard compounds: DHB-ser monomer (M), 2,3-dihydroxybenzoic acid (DHB), DHB-ser dimer (D) and trimer (T), and enterobactin (E). Enterobactin (20.2 min) is the sole serine-dependent product, formed at a rate of 122 min^{-1} (confirmed by co-injection with each of the standard compounds). The peak at 18.9 min is formed even in the absence of serine, EntF, or EntB and is likely a DHB-DTT adduct.

wild-type holo-EntF with serine, incubations were carried out at 0 °C at time points from 2 to 120 s.

(c) Autoradiography for the Detection of the Covalent Incorporation of Serine and Salicylate into EntF. Reaction mixtures included (100 μL final volume): 10 mM $MgCl_2$, 5 mM DTT, 75 mM Tris-HCl (pH 7.5), 10 mM ATP, 5 μM holo-EntB, 1 μM EntE, 1 μM holo-EntF (wild-type or S1138A mutant), and in some cases 410 μM [^{14}C]-L-serine (55.5 Ci/mol) (Moravek Biochemicals Inc.) or 144 μM [^{14}C]-salicylate (55.5 Ci/mol) (Dupont NEN) or 1.5 mM L-serine and 144 μM [^{14}C]salicylate (55.5 Ci/mol). Reactions were initiated by the addition of holo-EntF and incubated for 5 min at room temperature; reactions were quenched with 0.8 mL of 10% TCA. The precipitated proteins were pelleted by centrifugation, the TCA was removed, and the pellet was washed once with 0.8 mL of 10% TCA. The protein pellet was dissolved in SDS sample buffer (20 μL) and 1 M Tris

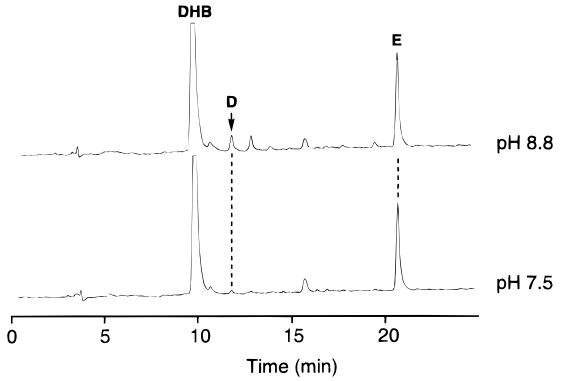


FIGURE 3: Detection of enterobactin biosynthetic intermediates released from holo-EntF. Holo-EntB (100 nM), EntE (200 nM), and holo-EntF (1 μM) were incubated for 10 min with L-serine, DHB, and ATP at pH 7.5 or pH 8.8. HPLC traces of the ethyl acetate-extractable material in the reaction mixtures show that while enterobactin is the major product, some DHB-ser dimer is also observed, 22% of the amount of enterobactin at pH 8.8. Peak identity was confirmed by co-injection with standard compounds. (Abbreviations are as described in Figure 2.)

base (2 μL) prior to loading and electrophoresis on a 4−20% Tris-glycine gel (Bio-Rad). The gel was stained with Coomassie blue, destained, and soaked in Amplify (Amersham) for 25 min prior to drying. The dried gel was exposed to film for 7 days.

Gel Filtration of the Ent Synthetase Components. Gel filtration chromatography was performed on a Superdex 200 HR 10/30 column (10 × 305 mm) as recommended by the manufacturer (Pharmacia). The column was calibrated with blue dextran 2000, thyroglobulin (698 kDa), ferritin (418 kDa), catalase (206 kDa), aldolase (167 kDa), albumin (66.1 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (15.6 kDa) available as a calibration kit and performed according to the manufacturer's instructions (Pharmacia). The standard proteins were dissolved in 50 mM sodium phosphate (pH 7.0), 150 mM NaCl (buffer C), loaded on the column in a 250 μL volume at 0.5 mL/min, and eluted from the column at 0.5 mL/min with buffer C. The ENT proteins were loaded and eluted using these same conditions. Holo-EntF (48 μM) was diluted to 4.8 μM in buffer C, holo-EntB (90 μM) was diluted to 14 μM in buffer C, and EntE (150 μM) was diluted to 18 μM in buffer C prior to their individual loadings on the gel filtration column. For the detection of any ent synthetase complex, the following reaction mixture was prepared (250 μL final volume): 20 mM $MgCl_2$, 10 mM DTT, 150 mM Tris-HCl (pH 7.5), 1 mM L-serine, 1 mM salicylate, 10 mM ATP, 18 μM holo-EntB, 7 μM EntE, and 4 μM holo-EntF; the reaction was incubated at room temperature for 15 min prior to loading and elution from the gel filtration column. Fractions (1 mL) were collected as the ent synthetase mixture was eluted from the column; 15 μL of each fraction was loaded on the 10% Tris-glycine gel shown in Figure 5.

RESULTS

Preparation of Holo-EntB and Holo-EntF by Pretreatment with the Phosphopantetheinyl Transferase EntD. EntD, which is required for the biosynthesis of enterobactin (*28*), has previously been shown to be the phosphopantetheinyl transferase which catalyzes the posttranslational attachment

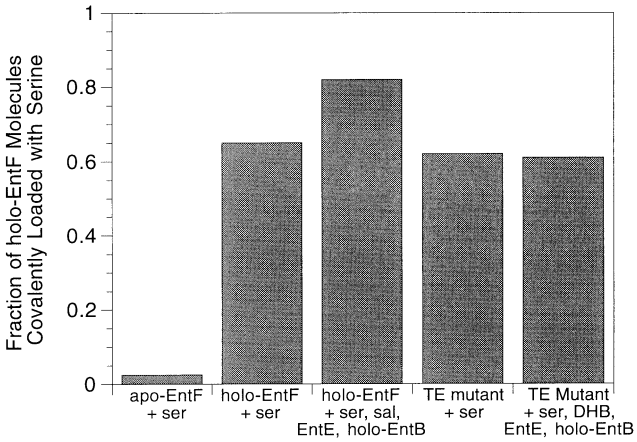


FIGURE 4: Stoichiometry of covalent loading of EntF with [^{3}H]-serine: (column 1) apo-EntF incubated with serine and ATP; (column 2) holo-EntF incubated with serine and ATP; (column 3) holo-EntF incubated with the complete enterobactin synthesis reaction mixture (serine, ATP, salicylate (sal), holo-EntB, EntE); (column 4) the holo-EntF thioesterase mutant (S1138A) incubated with serine and ATP; (column 5) S1138A holo-EntF incubated with serine, ATP, DHB, holo-EntB, and EntE.

