1993

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Burghoff, R. L., Pallesen, L., Krogfelt, K. A., Newman, J. V., Richardson, M., Bliss, J. L., Laux, D. C., & Cohen, P. S. (1993). Utilization of the Mouse Large Intestine To Select an *Escherichia coli* F-18 DNA Sequence That Enhances Colonizing Ability and Stimulates Synthesis of Type 1 Fimbriae. *Infect. Immun., 61*(4), 1293-1300. Retrieved from https://iai.asm.org/content/61/4/1293.
Available at: https://iai.asm.org/content/61/4/1293

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INFECTION AND IMMUNITY, Apr. 1993, p. 1293–1300
0019-9567/93/041293-08$02.00/0

Vol. 61, No. 4

Utilization of the Mouse Large Intestine To Select an *Escherichia coli* F-18 DNA Sequence That Enhances Colonizing Ability and Stimulates Synthesis of Type 1 Fimbriae

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Received 28 July 1992/Accepted 17 January 1993

***Escherichia coli* F-18, a normal human fecal isolate, is an excellent colonizer of the streptomycin-treated mouse large intestine. *E. coli* F-18 Col$^-$, a derivative of *E. coli* F-18 which no longer makes the *E. coli* F-18 colicin, colonizes the large intestine as well as *E. coli* F-18 when fed to mice alone but is eliminated when fed together with *E. coli* F-18. Random sequences of *E. coli* F-18 DNA were cloned into pRLB2, a *parB*-stabilized derivative of pHC79. The entire gene library was transformed into *E. coli* F-18 Col$^-$ and fed to streptomycin-treated mice. The mouse large intestine selected a predominant clone which contained a recombinant plasmid (pRLB7) that enhanced *E. coli* F-18 Col$^-$ colonizing ability 100-fold but did not stimulate colicin synthesis. Moreover, pRLB7 simultaneously improved the survival of *E. coli* F-18 Col$^-$ in stationary phase in vitro, utilizing nutrients derived from mouse cecal mucus, and stimulated synthesis of both type 1 fimbriae and three *E. coli* F-18 Col$^-$ outer membrane proteins (74, 71, and 69 kDa). The 6.5-kb *E. coli* F-18 DNA sequence in pRLB7 does not contain either the *fim* operon or *pilG* (*hns*), both known to be involved in type 1 fimbrial synthesis. The sequence encodes six proteins, all smaller than the three *E. coli* F-18 Col$^-$ outer membrane proteins whose synthesis it stimulates. Collectively, the results suggest that the cloned *E. coli* F-18 DNA sequence contains one or more regulators of *E. coli* F-18 Col$^-$ operons expressed in the mouse large intestine in vivo and in isolated mouse cecal mucus in vitro.**

When fed simultaneously to streptomycin-treated mice, *Escherichia coli* F-18, isolated from the feces of a healthy human, is a far better colonizer of the mouse large intestine than *E. coli* F-18 Col$^-$, a strain derived from *E. coli* F-18 (5) which no longer makes the *E. coli* F-18 colicin, colicin V (25). It is highly likely that both *E. coli* F-18 and *E. coli* F-18 Col$^-$ colonize the mouse large intestine by growing in intestinal mucus; i.e., both strains grow well when inoculated alone into cecal mucus in vitro, but neither grows in cecal luminal contents in vitro (31). Moreover, although both strains grow well alone in cecal mucus, together *E. coli* F-18 grows well and *E. coli* F-18 Col$^-$ grows poorly (31).

Recently, we reported that, when streptomycin-treated mice were simultaneously fed 10^8 CFU of *E. coli* F-18 Col$^-$ and 10^3 CFU of *E. coli* F-18, *E. coli* F-18 became the predominant strain within 5 days (31). This observation suggested the possibility of cloning random sequences of *E. coli* F-18 DNA into *E. coli* F-18 Col$^-$ and using streptomycin-treated mice to select strains which contain *E. coli* F-18 DNA sequences that enhance *E. coli* F-18 Col$^-$ colonization. In this report, we describe the isolation of an *E. coli* F-18 DNA sequence, selected in vivo, that results in enhanced *E. coli* F-18 Col$^-$ large intestine colonizing ability, enhanced survival in cecal mucus in stationary phase in vitro, and increased synthesis of both type 1 fimbriae and at least three outer membrane proteins.

MATERIALS AND METHODS

Bacterial strains. *E. coli* F-18 was isolated from the feces of a healthy human in 1977 and is an excellent colonizer of the streptomycin-treated mouse large intestine (5, 26). The *E. coli* F-18 strain used here is resistant to streptomycin (Str^r) and rifampin (Rif^r) and produces colicin V (25). Its serotype is rough:K1:H5 (25). *E. coli* F-18 Col$^-$ *fimA* was made (25) by bacteriophage P1 transduction into *E. coli* F-18 from *E. coli* ORN151, which contains the tetracycline resistance gene from Tn*10* inserted in the *fimA* (*pilA*) gene (25), making it type 1 fimbria negative. *E. coli* F-18 Col$^-$ was isolated in 1979 in the following way (5). A Str^r, nalidixic acid-resistant (Nal^r) mutant of *E. coli* F-18 was mated with *E. coli* RS2, which contains the R1 *drd*19 plasmid (R1$^+$), and an *E. coli* F-18 R1$^+$ strain was isolated. The *E. coli* F-18 R1$^+$ strain was cured of the R1 *drd* plasmid with acriflavine. One of the cured clones, *E. coli* F-18 Col$^-$, lost the ability to make the *E. coli* F-18 colicin (5). *E. coli* F-18 Col$^-$ is a poor mouse large intestine colonizer relative to *E. coli* F-18 (5, 26). At the time of isolation, *E. coli* F-18 Col$^-$ appeared to be missing an 86-kb plasmid (5). When recently reexamined, however, *E. coli* F-18 Col$^-$ was found to contain the 86-kb plasmid, but it still does not make colicin V and remains a poor colonizer (data not shown). The *col*V plasmid is known to be able to insert into the *E. coli* chromosome (12), which presumably accounts for its disappearance and reappearance in *E. coli* F-18 Col$^-$. *E. coli* F-18 Col$^-$ is resistant to colicin V (5). A spontaneous double mutant of *E. coli* HB101

