Genetic Analysis of the Enterobactin Gene Cluster in *Shigella flexneri*

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The genes for transport and synthesis of the phenolate siderophore enterobactin are present on the chromosomes of both Ent⁺ and Ent[−] clinical isolates of *Shigella flexneri*. To determine why Ent[−] *S. flexneri* isolates fail to express a functional enterobactin system, the structure and expression of enterobactin genes were examined. Several alterations may be responsible for the inability of *S. flexneri* to express enterobactin. (i) The mRNA levels produced from the *entC* and *fepB* genes were not derepressed in low-iron media. (ii) DNA sequence analysis of the *entC-fepB* intergenic region revealed an 83-bp noncontiguous deletion in the putative *fepB* leader sequence. The deleted sequences are in a region which would be capable of forming extensive stem-and-loop structures. (iii) An amber codon in the 5′ portion of the *entC* gene was also detected. (iv) An IS1 element, previously mapped to the Ent[−] *S. flexneri* enterobactin gene cluster, was found to lie within a potential transcriptional termination sequence in the *entF-fepE* intergenic region. (v) A mutation responsible for the inactivation of the *entF* gene was mapped to the *entF* coding region by using *entF* hybrid gene fusions. (vi) A comparison of outer membrane profiles from an *E. coli* strain harboring the cloned *fepA* gene from either an Ent⁺ or Ent[−] *Shigella* isolate revealed that the Ent[−] FepA protein is present in the outer membrane but at greatly reduced levels than that of the Ent⁺ FepA protein. This observation, along with additional studies, suggests that the Ent[−] FepA may be defective in translation and/or translocation.

In iron-deficient environments, bacteria may acquire iron by utilizing high-affinity iron transport systems which employ low-molecular-weight iron-chelating compounds, termed siderophores, and their cognate transport proteins. The enterobactin iron transport system, which has been found in *Escherichia coli* (30), *Salmonella typhimurium* (42), and other enteric species (21, 36), is the best characterized of the catechol-type siderophore systems. A general characterization of the genetics and biochemistry of almost all of the *E. coli* enterobactin genes and their products has been done (for a review, see reference 12). In *E. coli* there are thought to be as many as 14 distinct activities specifically required for synthesis and transport of enterobactin. The genes are clustered over approximately 20 kb of the genome, near 13 min on the *E. coli* chromosome. Seven genes (*entA-entG*) are required for the synthesis of enterobactin (8, 11, 14, 22, 39, 48). Several *fep* genes, whose products have been localized to the cytoplasmic membrane (FepC, FepE) (32, 40), periplasm (FepB) (41), and the outer membrane (FepA) (28), are required for transport of the ferrienterobactin complex through the cellular envelope. The product of the *fes* gene is thought to release iron from the ferrienterobactin complex once it has traversed the bacterial membranes (29).

Transcription of the enterobactin genes, which is regulated by the product of the *fur* gene and intracellular iron, initiates from two sets of tandem promoters situated between the *fepA* and *fes* genes (37, 38) and between *fepB* and *entC* (13, 31). It is likely that additional promoters exist.

Functional enterobactin transport systems are present in strains of *Shigella dysenteriae* and *Shigella sonnei*, but are only rarely detected in *Shigella flexneri* and *Shigella boydii*

(21). Studies examining siderophore systems in clinical isolates of *S. flexneri* revealed that while all the strains produced the native siderophore aerobactin, approximately 10% produced both aerobactin and enterobactin (35). Southern hybridization analysis revealed that the enterobactin genes were present on the chromosome of Ent[−] *S. flexneri*, but neither the siderophore nor the outer membrane receptor protein, FepA, were detected (34). In a previous study (44), the complete enterobactin gene clusters from Ent[−] and Ent⁺ *S. flexneri* isolates were cloned and characterized. The Ent[−] cloned genes were able to fully complement the defects in nine different *E. coli* mutants but failed to complement the *entF* mutant AN117 and only weakly complemented an *entE* mutant. An IS1 element, which appears to be conserved among Ent[−] isolates, was detected in the enterobactin gene cluster in the *entF-fepE* gene region. The mRNA levels produced from the Ent[−] *entB* and *entF* genes revealed that expression of these genes was significantly reduced relative to the levels in the Ent⁺ strain (44). Additionally, the mRNA levels of *entB* were unaffected by mutations in the Ent[−] *fur* gene, and *entF* was only partially derepressed in the Fur[−] strain.

Although the FepA protein is normally not detected in the outer membrane of Ent[−] *S. flexneri*, the cloned Ent[−] *fepA* gene when present on a multicopy plasmid is able to complement the defect in an *E. coli fepA* mutant (44). This observation suggests that the Ent[−] *fepA* gene product is functional but may be affected in expression or processing.

In this study, the enterobactin gene cluster present in an Ent[−] *S. flexneri* is further characterized. Specific mutations or genetic aberrations in the enterobactin genes and regulatory regions were mapped by the construction of gene fusions or identified by determining the nucleotide sequence of certain portions of the enterobactin gene cluster. Additionally, expression and synthesis of the Ent[−] FepA protein were examined.

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TABLE 1. Bacterial strains and plasmids used in the present study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>E. coli</i>		
AB1515	Ent ⁺	C. F. Earhart
MK1	<i>entF</i>	M. A. McIntosh
AN117	<i>entF</i>	C. F. Earhart
MT147	<i>entC::kan</i>	M. A. McIntosh
UT6900	<i>fepAΔ</i>	C. F. Earhart
UT481	M13 host	I. J. Molineux
<i>S. flexneri</i>		
SA101	Ent ⁻	S. M. Payne
SA301	Ent ⁺	S. M. Payne
Plasmids		
pACYC184	Cm ^r Tet ^r	10
pBR322	Cb ^r Tet ^r	4
pKK232-8	Promoterless <i>cat</i> gene	7
pFES1	SA101 <i>fepA-cat</i> fusion	This study
pFAS1	SA301 <i>fepA-cat</i> fusion	This study

MATERIALS AND METHODS

Strains. Bacterial strains, plasmids, and their sources are listed in Table 1. Recombinant plasmids are shown in Fig. 2 and 3.