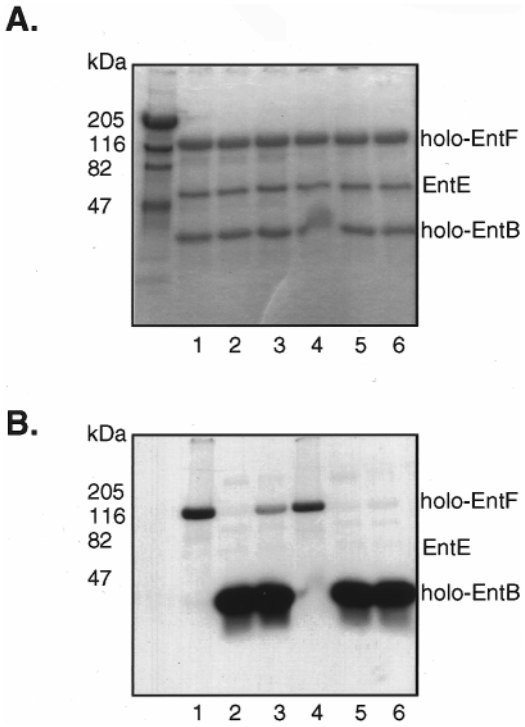


FIGURE 5: Demonstration of the covalent loading of wild-type and mutant holo-EntF with [^{14}C]serine as well as the serine-dependent loading of wild-type holo-EntF with [^{14}C]salicylate. (A) Coomassie-stained gel (4−20% gradient) of the TCA-precipitated reaction mixtures. (B) Autoradiograph of this gel. All reactions (100 μL) included 5 μM holo-EntB, 1 μM EntE, 1 μM holo-EntF, and 10 mM ATP. (lane 1) Wild-type EntF with 410 μM [^{14}C]serine (55.5 Ci/mol). (lane 2) Wild-type EntF with 144 μM [^{14}C]salicylate (55.5 Ci/mol). (lane 3) Wild-type EntF with 144 μM [^{14}C]salicylate and 410 μM unlabeled serine. (lanes 4−6) Same as lanes 1−3 but using S1138A EntF instead of wild-type.

of 4′-phosphopantetheine (Ppant) from coenzyme A to each of its substrate proteins, EntB and EntF, presumably at their conserved serine residues, Ser245 and Ser1006, respectively (*10, 14*). Conversion of apo-EntB and apo-EntF to their holo-forms makes these proteins competent for the activation of 2,3-dihydroxybenzoate (DHB) and L-serine, respectively,

in thioester attachment to Ppant, an obligatory step in nonribosomal peptide biosynthesis as delineated by the multiple carrier thiotemplate mechanism (reviewed in *29*). Purified EntD was incubated with coenzyme A and purified apo-EntB or apo-EntF at a molar ratio of approximately 100:1 EntB or EntF to EntD. The resulting holo-EntB or holo-EntF (10 mg) was then purified from the reaction mixture and used in all subsequent assays for enterobactin formation. Stoichiometric conversion of EntB and EntF to the holo-form was confirmed by incubating the holo-proteins again with EntD and [^{3}H]-CoASH; no additional radiolabel was incorporated into holo-EntB or holo-EntF, indicating their complete phosphopantetheinylation (data not shown). Conversion of EntB and EntF to holo-proteins thereby eliminated the requirement for EntD in the enterobactin synthetase (see below).

Reconstitution of Enterobactin Synthetase Activity. Incubation of the purified proteins holo-EntB, EntE, and holo-EntF with DHB, L-serine, and ATP resulted in the production of enterobactin. The reaction product, which comigrated on HPLC with a chemically synthesized enterobactin standard (MATERIALS AND METHODS section), was purified by HPLC and submitted for mass spectrometry analysis. Ion spray mass spectrometry gave a peak for the $[M - H]^-$ ion with $m/z = 668$ as expected for enterobactin (669 Da). This represents the first reported successful in vitro synthesis of enterobactin from DHB and serine by pure enterobactin synthetase enzyme components.

The rate of enterobactin formation by the ent synthetase components holo-EntB, EntE, and holo-EntF was investigated using both an ethyl acetate extraction radioassay and an HPLC assay. For the ethyl acetate extraction radioassay, previously used in the literature for the detection of ent synthetase activity (*12*, *22*, *30*), the ent synthetase enzymes were incubated with ATP, DHB, and [^{3}H]-L-serine. Reactions were quenched with 1 N hydrochloric acid, and the reaction products were then extracted into ethyl acetate; detection of [^{3}H] in the ethyl acetate layer indicated the formation of DHB-ser, either as enterobactin or one of its breakdown products (DHB-ser monomer, dimer, or linear trimer). The HPLC assay, based on the work of Winkelmann and co-workers (*23*), allowed the separation of enterobactin from authentic DHB-ser monomer, dimer, and trimer, permitting the reaction products and any intermediates to be precisely identified.

The ethyl acetate extraction radioassay was first used to determine the appropriate ratio of the holo-EntB, EntE, and holo-EntF components required to observe enterobactin formation under initial velocity conditions at pH 7.5. The velocity of enterobactin synthesis was monitored at varying concentrations of EntE and holo-EntB while holding the concentration of the holo-EntF catalyst constant at 10 nM (Figure 1). The requirement for EntE is quite low, with half-maximal activity at 2.4 nM EntE and a maximum velocity of 143 min^{-1} (Figure 1A). This is consistent with the previously identified function of EntE as a catalyst for the acylation of holo-EntB with DHB derived from DHB-AMP (*11*, *14*). Much more holo-EntB is required, with half-maximal activity observed at 0.73 μM holo-EntB and a maximum velocity of 170 min^{-1} (Figure 1B), again consistent with its proposed role as the aryl donor protein *substrate* for holo-EntF in the formation of DHB-ser (*14*). Holo-EntF appears to act as the sole catalyst for the formation of the three amide and three ester linkages found in enterobactin, using ATP, L-serine, and DHB-S-Ppant-EntB (acylated by EntE) as its substrates.

