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Nucleotide Sequence of a Gene Cluster Involved in Entry of E Colicins and Single-Stranded DNA of Infecting Filamentous Bacteriophages into *Escherichia coli*

TAI-PING SUN AND ROBERT E. WEBSTER*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Mutations in *fii* or *tolA* of the *fii-tolA-tolB* gene cluster at 17 min on the *Escherichia coli* map render cells tolerant to high concentrations of the E colicins and do not allow the DNA of infecting single-stranded filamentous bacteriophages to enter the bacterial cytoplasm. The nucleotide sequence of a 1,854-base-pair DNA fragment carrying the *fii* region was determined. This sequence predicts three open reading frames sequentially coding for proteins of 134, 230, and 142 amino acids, followed by the potential start of the *tolA* gene. Oligonucleotide mutagenesis of each open reading frame and maxicell analysis demonstrated that all open reading frames are expressed in vivo. Sequence analysis of mutant *fii* genes identified the 230-amino acid protein as the *fii* gene product. Chromosomal insertion mutations were constructed in each of the two remaining open reading frames. The phenotype resulting from an insertion of the chloramphenicol gene into the gene coding for the 142-amino acid protein is identical to that of mutations in *fii* and *tolA*. This gene is located between *fii* and *tolA*, and we propose the designation of *tolQRA* for this cluster in which *tolQ* is the former *fii* gene and *tolR* is the new open reading frame. The protein products of this gene cluster play an important role in the transport of large molecules such as the E colicins and filamentous phage DNA into the bacterium.

Transport of a number of compounds across the outer and cytoplasmic membranes of *Escherichia coli* is a multistep process. Some of these uptake systems have been shown to use a receptor in the outer membrane to bind the compound, followed by another system which mediates transport through the inner membrane. One such case is the uptake system for vitamin B_{12} which uses the product of the *btuB* gene as an outer membrane receptor and the *tonB* product to transport the vitamin into the periplasm (12, 27). Subsequent transport of vitamin B_{12} across the cytoplasmic membrane is thought to require a different group of proteins encoded by the *btuCED* region (6, 7). The E colicins also appear to use the product of the *btuB* gene for specific binding to the outer membrane. However, another set of proteins, coded by the *fii-tolAB* gene cluster, appears necessary to get these colicins to their respective targets (2, 24, 33).

Recently, we have shown that the penetration of the DNA of infecting filamentous bacteriophages (f1, fd, and M13) occurs by a similar multistep process (33). This process is initiated by the specific binding of one end of the bacteriophage to the tip of the F-conjugative pilus (5, 34). After this binding, it is postulated that progressive depolymerization of the pilus brings the phage to the cell surface where the capsid proteins integrate into the bacterial membrane and the DNA enters the cytoplasm (4, 16, 26). Although it is not clear how the DNA gets across the membrane, our recent evidence indicates that the products of the *fii* and *tolA* genes are necessary (33). Mutations in these genes not only render the cells tolerant to the E colicins, but also do not allow the filamentous phage DNA to be transported into the cytoplasm. However, the filamentous phages can still adsorb to the tip of the F pilus, and bacterial conjugation is normal in these mutant bacteria.

In this paper we present the nucleotide sequence of the *fii* region. In addition to the *fii* open reading frame (ORF), a second ORF which codes for a protein required for infection by the filamentous phages and the susceptibility of the bacteria to the E colicins was characterized. The two ORFs are proximal to *tolA*, and the region is designated *tolQRAB*, where *tolQ* is the *fii* gene. The proteins coded by this region form a system which permits transport of large molecules, such as the E colicins or filamentous phage DNA, into or across the bacterial membrane.

MATERIALS AND METHODS

Phages, bacterial strains, and plasmids. M13mp18 and M13mp19 phage, *E. coli* JM105, and plasmid pUC9 were obtained from J. Messing (25, 35). The F-specific bacteriophages f1 and f2 were obtained from N. Zinder at the Rockefeller University, while the P1 *vir* phage was a gift from R. Bell (Duke University). *E. coli* CH1330 (*polA1*) (9) and JW381, which contains a Tn*10* closely linked to *supD*, were obtained via M. Russel at the Rockefeller University. *fii* and *tolA* mutants TPS13, TPS30, TPS56, TPS58, TPS66, TPS94, and C131 (TPS13 *fii*$^+$ *zbg-1*::Tn*10*) were described previously (33). TPS13 *supD zed-508*::Tn*10* was produced by P1 transduction from JW381. The maxicell strain SE5000 (*recA*) was obtained from K. Ippen-Ihler at Texas A&M University. GM1 [*ara* Δ(*lac-pro*) *thi*/F′ *lac-pro*] was obtained from D. Steege (Duke University) (23). J. I. Horabin (this laboratory) supplied pJIH1 and pJIH12, plasmids which contain the left promoter and the *c*I857 repressor gene of phage λ (14). Plasmids pTPS202, pTPS301, and pTPS304 were described earlier (33).

* Corresponding author.

