JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 738–744 Vol. 177, No. 3
0021-9193/95/$04.00+0


# MyfF, an Element of the Network Regulating the Synthesis of Fibrillae in *Yersinia enterocolitica*

MAITE IRIARTE AND GUY R. CORNELIS*

*Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology and Faculté de Médecine, Université Catholique de Louvain, B-1200 Brussels, Belgium*

Received 2 August 1994/Accepted 21 November 1994

**The *Yersinia enterocolitica* surface antigen Myf is a fibrillar structure that resembles CS3 fimbriae. Gene *myfA* encodes the 21-kDa major subunit of the antigen, while genes *myfB* and *myfC* are required for the transport and assembly of pilin subunits at the bacterial cell surface. Here we show that the expression of Myf is regulated at the transcriptional level by temperature and pH. Gene *myfA* is transcribed only at 37°C and in acidic medium. The transcription start is preceded by a putative −10 box for the vegetative RNA polymerase as well as by sequences resembling the consensus sequence recognized by $\sigma^{28}$. Thus, *myfA* could be transcribed either from a classical $\sigma^{70}$ promoter or from a $\sigma^{28}$ promoter. Transcription of *myfA* requires at least two genes, *myfF* and *myfE*, situated immediately upstream from *myfA*. The *myfF* product does not show similarity to any known regulatory protein. It is an 18.5-kDa protein with no typical helix-turn-helix motif and a unique hydrophobic domain in the $NH_2$-terminal part. T7 expression, osmotic shock, fractionation experiments, and Tn*phoA* fusion analyses carried out in *Escherichia coli* suggest that MyfF is associated with the inner membrane by means of its hydrophobic domain whereas the hydrophilic part protrudes in the periplasm. These features strikingly evoke ToxS, a protein involved in regulation of Tcp pilus production in *Vibrio cholerae*. MyfE resembles PsaE, a protein involved in regulation of pH6 antigen in *Yersinia pestis*. Genes *myfF* and *myfE* are presumably part of a whole regulatory network. MyfF could be an element of the signal transducing system.**

*Yersinia enterocolitica* is an enteric human pathogen associated with a wide spectrum of clinical and immunological manifestations. The symptoms more currently associated with *Y. enterocolitica* infection in children under 5 years are diarrhea and abdominal pain (13). A mesenteric adenitis characterized by fever, abdominal pain, and leukocytosis is common in older children and adolescents (61). The pathogenicity depends on the presence of the pYV plasmid, which is responsible for resistance to nonspecific host defenses (for reviews, see references 10 and 12). In addition, *Y. enterocolitica* secretes a chromosome-encoded heat-stable enterotoxin called Yst (16, 47). The C-terminal domain of Yst displays a significant identity to the heat-stable toxin STI of *Escherichia coli* (57) and to guanylin, an intestinal paracrine peptide (14). At least in the young rabbit infection model, Yst is responsible for diarrhea (15). Other enterotoxinogenic bacteria such as enterotoxinogenic *E. coli* and *Vibrio cholerae* need to adhere to the intestinal mucosa to induce diarrhea, and this attachment is mediated by specific appendages called fimbriae (26, 33, 55). Likewise, *Y. enterocolitica* produces a fibrillar structure called Myf which closely resembles CS3 fimbriae of enterotoxinogenic *E. coli* (30, 36). Transport and assembly of the 21-kDa MyfA subunits require MyfB, a putative chaperone of the PapD family, and MyfC, a membrane usher protein related to PapC (30; for a review, see reference 24). The Myf fibrillae are the homologs of pH6 antigen of *Yersinia pestis*: MyfA is 44% identical to PsaA, the major subunit of pH6 antigen (4, 37, 38). Like pH6 antigen, Myf appears only when bacteria are incubated at 37°C in acidic conditions (30).

In the present work, we analyze the transcriptional organization of the *myfABC* genes and the conditions of expression of the Myf antigen. We show that transcription of *myfA* is dependent on temperature, pH, and the expression of at least two new additional genes called *myfE* and *myfF*. We describe *myfF*, which encodes a 18-kDa protein presumably involved in transduction of the signal triggering transcription of *myfABC*.