The formation of enterobactin versus time was investigated at pH 7.5 using 10 nM holo-EntF as the catalyst and saturating amounts of EntE (200 nM) and holo-EntB (6 μM); activity was measured using both the ethyl acetate extraction radioassay and the HPLC assay. Figure 2A shows that enterobactin formation is linear over the time points examined, with a rate of 121 min^{-1} calculated from the slope of this line. In Figure 2B are HPLC separations of reaction mixtures prepared in the same manner. At pH 7.5, only enterobactin (retention time = 20.2 min) is made and released by the ent synthetase enzymes (rate of 122 min^{-1}); no DHB-ser monomer, dimer, or trimer is evident in the HPLC trace. This result validates the use of the ethyl acetate extraction radioassay for the calculation of the velocity of enterobactin formation, at least at pH 7.5, because even though this assay cannot distinguish between enterobactin and its DHB-ser breakdown products, none of these DHB-ser compounds are observed in reactions carried out at this pH. The peak at 18.8 min, whose formation is independent of the presence of EntF or serine (data not shown), is most likely a DHB-DTT adduct (arising from the capture of DHB-AMP and/or DHB-Ppant-EntB by DTT) which will not be detected in the [^{3}H]serine-based extraction assay. In the same assays carried out at pH 8.8, some DHB-ser dimer is also observed in the HPLC trace (dimer:ent = 6:100) (data not shown), increasing when amounts of holo-EntB in the assay are subsaturating (dimer:ent = 22:100) (Figure 3). This DHB-ser dimer most likely results from premature hydrolysis of this intermediate from holo-EntF rather than hydrolysis of enterobactin itself given that under these conditions (pH 8.8 Tris-HCl buffer, 10 min incubation) enterobactin hydrolyzes to give about 8% DHB-ser linear trimer, but no dimer or monomer. To summarize, these data demonstrate that holo-EntF is an efficient catalyst for enterobactin formation with an average turnover number of 140 min^{-1}.

The rate of enterobactin formation was also examined in the presence of EntD to determine whether this enzyme had a role not only in phosphopantetheinylating the ArCP and PCP domains of EntB and EntF but also perhaps in facilitating productive interactions between the Ent proteins. However, addition of 500 nM EntD to the reaction mixture did not alter the rate of enterobactin biosynthesis (data not shown). Additionally, 2-hydroxybenzoate (salicylate) was substituted for DHB to see if this aromatic acid could be utilized by holo-EntF, as it had previously been shown to be adenylated by EntE (*11*) and used to acylate holo-EntB (*14*). Surprisingly, use of salicylate did not result in any product formation (data not shown) despite the fact that salicylate goes partway through the EntF catalytic cycle and may be observed covalently linked to EntF (see below, Figure 5).

Partial Reactions of Enterobactin Biosynthesis Catalyzed by EntF. (a) Reversible Formation of Seryl-AMP. EntF (purified predominantly in the apo-form) had been initially characterized as a seryl-AMP ligase by ATP-[^{32}P]PP$_i$ exchange activity with a turnover number for adenylation of 760 min^{-1} (*13*). To verify an equivalent adenylating ability of the holo-EntF used in these studies, its ATP-PP$_i$ exchange

rate was determined to be 1250 min^{-1} in the presence of 1.5 mM L-serine and 10 mM ATP (data not shown).

(b) Formation of Seryl-S-Ppant-EntF. According to the multiple carrier thiotemplate mechanism for nonribosomal peptide biosynthesis (*29*), amide bonds are formed between substrates while they are held in thioester linkages to the Ppant moiety of the synthetase's carrier protein domains. In the case of enterobactin biosynthesis, EntB has been shown to hold the DHB-Ppant thioester (Scheme 3) and, given that EntF is the seryl-AMP ligase and has an appropriately positioned PCP domain, EntF was presumed to hold the covalent seryl-Ppant thioester, setting the stage for DHB-ser-Ppant-EntF formation. This was confirmed by incubating holo-EntF with [^{3}H]serine and ATP, precipitating the reaction mixture with trichloroacetic acid (TCA), and counting the radioactivity that was incorporated into the protein (Figure 4). Holo-EntF could incorporate about 0.65 equiv of [^{3}H]-serine, consistent with the covalent attachment of up to 1 [^{3}H]serine molecule/PCP domain in each EntF molecule. We expected 1.0 equiv of serine/EntF molecule; however, factors such as errors in protein concentration and specific activity, incomplete recovery of the precipitated EntF, and inactivity of some percentage of the EntF population could all contribute to the fractional reduction in [^{3}H]serine incorporation observed here from that expected. For comparison, apo-EntF (as obtained following purification from an EntF-overproducing strain) was incubated with [^{3}H]serine and ATP; little covalent serine adduct was observed (2.5%), verifying the Ppant terminal thiol in the EntF PCP domain as the covalent attachment site for L-serine.

An attempt was made to determine the $t_{1/2}$ for self-catalyzed covalent loading of serine onto EntF as the Ser-S-Ppant-EntF species. However, this reaction was very rapid and even at 0 °C only a rough estimate of a $t_{1/2}$ of 2 s may be made (data not shown), corresponding to a rate of about 20 min^{-1} for the autocatalytic loading of serine onto EntF. Enterobactin synthesis was also observed at 0 °C with a rate of <5 min^{-1}.

(c) Formation of Aryl-Ser-S-Ppant-EntF. Following the self-catalyzed loading with serine, it is expected that EntF will catalyze the formation of an amide bond between intermolecularly Ppant-tethered DHB and serine moieties using the aryl donor EntB as substrate to yield the covalent DHB-ser-S-Ppant-EntF, a species detectable by autoradiography (Figure 5). Holo-EntF, EntE, and holo-EntB were incubated with [^{14}C]salicylate (since radiolabeled DHB was not available) or [^{14}C]serine and ATP, the reaction was quenched with TCA, and the protein precipitate was dissolved and subjected to SDS−PAGE. When [^{14}C]serine was used as the labeled substrate, holo-EntF was covalently radiolabeled (lane 1), confirming the results presented in Figure 4. When [^{14}C]salicylate was used, in the absence of unlabeled serine (lane 2), only holo-EntB was labeled as has been demonstrated previously (*14*). If, however, unlabeled serine was included in the reaction mixture (lane 3), both holo-EntB and holo-EntF were labeled with [^{14}C]salicylate. Given that the incorporation of salicylate into holo-EntF is dependent on serine, it is very likely that the labeled EntF band in lane 3 represents salicyl-ser-S-Ppant-EntF and by analogy provides evidence for the DHB-ser-S-Ppant-EntF intermediate.