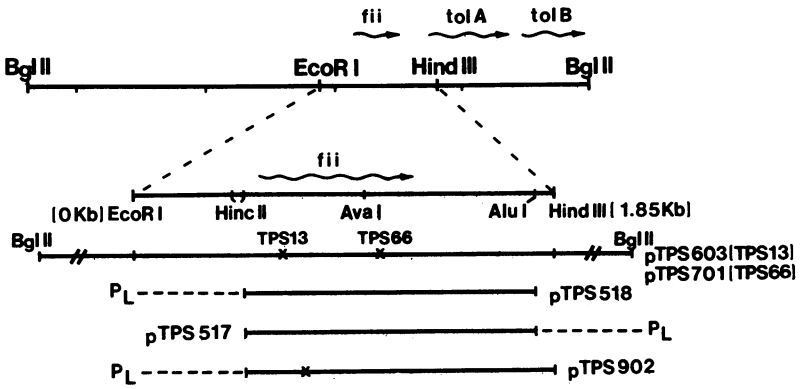


FIG. 1. Plasmid constructions. The top line shows the original 8.7-kb fragment isolated from *E. coli* containing the *fii*, *tolA*, and *tolB* region (33). Most construction were made with pUC9, pJIH1, or pJIH12 plasmids with inserts derived from the 1.85-kb *Eco*RI-*Hin*dIII fragment shown on the second line. Those plasmids containing the insert under control of the left promoter of λ are designated by a P_L to the left or right of the indicated insert. × indicates the position of the mutation. The amber mutation in pTPS603 (TPS13) is at position 654; in pTPS902 it is at position 753. The missense mutation in pTPS701 (TPS66) is at position 1087 (see Fig. 2).

Media and chemicals. L-[^{35}S]methionine (1,000 Ci/mmol), L-[4,5-^{3}H(N)]leucine (60 Ci/mmol), [α-^{35}S]dATP (1,000 Ci/mmol), [α-^{32}P]dATP (3,000 Ci/mmol), and En^{3}Hance were purchased from New England Nuclear Corp. (Boston, Mass.). Deoxynucleoside triphosphates and dideoxynucleoside triphosphates were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). M13 15-base universal primer was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Restriction and modifying enzymes used in the cloning and DNA-sequencing analysis were purchased from Bethesda Research Laboratories, P-L Biochemicals, and United States Biochemical Corp. Bacteria were grown in LB medium or 2 × YT medium (22). The Met$^-$ MTPA salt medium was that described by Vinuela et al. (36), but supplemented with 0.2% glucose and 1 mM each amino acid except methionine. Antibiotics were used in the following concentrations (micrograms per milliliter): ampicillin, 60; tetracycline, 20; kanamycin, 50; and chloramphenicol, 10. Colicins E1 and E3 were obtained from K. Jakes (The Rockefeller University).

DNA sequence analysis. The blunt-ended 4.2-kilobase (kb) *Eco*RI-*Bgl*II fragment and the 1.85-kb *Eco*RI-*Hin*dIII fragment from the originally isolated 8.7-kb fragment (33) (see top of Fig. 1) were cloned into the *Hin*cII site of replicative-form DNA of M13mp19 phage to sequence the region containing the *fii* gene. An ordered set of deletion clones was made by cleavage of the replicative-form DNA with *Sst*I and *Bam*HI followed by exonuclease III unidirectional digestion from the *Bam*HI site as described by Henikoff (13). By processing samples taken every 30 s after the addition of exonuclease III, we obtained a set of phage having deletions of increasing length in units of approximately 200 bases. The sequence of at least three deletion phage from each 30-s sample was determined starting from the M13 15-base universal primer by the dideoxy method of Sanger et al. (30) with [α-^{35}S]dATP as the radioactive label (3). Overlapping sequences were aligned by using the computer program of Staden (31). The computer program FASTP and the protein data bank of the National Biomedical Research Foundation were used in amino acid sequence homology searches (21, 37).

Oligonucleotide-directed mutagenesis. Single-stranded M13mp19 DNA containing the 1.85-kb *Eco*RI-*Hin*dIII fragment was used as the template DNA for mutagenesis. Amber mutations were placed in presumptive open reading frames in this DNA by using complementary heptadecanucleotides containing the appropriate one-base alteration as described by Zoller and Smith (38). The oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer. DNA fragments carrying the desired amber mutation were recloned into the *Hpa*I site of pJIH1, placing the inserted genes under control of the left promoter of λ (14). The resulting plasmids were transformed into different *fii* mutants and tested for their ability to complement the mutations. Successful complementation allowed the cells to grow f1 and made them susceptible to colicins E1 and E3 (33).

Cloning of *fii* genes from TPS13 and TPS66 *fii* mutants. Chromosomal DNAs from TPS13 and TPS66 digested with *Bgl*II were ligated into the *Bgl*II site in the *Eco*RI gene of pJIH12. The ligation mixture was used to transform TPS13 (*fii*) *recA* cells. pJIH12 contains the *c*I857 temperature-sensitive repressor gene of λ together with the gene for the *Eco*RI restriction endonuclease under the control of the left promoter of λ. Transformants were selected by spreading the cells on nitrocellulose filters laid on agar plates with ampicillin as described by Hanahan and Meselson (11). The Amp^r colonies were probed for the presence of the multicopy plasmid containing the *fii* region by using the nick-translated 1.3-kb *Hin*cII-*Alu*I fragment from pTPS304, which contains the wild-type *fii* gene (33), as described by Hanahan and Meselson (11). Two plasmids, pTPS603 and pTPS701, which carry the 8.7-kb *Bgl*II fragment from the *fii* mutants TPS13 and TPS66, respectively, were isolated (Fig. 1). The 1.85-kb *Eco*RI-*Hin*dIII fragments from pTPS603 and pTPS701 were both subcloned into the *Eco*RI-*Hin*dIII sites of pUC9 and M13mp18 phage. The plasmid derivatives for each were tested for their ability to complement *fii* mutants, while the phage were used for DNA sequencing analysis.