Media, chemicals, and enzymes. Bacterial stocks were maintained frozen at -70°C in Luria broth (L broth) with 20% glycerol. L broth was used as a complete medium for routine growth of all *Shigella* and *E. coli* strains. Tris-buffered medium without added iron was used as described previously (33) to determine siderophore production. Bacterial production of siderophores was also detected by growing strains on Chrome Azurol-S medium (45). M9 minimal medium was made as 10× stocks by the method of Miller (26). All chemicals used were reagent grade.

DNA manipulations. Gene fusions were constructed by digesting cloned DNA with the appropriate restriction endonucleases. After the digested DNA was separated by electrophoresis through a 0.9% agarose gel, the appropriate fragment was eluted from the gel and ligated with T4 DNA ligase (Promega Biotech, Madison, Wis.) into a suitable restriction-digested cloning vector and transformed (23) into the *E. coli* host HB101. The gene fusion constructs were then further mapped to determine if the proper orientation was obtained. Routine isolation of recombinant plasmid DNA for restriction mapping and other purposes was obtained by using the boiling method of Holmes and Quigley (16).

Complementation. Complementation assays to determine the ability of cloned genes to complement *E. coli* enterobactin mutants were performed as described previously (44). To determine complementation specifically for the *entC* gene, *E. coli* MT147 (*entC::kan*) was used. Since the insertion in *entC* has a polar effect on downstream genes, an additional compatible plasmid carrying functional *entE*, *entB*, *entG*, and *entA* genes was present in this strain during the complementation assays.

DNA sequencing. All of the DNA sequencing was performed by the dideoxy chain termination method developed by Sanger et al. (43). The specific procedure employed was the Sequenase system (U. S. Biochemical Corp., Cleveland, Ohio). All of the reagents and enzymes were obtained as a kit. The DNA to be sequenced was cloned into the modified

M13 cloning vectors MP18 and MP19 (25) which were grown in *E. coli* UT481. Sequencing reactions were performed according to the instructions specified in the Sequenase kit; [α -³⁵S]dATP was used as the label for all reactions. The reaction mixtures were electrophoresed through either 4 or 6% acrylamide denaturing gels at 44 W constant power. After electrophoresis, the gels were fixed to remove urea, dried under heat, and then autoradiographed.

Outer membrane preparation and SDS-PAGE. *S. flexneri* and *E. coli* strains grown in M9 media containing either 20 μ M FeCl₃ (+Fe) or 25 μ g of ethylenediamine di(*o*-hydroxyphenylacetic acid) (-Fe) per ml were harvested in mid-log phase. Outer membranes were prepared as described previously (35). Membranes were solubilized in Laemmli solubilization buffer (19), and the proteins were separated by the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system of Ames (1). Slab gels (0.75 mm) consisted of a 4% stacking gel and a 10% separating gel. Mini-gels (7 by 10 cm) were electrophoresed at 200 V until the tracking dye reached the bottom of the gel. The mini-gels were then stained with brilliant blue R (Coomassie blue), destained, and dried. Protein standards (Bio-Rad Laboratories, Richmond, Calif.) were included in each gel.

Chloramphenicol acetyl transferase assay. Bacterial cells were grown to mid-log phase in M9 media. The cells were pelleted by centrifugation, suspended in 0.25 M Tris hydrochloride (pH 7.8), and centrifuged again. The cells were resuspended in the Tris buffer and disrupted by sonication. Before the assay for enzyme activity was done, the total protein concentration of the cell extracts was determined by using a protein assay kit (Bio-Rad). To measure enzyme concentration, a portion of the cellular extract containing 1 μ g of protein was placed in an Eppendorf tube, and the total volume was brought to 100 μ l with the addition of 0.25 M Tris hydrochloride (pH 7.8). To this was added 79 μ l of 1 M Tris hydrochloride (pH 8.0), 20 μ l of 4 mM acetyl coenzyme A, and 1 μ l of [¹⁴C]chloramphenicol (1 μ Ci/ μ l). The mixture was incubated in a 43°C water bath for 30 min and extracted with 1 ml of cold ethyl acetate, and the upper phase was transferred to a new tube and dried. The dried sample was suspended in 30 μ l of ethyl acetate, spotted onto a thin-layer chromatography plate (0.25-mm silica gel; Brinkman Instruments, Westbury, N.Y.), and placed in a chromatography chamber containing methanol-chloroform (1:9). After the solvent front reached the top of the thin-layer chromatography plate (approximately 15 min), the plate was air dried and exposed to X-ray film.

RNA isolation and hybridization. RNA isolation and hybridization were performed as described previously (44).

RESULTS

IS1 element. In the previous study (44), an IS1 element was found in the *S. flexneri* SA101 (Ent⁻) enterobactin gene cluster near the 3' portion of the *entF* gene. While it was demonstrated that the IS1 was not the primary cause of the defect in the Ent⁻ *entF* gene, it could affect the expression of some of the Ent⁻ *S. flexneri* enterobactin genes. To locate precisely this IS1 element in the chromosome, the DNA sequence of the junction between chromosomal and IS1 sequences was determined. A comparison of the SA101 sequence with that of *E. coli* is shown in Fig. 1. The sequence from SA101 was determined on both strands. It initiates from an EcoRV site present at the 3' end of the *entF* gene and extends to a BstEII site present in the IS1 element. The *E. coli* enterobactin sequences were provided by M. A.

FIG. 1. (A) Nucleotide sequence identifying site of insertion of the IS1 element present in the SA101 enterobactin gene region. A comparison of *E. coli* and SA101 DNA sequences in the enterobactin region adjacent to the IS1 junction site is presented. *E. coli* sequences are shown on the top line while SA101 sequences are shown below. Asterisk indicates nucleotide homology. *E. coli* sequence was provided by M. A. McIntosh. Partial genetic and physical map of this region is shown below. TE indicates putative transcriptional termination site present in the *E. coli* sequence; arrows show regions of dyad symmetry which constitute a potential stem structure within the transcription termination region. (B) Partial comparison of nucleotide sequences between the *S. flexneri* SA101 IS1 and an *E. coli* IS1 sequence (18). *E. coli* IS1 sequences are shown on the top line. A thin bar over sequences indicates a ribosome binding site; translational start (arrow) and stop signals (solid bar) are shown for the *insA* gene.