The amount of [^{3}H]serine covalently linked to holo-EntF was also examined in the presence of the other ent synthetase components (EntE, holo-EntB, salicylate) using the TCA precipitation assay in the hopes of detecting multiple DHB-ser intermediates accumulating on each EntF molecule (i.e., >1 equiv of [^{3}H]serine/EntF molecule) as precursors to the assembly of the cyclic trimeric enterobactin product (Figure 4). Salicylate was used rather than DHB because we had previously observed that salicylate, while capable of being covalently linked to EntF, does not go on to product; [^{3}H]-enterobactin is precipitated by TCA and thus interferes with the quantitation of [^{3}H]serine covalently linked to EntF. In the presence of the other ent synthetase components, 0.82 equiv of [^{3}H]serine were incorporated into EntF, still approximately 1 equiv/EntF molecule; this may well be due to the inability of salicylate to go on to product. Given the rapidity of the enterobactin synthesis reaction ($2-3$ s^{-1}), rapid quench techniques will likely be required to search for any $(DHB\text{-}ser)_2$ or $(DHB\text{-}ser)_3$ enzyme-linked intermediates that may accumulate during turnover.

An EntF Thioesterase Domain Mutant (S1138A) Which Is Inactive for Enterobactin Formation. EntF is a multidomain protein with four domains identified by homology to other nonribosomal peptide synthetases, in order from N- to C-terminal as condensation (for making amide linkages), adenylation, thiolation (PCP), and thioesterase domains. A consensus sequence has been described for the active site serine residue responsible for acyl-*O*-enzyme intermediate formation in thioesterases (*19, 31, 32*); this corresponds to Ser1138 in the EntF protein sequence (*12*). $Serine_{1138}$ was therefore mutated to alanine, and the activity of the purified mutant holo-EntF was examined using the ethyl acetate extraction radioassay. As shown in Figure 2A, S1138A EntF is unable to catalyze enterobactin formation or even the release of DHB-ser intermediates using these assay conditions, to a lower limit of a rate of 1/4500 that of wild-type holo-EntF.

Despite the inability of S1138A EntF to accomplish catalytic assembly of enterobactin, the mutant enzyme could carry out several of the partial reactions of enterobactin biosynthesis. The ATP-PP_i exchange rate of S1138A holo-EntF was determined to be 1200 min^{-1} (data not shown), in good agreement with the 1250 min^{-1} obtained for the wild-type EntF. The S1138A mutant holo-EntF could also covalently incorporate [^{3}H]serine (Figure 4). In the presence of serine only or in the presence of all the ent synthetase reactants, 0.62 equiv of the mutant holo-EntF was covalently loaded with serine, also in good agreement with the wild-type results. Since the S1138A holo-EntF mutant accumulated the same amount of serine whether EntE, holo-EntB, and DHB were present or not, the EntF thioesterase mutant does not accumulate covalent $(DHB\text{-}ser)_2$ or $(DHB\text{-}ser)_3$ intermediates.

Finally, the ability of S1138A holo-EntF to form the amide-linked DHB-ser-S-Ppant-EntF species was investigated. As seen in the autoradiograph in Figure 5, while the mutant EntF is clearly covalently labeled with [^{14}C]serine (lane 4), surprisingly it is not definitively labeled with [^{14}C]-salicylate even in the presence of unlabeled serine (lane 6). Prolonged incubations of S1138A EntF (0.1 nmol) with the ent synthetase components, DHB, and high specific activity [^{3}H]serine (66.5 Ci/mol) were then performed in order to

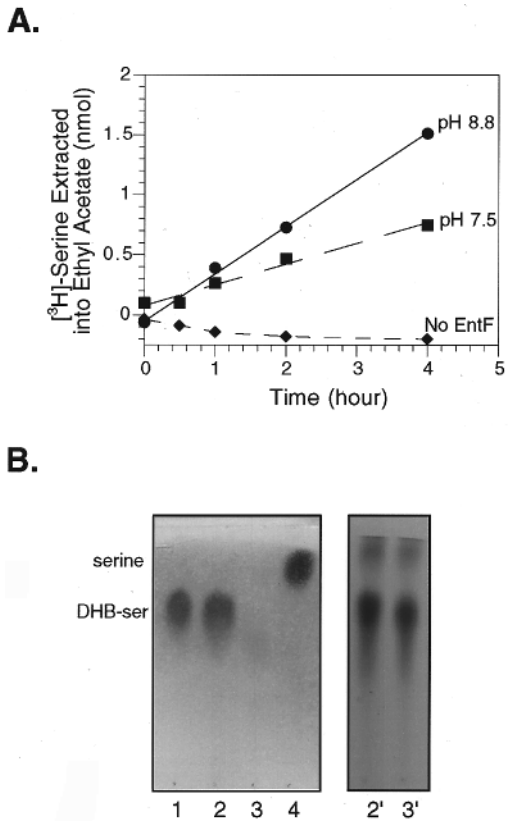


FIGURE 6: Conversion of [^{3}H]serine (66.5 Ci/mol) into an ethyl acetate extractable compound(s) dependent on the presence of the S1138A EntF mutant (0.1 nmol), possibly by hydrolysis of a DHB-ser covalent intermediate. (A) Time course generated using the ethyl acetate extraction radioassay. At pH 7.5 (squares) release is observed at a rate of 0.029 min^{-1}, while at pH 8.8 (circles) the rate increases to 0.066 min^{-1}. (B) Cellulose TLC analysis of the ethyl acetate extractable compound(s) obtained after a 4 h incubation. (lane 1) Synthetic DHB-ser monomer. (lane 2) Co-spot of lanes 1 and 3. (lane 3) Ethyl acetate extract of the reaction mixture. (lane 4) Unlabeled L-serine. Lanes 2′ and 3′ are autoradiographs of lanes 2 and 3.

detect any small amounts of DHB-ser compounds which may be released by or hydrolyzed from the mutant enzyme. Using the ethyl acetate extraction radioassay, after a 4 h reaction at pH 8.8 about 1.5 nmol of [^{3}H]serine could be extracted into ethyl acetate dependent on the presence of S1138A EntF (0.1 nmol) in the reaction mixture (Figure 6a), suggesting coupling of serine with DHB via amide bond formation at a turnover rate for DHB-ser monomer of 0.066 min^{-1} (at pH 8.8), some 2000-fold slower than the wild-type EntF turnover rate (at pH 7.5). The majority of this radiolabeled, ethyl acetate-extracted material co-chromatographed with a chemically synthesized DHB-ser monomer standard on a cellulose TLC plate (Figure 6B). Identification by HPLC of the compound(s) released by the mutant enzyme was hampered by the small quantities liberated as well as by the increased number of interfering peaks whose appearance was independent of the presence of EntF, likely a consequence of the long assay incubation times required.