* Corresponding author. Mailing address: Microbial Pathogenesis Unit, Avenue Hippocrate 74, UCL 74.49, B-1200 Brussels, Belgium. Phone: 32 2 764 74 49. Fax: 32 2 764 74 98. Electronic mail address: cornelis@mipa.ucl.ac.be.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *Y. enterocolitica* W1024 is a serotype O:9 strain. W1024-41 is a *myfB*::Tn*5*-Tc1 mutant (30). Strains W1024-33 and W1024-42 are a *myfE*::Tn*5*-Tc1 mutant and a *myfF*::Tn*5*-Tc1 mutant, respectively (as determined in this study).

*E. coli* LK111(F′ *lacZ*ΔM15), received from M. Zabeau (Ghent, Belgium), and *E. coli* XL1-blue (Stratagene, La Jolla, Calif.) were used for standard genetic manipulations. *E. coli* S17-1 (44) was used to deliver the mobilizable plasmids into *Y. enterocolitica*. *E. coli* CC102F′ (carrying the Tn*phoA* transposon in the chromosome) and KS272 (Δ*phoA*) (received from C. Parsot, Institut Pasteur, Paris, France) were used for Tn*phoA* mutagenesis and alkaline phosphatase assays, respectively, as described by Allaoui et al. (1). Plasmids are listed in Table 1.

Bacteria were routinely grown on Trypticase soy broth (Oxoid, Hampshire, England) containing 0.3% (wt/vol) yeast extract and on Trypticase soy agar (TSA) (Diagnostic Pasteur, Marnes la Coquette, France). To produce the Myf antigen, TSA was supplemented with 1% (wt/vol) glucose (TSA-glucose). $pYV^-$ variants were selected on TSA supplemented with 0.02 M sodium oxalate and 0.02 M $MgCl_2$. For the analysis of the effect of pH on Myf production, *Y. enterocolitica* was grown on brain heart infusion (Difco, Detroit, Mich.) supplemented with 0.5% yeast extract (Gibco, BRL, Paisley, England), 2.5 mM $CaCl_2$, and 0.2% xylose and adjusted to different pHs with HCl or NaOH as described by Lindler et al. (37) (SBHI). For the labelling of MyfF, recombinant *E. coli* was grown in M9 medium ($MgSO_4$ [1 mM], $CaCl_2$ [100 μM], NaCl [86 mM], $Na_2HPO_4$ [420 mM], $KH_2PO_4$ [220 mM], $NH_4Cl$ [190 mM], glucose (4 $mg \cdot ml^{-1}$). The antibiotics used for selection were nalidixic acid (35 $\mu g \cdot ml^{-1}$), tetracycline (10 $\mu g \cdot ml^{-1}$), ampicillin (400 $\mu g \cdot ml^{-1}$), and kanamycin (50 $\mu g \cdot ml^{-1}$).

**Mutagenesis with Tn*5*-Tc1 and analysis of the Myf antigen.** Mutagenesis was performed as described previously (30).

**DNA, RNA, and primer extension analysis.** DNA preparation, restriction enzyme digestions, ligation, labelling, and hybridization were performed by using standard methods.

RNA extractions and analysis were as described by Lambert de Rouvroit et al. (35). The probe used to detect the *myfA* transcript was prepared as described previously (30). Primer extension analysis of *myfA* was performed as described by

TABLE 1. Plasmids used in this study

| Plasmid | Characteristic | Reference |
|---|---|---|
| pIM98 | pTZ18R + 1.8-kb *Pst*I (right) junctional fragment of W1024-33::Tn*5*-Tc1; *myfF* is downstream from the T7 and *lac* promoters | This work |
| pIM106 | pTZ18R + 1.8-kb *Pst*I junctional fragment (right) of W1024-33::Tn*5*-Tc1; *myfF* is oriented opposite to the T7 and *lac* promoters | This work |
| pIM68 | pTZ18R + 1.6-kb *Pst*I (right) junctional fragment of W1024-42::Tn*5*-Tc1 | 30 |
| pTM100 | pACYC184 + ori$T_{RK2}$ cloned in the *Sst*II site (coordinate 832) | 41 |
| pIM122 | 1.8-kb *Hin*dIII-*Xba*I fragment of pIM106 cloned into the corresponding sites of pTM100; contains coordinates 186–1143 of *myf* system | This work |

Michiels and Cornelis (40). The primers used were MIPA 86 (CATCCCGC CACTAGCCAA), complementary to nucleotides 66 to 49 of *myfA*, and MIPA 131a (CGGTTCTGCATGTACCATA), complementary to nucleotides 90 to 72 of *myfA*.