McIntosh, while the *Shigella* IS1 sequences were compared with the nucleotide sequence of an IS1 obtained from an *E. coli* strain (18). The *E. coli* genetic features of this region are also shown. A potential Rho-independent transcriptional termination site (Fig. 1, designated TE) is present in the *E. coli* DNA sequence between the *entF* and *fepE* genes. The IS1 element appears to have inserted into a potential loop structure of the transcriptional termination sequence. Any secondary structure which could be created by the transcriptional termination sequence would be disrupted in SA101 by the insertion of the IS1. Neither the *entF* nor the *fepE* gene coding regions of SA101 are disrupted by the IS1.

Of the approximately 125 bp of enterobactin sequences which were examined, SA101 differed from *E. coli* at 3 bases (Fig. 1A). The IS1 DNA sequence comparison shows 31 base changes between the SA101 IS1 and the IS1 obtained

from the *E. coli* (Fig. 1B). The *insA* regulatory region and the entire *insA* gene coding region are also present in this sequence. There exists 91% DNA sequence homology between the *insA* genes from SA101 and *E. coli*.

Mapping a mutation in the *entF* gene. The results of complementation studies employing the *entF* gene fusion constructs present on plasmids pCRH1 and pCRF1 indicated that the IS1 element was not responsible for the failure of the cloned *entF* gene to complement an *E. coli entF* mutant (44). Previous mapping and hybridization studies of the *S. flexneri* SA101 *entF* gene region (44) indicated no detectable DNA deletion or rearrangement. It was suspected that the defect in the SA101 *entF* gene was either a small deletion or a point mutation occurring in the coding region or regulatory sequences. Hybrid gene fusions of the *entF* gene were constructed in an attempt to locate the defect (Fig. 2). All of the

FIG. 2. Chromosomal DNA of the enterobactin gene region carried by various recombinant plasmids obtained from *E. coli* (shaded bars), *S. flexneri* SA101 (Ent^-) (open bars), and *S. flexneri* SA301 (Ent^+) (striped bars). *entF* hybrid gene fusions were constructed for several of the plasmids shown. The ability of the cloned *entF* gene to complement *E. coli entF* mutants is shown to the right: +, positive complementation; -, failure to complement. Genetic map of this region is also shown: directly below genetic map is a shaded box which indicates the region where the SA101 *entF* mutation has been mapped. All recombinant plasmids shown complemented the *E. coli fes* mutant 1D2. Only restriction sites used to construct plasmids are shown and are abbreviated as follows: C, *Clal*; E, *EcoRI*; H, *HindIII*; P, *PvuII*; Ps, *PstI*; R, *EcoRV*.

gene fusions generated contained the *entF* and *fes* genes and the promoter and regulatory sequences essential for expression of both of these genes. The hybrid plasmid pPVR13 (Fig. 2) consists of SA101 sequences containing the promoter region, *fes* gene, and about 800 bp of the 5' region of the *entF* gene fused to SA301 sequences carrying the remaining 3' portion of the *entF* gene. This plasmid complemented both of the *E. coli entF* mutants, suggesting that SA101 sequences upstream of the *EcoRI* site, the restriction site used to join the two restriction fragments, were not involved in the failure to complement. However, the failure of plasmid pPSR31 to complement indicated that SA101 sequences 3' to the *EcoRI* site were associated with the *entF* defect. Finally, complementation results with plasmid pHRV1 suggested that the defect was present upstream of the *EcoRV* site located at the 3' end of the *entF* gene (Fig. 2). The complementation data obtained from these hybrid fusion constructions did not precisely map the defect of the cloned SA101 *entF* gene but defined it within a large area in the gene

coding region between the unique *EcoRI* site at the 5' end of the gene and the *EcoRV* site at the 3' end (Fig. 2, indicated by shaded area directly below genetic map).

Mapping a mutation in the SA101 *entC* gene. The *E. coli entC* mutant MT147 (31) was used to perform complementation studies with the cloned *Shigella entC* gene. Figure 3 shows various recombinant plasmids carrying the *entC* gene sequences and the results of complementation assays employing the *ent* mutant MT147. The original cloned chromosomal DNA present in plasmid pMPS12 (SA101 Ent^-) and pMPS32 (SA301 Ent^+) possesses the entire *entC* and *entE* coding sequences in addition to the promoter and regulatory sequences for these genes (Fig. 3). The SA101 cloned genes failed to exhibit any detectable complementing activity of the *entC* mutant MT147, while the SA301 cloned *entC* gene present on plasmid pMPS32 restored a normal wild-type phenotype to this strain (Fig. 3). The remaining four plasmids shown in Fig. 3 are *entC* hybrid gene fusion constructs which were used to define generally the location of the defect in the $Ent^- entC$ gene. The four fusions were generated by using the recombinant DNA present in plasmids pMPS12 and pMPS32. All of these fusions were generated by ligating the analogous 4-kb *EcoRV-SalI* fragment to the adjacent 3.2-kb *SalI* fragment. In addition to the promoter and regulatory sequences for *entC*, these fusions carried the complete coding regions for both *entC* and *entE*. Complementation studies with these plasmids revealed that the defect in the SA101 *entC* gene was located in sequences upstream of the *SalI* site used to join the two fragments (Fig. 3). The defect in the *entC* gene could lie either in coding sequences at the 5' portion of the gene or in the promoter or regulatory sequences. No defect appears to lie in sequences 3' to the *SalI* site since plasmid pRSS31 fully complements the *entC* mutation in MT147 (Fig. 3).

Expression of the cloned SA101 *entA*, *B*, and *C* genes. SA101 and MT147 fail to produce enterobactin and the precursor 2,3-dihydroxybenzoic acid (DHBA) when grown in low-iron media. The synthesis of DHBA requires the gene products of the *entA*, *entB*, and *entC* genes (48). To further investigate the nature of the defect in the *entC* gene and evaluate what affect this mutation may have on downstream genes, various plasmids containing the cloned *entA*, *entB*, and *entC* genes were transformed into SA101 and MT147 and production of DHBA was examined.