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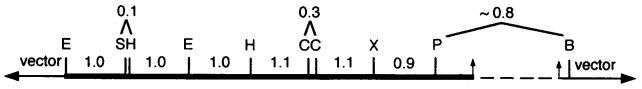

FIG. 1. Restriction map of pRLB7. P, *Pst*I; X, *Xho*I; C, *Cla*I; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I. Numbers between restriction sites are in kilobase pairs. The dashed line indicates the approximate 2-kb deletion in pRLB2, and the vertical arrows delineate the undefined ends of the *E. coli* F-18 sequence and pRLB2. The *Bgl*II (B) site is a pRLB2 site.

resistant to both nalidixic acid and streptomycin was used for characterization of relevant constructions. *E. coli* K38 (28) was used for identifying proteins encoded by the *E. coli* F-18 DNA sequence which enhances the colonizing ability of *E. coli* F-18 Col$^-$. *E. coli* JM109, obtained from Promega (Madison, Wis.), makes β-galactosidase when complemented by the pGEM-Zf(+) plasmid which encodes the *lacZ* α-peptide. Therefore, *E. coli* JM109 colonies containing recombinant pGEM-Zf(+) vector with inserts into the *lacZ* α-peptide gene fail to make β-galactosidase.

***E. coli* F-18 gene library construction.** *E. coli* F-18 DNA, isolated by the method of Marmur (23), was partially digested with *Eco*RI. Digestion products in the 5- to 15-kb range were used for subsequent ligation to *Eco*RI-restricted pRLB2, a pHC79 derivative described previously (4). *E. coli* F-18 Col$^-$ was found to be a poor recipient for the relaxed-circular ligation products generated during plasmid bank construction. For this reason, ligation products were initially transformed into *E. coli* HB101. These colonies (10,000 CFU) were then mixed and added to 1 liter of L-broth containing ampicillin (100 μg/ml) grown to an optical density at 600 nm of 0.4, and plasmid DNA was amplified following addition of chloramphenicol (200 μg/ml). Supercoiled plasmid DNA was then isolated by cesium chloride-ethidium bromide density centrifugation (22). The resulting purified supercoiled plasmid DNA was assayed for variability by restriction analysis and then transformed into *E. coli* F-18 Col$^-$ at high frequency (approximately 10^6 transformants per μg of DNA). These transformants, representing the shotgun-cloned *E. coli* F-18 gene library in *E. coli* F-18 Col$^-$, were used for all subsequent in vivo selection experiments.

Small-scale isolation of plasmid DNA was carried out by the method of Birnboim and Doly (2). Agarose gel electrophoresis was carried out at a concentration of 0.8% agarose. All restriction endonuclease and T4 DNA ligase reactions were performed as described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). Calf intestinal phosphatase and RNase I treatments were performed as specified by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). All restriction fragments were purified with GENECLEAN, using the instructions provided by the manufacturer (Bio 101, Inc., La Jolla, Calif.).

Plasmid pRLB7 deletion construction. The *parB* stabilized plasmid pRLB2, described previously (4), was used as the *E. coli* F-18 gene library recipient plasmid. Plasmid pRLB7 was selected by the mouse large intestine in vivo from the *E. coli* F-18 gene library in *E. coli* F-18 Col$^-$. The restriction map of pRLB7 is presented in Fig. 1. pRLB18 is a deletion which extends from the *Xho*I site to the *Sal*I site of pRLB7 and was constructed by digesting pRLB7 with *Xho*I and *Sal*I and religating the deleted plasmid by virtue of *Xho*I and *Sal*I restriction site compatibility. All plasmids used in this study are listed in Table 1.

Mouse colonization experiments. The method used to distinguish the relative colonizing abilities of *E. coli* strains in mice has been described in detail previously (5, 31). Briefly, after 1 day of being fed streptomycin sulfate in their drinking water (5 g/liter), male CD-1 mice (5 to 8 weeks old) were starved for food (Charles River Valley Rat, Mouse, and Hamster Formula) and water for 18 to 24 h and fed 10^{10} CFU each of the L-broth-grown *E. coli* strains to be tested in 1 ml of sterile 20% (wt/vol) sucrose. The mice drank the bacterial suspension almost immediately and were then given food and streptomycin-containing drinking water. The next day and at 48-h intervals, fecal samples, no older than 24 h, were collected, homogenized, diluted, and plated on selective media as described below. In all colonization experiments, plates were incubated at 37°C for 18 to 24 h. Colonizing ability was assessed by the level at which a strain persisted in feces. Each experiment was performed at least twice, with essentially identical results.

To differentiate between *E. coli* F-18 Col$^-$ strains carrying recombinant plasmids and *E. coli* F-18 Col$^-$ *fimA*(pRLB2), fecal samples were plated on MacConkey agar containing 100 μg of streptomycin sulfate and 100 μg of ampicillin per ml and on MacConkey agar containing 100 μg of streptomycin sulfate, 100 μg of ampicillin, and 10 μg of tetracycline hydrochoride per ml. The former medium grows both strains being tested, whereas the latter medium only grows the *E. coli* F-18 Col$^-$ *fimA*(pRLB2) strain. When necessary, i.e., when the numbers of CFU on the two media were difficult to distinguish as different, 100 colonies were typed by transferring samples of each by toothpick from the plates without tetracycline to the plates containing tetracycline.

Growth in cecal mucus dialysates. Cecal mucus dialysates were prepared from CD-1 mouse crude cecal mucus as

TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pRLB2	*parB* inserted in *tet* of pHC79	4
pRLB7	Contains an approximately 6.5-kb *E. coli* F-18 DNA sequence that enhances *E. coli* F-18 Col$^-$ colonization, inserted in the *Eco*RI site of pRLB2	This study
pRLB18	*Xho*I-*Sal*I deletion of pRLB7	This study
pPKL4	Contains the *E. coli* K-12 *fim* operon in pBR322	16
pTHK113	Contains *pilG* cloned into pBR322	13
pGP1-2	Contains the RNA polymerase gene of phage T7 under the heat-inducible control of the p_L promoter cloned into pBR322	30
pGEM-7Zf(+)	Contains the phage T7 promoter	Promega (Madison, Wis.)
pLPA213	Contains the *Pst*I-*Sal*I fragment of pRLB7 inserted into pGEM-7Zf(+) such that the *Pst*I-*Sal*I fragment is transcribed in that direction, using the phage T7 promoter	This study
pLPA214	As pLPA213, but with the *Pst*I-*Sal*I fragment inserted in the opposite orientation	This study

described previously (10). Dialysates (0.5 ml) were inoculated with 10^4 CFU each of the plasmid-containing strains to be tested, incubated standing at 37°C, and plated on MacConkey agar containing streptomycin sulfate (100 μg/ml), nalidixic acid (50 μg/ml), and ampicillin (100 μg/ml) at 0, 4, 8, 12, 16, 20, 24, 48, and 120 h. The plates were incubated at 37°C for 18 to 24 h; 150 colonies from each time point were transferred by toothpick to MacConkey agar containing streptomycin sulfate (100 μg/ml), nalidixic acid (50 μg/ml) and tetracycline hydrochloride (10 μg/ml), and after incubation colonies were scored for either resistance or sensitivity to tetracycline.