**Sequence analysis.** DNA and protein sequences were analyzed with the FASTA program (48). The isoelectric point was calculated by using the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison) computer program. The mean alpha-helical hydrophobic moment and the mean hydrophobicity were calculated for windows of 11 amino acids and plotted against each other as described by Eisenberg et al. (19). The mean hydrophobicity for windows of seven amino acids was plotted by using the Kyte and Doolittle scale (34). The secondary structure of MyfF was calculated by using the Alb program (20, 50).

**Protein expression and topology analysis.** The *myfF* gene product was identified with the bacteriophage T7 promoter/polymerase system of Tabor and Richardson (56), using *E. coli* LK111 containing pGP1-2 and either pTZ18R or pIM98. The periplasmic proteins were isolated by a modification of the method of Yim and Villarejo (62). After expression of *myfF* by using the T7 system, cells were pelleted at 4,000 × *g* for 5 min, resuspended in ice-cold solution containing 33 mM Tris-HCl, 0.5 M sucrose, and 1 mM EDTA (pH 7.6), and put in ice for 10 min. The cells were spun down again, osmotically shocked by resuspension in ice-cold 0.5 mM $MgCl_2$, and incubated for 10 min on ice. The cells were pelleted, and the periplasmic proteins in the supernatant were precipitated with acetone at −80°C. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 30 min at 4°C and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Mutagenesis of *myfF* by Tn*phoA* was done by using plasmid pIM98 as described by Allaoui et al. (1). The junctions between Tn*phoA* and *myfF* were sequenced also as described by Allaoui et al. (1). Alkaline phosphatase activity was analyzed by using *p*-nitrophenylphosphate as described by Manoil and Beckwith (39).

**Nucleotide sequence accession number.** The sequence of *myfF* and part of *myfE* has been submitted to the GenBank Nucleotide Sequence Data Libraries under accession number U12766.

## RESULTS

**Expression of the Myf antigen is regulated at the transcriptional level.** To determine the role of temperature and pH on transcription of *myf*, we extracted total RNA from *Y. enterocolitica* W1024 grown at 22 and 37°C at various pHs, and we carried out a Northern (RNA) blot analysis using a *myfA* probe consisting of the central 280 bp of the gene. This probe revealed a very abundant transcript with an estimated molecular size of 650 nucleotides. This size corresponds to that of the *myfA* gene, suggesting that *myfA* constitutes a transcriptional unit independent from the downstream *myfB* and *myfC* genes as well as from the upstream region. The *myfA* transcript was detected only in bacteria grown at 37°C and at acidic pH (Fig. 1). This could result either from increased transcription or from increased transcript stability in those conditions. The most likely hypothesis is that expression of *myfA* is regulated at the transcriptional level by temperature and pH. Since the intestine is a largely anaerobic medium, we also analyzed by SDS-PAGE and immunoblotting, the expression of Myf in bacteria grown in anaerobic conditions. Myf was expressed in anaerobiosis as well as in aerobiosis but also at 37°C and at acid pH (data not shown).

**Mapping of the *myfA* promoter.** To identify the promoter of *myfA*, we mapped the 5′ end of the *myfA* transcript by primer extension on total RNA extracted from *Y. enterocolitica* W1024 grown at low pH and at 37 or 22°C. We used two different oligonucleotide primers complementary to mRNA sequences within the coding region of *myfA*. The extension products obtained with both primers ended at the GC residues at positions 98 and 99 bp upstream from the *myfA* initiation codon (Fig. 2). Two longer extension products were also observed (not present in Fig. 2). Since their origin differed depending on the primer used, these two additional extension products were considered nonspecific and were not taken into account. No primer extension products were detected in reaction mixtures containing mRNA extracted from W1024 grown at 22°C.

We identified 7 bp upstream from the transcription start a putative -10 box for the vegetative RNA polymerase, but no sequence similar to the -35 box could be identified upstream from this -10 box. Upstream from the transcription start, we also identified a TAAA($N_{15}$)GCCAGTAT sequence that matches six of eight nucleotides of the -10 box and four of four nucleotides of the -35 box from $\sigma^{28}$ promoters (25). The distance between the putative -10 and -35 boxes (15 bp) and the separation between the -10 box and the transcription start point (8 bp) fit the consensus sequence for $\sigma^{28}$ promoters (TAAA[$N_{15}$] GCCGATAT) (25). Thus, *myfA* could be transcribed either from a classical $\sigma^{70}$ promoter or from a $\sigma^{28}$ promoter.