Identification of Fii protein. The 1.3-kb *Hin*cII-*Alu*I fragment containing *fii* (Fig. 1) was inserted in both orientations into the *Hpa*I site of pJIH1 to form pTPS517 and pTPS518. pTPS518 carries the *fii* gene under the control of the left promoter of λ and also carries the gene for the *c*I857 repressor. pTPS902 contains the 1.4-kb *Hin*cII-*Hin*dIII fragment (Fig. 1), which contains an amber mutant *fii* gene (position 753, see Fig. 2), under control of the left promoter of λ in the *Hpa*I site of pJIH1. TPS13 *recA* carrying pTPS517, pTPS518, or pTPS902 and TPS13 *supD* carrying pTPS902 were grown in Met$^-$ MTPA medium to 4 × 10^8/ml at 35°C. The cultures were then shifted to 41°C for 10 min,

and 10 μCi of [^{35}S]methionine per ml was added. After a 20-s pulse, 1 ml of cells was immediately pelleted and suspended in 200 μl of sample buffer (8% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, 5% glycerol, 0.004% bromophenol blue, 0.125 M Tris hydrochloride, pH 6.8). A 20-μl portion of each sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and treated with En^{3}Hance for fluorography (33).

Maxicell preparation and labeling of proteins. The 1-kb *Eco*RI-*Ava*I fragment from pTPS603 which contains the gene for ORF1 and an amber mutation in *fii* (from TPS13) was blunt-end ligated into the filled-in *Bam*HI site of pUC9 to produce pTPS811 (see Fig. 4). pTPS813 contains the 1-kb *Eco*RI-*Ava*I fragment which was filled in and inserted in the filled-in *Bam*HI site of pUC9. This fragment contains amber mutations in both *orf-1* and *fii* made by oligonucleotide-directed mutagenesis. pTPS817 has the 1.4-kb *Hin*cII-*Hin*dIII fragment containing wild-type *fii* and an amber mutation in *orf-3* (by oligonucleotide-directed mutagenesis) in the *Hin*cII site of pUC9 (see Fig. 4).

SE5000 (*recA*) and its derivative containing pUC9, pTPS811, pTPS813, pTPS304, and pTPS817 were used to prepare maxicells. Maxicells were prepared as described earlier (29, 33) except that UV-irradiated cultures were incubated in the dark and the cycloserine concentration was 200 μg/ml. Proteins encoded by these plasmids in maxicells were labeled with [^{35}S]methionine (33), and the products were analyzed on either 12% SDS-polyacrylamide gels (18) or 19.3% SDS-urea-polyacrylamide gels (15).

Isolation of insertion mutations in *orf-1* and *orf-3*. The *Ava*I-*Bgl*II fragment carrying *orf-2* (*fii*) and *orf-3* and the *Alu*I fragment carrying *orf-1*, *orf-2* (*fii*), and *orf-3* were blunt-end ligated into the *Bam*HI site of pUC9 to give pTPS301 and pTPS303, respectively (see Fig. 5). The 1.3-kb *Hae*II fragment of pACYC184 which contains the chloramphenicol resistance gene (Cm) was cloned into the blunt-ended *Nco*I site of pTPS301 and the *Mlu*I site of pTPS303. Replacement of the chromosomal *orf-1* and *orf-3* regions by *orf-1*::Cm and *orf-3*::Cm was done as described by Gutterson and Koshland (10) and Russel and Model (28) with slight modifications. pTPS301-*orf-3*::Cm and pTPS303-*orf-1*::Cm were transformed into CH1330 *polA*, and Amp^r Cam^r transformants were selected. P1 *vir* lysates were made from purified Amp^r Cam^r transformants and were used to transduce Gm1 carrying pTPS401. pTPS401 contains the *Alu*I fragment with the wild-type *orf-1*, *-2*, and *-3* regions in the *Hin*cII site of pACYC177. Kan^r (from pACYC177) Cam^r transductants were selected, and several hundred of these transductants were replica plated to select for Amp^s Cam^r colonies. P1 *vir* lysates were made from Amp^s Cam^r transductants and used to transduce GM1 or GM1 with pTPS401. Cam^r transductants were selected and tested for the sensitivity to f1, f2, and P1 phages and colicins E1 and E3 (33).

RESULTS

DNA sequence of *fii* region. DNA fragments containing the *fii* gene were inserted into M13mp19, and deletions were constructed with exonuclease III as described in Materials and Methods. The DNA of the phage derived from these deletions was sequenced by the dideoxy method of Sanger et al. (30) with [^{35}S]dATP as the radioactive label (3). The sequence of one strand was determined by using phage carrying deletions in the 4.2-kb *Eco*RI-*Bgl*II fragment (Fig. 1), and the sequence of the other strand was determined by using phage with deletions in the 1.85-kb *Eco*RI-*Hin*dIII fragment. The DNAs from several isolated phage clones were sequenced to obtain a completely overlapping set of sequences for both strands. The nucleotide sequence of the *Eco*RI-*Hin*dIII fragment and the amino acids coded by the three potential ORFs are shown in Fig. 2. Three of the ORFs (ORF1, -2, and -3) are on one strand, while another, ORF4, is on the complementary strand. ORF1 starts at a GTG at position 145 and codes for a 134-amino acid protein. The designated potential start for ORF2 is a GTG at position 546 (see below), and it codes for a protein of 230 amino acids. ORF3 starts with an ATG at position 1242 and codes for a 142-amino acid protein. ORF4 is on the complementary strand and potentially starts at a GTG at position 1199 and would code for a peptide of 190 amino acids. The potential initiation codon for *tolA* protein is a GTG at position 1735.