Plate test for colicin production. The indicator strain used to detect colicin production was *E. coli* ORN152 (25). Approximately 10^6 CFU from an aerobically grown L-broth overnight culture (37°C) was spread on the surface of an L-agar plate. The strain to be examined for colicin production was transferred by toothpick from a fresh streak to the plate containing the indicator strain. After overnight incubation at 37°C, colonies of the test strain were examined for zones of inhibition.

Radioactive labeling of *E. coli* F-18 Col⁻ strains during growth in mouse cecal mucus. *E. coli* F-18 Col⁻ strains and their plasmid-containing derivatives were grown in L-broth as described previously (25) and diluted 10^4-fold in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-Hanks buffer (pH 7.4) into 2 ml of crude cecal mucus (4 mg of protein per ml) containing 10 μCi of Tran^{35}S-label (1,100 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, Calif.) per ml. Cultures were incubated standing for 16 h at 37°C, washed twice in HEPES-Hanks buffer (pH 7.4), and resuspended in the same buffer at a concentration of about 10^9 CFU/ml. The specific activity of the cultures was routinely between 2×10^{-3} and 5×10^{-3} cpm/CFU.

Adhesion assay. The adhesion assay has been described in detail previously (25). Briefly, ^{35}S-labeled cells (0.2 ml) in HEPES-Hanks buffer (pH 7.4) or ^{35}S-labeled cells (0.2 ml) in HEPES-Hanks buffer (pH 7.4) containing α-methyl-D-mannoside (100 mM) were added to multiwell polystyrene plates containing an immobilized mannose-bovine serum albumin (BSA) glycoconjugate (Carbohydrates International, Arlov, Sweden). The plates were incubated for 1 h at 37°C, and the wells were then washed twice with HEPES-Hanks buffer (pH 7.4) to remove unbound bacteria. Adherent bacteria were released by adding 0.5 ml of 5% sodium dodecyl sulfate (SDS) to each well and then incubating the plates for 3 h at 37°C. The SDS was removed from each well, and the level of radioactivity was determined by scintillation counting.

Isolation of outer membranes and periplasm. The periplasm of ^{35}S-labeled cells grown in cecal mucus was released from spheroplasts (21). Spheroplasts were centrifuged at 15,000 × *g* for 10 min at 4°C, resuspended in 30 mM Tris buffer (pH 7.3) containing 3 mM EDTA, and lysed by sonication. Surviving spheroplasts were removed by centrifugation at 15,000 × *g* for 5 min at 4°C, and the supernatant was centrifuged at 40,000 × *g* for 90 min at 4°C to collect whole cell membranes. Outer membranes were then prepared from whole membranes by differential centrifugation, using the detergent sodium dodecyl sarcosinate as described by Filip et al. (9).

Purification and quantitation of type 1 fimbriae. *E. coli* F-18 Col⁻ strains were grown in 100 ml of Minimal Broth Davis (Difco) containing 1% (wt/vol) D-glucose as described previously (25). Fimbriae were purified from each strain as described by Eisenstein and Dodd with minor modifications (7). Briefly, bacteria were collected by centrifugation and were then washed and resuspended in Tris-HCl buffer (pH 7.4). The fimbriae were sheared off by blending. Bacterial cells and membrane debris were removed by centrifugation, and the supernatant was subjected to ultracentrifugation (227,000 × *g*, 2 h, 4°C). The pellet was resuspended in 5 M urea, diluted to 1 M urea, and layered over a 1 M urea–1 M sucrose–5 mM Tris cushion. Pellets of pure fimbriae were obtained by ultracentrifugation of the samples at 4°C for 16 h at 200,000 × *g*. The fimbrial preparations were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (20), and the purity of the fimbriae was assessed by silver staining (3). The preparations contained a small amount of a high-molecular-weight protein in addition to the 17-kDa FimA protein. Therefore, type 1 fimbrial protein in each preparation was calculated by subtracting the amount of protein in preparations derived from *E. coli* F-18 Col⁻ *fimA*, which does not contain type 1 fimbriae (25). Protein was quantitated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Assays were performed in accordance with package instructions, using BSA as the standard.

Insertion of the *Pst*I-*Sal*I sequence of pRLB7 into pGEM-7Zf(+). pGEM-7Zf(+) (Promega Corp.) was digested with *Sma*I and treated with calf intestinal phosphatase. pRLB7 was digested with *Pst*I and *Sal*I and then incubated with the Klenow fragment of *E. coli* DNA polymerase in the presence of the four deoxynucleotide triphosphates to fill in recessed 3′ ends. Phosphatase-treated pGEM-7Zf(+) was then ligated to the pRLB7 fragments that had been incubated with Klenow fragment, and ligation products were transformed into *E. coli* JM109 and plated on L-agar containing IPTG (isopropyl-β-D-thiogalactopyranoside; 24 μg/ml), ampicillin (100 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml). White colonies were screened for the *Pst*I-*Sal*I insert into pGEM-7Zf(+), and its orientation with respect to the T7 promoter was determined by digesting the recombinant plasmids with *Xho*I, *Hin*dIII, and *Cla*I.