The intercistronic DNA region upstream from the promoter of *myfA* contains a TTTATTAAATAT sequence which closely resembles the possible consensus for the leucine-responsive regulatory protein-binding sites, TTTATTCtNaAT (51). The leucine-responsive regulatory protein (Lrp), which is known to regulate genes involved in nitrogen assimilation, amino acid biosynthesis and degradation, and oligopeptide transport (46), has been also shown to regulate the expression of Pap (8, 9),

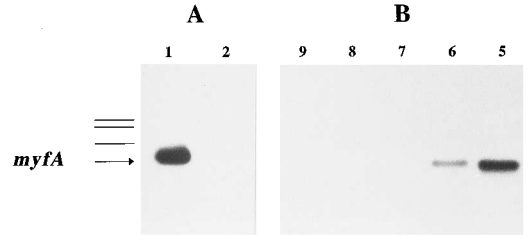


FIG. 1. (A) Influence of temperature on transcription of *myfA*. Total RNA was extracted from bacteria grown on SBHI (pH 6) at 37°C (lane 1) and 22°C (lane 2). (B) Influence of pH on *myfA* transcription. Total RNA was extracted from bacteria grown at 37°C on SBHI adjusted to various pHs, as indicated above the lanes. Transcripts were detected with a PCR probe corresponding to the central part of *myfA* amplified with primers MIPA 114 and MIPA 112. The bars on the left indicate the positions of the three *Y. enterocolitica* rRNA bands (1,750, 1,550, and 1,150 nucleotides) (54). In each experiment, the same amount of RNA was loaded in each lane. Note, however, that panels A and B represent different experiments.

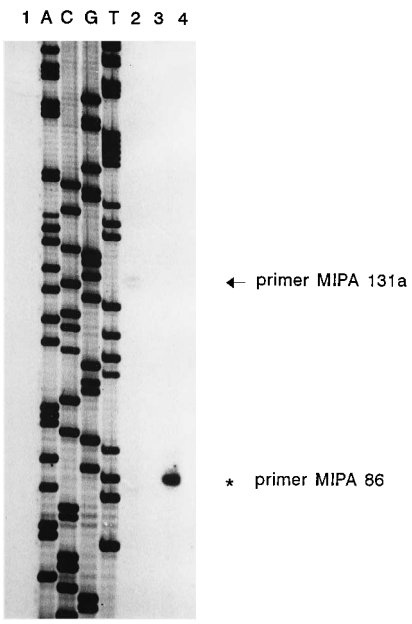


FIG. 2. Determination of the 5′ end of *myfA* transcript by primer extension analysis. The primers used were MIPA 131a (lane 2) and MIPA 86 (lane 4). The sequence corresponding to part of the *myfA* gene and upstream region was extended with primer MIPA 131a. The extension product obtained with MIPA 131a is shown by an arrow at the right. The extension product obtained with primer MIPA 86 is shown by an asterisk. The negative controls were extension analysis with primer MIPA 131a and RNA extracted from wild-type cells grown at 22°C (lane 1) and extension analysis with primer MIPA 131a and RNA extracted from mutant W1024-42 grown at 37°C (lane 3).

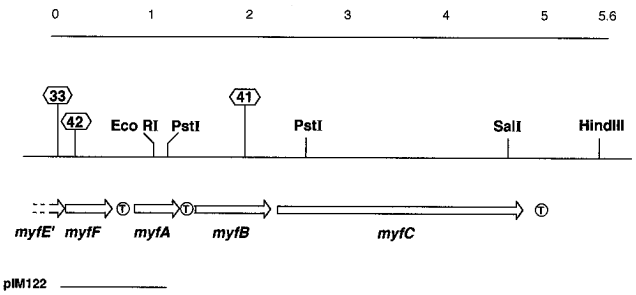


FIG. 3. Schematic representation of the chromosomal locus containing some of the genes involved in the synthesis of the Myf antigen. ⬭, insertion point of Tn*5*-Tc1. Numbers identify the different mutants. The arrows indicate the direction of transcription of the different genes. T, transcription terminator.

K99 (9), F1845 (5, 60), type 1 (6), K88 (28), and S (60) fimbriae of *E. coli.*

Finally, we could not identify in the regulatory region any sequences resembling the *E. coli* consensus binding sites for integration host factor or H-NS, two global regulators often involved in the regulation of fimbrial genes.