Table 2 shows DHBA concentrations detected in culture supernatants from SA101 and MT147 grown in either high- or low-iron media. No increased levels of DHBA are apparent when SA101 carries either plasmid pMPS11 (*entA*⁺, *entB*⁺) or pMPS32 (*entC*⁺). This suggests that SA101 does not produce the *entC* gene product from the chromosomal gene in levels adequate to allow the detection of DHBA when harboring only plasmid pMPS11 (Table 2). This observation is consistent with the complementation data in which the cloned SA101 *entC* gene failed to complement an *E. coli entC* mutant (Fig. 3). Furthermore, the inability of SA101 to synthesize DHBA when harboring only pMPS32 (*entC*⁺) suggests that the gene products produced from the chromosomal *entA* and *entB* genes either are not present or are synthesized at very low levels in this strain. Interestingly, the cloned *entA* and *entB* gene products from SA101, unlike *entC*, are functional and are expressed at adequate levels as determined by their ability to complement *E. coli* mutants (44). When both pMPS11 and pMPS32 are present in either SA101 or MT147, significant levels of DHBA are detected and the synthesis is iron regulated (Table 2).

DNA sequence of the 5' portion of the SA101 *entC* gene and

FIG. 3. DNA of the enterobactin gene cluster carried by various recombinant plasmids obtained from *S. flexneri* SA101 (Ent^-) (open bars) and SA301 (Ent^+) (striped bars). *entC* complementation results with recombinant plasmids are shown to the right: +, positive complementation; -, failure to complement. A partial genetic map of this region is shown above. Only restriction sites used to construct these recombinant plasmids are shown: P, *PvuII*; R, *EcoRV*; S, *SalI*.

the *entC-fepB* regulatory region. To specifically identify the defect in the SA101 *entC* gene, the nucleotide sequence of the 5' coding region and regulatory region of the *entC* gene was determined (Fig. 4). The sequence shown in Fig. 4 extends from the start codon of the *fepB* gene (CAC; *fepB* is coded for on the DNA strand opposite from *entC*) to a unique *EcoRV* site in the *entC* gene. All but a small portion of the sequence (near the *fepB* gene) was determined for both DNA strands. The previously determined *E. coli* sequence of this region (6, 13, 31), shown on the top strand, is compared with the SA101 sequence shown below it. Several interesting genetic features which may have functions in the regulation of both the *fepB* and *entC* genes lie in the region between these two genes. Putative promoter sequences, Fur binding sites, regions of secondary structure, and other genetic elements are indicated. A striking feature of the SA101 sequence is that a noncontiguous 83-bp deletion occurred in a large region of potential secondary structure

situated between the *fepB* and *entC* genes. In *E. coli*, this region of secondary structure can be drawn into various stable stem-and-loop configurations (13). Sequences sharing homology with this region have been found in *E. coli* in the intergenic region of the *pstA* and *pstB* genes and downstream of the *adk* gene (13). No role or activity for these sequences has been determined. Additionally, an open reading frame has been found in this area of secondary structure preceding the *E. coli fepB* gene (13). The deletion in SA101 removes a large segment of this reading frame. Although no function has been assigned to this region, the presence of a large region of secondary structure associated with an open reading frame suggests that these sequences may have a regulatory role. Transcription from the promoters present in the *entC-fepB* regulatory region has been measured, and only a very low level expression of either *entC* or *fepB* was detected in SA101 (44). In contrast, SA301 strongly depresses transcription of these genes in low-iron conditions (44).

The *fepB* gene product produced from the cloned SA101 *fepB* gene is a functional protein as determined by its ability to complement the *E. coli fepB* mutant DK214 (44). However, mRNA levels produced from the chromosomal SA101 *fepB* gene are reduced relative to those in SA301 (44). No nucleotide differences were detected between SA101 and *E. coli* in the putative Fur binding site, promoter sequence, or in the translational start site for the *fepB* gene. Because the region of potential secondary structure, which has been deleted in SA101, lies between the *fepB* promoter and the start of the *fepB* gene, it is possible that the deletion is responsible for the reduced expression of *fepB*.

The 83-bp deletion found in SA101 lies outside of the region where all known *entC* regulatory features are located. A difference of only one base exists in the putative *entC* Fur binding site between the SA101 and *E. coli* DNA sequences. In a comparison with other suspected Fur binding sequences, this base appears strongly conserved (9). Whether

TABLE 2. Synthesis and regulation of DHBA from cloned *ent* genes

Strain and plasmid	Genes ^a	DHBA (OD ₅₁₅) ^b		Ratio (-Fe/+Fe)
		+Fe	-Fe	
SA101 (Ent^-)		<0.01	<0.01	
SA101, pMPS11	<i>entA</i> ⁺ , <i>entB</i> ⁺	<0.01	<0.01	
SA101, pMPS32	<i>entC</i> ⁺	<0.01	<0.01	
SA101, pMPS11 + pMPS32	<i>entA</i> ⁺ , <i>entB</i> ⁺ , <i>entC</i> ⁺	0.089	0.240	2.7
MT147 ($entC^-$)		<0.01	<0.01	
MT147, pMPS11 + pMPS32	<i>entA</i> ⁺ , <i>entB</i> ⁺ , <i>entC</i> ⁺	0.119	0.670	5.6

^a Genes present on recombinant plasmids; only genes required for DHBA production (*entA*, *entB*, *entC*) are indicated.

^b DHBA determined by optical density at 515 nm (OD₅₁₅) by the method of Arnow (2).