Labeling of plasmid-encoded proteins by the T7 RNA polymerase/promoter system. *E. coli* K38 cells containing both plasmid pGP1-2 and the plasmid to be tested were grown in L-broth containing 40 μg of ampicillin and 40 μg of kanamycin sulfate per ml at 30°C. At A_{590} = 0.5, 0.2 ml of cells was centrifuged and washed with 5 ml of M9 medium, recentrifuged, and resuspended in 1 ml of M9 medium supplemented with 20 μg of thiamine per ml and 0.01% of 18 amino acids (i.e., no cysteine or methionine). Cells were grown with shaking at 30°C for 60 min and then shifted to 42°C for 15 min to lift repression on T7 RNA polymerase synthesis (30). Rifampin was added to a final concentration of 200 μg/ml to block *E. coli* K38 RNA polymerase activity (30), and the incubation was continued at 42°C for 10 min. The temperature was then shifted to 30°C for 20 min, and the cells were pulsed with 10 μCi of Tran^{35}S-label per ml for 10 min at 30°C. Finally, the cells were centrifuged for 20 s in an Eppendorf 5415 centrifuge and subjected to SDS-PAGE and autoradiography, as described previously (32).

Labeling of DNA with ^{32}P. The various probes used were labeled with ^{32}P, using [α-^{32}P]dCTP (3,000 Ci/mmol; NEN Research Products, Boston, Mass.) and the Boehringer Mannheim Random Primed DNA Labeling Kit, according to instructions.

Southern blots. DNA fragments were separated in 1% agarose gels and transferred to GeneScreen filters (NEN Dupont, Boston, Mass.) as described by the manufacturer. Prior to transfer, the gels were placed in 0.37% (vol/vol) HCl for 10 min, washed twice with water, placed in denaturation

buffer (1.5 M sodium chloride, 0.5 M sodium hydroxide) for 40 min, washed twice with water, and finally placed in neutralization buffer (3 M sodium citrate, pH 5.5) for 60 min. The filters were prehybridized in 0.25 M phosphate buffer (pH 7.2) containing 6% SDS, 1 mM EDTA, and 50 μg of denatured salmon sperm DNA per ml at 65°C for 60 min. The filters were then hybridized with probe in the same solution as described above, in the absence of salmon sperm DNA, for 18 h at 65°C. The filters were then washed four times for 15 min each time at 65°C in 20 mM phosphate buffer (pH 7.2) containing 1% SDS and then washed once for 15 min at 65°C in 10 mM phosphate buffer (pH 7.2) containing 0.5% SDS. Autoradiography was performed as described previously (32).

RESULTS

In vivo selection of an *E. coli* F-18 DNA fragment that enhances *E. coli* F-18 Col⁻ colonizing ability. Three streptomycin-treated mice were fed 10^{10} CFU of *E. coli* F-18 Col⁻ cells containing random *E. coli* F-18 DNA fragments cloned into pRLB2. On day 9 postfeeding, 16 colonies were selected from the feces of each mouse and characterized. Of the 48 colonies, 44 contained a recombinant plasmid which by restriction enzyme analysis appeared to contain the same insert (Fig. 1). One plasmid, termed pRLB7, was used in further experiments.

pRLB7 was transformed into a fresh *E. coli* F-18 Col⁻ background, and one colony was selected and designated *E. coli* F-18 Col⁻(pRLB7). This strain (10^{10} CFU) was fed to eight streptomycin-treated mice along with 10^{10} CFU of *E. coli* F-18 Col⁻ *fimA*(pRLB2), which colonizes as well as *E. coli* F-18 Col⁻(pRLB2) (data not shown). *E. coli* F-18 Col⁻(pRLB7) colonized at about 5×10^7 CFU/g of feces, whereas *E. coli* F-18 Col⁻ *fimA*(pRLB2) colonized at a level of only 5×10^5 CFU/g of feces; i.e., pRLB7 contained a sequence that appeared to improve the colonizing ability of *E. coli* F-18 Col⁻ 100-fold (Fig. 2A).

Additional control experiments were performed to be sure that pRLB7 enhanced *E. coli* F-18 Col⁻ colonizing ability. First, 10^{10} CFU of *E. coli* F-18 Col⁻ containing a random 4.5-kb *E. coli* F-18 DNA insert in pRLB2 and 10^{10} CFU of *E. coli* F-18 Col⁻ *fimA*(pRLB2) were simultaneously fed to three streptomycin-treated mice. Both strains colonized equally well at about 10^7 CFU/g of feces (data not shown). Second, and of greater importance, a 4.5-kb *Xho*I to *Sal*I deletion of pRLB7, designated pRLB18 (Fig. 1), was transformed into *E. coli* F-18 Col⁻. This strain, *E. coli* F-18 Col⁻(pRLB18), and *E. coli* F-18 Col⁻ *fimA*(pRLB2) were simultaneously fed (10^{10} CFU of each) to nine streptomycin-treated mice. Both strains colonized equally well at about 5×10^6 CFU/g of feces (Fig. 2B). These control experiments supported the idea that pRLB7 contains a specific *E. coli* F-18 DNA sequence that enhances *E. coli* F-18 Col⁻ mouse large intestine colonizing ability.

In the colonization experiments described above, we used *E. coli* F-18 Col⁻ *fimA*(pRLB2) because it has the tetracycline resistance gene from Tn*10* inserted into *fimA* and we could use the tetracycline resistance phenotype in cofeeding experiments to determine whether *E. coli* F-18 Col⁻(pRLB7) was the better colonizer. However, it might be argued that *E. coli* F-18 Col⁻ *fimA*(pRLB2) was a bad choice since the *E. coli* F-18 DNA sequence in pRLB7 was subsequently shown to result in increased synthesis of type 1 fimbriae in *E. coli* F-18 Col⁻ (see below). Therefore, *E. coli* F-18 Col⁻ *fimA*(pRLB7) was constructed and fed to six mice simultaneously with *E. coli* F-18 Col⁻(pRLB2). The two strains were differentiated in mouse feces by transferring by toothpick 100 colonies from each mouse at each time of sampling to MacConkey plates containing tetracycline (10 μg/ml). Under these conditions, *E. coli* F-18 Col⁻ *fimA*(pRLB7) colonized at a level of about 5×10^7 CFU/g of feces, whereas *E. coli* F-18 Col⁻(pRLB2) colonized at some level below 5×10^5 CFU/g of feces, i.e., at least 2 orders of magnitude lower (data not shown). Therefore, pRLB7 contains an *E. coli* F-18 DNA sequence which increases the colonizing ability of both *E. coli* F-18 Col⁻ and *E. coli* F-18 Col⁻ *fimA*.