**Isolation of genes involved in the regulation of transcription of *myfA*.** Regulation of *myf* transcription by pH presumably involves specific regulators and signal transduction. *Y. enterocolitica* W1024 was mutagenized with transposon Tn*5*-Tc1 in order to identify genes involved in this regulatory pathway. Myf-defective mutants affected in the previously characterized *myfA*, *myfB*, or *myfC* gene were discarded. Two Myf-negative mutants, W1024-42 and W1024-33, carried the transposon inserted in a region situated immediately upstream from *myfA*. We took advantage of the presence of a *Pst*I site in Tn*5*-Tc1 and in *myfA* to clone the mutated gene(s). We digested chromosomal DNA of W1024-42 and W1024-33 with *Pst*I, and we cloned the fragments in the range of 2 kb into pTZ18R. The clones were identified by hybridization with Tn*5*-Tc1 DNA. Plasmid pIM68 contained the fragment from W1024-42 cloned in one orientation, and plasmids pIM98 and pIM106 contained the fragment from W1024-33 cloned in both orientations. Using these two plasmids, we sequenced a continuous 700-bp region downstream from the insertion point of the transposon in mutant W1024-33. The sequence revealed two open reading frames (ORFs) with the same transcription polarity as *myfA*, *myfB*, and *myfC*. In mutant W1024-42, the insertion occurred in ORF1, 648 bp upstream from the ATG start codon of *myfA*, while in mutant W1024-33, the insertion occurred in ORF2, 834 bp upstream from *myfA* (Fig. 3).

**Sequence analysis of *myfF* and *myfE*.** The first ORF, immediately upstream of *myfA*, was called *myfF* (Fig. 4). This ORF, separated from *myfA* by a 48-bp palindromic sequence, encodes a putative protein of 163 amino acids with a predicted molecular mass of 18.46 kDa and a calculated pI of 8.6. The predicted amino acid sequence suggests the presence of a signal peptide. This N-terminal sequence represents the only hydrophobic domain of the protein. Comparison of the predicted amino acid sequence of MyfF with the PIR and GenBank sequence databases did not reveal sequence similarity with any other known protein. We compared in detail the

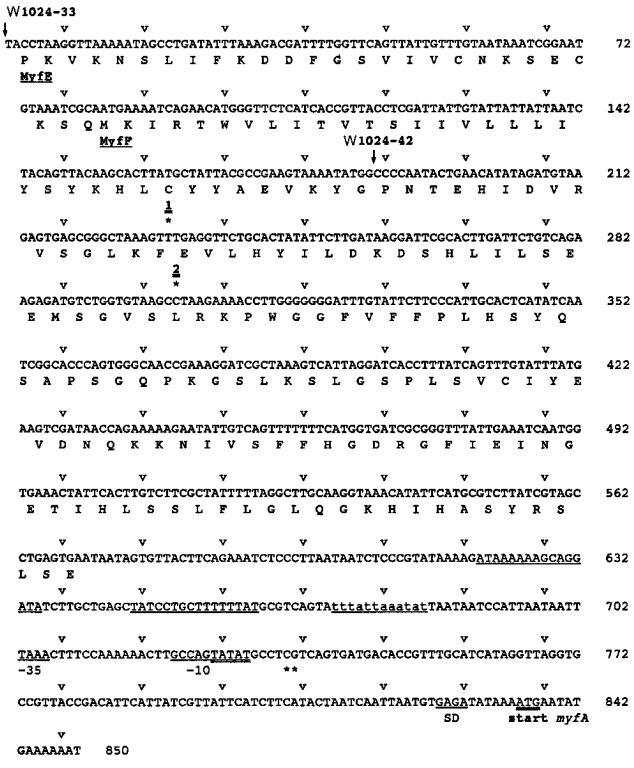


FIG. 4. Nucleotide sequence of the *myfA* upstream region containing the deduced amino acid sequence of MyfF and part of MyfE. The Tn*5* insertion sites in mutants W1024-33 and W1024-42 are shown by an arrow. The sites of two Tn*phoA* insertions in *myfF* are also given (labelled 1 and 2). The terminator of MyfF is underlined. A putative Lrp fixation site is printed in lowercase and underlined. The transcription start point for *myfA* is shown by two asterisks. Putative -10 and -35 boxes for a $\sigma^{28}$ promoter upstream from *myfA* are underlined once. The -10 box for the vegetative sigma factor is underlined twice. The putative Shine-Dalgarno ribosome binding site for *myfA* is designated SD.