Identification of ORF2 protein as the product of the *fii* gene. To determine which ORF codes for the *fii* protein, we used specific heptadecanucleotides containing one-base alterations to create C-to-T transitions. In this way, amber mutations were generated in each potential ORF as shown in Fig. 2. Plasmids containing the different amber mutations were transformed into the TPS13 class of mutants, which contains an amber mutation in *fii*, or the TPS58 class of mutants, which contains a missense mutation in *fii* (33). The resulting transformants were tested for their ability to complement the *fii* mutations in a non-amber-suppressing host by assaying for their ability to grow f1 phage and their sensitivity to colicins E1 and E3. Plasmids containing amber mutations in *orf-1*, *-3*, or *-4* were able to complement both *fii* mutations, while the plasmid with the mutation in *orf-2* would not. These data suggest that *orf-2* encodes the Fii protein and that the amber mutation in *orf-1* does not prevent some expression of the downstream *orf-2*. To further check that ORF2 codes for the Fii protein, the region of chromosomal DNA containing the *fii* gene was cloned and sequenced from TPS13 and TPS66, which contained an amber and missense mutation in *fii*, respectively. In TPS13, there are two C-to-T transitions (at positions 653 and 654; Fig. 2) which alter a glutamine codon to an amber codon in ORF2. There is a G-to-A transition at position 1087 in TPS66, and it converts a glycine residue to an aspartic acid residue in ORF2 (Fig. 2). These data confirm that ORF2 encodes the Fii protein.

We placed the potential start of the *fii* ORF at the GTG at position 546. There are two other potential ATG starts at positions 555 and 606. However, the best potential ribosome-binding site with a good Shine-Dalgarno sequence 8 nucleotides away from the initiation codon appears to be at position 546 (8, 32). Synthesis starting from this site would lead to a protein having a calculated molecular weight of 25,487 (minus the initiating methionine). To visualize this protein, we placed a wild-type *orf-2* and one containing an amber mutation in plasmids under control of the left promoter of λ (pTPS517, -518, -902 in Fig. 1). These plasmids also contain the *c*I857 temperature-sensitive λ repressor gene. Each plasmid was transformed into TPS13 containing an *fii* amber mutation, in the presence or absence of the *supD* amber suppressor. Transcription from p_L was induced at high temperature, proteins were pulse-labeled with [^{35}S]methionine, and the resulting radioactive proteins were analyzed by SDS-PAGE as described in Materials and Methods (Fig. 3). A protein with an approximate molecular weight of 24,000 was present in strains carrying wild-type *fii* in the proper orientation to p_L (pTPS518) but absent when the gene was in the opposite orientation with respect to p_L (pTPS517). Little if any protein of this molecular weight was