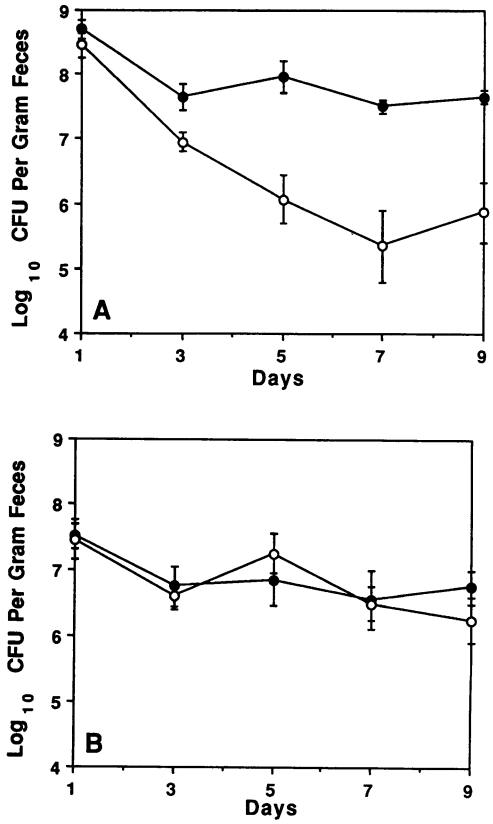

FIG. 2. Colonization of *E. coli* F-18 Col⁻(pRLB7) in mice. (A) (●) *E. coli* F-18 Col⁻(pRLB7); (○) *E. coli* F-18 Col⁻ *fimA*(pRLB2). (B) (●) *E. coli* F-18 Col⁻(pRLB18); (○) *E. coli* F-18 Col⁻ *fimA*(pRLB2).

Colicin test. *E. coli* F-18 Col⁻, *E. coli* F-18 Col⁻(pRLB2), *E. coli* F-18 Col⁻(pRLB7), *E. coli* F-18 Col⁻(pRLB18), and *E. coli* F-18 were tested for colicin production (see Materials and Methods). Only *E. coli* F-18 produced the colicin. Moreover, *E. coli* F-18 Col⁻(pRLB7) and *E. coli* F-18 were fed separately to three mice and were allowed to colonize for 30 days, and then colonies from each mouse were tested for colicin production. *E. coli* F-18 Col⁻(pRLB7) was colicin negative, whereas the zones of inhibition around the *E. coli* F-18 colonies were far larger than those produced by *E. coli* F-18 colonies isolated from frozen stocks. These results suggest that pRLB7 does not enhance *E. coli* F-18 Col⁻ colonizing ability by inducing the synthesis of the *E. coli* F-18 colicin, colicin V.

Restriction mapping of pRLB7. The *E. coli* F-18 DNA in pRLB7 was mapped to an approximately 6.5-kb *Pst*I-*Eco*RI fragment (Fig. 1). The actual border on the *Pst*I side of the *E. coli* F-18 insertion could not be determined because residual

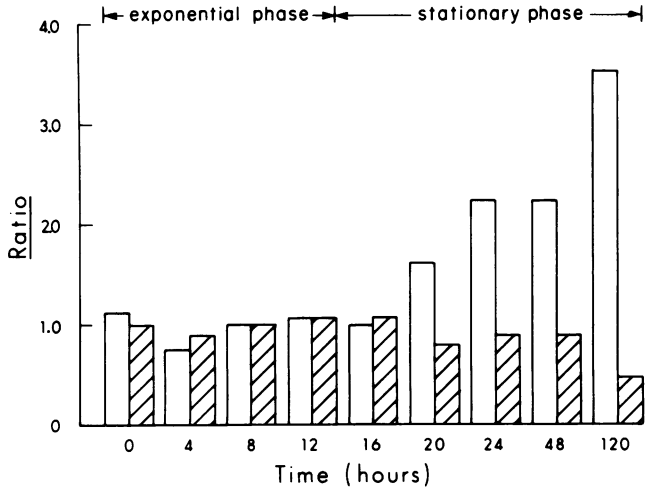

FIG. 3. Growth and survival of *E. coli* F-18 Col⁻(pRLB7) relative to those of *E. coli* F-18 Col⁻ *fimA*(pRLB2) in mouse cecal mucus dialysates. □, ratio of *E. coli* F-18 Col⁻(pRLB7) CFU to *E. coli* F-18 Col⁻ *fimA*(pRLB2) CFU. The total viable counts at each of the times listed beginning with time zero were as follows: 1.1×10^5, 4.5×10^5, 4.2×10^7, 4.6×10^8, 1.1×10^8, 9.7×10^7, 2.4×10^8, 1.8×10^8, and 5.3×10^7 CFU/ml. ▨, ratio of *E. coli* F-18 Col⁻(pRLB2) CFU to *E. coli* F-18 Col⁻ *fimA*(pRLB2) CFU. The total viable counts at each of the times listed beginning with time zero were as follows: 9.5×10^4, 4.0×10^5, 2.3×10^7, 1.6×10^8, 1.1×10^8, 1.0×10^8, 1.3×10^8, 8.8×10^7, and 2.3×10^7 CFU/ml.

amounts of an extremely active *E. coli* F-18 nuclease present during construction of the gene library (unpublished observations) deleted the pRLB2 plasmid sequence in pRLB7 from the *Eco*RI site to somewhere near the first *Bgl*II site, a loss of approximately 2.0 kb. The nuclease-affected end of the plasmid apparently blunt-end ligated with the *E. coli* F-18 DNA approximately 1 kb upstream of the *Pst*I site to form pRLB7 (Fig. 1). Sequence analysis of this region will be necessary to locate the exact position defining the plasmid-insert border.