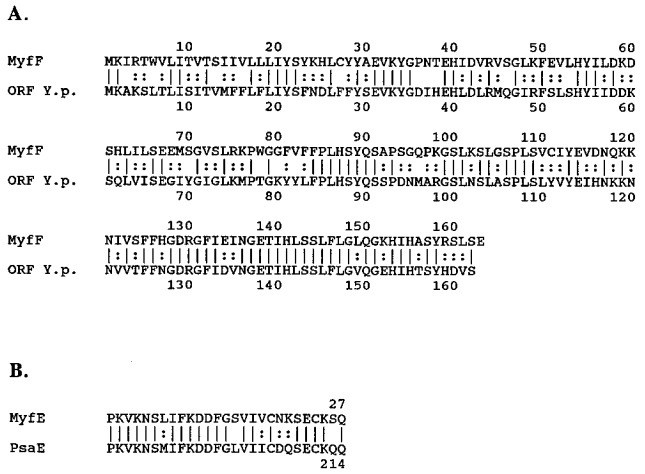


FIG. 5. (A) Amino acid sequence comparison of MyfF from *Y. enterocolitica* and the corresponding protein in the *psa* locus of *Y. pestis* (Y.p.). (B) Amino acid sequence comparison of the partial *myfE′* product and the carboxy-terminal end of the PsaE protein of *Y. pestis* (38). Vertical bars indicate identical amino acids, and colons indicate amino acid similarity.

nucleotide sequences of the *myf* locus with the *psa* locus responsible for the expression of the pH6 antigen in *Y. pestis* (38). According to this analysis, the *psa* sequence contains a putative ORF (nucleotides 1038 to 1526) which presents 54% identity with MyfF (Fig. 5A).

Upstream from *myfF* lies ORF2, an incomplete 27-amino-acid sequence ending at nucleotide 86 with a TGA stop codon that overlaps the ATG codon of *myfF*. Comparison of this incomplete putative protein with the PIR and GenBank databases indicated a 77.8% identity to the carboxy-terminal end of the PsaE protein described by Lindler and Tall (38) (Fig. 5B). In agreement with this observation, ORF2 is situated within the *myf* locus in a position similar to that of the *psaE* gene in the *psa* locus of *Y. pestis* (38). On the basis of these similarities, we designated ORF2 *myfE′*.

**Roles of *myfF* and *myfE* in transcription of *myfA*.** To demonstrate that mutation in *myfF* was responsible for the Myf-negative phenotype of mutant W1024-42, we attempted to complement the mutation with a cloned *myfF* gene. We subcloned a 1.8-kb *Hin*dIII-*Xba*I fragment from pIM106 into pTM100, a moderate-copy-number vector derived from pACYC184 (41). In this construct, called pIM122, *myfF* is transcribed from the P1 promoter of pACYC184. We introduced pIM122 by conjugation in *Y. enterocolitica* W1024-42, and we monitored the production of the Myf antigen by SDS-PAGE and immunoblotting. As shown in Fig. 6, mutant W1024-42 carrying pIM122 synthesized the Myf antigen, indicating that *myfF* acts in *trans* on the production of Myf.

Taking into account that *myfE* and *myfF* seem to form an operon, the Myf-negative phenotype of mutant W1024-33 could be due either to the disruption of the *myfE* gene itself or to a polar effect on *myfF* transcription. To define the role of *myfE*, we tried to complement W1024-33 with pIM122, which contains *myfF*. As can be seen in Fig. 6, pIM122 could not complement the *myfE* mutation of W1024-33, although it did complement W1024-42. Gene *myfE* itself is thus also required for the expression of the Myf antigen.