FIG. 4. Comparison of nucleotide sequences of *E. coli* and *S. flexneri* SA101. DNA sequence extends from the start codon (CAC) for the *fepB* gene to an *EcoRV* site located in the 5' coding region of the *entC* gene. Top line shows *E. coli* DNA sequence and associated regulatory elements as determined by others (6, 13, 31). SA101 sequence is depicted below the *E. coli* sequence: asterisk indicates nucleotide identity, and hyphens represent nucleotide deletions. Genetic features are represented as follows: transcriptional initiation signals, -10 and -35; transcriptional start sites, +1; boxed sequences show Fur recognition sequences; horizontal bars above sequences, represent regions involved in the formation of putative stem-and-loop structures. The amber codon in the SA101 *entC* gene is also indicated. Restriction sites: Eco, *EcoRI*; Av, *AvaI*; H III, *HindIII*; S, *SalI*; RV, *EcoRV*. SD, Shine-Dalgarno sequence.

this base change affects expression or regulation of the *entC* gene has not been determined.

A comparison of the coding region of the *entC* gene between *E. coli* and SA101 revealed the presence of an amber codon in the SA101 gene (Fig. 4). This stop codon would result in the synthesis of a small truncated protein that would be unlikely to possess any *entC* complementing activity. The presence of this amber codon may have a polar effect on the expression of downstream genes in this operon,

since it has been demonstrated in other systems that the presence of a translational termination signal can cause reduced expression of downstream genes in an operon (17).

Expression of the cloned *Shigella fepA* gene. Although the cloned SA101 *fepA* gene can complement the defect in *E. coli fepA* mutants (44), the FepA protein is not normally detected in the outer membrane of Ent⁻ *S. flexneri* strains (35). To determine if reduced transcription of the *fepA* gene was the reason why the SA101 FepA protein was absent

FIG. 5. (A) Physical map of *feaA* promoter-*cat* gene fusion constructs. The analogous 6.4-kb *EcoRV* fragment from SA101 and SA301 cloned DNA was used to construct the *cat*-promoter fusions shown. (B) Assay of chloramphenicol acetyl transferase activity in an *E. coli* host harboring either plasmid pFES1 (SA101, *feaA-cat* fusion) or pFAE1 (SA301, *feaA-cat* fusion). Strains were grown in either high- or low-iron media. Cellular extracts were prepared and samples were spotted onto thin-layer silica gels. [14 C]chloramphenicol (darkest spot at top of autoradiograph) and its acetylated forms are shown. Lanes: 1, +Fe, pFES1; 2, -Fe, pFES1; 3, +Fe, pFAE1; 4, -Fe, pFAE1.

from the outer membrane, expression from the cloned SA101 *feaA* gene was examined by constructing *feaA* promoter fusions to the chloramphenicol acetyl transferase (*cat*) gene. Fusions with the SA301 *feaA* gene were also constructed as a positive control. Plasmid pKK232-8 (7), which carries a promoterless *cat* gene, was employed in the construction of these fusions. Promoter strength can be measured by assaying for the production of the *cat* gene product.

The analogous 6.4-kb *EcoRV* DNA fragments were used to construct the promoter fusions for SA101 and SA301 (Fig. 5A). The autoradiograph in Fig. 5B shows the result of a *cat* assay in which expression from the SA101 and SA301 *feaA* promoter-*cat* gene fusions was measured. Qualitative examination of the autoradiograph reveals that the SA101 and SA301 *feaA* promoters are equivalent in strength and are similarly regulated by iron. Quantitative analysis indicated that the level of expression from the SA101 *feaA* promoter was approximately 80% of that detected from the SA301 *feaA* promoter (data not shown).

Analysis of FepA protein by acrylamide gels. Outer membrane preparations were obtained from the *feaA* deletion mutant UT6900 (grown in low iron) harboring plasmids

FIG. 6. SDS-PAGE of outer membrane proteins obtained from either *E. coli* *feaA* deletion mutant UT6900 (lanes 1 to 6) or *S. flexneri* SA101 (lanes 7 to 9) harboring various recombinant plasmids. Strains were grown in either high- or low-iron media. Lanes: 1, +Fe, pMPS13 (SA101 FepA); 2, +Fe, no plasmid; 3, -Fe, no plasmid; 4, -Fe, pMPS13 (SA101 FepA); 5, -Fe, pMPS33 (SA301 FepA); 6, +Fe, pMS101 (*E. coli* FepA) (9); 7, -Fe, pMPS13 (SA101 FepA); 8, -Fe, no plasmid; 9, +Fe, pMPS13 (SA101 FepA).

pMPS33 (SA301 Ent⁺) and pMPS13 (SA101 Ent⁻). These plasmids contain the analogous insert DNA from the two *Shigella* strains, and both are able to complement the *feaA* mutation present in UT6900 (44). The purpose of this analysis was to determine if any detectable FepA protein was synthesized from the cloned SA101 *feaA* gene and, if so, at what levels would it be present in the outer membrane relative to the SA301 protein. A comparison of the amount of the 81-kDa FepA protein produced by the two plasmids under identical growth conditions is shown in Fig. 6, lanes 4 (pMPS13; SA101, Ent⁻) and 5 (pMPS33; SA301, Ent⁺). The SA301 FepA protein is clearly produced in substantially higher quantities than the SA101 protein. Nevertheless, SA101 FepA appears to be similar in size to that of the SA301 protein, and while it is produced at levels relatively lower than that of the overproduced SA301 protein, it is clearly as abundant in the outer membrane as other iron-regulated proteins synthesized from the UT6900 chromosomal genes, namely, *fiu*, (83-kDa Fiu) and *cir* (74-kDa Cir) (Fig. 6, lane 3) (24). This sufficient level of expression may explain why the SA101 cloned *feaA* gene present on this plasmid is able to fully complement the *feaA* defect in *E. coli* mutants (44). The SA101 FepA protein produced from plasmid pMPS13 was not detected at significant levels in either the cytoplasmic membrane or cytosol (data not shown).

Additional outer membrane preparations were obtained from SA101 and UT6900 grown in either high- or low-iron media (Fig. 6). The only iron-regulated outer membrane protein detected in SA101 is the 77-kDa aerobactin receptor IutA (Fig. 6, lane 8) (21). Outer membrane preparations from SA101 harboring plasmid pMPS13, which carries the SA101 *feaA* gene, revealed that two proteins, corresponding to the 81-kDa FepA and the 77-kDa IutA, were expressed when cells were iron deficient (lane 7) but were repressed in iron-replete environments (lane 9). The 77-kDa IutA protein present in lane 8 had a slightly slower mobility than its counterpart in lane 7. This was thought to be due to an aberration in the electrophoresis process and not to any difference in size between the two proteins. Fig. 6 (lane 6) shows the outer membrane proteins expressed from UT6900 harboring the *E. coli* plasmid pMS101 (*feaA*⁺). This preparation is shown as a size marker to allow comparison of the FepA protein in *E. coli* (lane 6) with that of SA101 (lane 7).