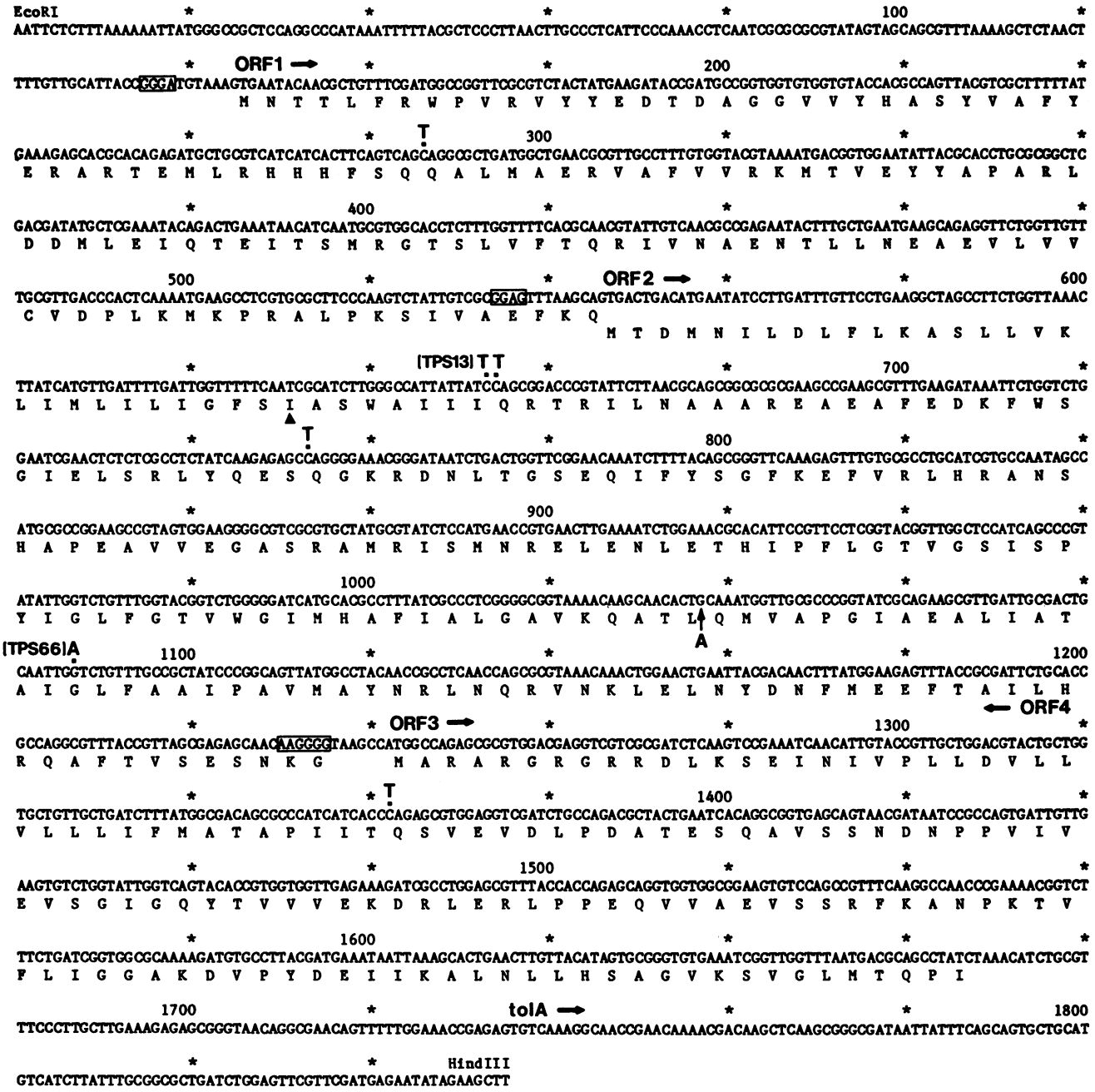


FIG. 2. DNA sequence of the 1.85-kb cloned fragment. Potential ORFs are indicated by ORF→ and were determined by locating the best consensus ribosome-binding site as previously described (8, 32). The boxes enclose putative Shine-Dalgarno sequences. The one-letter amino acid code is indicated for ORF1, -2, and -3. ▲, Position of the last amino acid code of ORF4. Positions of specific C-to-T transitions generated by oligonucleotide-directed mutagenesis as described in Materials and Methods are indicated by T's above the sequence for the DNA strand shown. The C-to-T transition created by the same technique in the complementary strand is indicated by an A below the sequence. Each transition generated an amber mutation in the presumptive ORF in which it was generated. The position and nucleotide change in the *fii* mutants TPS13 and TPS66 is also noted.

present in cells containing the plasmid with the amber mutant (pTPS902). However, a protein of 24-kilodaltons (kDa) was synthesized from pTPS902 in a strain containing the *supD* amber suppressor. These data strongly suggest that this protein is the product of ORF2 and consequently the Fii protein.

Characterization of ORF3 protein. DNA fragments containing wild-type *fii* and *orf-3* or wild-type *fii* and amber mutant *orf-3* were cloned into pUC9 to give pTPS304 and pTPS817, respectively (Fig. 4, top). Maxicells containing these plasmids were labeled with [^{35}S]methionine, and the products were analyzed by SDS-PAGE (Fig. 4B). In addition to the 29- and 31-kDa β-lactamase bands coded by pUC9 and the 24-kDa *fii* gene product, there was a band migrating at a position expected for a protein of 17 kDa in cells harboring pTPS304. This protein was absent when the maxicells con-

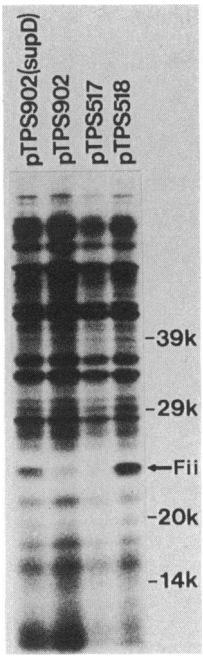


FIG. 3. Autoradiograms of [^{35}S]methionine-pulse-labeled proteins in plasmid-containing bacteria. Plasmids containing the wild-type (pTPS517, -518) or amber mutant (pTPS902) *fii* gene were constructed as described in Materials and Methods and diagrammed in Fig. 1. Each plasmid was placed in TPS13 *recA*, induced at 41°C for 10 min, and labeled with [^{35}S]methionine for 20 s. TPS13 contains an amber mutation in *fii*. The bacterial proteins were solubilized and analyzed by SDS-PAGE as described in Materials and Methods. The same experiment was done with TPS13 *supD zed-508*::Tn*10* containing pTPS902. The arrow denotes the position of the *fii* protein and molecular weight standards (k, 10^3).

tained the plasmid pTPS817 with an amber mutation in *orf-3*. These data are consistent with the 17-kDa protein being the product of the predicted ORF3 which codes for a 141-amino acid protein (minus the initiating methionine) with a calculated molecular weight of 15,385.