We analyzed transcription of *myfA* in *Y. enterocolitica* W1024 and in various mutants affected in *myfF* (W1024-42), *myfB* (W1024-41), and *myfE* (W1024-33). As can be seen in Fig. 7, the *myfA* transcript was detected in the *myfB* mutant as

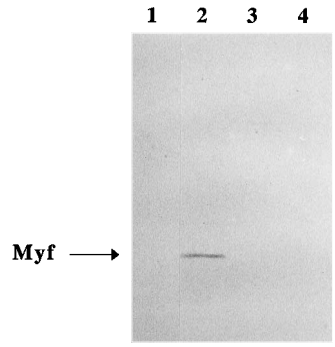


FIG. 6. (A) Immunoblot analysis of Myf antigen production. Lanes: 1, mutant W1024-42; 2, mutant W1024-42(pIM122); 3, mutant W1024-33; 4, mutant W1024-33(pIM122). Bacteria were grown on TSA-glucose at 37°C. The extraction of Myf and the Myf-specific antiserum were as described previously (30).

well as in the wild type, but it was absent from the *myfF* and *myfE* mutants. The *myfA* gene is thus positively regulated, and *myfF* and *myfE* are both required for its transcription.

**Detection of MyfF.** We analyzed the *myfF* gene product by exclusive $^{35}S$ labelling using the T7 RNA polymerase system and plasmid pIM98. We detected by SDS-PAGE a faint band corresponding to a protein with an apparent molecular mass of 18 kDa that was absent in the extract of the control strain containing the vector pTZ18R instead of pIM98. This molecular mass is in agreement with that predicted from the DNA sequence of *myfF* including the 30 $NH_2$-terminal amino acids. This result suggests that the hydrophobic domain is not cleaved and may constitute a transmembrane domain rather than a signal sequence (Fig. 8).

**Localization of MyfF.** As we already mentioned, MyfF has a unique $NH_2$-terminal hydrophobic domain. This domain, which has the characteristics of a signal peptide, could direct the protein toward the periplasm. To test this hypothesis, we constructed and analyzed MyfF-PhoA hybrid proteins. We mutagenized plasmid pIM98 carrying *myfF* with Tn*phoA* and selected the clones giving blue colonies on plates containing X-phosphate. Two transposon insertion sites were determined by sequencing the junctions. Tn*phoA* was found to be inserted after codon 50 and after codon 74. These two fusions give alkaline phosphatase activities in *E. coli* KS272 of 98 ± 19 and

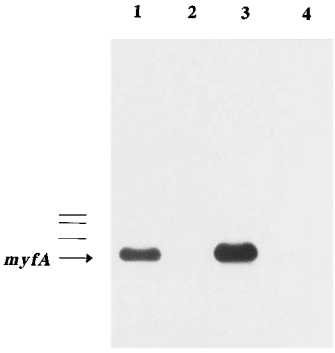


FIG. 7. Northern blot analysis of the influence of *myfF* and *myfE* on *myfA* transcription. Total RNA was extracted from bacteria grown at 37°C on SBHI (pH 6). Lanes: 1, wild-type strain W1024; 2, mutant W1024-33 (*myfE*::Tn*5*-Tc1); 3, mutant W1024-41 (*myfB*::Tn*5*-Tc1); 4, mutant W1024-42 (*myfF*::Tn*5*-Tc1). The transcript was detected with the same PCR fragment as in Fig. 1. The bars on the left indicate the positions of the three typical *Y. enterocolitica* rRNA bands (54).

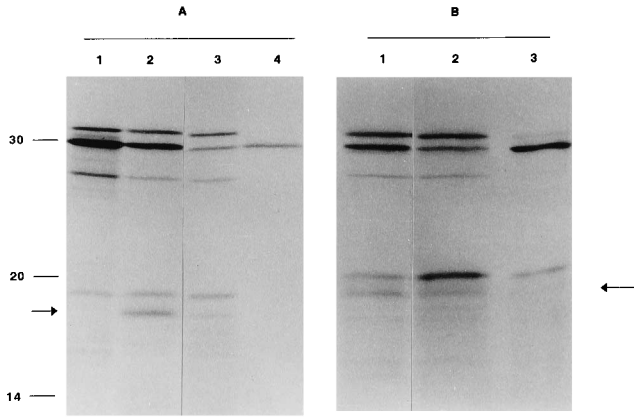


FIG. 8. Expression of the *myfF* gene with the T7 RNA polymerase system in *E. coli* LK111. (A) Osmotic shock experiment. Lanes: 1, total cell proteins from *E. coli* LK111(pGP1-2)(pTZ18R); 2, total cell proteins from *E. coli* LK111 (pGP1-2)(pIM98); 3, *E. coli* LK111(pGP1-2)(pIM98) cell pellet after osmotic shock; 4, *E. coli* LK111(pGP1-2)(pIM98) periplasmic fraction after osmotic shock. Positions of molecular markers are indicated in kilodaltons. (B) Fractionation experiment. Lanes: 1, total cell proteins from *E. coli* LK111(pGP1-2)(pIM98); 2, *E. coli* LK111(pGP1-2)(pIM98) membrane fraction after sonication; 3, *E. coli* LK111(pGP1-2)(pIM98) soluble fraction after sonication. The arrows point to MyfF. Upper bands are *bla* gene products.