Gel Filtration Chromatography of the Enterobactin Synthetase Components. The formation of the DHB-serine portion of enterobactin (i.e., the *amide* bonds) may be readily explained by the tenets of the multiple carrier thiotemplate model for nonribosomal peptide biosynthesis (*29*), but a mechanism for the formation of the *ester* linkages between the three DHB-ser units of enterobactin has not been well-defined. Given that EntF has only one PCP domain, one possible mechanism for assembling 3 equiv of DHB-ser and accomplishing the formation of the three ester linkages would be for holo-EntF to oligomerize as a trimer, with ester bond formation occurring between the three DHB-ser units each held by the Ppant cofactor attached to the 1 PCP domain/EntF monomer. To test this, gel filtration chromatography on Superdex 200 HR was used to detect any oligomerization of EntF or any complex with the other ent synthetase proteins, EntE and holo-EntB. When holo-EntF was chromatographed alone, its elution volume corresponded to a mass of 192 kDa (relative to the globular proteins used to calibrate the column), about 1.4 times its calculated mass of 142 kDa (data not shown). Given that EntF is a multidomain protein and therefore possibly nonspherical in structure, this peak most likely represents an EntF monomer. This is further supported by the observation that, in assays for enterobactin production, the rate of enterobactin synthesis is not slowed by inclusion of a 10-fold excess of apo-EntF over holo-EntF in the reaction mixture (data not shown); since the activity of EntF requires phosphopantetheinylation, the presence of apo-EntF in the reaction mixture would be expected to slow enterobactin production if multimers of EntF were required to accomplish its biosynthesis (a dominant negative effect). Holo-EntB (32.6 kDa calculated mass) and EntE (59 kDa calculated mass) were also each chromatographed alone on the gel filtration column, eluting as a trimer (90 kDa) and a monomer (66 kDa), respectively (data not shown). This is in contrast to previous gel filtration data which indicated EntE is a dimer (*11*) and EntB is a pentamer (*8*).

Holo-EntF was then incubated with holo-EntB, EntE, ATP, serine, and salicylate prior to loading and elution from the gel filtration column (Figure 7). Holo-EntF eluted at approximately the same volume as when run alone, with an elution volume corresponding to a mass of 216 kDa, 1.5 times its calculated mass. The peaks containing holo-EntB and EntE were somewhat overlapping as shown in the SDS−PAGE gel of the gel filtration column fractions (Figure 7B). The position of the holo-EntB peak (fraction 27) is again consistent with trimerization of holo-EntB (100 kDa). Because of the overlap of the holo-EntB and EntE peaks, the EntE peak is difficult to pinpoint, but the elution volume corresponds to a mass of approximately 79 kDa. There may be some association of the holo-EntB and EntE proteins, but neither of these proteins displays a stable interaction with holo-EntF.

DISCUSSION

The mechanism for the assembly of three 2,3-dihydroxybenzoate and three L-serine moieties by amide and ester bond-forming steps into the iron-chelating cyclic trilactone enterobactin has been a subject of study for more than 25 years. Early genetic complementation studies established that four gene products were required for the conversion of DHB into enterobactin: *entD*, *entE*, *entF*, and *entG* (*28*, *33*). Enterobactin biosynthesis was detected in partially purified *E. coli* cell extracts, and the EntD−G activities were observed by their ability to complement mutant cell extracts (*22*, *30*, *34*). Genes encoding EntE (*11*) and EntF (*12*) were cloned,

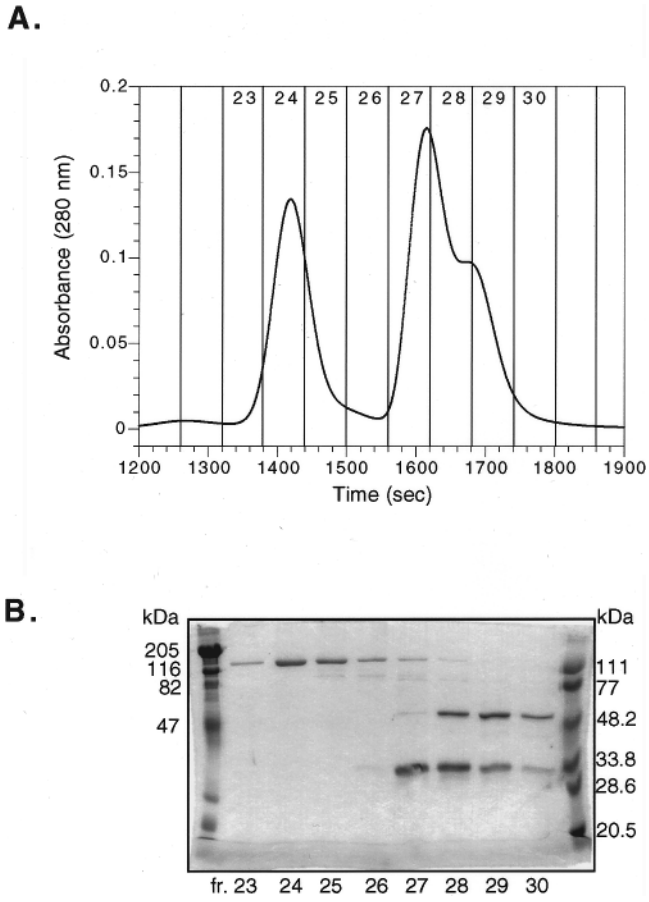


FIGURE 7: Gel filtration chromatography provides no evidence for a long-lived association of the enterobactin synthetase proteins. (A) Elution profile of the ENT proteins from a Superdex 200 HR 10/30 column. Holo-EntB (4.5 nmol), EntE (1.8 nmol), and EntF (1 nmol) were incubated for 15 min with ATP, salicylate, and serine prior to loading to and elution from the Superdex column. (B) Coomassie-stained 10% SDS−PAGE gel of Superdex column fractions 23−30. Holo-EntF (142 kDa) elutes first (fractions 23−26) followed by holo-EntB (32.6 kDa, eluting as a trimer) (fractions 27−30) and EntE (59 kDa) (fractions 28−30).

and DHB-AMP ligase and serine-AMP ligase activity, respectively, were demonstrated for these proteins. Until recently, however, the precise function of the enterobactin synthetase components, particularly EntD and EntG, has remained elusive. EntD has now been identified as a phosphopantetheinyl transferase, catalyzing the posttranslational covalent attachment of a phosphopantetheine cofactor, derived from coenzyme A, onto a serine residue of its substrate proteins, EntB and EntF (*10, 14*). Genetic evidence had indicated that *entG* resided within *entB* (*35*) and recent biochemical studies have confirmed that the C-terminal domain of EntB functions as an aryl carrier protein (ArCP) domain, accepting DHB from EntE as a covalent thioester linked to its Ppant cofactor (*14*).