Effect of pRLB7 on *E. coli* F-18 Col⁻ growth and survival in vitro. *E. coli* F-18 grows as well in a mouse cecal mucus dialysate as in crude mouse cecal mucus (10). *E. coli* F-18 Col⁻(pRLB7) and *E. coli* F-18 Col⁻ *fimA*(pRLB2) were inoculated simultaneously into the cecal mucus dialysate, each at a level of approximately 10^4 CFU/ml. The strains grew equally well during the exponential phase of growth from 10^4 to about 10^8 CFU/ml, with doubling times of about 36 min. However, significant differences were noted during stationary phase. That is, in a typical experiment illustrated in Fig. 3, the ratio of *E. coli* F-18 Col⁻(pRLB7) to *E. coli* F-18 Col⁻ *fimA*(pRLB2) changed from 1.0 during the exponential phase of growth to 3.6 during stationary phase; i.e., pRLB7 appeared to give *E. coli* F-18 Col⁻ survival advantage over *E. coli* F-18 Col⁻ *fimA*(pRLB2) in stationary phase. In support of this view, pRLB7 also afforded *E. coli* F-18 Col⁻ *fimA* the same survival advantage in stationary phase when grown together with *E. coli* F-18 Col⁻(pRLB2) in cecal mucus dialysates (data not shown). Moreover, the survival advantage in stationary phase was abolished when *E. coli* F-18 Col⁻(pRLB18), the *Xho*I-*Sal*I deletion derivative of pRLB7, was grown together with *E. coli* F-18 Col⁻ *fimA*(pRLB2) in cecal mucus dialysates (data not shown).

pRLB7-directed stimulation of synthesis of type 1 fimbriae. *E. coli* F-18 makes type 1 fimbriae in L-broth in vitro (19), and *E. coli* F-18 type 1 synthesis appears to be stimulated during growth in mouse cecal mucus in vivo (19). pRLB7 was therefore tested for its ability to stimulate type 1 fimbrial synthesis in *E. coli* F-18 Col⁻. *E. coli* F-18 Col⁻(pRLB7), *E. coli* F-18 Col⁻(pRLB18), *E. coli* F-18 Col⁻(pRLB2), *E. coli* F-18 Col⁻, and *E. coli* F-18 *fimA* were grown in cecal mucus in vitro and tested for abilities to bind specifically to a mannose-BSA glycoconjugate (see Materials and Methods). pRLB7 stimulated adhesion to mannose-BSA greater than 10-fold relative to pRLB2 (Table 2). The *Xho*I-*Sal*I deletion, pRLB18, which does not enhance *E. coli* F-18 Col⁻ colonizing ability (Fig. 1B), did not stimulate adhesion to mannose-BSA (Table 2).

TABLE 2. Mannose-specific adhesion of *E. coli* F-18 Col⁻ strains

E. coli strain	Adhesion (%, mean ± SE)[a] to:		Mannose-specific adhesion (%)[b]
	Mannose-BSA	Mannose-BSA + αMM[c]	
F-18 Col⁻(pRLB2)	2.04 ± 0.09	0.80 ± 0.04	1.24
F-18 Col⁻(pRLB7)	18.30 ± 0.89	2.22 ± 0.14	16.08
F-18 Col⁻(pRLB18)	1.97 ± 0.05	1.37 ± 0.13	0.60
F-18 Col⁻	2.37 ± 0.05	1.5 ± 0.18	0.87
F-18 Col⁻ *fimA*	0.66 ± 0.04	0.73 ± 0.02	0.00

[a] All adhesion assays were performed in triplicate.
[b] Calculated as percent adhesion to mannose-BSA minus percent adhesion to mannose-BSA in the presence of 100 mM α-methyl-D-mannoside.
[c] α-Methyl-D-mannoside (αMM; 100 mM) was added with the labeled bacteria.

When the *E. coli* F-18 Col⁻ strains containing the recombinant plasmids were grown in Minimal Broth Davis containing 1% (wt/vol) D-glucose and tested for their abilities to bind to mannose-BSA, results similar to those depicted in Table 2 were obtained (not shown). Therefore, type 1 fimbriae were purified from these cultures and quantified. *E. coli* F-18 Col⁻(pRLB7) produced three to four times as much type 1 fimbrial protein as *E. coli* F-18 Col⁻, *E. coli* F-18 Col⁻(pRLB2), and *E. coli* F-18 Col⁻(pRLB18) (Table 3), further supporting the idea that the *E. coli* F-18 DNA in pRLB7 encodes a gene(s) which stimulates the synthesis of type 1 fimbriae.

pRLB7 does not contain the *fim* region or *pilG* (*hns*). The *fim* (*pil*) operon contains both regulatory and structural genes for type 1 fimbrial synthesis (17). *pilG* (*hns*), which maps outside the *fim* operon, is thought to regulate the frequency of switching of the *fimA* invertible promoter (29). As shown by Southern blot analysis, neither *fim* nor *pilG* sequences are contained in pRLB7 (Fig. 4).

***E. coli* F-18 Col⁻ contains DNA homologous to the *E. coli* F-18 DNA fragment in pRLB7.** DNAs isolated from *E. coli*

TABLE 3. Type 1 fimbrial protein on *E. coli* F-18 Col⁻ strains

E. coli strain	Type 1 fimbrial protein (μg/CFU [10^{10}])[a]	
	Expt 1	Expt 2
F-18 Col⁻	0.52	0.47
F-18 Col⁻(pRLB2)	0.49	0.55
F-18 Col⁻(pRLB7)	2.21	1.63
F-18 Col⁻(pRLB18)	0.56	0.38

[a] Values given have been corrected for the protein present in *E. coli* F-18 Col⁻ *fimA* preparations (i.e., 0.29×10^{10} μg/CFU in experiment 1 and 0.35×10^{10} μg/CFU in experiment 2).

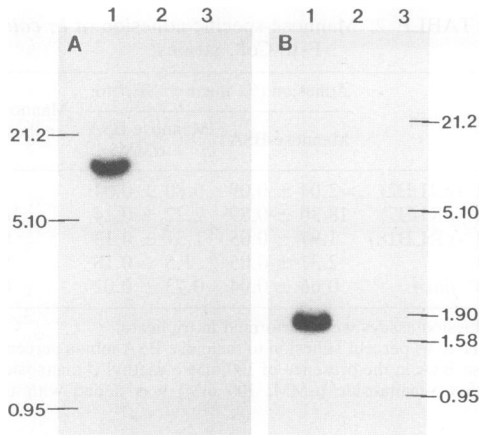

FIG. 4. Detection of the *fim* operon and *pilG* by Southern hybridization. (A) Plasmid DNA digested with *Cla*I and probed with ^{32}P-labeled *fim* operon DNA (i.e., the 8.7-kb *Cla*I fragment of pPKL4 [16]): lane 1, pPKL4; lane 2, pRLB2; lane 3, pRLB7. (B) Plasmid DNA digested with *Eco*RI and *Sal*I and probed with ^{32}P-labeled *pilG* (i.e., the 1.8-kb *Eco*RI-*Sal*I fragment of pTHK113 [13]): lane 1, pTHK113; lane 2, pRLB2; lane 3, pRLB7.