DISCUSSION

Although most clinical isolates of *S. flexneri* fail to produce the siderophore enterobactin (35), all of the strains examined were found to possess the enterobactin genes on their chromosome (34, 44). In an earlier study (44), we initiated an investigation examining the genetic organization and expression of the enterobactin genes from Ent⁺ and Ent⁻ isolates of *S. flexneri*. In this study, we have extended our analysis and provide a more detailed description of the various genetic defects and other genetic aberrations present in the enterobactin gene cluster in an Ent⁻ *S. flexneri* (SA101).

Complementation results employing *S. flexneri* SA101-*E. coli* *entF* hybrid gene fusions indicated that the IS1 element, located near the *entF-fepE* intergenic region, did not interfere with the ability of the cloned *entF* gene to complement an *E. coli* *entF* mutant (44). However, the unexpected presence of an IS1 element in the middle of the enterobactin gene cluster encouraged us to examine this phenomenon more closely. The nucleotide sequence of the junction of IS1 sequences and the enterobactin genes revealed that the IS1 element had integrated into a potential Rho-independent transcriptional termination sequence, which would likely interrupt any secondary structure which could be formed in the RNA transcript of this region. This transcriptional terminator may function to terminate transcripts over the *fes-entF* operon. In other systems (5), it has been proposed that the secondary structure created by a transcriptional termination sequence in the RNA can function to stabilize and prolong the half-life of an mRNA by preventing degradation of the RNA by 3' exonucleases. It is conceivable that the presence of the IS1 may be affecting the stability of the *fes-entF* transcript. Additionally, the mutation within the coding region of the gene may reduce message stability. It has also been suggested that the *fepE* gene, which lies downstream of *entF*, may be present in the same transcriptional unit as *fes* and *entF* (38). IS1 elements have been shown to have polar effects on downstream genes when inserted into an operon (46). However, it was not determined from this study what affect the IS1 has on expression of the *fepE* gene.

The SA101 *entC* gene, like *entF*, failed to produce a functional gene product and appeared to possess a mutation which affected both expression of the gene and the activity of the gene product. *entC* hybrid genes, analogous to those generated for the *entF* gene, mapped the defect in the *entC* gene to either the 5' coding region or the regulatory sequences. Analysis of mRNA levels produced from the chromosomal *entC* gene indicated that this gene was expressed at very low levels and was poorly iron regulated. In a previous study, the SA101 chromosomal *entB* gene, present in the same operon as *entC*, was found to be similarly expressed (44). Interestingly, the SA101 cloned *entB* gene and the adjacent *entA* gene are both capable of fully complementing *E. coli* mutants (44). However, no DHBA is detected in SA101 when the functional SA301 *entC* gene is placed into that strain, although only the products of the *entA*, *entB*, and *entC* genes are required to produce DHBA. This suggests that the chromosomal *entA* and *entB* genes are not expressed at levels adequate to synthesize detectable quantities of DHBA. It is suspected that the cloned *entB* and *entA* genes are transcribed from vector promoters, since no chromosomal promoters are present on the plasmids carrying these genes (27). It is likely that the complementation data for these genes, and perhaps other cloned enterobactin genes,

reveal little concerning the native chromosomal expression but rather are only indicative of whether a gene product is functional.

To determine more precisely the nature of the mutation(s) affecting the SA101 *entC* gene, the nucleotide sequence of the 5' coding region of the *entC* gene and the intercistronic region between *fepB* and *entC* was determined. In *E. coli*, the region between the *fepB* and *entC* genes is approximately 370 bp, which contains the *fepB* and *entC* promoters (6, 13, 31). These promoters are present on opposite DNA strands and separated by about 30 bp. Directly downstream from each promoter is located a putative Fur recognition sequence. The *fepB* gene contains a 215-bp leader sequence which possesses a region of potential secondary structure and an open reading frame. Although this open reading frame would be transcribed by the *fepB* promoter, there is no evidence as yet for the synthesis of a gene product or for any type of attenuator-mediated regulation of *fepB*. However, these potential regulatory features in the *fepB-entC* intergenic region suggest that regulatory factors in addition to Fur may influence expression of these genes.

The SA101 nucleotide sequence of the *fepB* leader region revealed the presence of an 83-bp deletion which interrupts both the extensive area of secondary structure and the open reading frame. The promoters, Fur recognition sequences, ribosome binding sites, and coding regions for the *fepB* and *entC* genes, however, were not affected by the deletion. Analysis of mRNA levels of the SA101 *fepB* gene indicated that, like *entC*, it was expressed at low levels and was poorly iron regulated. These results suggest that the deletion in the *fepB* leader sequence is responsible for the reduced expression of the *fepB* gene, implying that the *fepB* leader sequence has a direct role in the regulation of the *fepB* gene.

Despite the reduced levels of mRNA detected from the chromosomal *fepB* gene, the cloned SA101 *fepB* gene fully complements an *E. coli* *fepB* mutant (44). This may reflect increased expression resulting from cloning into a high-copy-number vector. However, since FepB is required for transport functions, and SA101 is capable of transporting the ferrienterobactin complex (albeit at low levels) (35), it is assumed that the FepB protein in *S. flexneri* is synthesized sufficiently to allow for transport.

An amber codon found in the 5' portion of the SA101 *entC* gene would result in a polypeptide that would be approximately 25% of the full-size EntC protein. It is suspected that the truncated *entC* gene product is inactive. Studies with the tryptophan operon in *E. coli* revealed that nonsense mutations affect the transcription of downstream genes (17). The decrease in transcription was found to affect all downstream genes equally. It was further revealed that the larger the untranslated region in the mRNA was, the greater the decrease in transcription of downstream genes. Since approximately 75% of the *entC* mRNA would remain untranslated, it is possible that this nonsense mutation has a strong polar affect on all genes in this operon.