With this new information, the stage was set to attempt the in vitro reconstitution of enterobactin biosynthesis using purified enzyme components. Indeed, incubation of EntB, EntD, EntE, and EntF with CoASH, DHB, serine, and ATP results in the enzymatic production of enterobactin. By pretreating apo-EntB and apo-EntF with EntD and CoASH and isolating the resulting holo-EntB and holo-EntF, the requirement for EntD in enterobactin biosynthesis is eliminated. Thus, only three proteins are necessary and sufficient to observe enterobactin biosynthesis in vitro at the rate of 140 min^{-1}: holo-EntB, EntE, and holo-EntF. Holo-EntF functions as the catalyst for creating the three amide and three ester linkages in the cyclic enterobactin, with EntE and holo-EntB serving priming (acylation of holo-EntB with DHB) and substrate functions, respectively. Presumably, isolation of and reconstitution with the DHB-S-Ppant-EntB species would eliminate the requirement for EntE as well.

It has been proposed in the literature that the enterobactin synthetase is a multienzyme complex perhaps associated with the cytoplasmic membrane through the EntD component (*22, 34, 36, 37*). Of particular interest to us was the number of EntF multimers found in any such enterobactin synthetase complex. Gel filtration chromatography of holo-EntF alone or in combination with holo-EntB and EntE did not provide evidence for the oligomerization of EntF or for the formation of any stable enterobactin synthetase complex. In fact, the gel filtration profile (Figure 7) of the pure holo-EntB, EntE, and holo-EntF components compares very well with the profile generated by Greenwood and Luke (*22*) for the fractionation of Ent^+ cell extract on Sephadex G-200 with detection of EntD−G activity by complementation of mutant cell extracts. Previous gel filtration studies indicated complexes between EntD with EntF and/or EntB (EntG) (*22, 34, 36*); EntD was omitted from the studies presented here as it is superfluous to enterobactin biosynthesis provided EntF and EntB have been modified to the holo-form. In a recent study, antibodies to EntB, EntE, EntF, and an epitope-tagged EntD failed to immunoprecipitate any enterobactin synthetase complex from *E. coli* cell extracts (*38*). Therefore, it seems unlikely that any enterobactin synthetase complex persists in vitro, and if such a complex does form in vivo, its assembly is not required for enterobactin biosynthetic activity. It appears that one holo-EntF molecule, acting alone, is capable of the remarkable feat of catalyzing three amide and three ester bond-forming steps to yield cyclic enterobactin in the presence of its substrates ATP, serine, and DHB-S-Ppant-EntB (acylated by EntE).

The four-domain, 142 kDa holo-EntF catalyzes a number of different reactions in the assembly of enterobactin. EntF activates serine by adenylation (*12, 13*) and as demonstrated here then forms the covalent Ser-S-Ppant-EntF species. Previous attempts to detect covalent incorporation of serine into purified EntF were unsuccessful (*13*), undoubtedly due to substoichiometric phosphopantetheinylation of the EntF preparation. It is likely that an amide linkage between DHB and serine is then fashioned prior to the formation of the ester linkages between serine moieties, giving a DHB-ser-S-Ppant-EntF intermediate by interaction of holo-EntF with its substrate acyl-holo-EntB. When [^{14}C]salicylate is used in place of DHB in the enterobactin synthesis reaction, covalent incorporation of radioactivity into EntF is observed dependent on the presence of serine, this despite the fact that salicylate is not converted to a released cyclic trilactone product—a strong indication that (salicyl-ser)$_1$-S-Ppant-EntF forms but cannot elongate. Observation of DHB-ser dimer production in reactions carried out at basic pH (8.8) and/or at subsaturating levels of holo-EntB also indicates that two amide bonds form by the time the first ester bond is made. Indeed, early studies with partially purified enterobactin

enzymes reported the isolation of an enzyme-linked DHB-ser species (*30*). Since catalysis of enterobactin assembly occurs so rapidly (140 min^{-1}), rapid quench studies will be required to probe for and quantitate any intermediates which accumulate on EntF during turnover (e.g., $(DHB\text{-}ser)_1$-EntF, $(DHB\text{-}ser)_2$-EntF, $(DHB\text{-}ser)_3$-EntF). Release of small amounts of $(DHB\text{-}ser)_2$ at high pH may reflect diversion of a fraction of a $(DHB\text{-}ser)_2$-EntF intermediate released due to base lability of the covalent linkage.

Since gel filtration studies indicate that EntF acts as a catalytic monomer, the question arises as to how many sites are available on the enzyme for covalent attachment and accrual of DHB-ser intermediates. By sequence analysis there should be only 1 PCP domain with its attached Ppant cofactor at Ser1006/EntF chain. Indeed, analysis of the covalent loading of holo-EntF with [^{3}H]serine indicates 0.6−0.8 equiv of serine are incorporated/enzyme whereas little serine is incorporated into apo-EntF. How do the second and third serines proceed to load as covalent acyl-*S*-enzyme species if the cysteaminethiol terminus of the Ppant group is already derivatized by the first DHB-ser unit docked there? To address this issue, we focused our attention on the C-terminal domain of EntF (approximately 30 kDa) which shows sequence similarity to thioesterase domains typically seen at the C-terminus of multidomainal fatty acid synthases (FAS), polyketide synthases, and nonribosomal peptide synthetases including those responsible for the biosynthesis of the antibiotics gramicidin and pristinamycin and the penicillin precursor ACV (*17*, *19*).

The mechanism of thioesterase action, best studied for the vertebrate FAS type I and type II thioesterases, is believed to involve a serine−histidine−aspartate catalytic triad, with hydrolysis of the fatty acyl chain proceeding through a covalent acyl−enzyme intermediate at the active site serine, analogous to the mechanism of serine proteases; this mechanism has been supported by a variety of mutagenic studies (*39*−*42*) as well as by the first reported crystal structure of a thioesterase, myristoyl-ACP-specific thioesterase from *Vibrio harveyi* (*43*). The precise role of the thioesterase domain in nonribosomal peptide biosynthesis is less well understood. For the nonribosomal surfactin synthetase, insertions or deletions in the portion of the *srfA* operon encoding the thioesterase domain cause cells to lose their ability to produce surfactin (*44*); movement of this thioesterase domain from the C-terminus of srfAORF3 of the synthetase to a position within srfAORF2 results in the release of truncated peptides from the altered surfactin synthetase (*45*). Studies of the *entF* gene have also indicated that a truncation corresponding to deletion of the C-terminal 30 amino acids of the EntF thioesterase domain gives a loss of $EntF^+$ activity (*46*). EntF possesses a thioesterase consensus motif at Ser1138 (*12*), so this residue was targeted for mutagenesis to alanine to discern the importance of the thioesterase domain in the mechanism of enterobactin assembly.