F-18 and *E. coli* F-18 Col$^-$ were restricted with *Pst*I and *Sal*I and probed with the ^{32}P-labeled *Pst*I-*Sal*I fragment of pRLB7. Both *E. coli* F-18 and *E. coli* F-18 Col$^-$ contain the same-size *Pst*I-*Sal*I fragment (Fig. 5). Therefore, although *E. coli* F-18 Col$^-$ may be defective in the *Pst*I-*Sal*I region, it contains the region.

pRLB7-directed synthesis of outer membrane proteins. pRLB7 appeared to increase the synthesis of type 1 fimbriae, enhance survival of *E. coli* F-18 Col$^-$ in stationary phase, and enhance its colonizing ability in the streptomycin-treated mouse large intestine. To determine whether pRLB7 might also direct the synthesis of unique outer surface proteins, *E. coli* F-18 Col$^-$(pRLB2), *E. coli* F-18 Col$^-$(pRLB7), and *E. coli* F-18 Col$^-$(pRLB18) were grown to

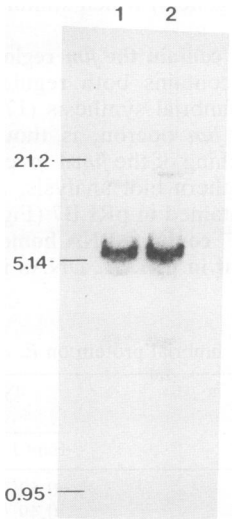

FIG. 5. Detection of the *Pst*I-*Sal*I fragment of pRLB7 in *E. coli* F-18 Col$^-$ by Southern hybridization. Total DNA was digested with *Pst*I and *Sal*I and probed with the 5.4-kb ^{32}P-labeled *Pst*I-*Sal*I fragment of pRLB7. Lane 1, *E. coli* F-18 DNA; lane 2, *E. coli* F-18 Col$^-$ DNA.

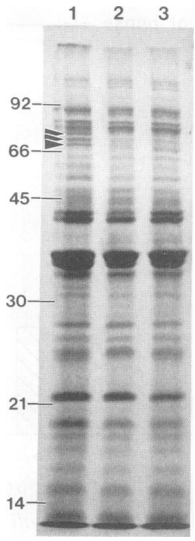

FIG. 6. Outer membrane protein profile. Outer membrane protein preparations (50,000 cpm) were applied to each lane; the membrane proteins were separated by SDS-PAGE and detected by autoradiography. Lane 1, *E. coli* F-18 Col$^-$(pRLB7); lane 2, *E. coli* F-18 Col$^-$(pRLB18); lane 3, F-18 Col$^-$(pRLB2). The arrowheads, from top to bottom, point to the 74-, 71-, and 69-kDa proteins, respectively.

stationary phase in mouse cecal mucus in the presence of ^{35}S-methionine and ^{35}S-cysteine (see Materials and Methods), and the outer membrane and periplasmic fractions of each strain were isolated, subjected to SDS-PAGE, and autoradiographed. No clear differences were found in the periplasmic fraction (not shown), but synthesis of three outer membrane proteins (74, 71, and 69 kDa) clearly appeared to be stimulated by pRLB7 (Fig. 6). Moreover, pRLB18, the *Xho*I-*Sal*I deletion derivative, did not stimulate synthesis of the three *E. coli* F-18 Col$^-$ outer membrane proteins (Fig. 6). Therefore, the enhancement of synthesis of the three outer membrane proteins was due at least in part to the *Xho*I-*Sal*I *E. coli* F-18 sequence in pRLB7.

Proteins encoded by the *Pst*I-*Sal*I fragment of pRLB7. The *Pst*I-*Sal*I fragment of pRLB7 was cloned into pGEM7Zf(+) in both directions immediately upstream of the bacteriophage T7 promoter, and the recombinant plasmids were transformed into *E. coli* K38(pGP1-2) and tested for the proteins encoded by the fragment (see Materials and Methods). When transcription occurred in the *Pst*I-*Sal*I direction, three proteins were made (26, 22, and 20 kDa) (Fig. 7). When transcription occurred in the *Sal*I-*Pst*I direction, three different proteins were made (32.5, 28.5, and 27 kDa) (Fig. 6). The *Pst*I-*Sal*I fragment of pRLB7 contains approximately 5,400 bp, and the six proteins require only about 4,300 bp. Since there may be as much as 1 kb of *E. coli* F-18 DNA upstream of the *Pst*I site and about 1 kb downstream of the *Sal*I site in pRLB7, it is possible that the two largest proteins encoded by the *E. coli* F-18 sequence in pRLB7 could be 65.5 kDa (i.e., 32.5 + 33.0 kDa) transcribed in the *Sal*I-*Pst*I direction and 59 kDa (i.e., 26 + 33.0 kDa) transcribed in the *Pst*I-*Sal*I direction. Even if this were the case, the three outer membrane proteins whose synthesis is stimulated by pRLB7 are all larger than the largest proteins that could be encoded by pRLB7, suggesting that the proteins encoded by pRLB7 are not the outer membrane proteins whose synthesis is stimulated by pRLB7 (Fig. 7).

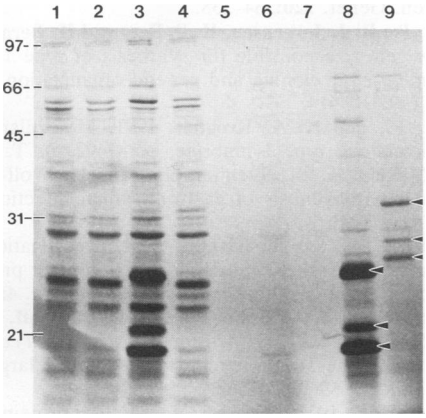

FIG. 7. Detection of proteins encoded by the *Pst*I-*Sal*I fragment of pRLB7 in *E. coli* K38. Lanes 1 to 4, uninduced T7 RNA polymerase, cells labeled for 20 min; lanes 5 to 9, induced T7 RNA polymerase, cells labeled for 10 min. *E. coli* K38 contained the following: lane 1, pGP1-2; lane 2, pGP1-2 plus pGEM-7Zf(+); lane 3, pGP1-2 plus pLPA213; lane 4, pGP1-2 plus pLPA214; lane 5, no plasmid; lane 6, pGP1-2; lane 7, pGP1-2 plus pGEM-7Zf(+); lane 8, pGP1-2 plus pLPA213; lane 9, pGP1-2 plus pLPA214. Arrowheads on the right, from top to bottom, point to the 32.5-, 28.5-, 27-, 26-, 22-, and 20-kDa proteins, respectively.