The SA101 FepA protein is not produced in quantities large enough to be detected on a Coomassie blue-stained polyacrylamide gel (35). Others, using FepA-specific antibodies, showed that small amounts of the 81-kDa FepA protein were present in the outer membrane of an Ent⁻ *S. flexneri* (15). The cloned SA101 *fepA* gene present on a multicopy plasmid fully complemented the *E. coli* *fepA* mutants UT6600 and UT6900 (44). Attempts at measuring the expression of the SA101, and of the SA301 chromosomally encoded *fepA* gene by examining mRNA levels as was done for the *entC* and *fepB* genes, produced ambiguous

results in which high levels of RNA were detected in both low- and high-iron conditions (43a). We have not determined at this time why the mRNA analysis of the *fepA* gene failed to reveal iron regulation.

fepA-lacZ translational gene fusions, using the cloned *fepA* gene, have also been constructed to measure expression of the *Shigella fepA* gene (43a). However, an accurate comparison of promoter strength between the Ent⁻ and Ent⁺ *Shigella* strains could not be achieved because of the deleterious effect on cell growth caused by the Ent⁺ *Shigella* FepA-LacZ translational fusion protein. Other researchers have made similar observations with translational protein fusions when LacZ is fused to membrane or transported proteins (3). Interestingly, the SA101 fusion protein was strongly expressed and did not affect cell growth. This observation suggests that the SA101 protein may be transported improperly or with greatly lower efficiency.

Fusions of the SA101 and SA301 *fepA* promoter to the *cat* gene revealed that transcription from the SA101 promoter was about 80% as efficient as the SA301 *fepA* promoter. This difference in transcriptional efficiencies is not sufficient to account for the difference in FepA protein detected in the outer membrane of these two strains (35).

Production of the SA101 and SA301 FepA protein from the cloned *fepA* gene present on pACYC184 was examined. PAGE analysis of outer membrane preparations indicated that while detectable amounts of the SA101 FepA protein were produced, severalfold greater quantities of the SA301 FepA protein were present. No significant amount of the SA101 or SA301 FepA protein were detected in the cytosol or cytoplasmic membrane fractions. The cumulative data from this study and previous studies concerning the SA101 FepA protein suggest that the *fepA* gene is expressed normally but that a mutation may exist in the gene so that the transcript is translated inefficiently or the protein fails to be transported efficiently through the bacterial envelope. This mutation does not appear to impair the ferrienterobactin transport activity of the FepA protein.

Although most *S. flexneri* utilize only the aerobactin siderophore system, they appear to have preserved the genes of the enterobactin cluster. Many virulent isolates of *E. coli* and some *Shigella* species utilize both the aerobactin and enterobactin high-affinity iron transport systems. Why these strains possess two functional high-affinity iron transport systems is not clear. It has been shown that the aerobactin system in certain pathogenic *E. coli* enhances the virulence of these organisms (47). However, the aerobactin system in *S. flexneri* has not been shown to be a virulence factor for this organism (20). Additionally, the enterobactin iron transport system has not been shown to be required for virulence in any species of *Shigella* or in *E. coli*, and it appears to be completely dispensable in most strains of *S. flexneri*. It is likely that the enterobactin genes present in Ent⁻ *S. flexneri* represent a genetic system which in an ancestral strain may have been functional, but became dispensable, perhaps after the acquisition of the aerobactin genes by *S. flexneri*. It appears that the enterobactin gene cluster in Ent⁻ *S. flexneri* has accumulated a variety of mutations and genetic alterations throughout the entire genetic system. Interestingly, many of these characteristics appear to have been conserved among different strains and serotypes of *S. flexneri* (44, 34). These common findings include (i) the inability to synthesize both enterobactin and its precursor DHBA, (ii) the presence of the enterobactin genes on the chromosome; (iii) the existence of an IS1 element in the *entF-fepE* region; (iv) the absence or greatly

reduced levels of the FepA protein in the outer membrane, and (v) the presence of several unique restriction sites within the enterobactin gene region of Ent⁻ *S. flexneri*. It is not clear why these particular characteristics are preserved among evolutionary divergent strains of *S. flexneri*. It is hoped that the study of these naturally occurring genetic alterations present in the Ent⁻ *S. flexneri* enterobactin genes may facilitate the study and understanding of functional enterobactin systems in other organisms.

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REFERENCES

- Ames, G. F. L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J. Biol. Chem.* **249**:634-644.
- Arnold, L. E. 1937. Colorimetric determination of the components of 3,4 dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem.* **228**:531-537.
- Bassford, P. J., T. J. Silhavy, and J. R. Beckwith. 1979. Use of a gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. *J. Bacteriol.* **139**:19-31.
- Bolivar, F., R. Rodriguez, M. Betlach, and H. Boyer. 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**:75-93.
- Brawerman, G. 1987. Determinants of messenger RNA stability. *Cell* **48**:5-6.
- Brickman, T. J., B. A. Ozenberger, and M. A. McIntosh. 1990. Regulation of divergent transcription from the iron-responsive *fepB-entC* promoter-operator regions in *Escherichia coli*. *J. Mol. Biol.* **212**:669-682.
- Brosius, J. 1984. Plasmid vectors for the selection of promoters. *Gene* **27**:151-160.
- Bryce, G. F., and N. Brot. 1972. Studies on the enzymatic synthesis of the cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine in *Escherichia coli*. *Biochemistry* **11**:1708-1715.
- Calderwood, S. B., and J. J. Mekalanos. 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. *J. Bacteriol.* **170**:1015-1017.
- Chang, C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**:1141-1156.
- Coderre, P. E., and C. F. Earhart. 1984. Characterization of a plasmid carrying the *Escherichia coli* K-12 *entD*, *fepA*, *fes*, and *entF* genes. *FEMS Microbiol. Lett.* **25**:111-116.
- Earhart, C. F. 1987. Ferrienterobactin transport in *Escherichia coli*, p. 67-81. In G. Winkelmann, D. Van der Helm, and J. B. Neilands (ed.), *Iron transport in microbes, plants and animals*. VCH Publishers, New York.
- Elkins, M. F., and C. F. Earhart. 1989. Nucleotide sequence and regulation of the *Escherichia coli* gene for ferrienterobactin transport protein FepB. *J. Bacteriol.* **171**:5443-5451.
- Greenwood, K. T., and R. K. J. Luke. 1976. Studies on the enzymatic synthesis of enterochelin in *Escherichia coli* K-12: four polypeptides involved in the conversion of 2,3 dihydroxybenzoate to enterochelin. *Biochim. Biophys. Acta* **454**:285-297.
- Griffiths, E., P. Stevenson, T. L. Hale, and S. B. Formal. 1985. Synthesis of aerobactin and a 76,000-dalton iron-regulated outer membrane protein by *Escherichia coli* K-12-*Shigella flexneri* hybrids and by enteroinvasive strains of *Escherichia coli*. *Infect. Immun.* **49**:67-71.
- Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**:193.
- Imamoto, F., and C. Yanofsky. 1967. Transcription of the