The Ser1138Ala mutant EntF was indeed incompetent for enterobactin formation, less than 1/4500 wild-type activity. However, this mutant could still catalyze serine adenylation at the wild-type rate as well as autoacylate itself with the covalent attachment of serine (derived from Ser-AMP) to its Ppant cofactor. Moreover, the defect in the EntF S1138A mutant was not merely in cyclization and release of enterobactin as the mutant EntF accumulated only 0.62 equiv of serine when incubated with the complete enterobactin synthesis reaction mixture, indicating the failure of $(DHB\text{-}ser)_2$ and $(DHB\text{-}ser)_3$ intermediates to accumulate on the enzyme. Surprisingly, we were unable to show definitively that [^{14}C]salicylate is incorporated into the mutant EntF as detected by autoradiography in contrast to its formation on wild-type holo-EntF; a priori it is not expected that a defect in the thioesterase domain would affect catalysis of the amide linkage between salicylate or DHB and seryl-EntF. Very slow release of a compound from S1138A EntF was observed which co-chromatographs with the DHB-ser monomer on cellulose TLC, but definitive identification of this compound by HPLC purification and mass spectrometry awaits its isolation in larger quantity. In summary, the EntF thioesterase domain mutant is impaired in forming and/or elongating DHB-ser species into the final enterobactin product. Whether the defect occurs at the stage of amide bond formation between DHB and seryl-EntF, at the stage of ester bond formation between DHB-ser-EntF units, or both awaits further study; based on sequence similarity of the EntF thioesterase domain to other esterases and acyltransferases, we would expect the primary defect to be in the catalysis of ester bond formation.

Assuming that the mutation of Ser1138 to alanine does indeed eliminate ester bond formation in the mutant EntF, this serine residue is a good candidate for a second, "peripheral" site for the acceptance of DHB-ser units prior to ester bond formation. After DHB-ser is assembled on the "central" acylation site of the phosphopantetheine cofactor (in turn attached at Ser1006), it could be passed to and held covalently by Ser1138 of the thioesterase domain, a net S → O acyl shift. This would free up the Ppant thiol for formation of another DHB-ser unit; ester bond formation could then proceed by attack of the serine hydroxyl of the DHB-ser moiety held at the "peripheral" site on the thioester linkage of the DHB-ser moiety held at the "central" Ppant site, yielding the DHB-ser dimer covalenty attached to the serine residue of the thioesterase domain active site (Figure 8). Repetition of this process would give the $(DHB\text{-}ser)_3$-EntF species and finally the release of cyclic enterobactin. This model would account for the need to successively load 3 equiv of DHB-ser onto EntF and has precedent in the mechanism of fatty acid and polyketide chain elongation. In those processes, the growing acyl chain is passed from the Ppant cofactor of the ACP domain to the active site cysteine of the β-ketoacyl synthase prior to nucleophilic attack by the next malonyl extender unit (*47*). Future studies will concentrate on the identification of any such $(DHB\text{-}ser)_n$ species covalently linked to the wild-type EntF TE domain.

With the reconstitution of the enterobactin synthetase from a small number of pure enzyme components, we are poised to learn much about the mechanism of amide and ester bond formation in nonribosomal peptide and depsipeptide biosynthesis. Acyl transfer of an aryl group from a Ppant thioester (in this case intermolecularly from DHB-EntB) to the amino group of a second Ppant thioester (here seryl-EntF) is likely to be a prototypical strategy for the formation of other aryl-N-capped peptides, including the antibiotics actinomycin D (*48*) and pristinamycin (*49*) as well as for such siderophores as anguibactin (*50*), mycobactins/exochelins (*51*), and yers-

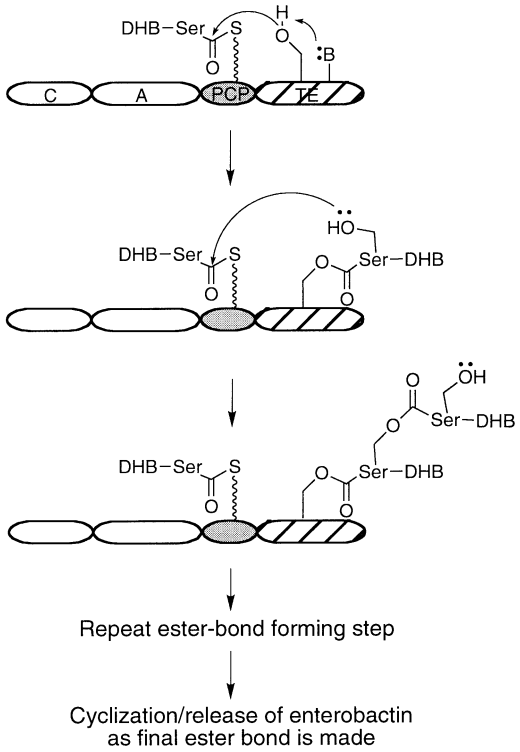


FIGURE 8: Model for the loading of three DHB-ser moieties and the formation of the ester bonds by monomeric holo-EntF in the assembly of enterobactin. DHB-ser formed on the EntF PCP (shaded) domain's Ppant cofactor may be passed to the nucleophilic active site serine$_{1138}$ residue in the adjacent thioesterase (TE) domain (hatched), freeing up the Ppant thiol for formation of the second DHB-ser unit. Nucleophilic attack by the hydroxyl group of the first DHB-ser covalently linked to the TE domain on the acyl thioester linkage of DHB-ser-Ppant would give the (DHB-ser)$_2$ oxo−acyl enzyme with covalent attachment to the TE domain. Repetition of this process would yield the (DHB-ser)$_3$−TE intermediate, and intramolecular attack of the free serine hydroxyl group of the third DHB-ser on the oxoester linkage to the TE domain serine$_{1138}$ residue would result in cyclization, release of enterobactin, and regeneration of enzyme (C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein domain; TE, thioesterase domain.)

iniabactin (*52*). Further studies of the EntF thioesterase domain should be rewarding for assessing its role as a peripheral acylation site for the growing tethered product as well as for deconvoluting chain length and macrocyclic size preferences for other peptide, depsipeptide, and polyketide synthases.