Since the gene for this protein is between *fii* and *tolA*, it is possible that its product is necessary for the entrance of the E colicins or single-stranded phage DNA into the cell. To test this, we inserted the gene for chloramphenicol resistance into the chromosomal ORF3 gene to give an ORF3 mutant strain in the following way. Plasmid pTPS301-*orf-3*::Cm (Fig. 5) was constructed and transformed into a *polA* strain as described in Materials and Methods. The plasmid integrants were isolated as Amp^r Cam^r transformants, and P1 *vir* lysates were made on these plasmid integrants. The lysates were used to transduce Gm1 containing pTPS401, and an Amp^s Cam^r transductant was isolated, thus creating a strain containing only the *orf-3*::Cm insertion mutation in the chromosome. Plasmid pTPS401 was present to supply wild-type *orf-3* function in case the product was essential for cell viability. When the *orf-3*::Cm insertion gene was transduced into GM1 or GM1 containing pTPS401 by using P1, similar numbers of transductants were obtained with both strains, suggesting that *orf-3* is not essential for bacterial growth.

GM1 containing the *orf-3*::Cm insertion mutation was designated strain TPS300. Two experiments were done to confirm the gene replacement in this strain. We had previously observed that P1 lysates from strain C131 would cotransduce Fii$^+$ and Tet^r with a 90% frequency (33). Using this same lysate to transduce TPS300, we found 94% of the Tet^r transductants had become f1 and chloramphenicol sensitive indicating that the Cam^r marker in TPS300 is located very close to *fii*. The second experiment was a Southern hybridization analysis of *Eco*RI-digested chromosomal DNA of TPS300 by using the 1.85-kb nick-translated *Eco*RI-*Hin*dIII fragment containing the *orf-1-fii-orf-3* region (Fig. 1) as a probe. Insertion of the Cam^r gene into this region should generate a new *Eco*RI site and thus give two, rather than one, chromosomal *Eco*RI fragments which would hybridize to the probe. Such analysis showed that the insertion generated a new *Eco*RI site at the predicted site in TPS300 (data not shown).

TPS300 was resistant to f1 phage, tolerant to colicins E1 and E3, and sensitive to f2 phage. Thus cells with the *orf-3*::Cm insertion mutation have the same phenotype observed for *tolA* and *fii* mutations. A complementation test was performed with plasmids carrying different regions of DNA to confirm that the mutant phenotype of TPS300 does not result from the disruption of adjacent *fii* or *tolA* gene. TPS300 could be complemented by any plasmid carrying wild-type ORF3 regardless of the absence of wild-type *fii* or *tolA* gene (Fig. 5, bottom). The only two plasmids which failed to complement the TPS300 mutation contained the *orf-3* gene inactived either by Cm^r gene insertion (pTPS301-*orf-3*::Cm) or by creation of an amber mutation by oligonucleotide-directed mutagenesis (pTPS806). These data suggest that the resistance of TPS300 to f1 and colicins E1 and E3 is due to the absence of the ORF3 protein. These results also indicate that *orf-3*::Cm does not totally prevent the expression of *tolA* by a polar effect because pTPS301-*orf-3*::Cm still can complement the TPS94 mutation (a *tolA* mutation). Furthermore, since pTPS902 can complement the TPS300 mutation, amber mutations in *orf-2* are not functionally polar for *orf-3* expression.

Characterization of ORF1 protein. Plasmids which contain a wild-type *orf-1* (pTPS811) or an amber mutant *orf-1* (pTPS813) were constructed from pUC9 as shown at the top of Fig. 4. Maxicells containing these plasmids were labeled with [^{35}S]methionine, and the products were analyzed by SDS-PAGE (Fig. 4A). A protein migrating at a position expected for a protein of approximately 16 kDa was synthesized only in the maxicells containing pTPS811 which contained the wild-type *orf-1*. The other bands unique to maxicells containing pTPS811 or -813 probably represent the amber fragments of *orf-1* and *fii* which are present in the respective plasmids. The reduced amount of the 6-kDa band representing the *fii* or *orf-1* amber fragments coded for by pTPS813 relative to the large amount of the smaller *fii* amber fragment in pTPS811 indicates a slight polar effect of the *orf-1* amber mutation on *fii* expression.

The size of the protein synthesized in pTPS811-containing maxicells corresponds well with the 15,477 molecular weight calculated for the protein defined by ORF1 (Fig. 2). To test whether this protein was necessary for f1 infection or E colicin sensitivity, the Cm^r gene was inserted into *orf-1* in plasmid pTPS303 as shown in Fig. 5. This plasmid was used to create a chromosomal insertion mutation by the same technique as described above to create the *orf-3*::Cm chromosomal insertion mutation. The resulting strain, TPS400, which contained an *orf-1*::Cm insertion mutation, was able to plaque f1 phage and was sensitive to colicins E1 and E3. Thus, although ORF1 does define a gene in *E. coli*, its function is unknown. However, the ability of pTPS303-*orf-1*::Cm to complement the TPS13 *fii* mutation (Fig. 5) sug-