DISCUSSION

We have described the selection and partial characterization of an *E. coli* F-18 DNA sequence that enhances the mouse large intestine colonizing ability of *E. coli* F-18 Col⁻, a poor colonizing derivative of *E. coli* F-18 (Fig. 2). This sequence simultaneously enhances the ability of *E. coli* F-18 Col⁻ to survive in stationary phase, utilizing nutrients derived from mouse cecal mucus (Fig. 3), and stimulates synthesis of type 1 fimbriae (Table 2). Also, the sequence appears to stimulate the synthesis of three outer membrane proteins in *E. coli* F-18 Col⁻ (Fig. 6).

How might survival in stationary phase be a factor in *E. coli* F-18 Col⁻ mouse large intestine colonizing ability? As suggested by Freter (11), it is possible that maintenance of a microorganism in the mouse large intestine depends on its ability to sequester small amounts of one or more nutrients which that microorganism utilizes exceptionally well. If so, as those nutrients become limiting, growth would slow considerably, i.e., a situation in the intestine perhaps akin to stationary phase. Of two nearly isogenic strains, the one that can utilize the preferred nutrients better under limiting conditions would become predominant. Alternatively, it is possible that expression of the *E. coli* F-18 DNA sequence in *E. coli* F-18 Col⁻ improves its ability to survive in the presence of its own metabolic products, which again would enhance its large intestine colonizing ability. However, in this context, it should be noted that *E. coli* F-18 Col⁻(pRLB7) does not make colicin V, the colicin made by *E. coli* F-18 in vitro. It would therefore appear that pRLB7 does not enhance *E. coli* F-18 Col⁻ colonizing ability or survival in stationary phase by inducing colicin V synthesis and then killing *E. coli* F-18 Col⁻(pRLB2).

The expression of type 1 fimbriae is subject to phase variation, i.e., oscillation between the fimbriate and afimbriate states, due to the stochastic inversion of a 314-bp DNA segment which contains the *fimA* promoter (1, 14). The products of two *fim* genes, *fimB* and *fimE* (*hyp*), influence the frequency of inversion (14, 24, 27). The gene product of *fimB* appears to mediate inversion in both directions, with a moderate preference for the "on" orientation (24), such that in the presence of a large excess of *fimB* product all cells express type 1 fimbriae (14). The *fimE* product has recently been shown to promote inversion from "on" to "off" (24). While we have shown here that the sequence we have isolated has no homology with the *fim* region (Fig. 4), it is still possible that it stimulates inversion to the "on" orientation by either increasing the rate of synthesis of the *fimB* protein or decreasing the rate of synthesis of the *fimE* protein.

Three other genes are known to play a role in the frequency of inversion. One of these genes is *pilG* (*hns*) (29). The sequence we describe here is not *pilG* (Fig. 4), but it is still possible that it regulates *pilG* expression. The other two genes are *himA* and *himD* (*hip*), which encode the α and β subunits of integration host factor, respectively, which is required for efficient recombination of the *fim* invertible element (6, 8). While we have not unequivocally ruled out that our sequence contains a *himA* or *himD* gene, it is clear from transmission electron microscopy studies that all modified Davis minimal medium (32)-grown *E. coli* F-18 Col⁻ cells which contain an extra *fimB* gene in *trans* express type 1 fimbriae compared with expression by only 0.5% of cells in its absence (unpublished observations). Therefore, it is highly likely that *E. coli* F-18 Col⁻ contains functional *himA* and *himD* (*hip*) genes and that the sequence we have isolated contains a previously unidentified regulator of type 1 fimbrial synthesis.

The sequence we describe here appears to regulate the synthesis of not only type 1 fimbriae, but also three outer membrane proteins (Fig. 6). The evidence for this is twofold. First, the three outer membrane proteins that are affected by the sequence appear to be made in its absence in *E. coli* F-18 Col⁻, but at reduced levels (Fig. 6). Second, the six proteins encoded by the sequence are all smaller than the three outer membrane proteins and are therefore likely to be playing a regulatory rather than a structural role. The three outer membrane proteins do not appear to be *fim*-encoded surface proteins which are either too small, i.e., FimA, FimF, FimG, and FimH (18), or too large, i.e., FimD (15). Whether one or more of the three outer membrane proteins play an important role in the colonization process is presently under investigation.

At the present time, we do not know whether the genes in the sequence that regulate synthesis of type 1 fimbriae are the same genes that enhance *E. coli* F-18 Col⁻ large intestine colonizing ability. It is possible, for example, that colonizing ability is enhanced by expression of the genes in the sequence that are transcribed in one direction and that type 1 fimbrial synthesis is regulated by the genes in the sequence that are transcribed in the opposite direction (Fig. 7). Alternatively, it is possible that the sequence we have isolated contains a gene or genes that coordinately regulate both type 1 fimbrial synthesis and enhanced colonizing ability.

In summary, we have used the streptomycin-treated mouse large intestine to select an *E. coli* F-18 DNA sequence which enhances the colonizing ability of *E. coli* F-18 Col⁻. The enhancement in colonizing ability is accompanied by an increase in the ability of *E. coli* F-18 Col⁻ to survive stationary phase in vitro, utilizing cecal mucus nutrients. Simultaneously, the sequence stimulates the synthesis of both type 1 fimbriae and three *E. coli* F-18 Col⁻ outer membrane proteins.

ACKNOWLEDGMENTS

We gratefully acknowledge T. H. Kawula for pTHK113 and P. Klemm for pPKL4. We also thank Carolyn Lunt for help in preparing the manuscript and Frederick M. Croy for excellent technical assistance.

This research was supported by Public Health Service grant AI16370 to P.S.C. and D.C.L. from the National Institute of Allergy and Infectious Diseases.

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