REFERENCES

1. Neilands, J. B. (1981) *Annu. Rev. Biochem. 50*, 715−731.
2. Crosa, J. H. (1989) *Microbiol. Rev. 53*, 517−530.
3. Crosa, J. H. (1997) *Microbiol. Mol. Biol. Rev. 61*, 319−336.
4. Walsh, C. T., Liu, J., Rusnak, F., and Sakaitani, M. (1990) *Chem. Rev. 90*, 1105−1129.
5. Harris, W. R., Carrano, C. J., Cooper, S. R., Sofen, S. R., Avdeef, A. E., McArdle, J. V., and Raymond, K. N. (1979) *J. Am. Chem. Soc. 101*, 6097−6104.
6. Liu, J., Duncan, K., and Walsh, C. T. (1989) *J. Bacteriol. 171*, 791−798.
7. Sakaitani, M., Rusnak, F., Quinn, N. R., Tu, C., Frigo, T. B., Berchtold, G. A., and Walsh, C. T. (1990) *Biochemistry 29*, 6789−6798.
8. Rusnak, F., Liu, J., Quinn, N., Berchtold, G. A., and Walsh, C. T. (1990) *Biochemistry 29*, 1425−1435.
9. Liu, J., Quinn, N., Berchtold, G. A., and Walsh, C. T. (1990) *Biochemistry 29*, 1417−1425.
10. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) *Chem. Biol. 3*, 923−936.
11. Rusnak, F., Faraci, W. S., and Walsh, C. T. (1989) *Biochemistry 28*, 6827−6835.
12. Rusnak, F., Sakaitani, M., Drueckhammer, D., Reichert, J., and Walsh, C. T. (1991) *Biochemistry 30*, 2916−2927.
13. Reichert, J., Sakaitani, M., and Walsh, C. T. (1992) *Protein Sci. 1*, 549−556.
14. Gehring, A. M., Bradley, K. A., and Walsh, C. T. (1997) *Biochemistry 36*, 8495−8503.
15. de Crécy-Lagard, V., Marlière, P., and Saurin, W. (1995) *C. R. Acad. Sci., Ser. III 318*, 927−936.
16. Stachelhaus, T., and Marahiel, M. A. (1995) *J. Biol. Chem. 270*, 6163−6169.
17. Marahiel, M. A. (1997) *Chem. Biol. 4*, 561−567.
18. Stachelhaus, T., Hüser, A., and Marahiel, M. A. (1996) *Chem. Biol. 3*, 913−921.
19. de Crécy-Lagard, V., Saurin, W., Thibaut, D., Gil, P., Naudin, L., Crouzet, J., and Blanc, V. (1997) *Antimicrob. Agents Chemother. 41*, 1904−1909.
20. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
21. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
22. Greenwood, K. T., and Luke, R. K. J. (1976) *Biochim. Biophys. Acta 454*, 285−297.
23. Winkelmann, G., Cansier, A., Beck, W., and Jung, G. (1994) *BioMetals 7*, 149−154.
24. O'Brien, I. G., Cox, G. B., and Gibson, F. (1969) *Biochim. Biophys. Acta 177*, 321−328.
25. Ramirez, R. J. A., Karamanukyan, L., Ortiz, S., and Gutierrez, C. G. (1997) *Tetrahedron Lett. 38*, 749−752.
26. O'Brien, I. G., and Gibson, F. (1970) *Biochim. Biophys. Acta 215*, 393−402.
27. Rastetter, W. H., Erickson, T. J., and Venuti, M. C. (1981) *J. Org. Chem. 46*, 3579−3590.
28. Luke, R. K. J., and Gibson, F. (1971) *J. Bacteriol. 107*, 557−562.
29. Kleinkauf, H., and von Döhren, H. (1996) *Eur. J. Biochem. 236*, 335−351.
30. Bryce, G.F, and Brot, N. (1972) *Biochemistry 11*, 1708−1715.
31. Hardie, D. G., Dewart, K. B., Aitken, A., and McCarthy, A. D. (1985) *Biochim. Biophys. Acta 828*, 380−382.
32. Brenner, S. (1988) *Nature 334*, 528−530.
33. Woodrow, G. C., Young, I. G., and Gibson, F. (1975) *J. Bacteriol. 124*, 1−6.
34. Woodrow, G. C., Young, I. G., and Gibson, F. (1979) *Biochim. Biophys. Acta 582*, 145−153.
35. Staab, J. F., and Earhart, C. F. (1990) *J. Bacteriol. 172*, 6403−6410.
36. Greenwood, K. T., and Luke, R. K. J. (1980) *Biochim. Biophys. Acta 614*, 185−195.
37. Armstrong, S. K., Pettis, G. S., Forrester, L. J., and McIntosh, M. A. (1989) *Mol. Microbiol. 3*, 757−766.
38. Hantash, F. M., Ammerlaan, M., and Earhart, C. F. (1997) *Microbiology 143*, 147−156.
39. Pazirandeh, M., Chirala, S. S., and Wakil, S. J. (1991) *J. Biol. Chem. 266*, 20946−20952.
40. Witkowski, A., Witkowska, H. E., and Smith, S. (1994) *J. Biol. Chem. 269*, 379−383.
41. Tai, M.-H., Chirala, S. S., and Wakil, S. J. (1993) *Proc. Natl. Acad. Sci. USA 90*, 1852−1856.
42. Witkowski, A., Naggert, J., Wessa, B., and Smith, S. (1991) *J. Biol. Chem. 266*, 18514−18519.
43. Lawson, D. M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E. A., and Derewenda, Z. S. (1994) *Biochemistry 33*, 9382−9388.

44. Galli, G., Rodriguez, F., Cosmina, P., Pratesi, C., Nogarotto, R., de Ferra, F., and Grandi, G. (1994) *Biochim. Biophys. Acta 1205*, 19−28.
45. de Ferra, F., Rodriguez, F., Tortora, O., Tosi, C., and Grandi, G. (1997) *J. Biol. Chem. 272*, 25304−25309.
46. Pettis, G. S., and McIntosh, M. A. (1987) *J. Bacteriol. 169*, 4154−4162.
47. Hopwood, D. A., and Sherman, D. H. (1990) *Annu. Rev. Genet. 24*, 37−66.
48. Stindl, A., and Keller, U. (1993) *J. Biol. Chem. 268*, 10612−10620.
49. Thibaut, D., Bisch, D., Ratet, N., Maton, L., Couder, M., Debussche, L., and Blanche, F. (1997) *J. Bacteriol. 179*, 697−704.
50. Tolmasky, M. E., Actis, L. A., and Crosa, J. H. (1993) *Infect. Immun. 61*, 3228−3233.
51. Wong, D. K., Gobin, J., Horwitz, M. A., and Gibson, B. W. (1996) *J. Bacteriol. 178*, 6394−6398.
52. Bearden, S. W., Fetherston, J. D., and Perry, R. D. (1997) *Infect. Immun. 65*, 1659−1668.

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