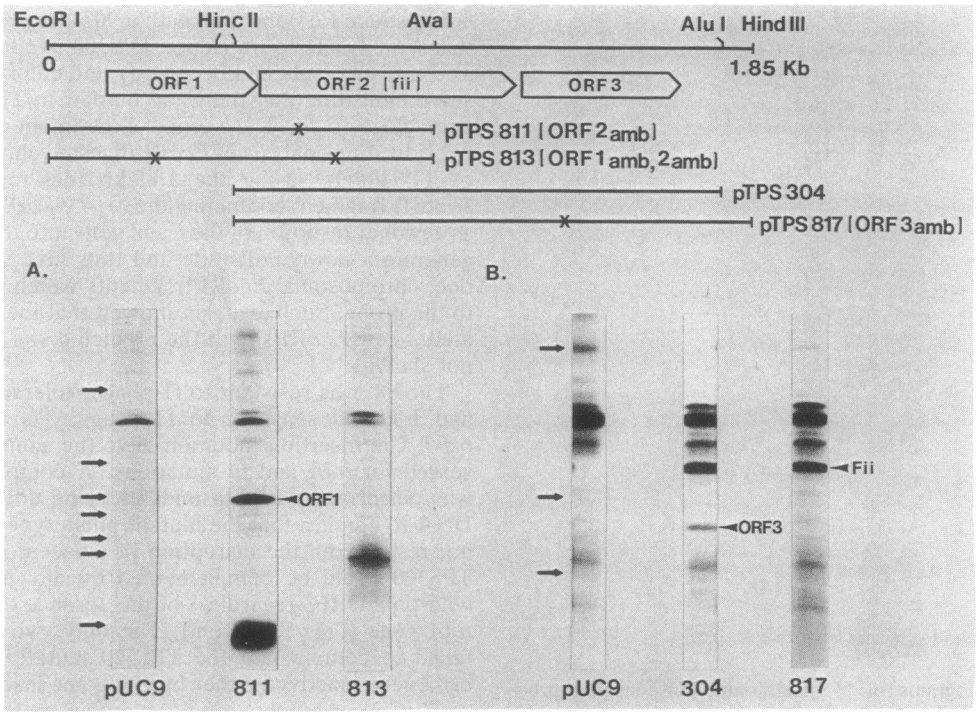
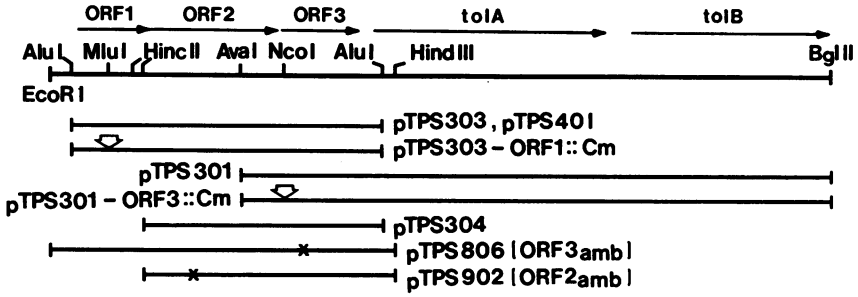


FIG. 4. Expression of the proteins designated by ORF1 and ORF3 in maxicells. The top of the figure shows the inserts of the region which were cloned into pUC9 as described in Materials and Methods. The pTPS designation to the right is the name of the resulting plasmid containing the respective insert. × indicates the position of the amber mutation. The amber mutation in pTPS811 is at position 654; in pTPS813 they are at positions 286 and 753; and in pTPS817 it is at position 1362. The bottom portion shows autoradiograms of the analysis by SDS-PAGE of the [^{35}S]methionine-labeled proteins synthesized in maxicells carrying the designated plasmids. (A) Autoradiograms of proteins displayed on a 19.3% SDS-urea-polyacrylamide gel (15). Arrows indicate positions of molecular weight standards: turkey ovalbumin (42,000), soybean trypsin inhibitor (20,000), and myoglobin standards from Sigma Chemical Co. (17,000, 14,400, 8,160, 6,210, and 2,510). (B) Autoradiograms of proteins displayed on a 12% SDS-polyacrylamide gel (18). Arrows indicate positions of molecular weight standards: ovalbumin (42,000), soybean trypsin inhibitor (20,000), and lysozyme (14,300).



Strain complemented	Plasmid (pTPS) 401	304	301	301-orf3::Cm	806	902	303-orf1::Cm
TPS13 (fii)	+	+	-	-	+	-	+
TPS94 (tolA)	-	-	+	+	-	-	-
TPS300 (orf3::Cm)	+	+	+	-	-	+	+

FIG. 5. Construction and phenotype of insertion mutation into the chromosomal *orf-1* and *orf-3* regions. The top line shows the position of the genes in the 4.2-kb isolated chromosomal segment and the position of the sites used to construct the plasmids shown below. The open arrows show the positions where the gene for chloramphenical resistance (Cm) was inserted into the *Nco*I and *Mlu*I sites of pTPS301 and pTPS303, respectively. The plasmids containing the Cm insert were used to construct the chromosomal insertion mutations in *orf-1* and *-3* as described in the text. × indicates the position of the amber mutation. The amber mutation in pTPS806 is at position 1362, and in pTPS902 it is at position 753. The bottom of the figure shows the ability of the plasmids containing the inserts shown above to complement mutations in *fii*, *tolA*, and *orf-3* in strains TPS13, -94, and -300, respectively. + means the cell can be infected by f1 and is susceptible to colicins E1 and E3 (33).

gests that *orf-1*::Cm is not completely polar for *orf-2* (*fii*) expression.

We have no data indicating that ORF4 codes for a bacterial protein. There is no strong ribosome-binding site before the potential start site for ORF4. We could not detect any protein product for this ORF by doing the same maxicell experiments as those for ORF1, -2, and -3. From oligonucleotide-directed mutagenesis, we showed that an amber mutation in *orf-4* does not affect the complementation of *fii* mutations. Therefore, it is not clear whether *orf-4* encodes a protein or not.