- tryptophan operon in polarity mutants of *Escherichia coli*. J. Mol. Biol. 28:25-35.
18. Johnsrud, L. 1979. DNA sequence of the transposable element IS1. Mol. Gen. Genet. 169:213-218.
 19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
 20. Lawlor, K. M., P. A. Daskaleros, R. Robinson, and S. M. Payne. 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. 55:594-599.
 21. Lawlor, K. M., and S. M. Payne. 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. 160:266-272.
 22. Luke, R. K. J., and F. Gibson. 1971. Location of the genes concerned with the conversion of 2,3-dihydroxybenzoate into enterochelin in *Escherichia coli* K-12. J. Bacteriol. 107:557-562.
 23. Mandel, M., and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53:159-162.
 24. McIntosh, M. A., and C. F. Earhart. 1977. Coordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli*. J. Bacteriol. 131:331-339.
 25. Messing, J., and J. Vieira. 1982. A new pair of M13 vectors for selecting either DNA strand of double digested restriction fragments. Gene 19:269-276.
 26. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 27. Nahlik, M. S., J. J. Brickman, B. A. Ozenberger, and M. A. McIntosh. 1989. Nucleotide sequence and transcriptional organization of the *Escherichia coli* enterobactin biosynthesis cistrons *entB* and *entA*. J. Bacteriol. 171:784-790.
 28. Neilands, J. B. 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. 36:285-309.
 29. O'Brien, I. G., G. B. Cox, and F. Gibson. 1971. Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim. Biophys. Acta 237:537-549.
 30. O'Brien, I. G., and F. Gibson. 1970. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochem. Biophys. Acta. 215:393-402.
 31. Ozenberger, B. A., T. J. Brickman, and M. A. McIntosh. 1989. Nucleotide sequence of *Escherichia coli* isochorismate synthetase gene *entC* and evolutionary relationship of isochorismate synthetase and other chorismate-utilizing enzymes. J. Bacteriol. 171:775-783.
 32. Ozenberger, B. A., M. S. Nahlik, and M. A. McIntosh. 1987. Genetic organization of multiple *fep* genes encoding ferric enterobactin transport functions in *Escherichia coli*. J. Bacteriol. 169:3638-3646.
 33. Payne, S. M. 1980. Synthesis and utilization of siderophores by *Shigella flexneri*. J. Bacteriol. 143:1420-1424.
 34. Payne, S. M. 1987. Iron transport in *Shigella* and *Vibrio* species, p. 99-109. In G. Winkelmann, D. Van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Publishers, New York.
 35. Payne, S. M., D. W. Niesel, S. S. Peixotto, and K. M. Lawlor. 1983. Expression of hydroxamate and phenolate siderophores by *Shigella flexneri*. J. Bacteriol. 155:949-955.
 36. Perry, R. D., and C. L. San Clemente. 1979. Siderophore synthesis in *Klebsiella pneumoniae* and *Shigella sonnei* during iron deficiency. J. Bacteriol. 140:1129-1132.
 37. Pettis, G. S., T. J. Brickman, and M. A. McIntosh. 1988. Transcriptional mapping and nucleotide sequence of the *Escherichia coli fepA-fes* enterobactin region. J. Biol. Chem. 263:18857-18863.
 38. Pettis, G. S., and M. A. McIntosh. 1987. Molecular characterization of the *Escherichia coli* enterobactin cistron *entF* and coupled expression of *entF* and the *fes* gene. J. Bacteriol. 169:4154-4162.
 39. Pickett, C. L., L. Hayes, and C. F. Earhart. 1984. Molecular cloning of the *Escherichia coli* K-12 *entACGBE* genes. FEMS Microbiol. Lett. 24:77-80.
 40. Pierce, J. R., and C. F. Earhart. 1986. *Escherichia coli* K-12 envelope proteins specifically required for ferrienterobactin uptake. J. Bacteriol. 166:930-936.
 41. Pierce, J. R., C. L. Pickett, and C. F. Earhart. 1983. Two *fep* genes are required for ferrienterobactin uptake in *Escherichia coli* K-12. J. Bacteriol. 155:330-336.
 42. Pollack, J. R., and J. B. Neilands. 1970. Enterobactin an iron transport compound from *Salmonella typhimurium*. Biochim. Biophys. Res. Commun. 38:989-992.
 43. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
 - 43a. Schmitt, M. P. Unpublished results.
 44. Schmitt, M. P., and S. M. Payne. 1988. Genetics and regulation of enterobactin genes in *Shigella flexneri*. J. Bacteriol. 170:5579-5587.
 45. Schwyn, B., and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160:47-56.
 46. Starlinger, P. 1980. IS1 elements and transposons. Plasmid 3:241-259.
 47. Warner, P. J., P. H. Williams, A. Binderief, and J. B. Neilands. 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. Infect. Immun. 33:540-545.
 48. Young, I. G., L. Langman, R. K. J. Luke, and F. Gibson. 1971. Biosynthesis of the iron transport compound enterochelin: mutants of *Escherichia coli* unable to synthesize 2,3-dihydroxybenzoate. J. Bacteriol. 106:51-57.