DISCUSSION

The nucleotide sequence of a 1,854-base-pair fragment carrying the *fii* region revealed the presence of three ORFs (ORF1, -2, and -3) on one strand and one ORF (ORF4) on the complementary strand. Analysis of the proteins produced in maxicells from plasmids containing a wild-type or an amber mutation in each ORF showed that ORF1, -2, and -3 coded for proteins of approximately 16, 24, and 17 kDa, respectively, indicating that each was representative of a separate gene. No protein product was detected for ORF4 by the same technique. The 24-kDa protein coded by ORF2 is the product of the *fii* gene by the following criteria. (i) A plasmid carrying an amber mutation in the *orf-2* gene generated by oligonucleotide-directed mutagenesis failed to complement *fii* mutations and could not direct the synthesis of the 24-kDa protein. (ii) DNA sequence analysis of the *fii* region cloned from an *fii* amber (TPS13) or *fii* missense (TPS66) mutant strain showed both mutations located in ORF2. The phenotype of mutations in *orf-2* (*fii*) is an increased tolerance to colicins E1, E2, and E3, resistance to infection by the filamentous phages, and an increased sensitivity to deoxycholate (33). This is the same phenotype previously observed for mutations in the *tolA* locus (2, 24). Replacement of the chromosomal *orf-3* by an *orf-3*::Cm insertion mutation leads to a bacterial strain which has the same phenotype as bacteria carrying an *orf-2* (*fii*) or a *tolA* mutation. Since the *orf-3* gene is between *orf-2* (*fii*) and *tolA*, these three contiguous genes define a cluster whose products function to allow the E colicins or single-stranded DNA of the filamentous phages to enter or cross (or both) the inner membrane. None of these phenotypes was observed when the chromosomal *orf-1* gene was replaced by an *orf-1*::Cm insertion mutation.

Complementation studies between *fii*, *tolA*, and *tolB* mutations and plasmids containing various cloned portions of this region (33) together with the sequence data in this paper define a gene order of *orf-1-orf-2* (*fii*)-*orf-3-tolA-tolB*. This is in agreement with the gene order *tolP-tolA-tolB* previously assigned by Bernstein (1). It is not known whether the *tolP* mutations were alleles of *orf-2* (*fii*) or *orf-3*. As described above, the phenotypes exhibited by strains containing mutations in each of the *orf-2* (*fii*), *orf-3*, and *tolA* genes are indistinguishable. Mutations in *tolB* differ in that they are sensitive to E1 (2, 24) and to the filamentous phages (33) but are still tolerant to colicins E2 and E3. Since the mutations described in both *orf-2* (*fii*) and *orf-3* exhibit tolerance to colicin E1 and are resistant to infection by the filamantous phages, the same phenotype exhibited by mutants in *tolA* (33; this paper), we propose to name this gene cluster *tolQRAB*, where *tolQ* is *orf-2* (*fii*) and *tolR* is *orf-3*.

Although the direction of transcription of this gene cluster is from *tolQ* to *-B*, the pattern of expression is not clear. Preliminary attempts to detect mRNA from this region have been ambiguous, perhaps because of the low level of mRNA expressed (data not shown). The presence of amber mutations or Tn*5* insertions in any gene in the cloned gene cluster does not affect the ability of the distal genes in the cluster to complement mutations in their respective chromosomal loci (33) (Fig. 5). This suggests that each of these genes is transcribed independently, although it does not rule out the possibility that an operon exists in this region which cannot be detected owing to the presence of internal promoters. It is also possible that nonsense mutations in one gene may be slightly polar on the expression of downstream genes but that the reduced amount of distal gene product may still be sufficient for full function. Bernstein (1) proposed that *tolP* (*tolQR*) and *tolA* were in the same operon based on studies analyzing bacteriophage Mu insertions into the *tolPAB* cluster. However, the fact that insertions in *tolQ*, *tolR*, or *tolA* all have the same phenotype suggests that this interpretation is not correct.

The products of the *tolQRA* gene cluster are responsible for the translocation of single-stranded phage DNA and E colicins into or across the cytoplasmic membrane. This is at least the second step in the uptake of these molecules into the bacteria as they each have separate receptors: the conjugative pilus for the filamentous phages (26) and the *btuB* gene product for the colicins (12, 27). Previous studies have shown that the *tolQ* (*fii*) product remains associated with the membrane in all fractionation experiments (33). Hydropathic predictions (17) suggest that this protein has three hydrophobic regions (positions 561 to 653, 927 to 1016, and 1038 to 1112 in Fig. 2) which could be membrane-spanning domains of this protein. Similarly, the TolR protein has a hydrophobic region (positions 1293 to 1367 in Fig. 2) which could interact with the membrane. Such an interaction of these proteins might predict that mutations in these genes would show other membrane-related phenotypes. Lazzaroni and Portalier (20) have isolated periplasmic leaky mutants which released periplasmic enzymes into the medium. One of these, *lkyB*, maps at 16.5 min and confers resistance to colicin E1 (19), a phenotype which is consistent with it being a mutation in the *tolQRA* cluster.

The role that the *tolQRAB* proteins play in normal cell metabolism is unknown. They may be part of a general mechanism which allows charged macromolecules to enter the cell. Examination of the amino acid sequences of the *tolQ* and *tolR* proteins showed no significant homology with any protein sequences in the data bank (21, 37). Further studies on the nature of the products of the *tolQRAB* gene cluster may help us understand how macromolecules such as colicins and phage DNA can be translocated across the bacterial membrane.

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