$108 \pm 17$ U per optical density at 600 nm ($OD_{600}$), respectively. These values can be compared with values obtained in the same conditions and in the same host but with different PhoA fusions. For instance, fusions between YscU, an inner membrane protein of the Yop secretion machinery, and PhoA give values of $350 \pm 77$ U per unit of OD if PhoA is localized in the periplasm and $49 \pm 17$ U per unit of OD if PhoA is localized in the cytoplasm (1). This finding suggests that at least the hydrophilic domain of MyfF resides in the periplasm.

To determine whether MyfF is soluble in the periplasm or anchored in the cytoplasmic membrane, we performed osmotic shocks in *E. coli* LK111(pGP1-2)(pIM98) after T7 promoter induction. In agreement with our observation that the signal sequence is not cleaved, MyfF remained in the cell pellet while β-lactamase was largely released (Fig. 8A). To confirm these results, we sonicated *E. coli* LK111(pGP1-2)(pIM98) after T7 induction and separated the soluble fraction from the membrane fraction by centrifugation; MyfF was detected in the membrane fraction (Fig. 8B).

## DISCUSSION

Myf forms fibrillae which closely resemble CS3 of human enterotoxinogenic *E. coli* strains (30, 36). This similarity suggests that Myf could favor the adhesion of *Y. enterocolitica* to enterocytes and, in doing so, allow the action of the Yst enterotoxin. However, Myf also appears as the *Y. enterocolitica* counterpart of *Y. pestis* pH6 antigen, whose function remains elusive. Expression of pH6 is induced inside macrophages, which may allow bacteria to interact with other uninfected macrophages or with other host cells after they are released from the infected cells (38).

To gain a better insight on the possible function of Myf, we determined the conditions of its expression. In vitro, Myf production turned out to be regulated by temperature and pH. Like the Yops and YadA, Myf is produced only at 37°C. In contrast, when bacteria are grown in usual culture medium, the enterotoxin Yst can be detected only at temperatures lower than 30°C (2, 7). This finding might argue against a coordinate action of Myf and Yst in inducing diarrhea, but recently Mikulskis et al. (42) showed that *yst* transcription can be induced at 37°C by increasing osmolarity of the culture medium up to values observed in the intestinal lumen close to the mucosa. Myf and Yst could thus be produced in vivo at the same stage of the infection. Myf production also requires an acid pH. This may fit with the fact that after entering the host, bacteria will find an acidic pH in the stomach. The pH will then increase after passage to the intestine (21), but nondividing bacteria might keep their fimbriae. It is interesting that the Tcp pili of *V. cholerae*, which are required for the onset of cholera, are also produced at pHs below 7 (58). The general pattern of Myf production seems thus to fit quite well with the hypothesis of an adhesion factor. However, this pattern of expression, in particular the acid dependence, is of course strikingly reminiscent of pH6, the Myf counterpart in *Y. pestis*. It is also reminiscent of invasin, which is also produced at 37°C provided that the pH is acidic (49).

Regulation of fimbrial expression has been extensively analyzed in other bacteria. It is a highly complex phenomenon which involves several proteins, including Lrp, Dam, integration host factor, H-NS, and catabolite gene activator protein. In addition to these global regulatory proteins, specific regulators are also involved. Most fimbrial regulatory systems can be classified in three groups. The system regulating the expression of Pap pili is the archetype of the first group. It involves two divergent genes, *papB* and *papI*, coding for small (11- and 8.5-kDa) DNA-binding proteins (3). Homologs to these genes have been shown to regulate expression of S, F1845, K99, and K88 pili (5, 23, 52, 59).

The second family of fimbrial regulators includes transcriptional activators of the AraC family with a molecular mass of 30 kDa and a helix-turn-helix motif in their carboxy-terminal ends. This family includes Rns (CFAII pili [11]), CfaD (CFAI pili [31, 53]), and FapR (987P [32]) in *E. coli*. These two groups of fimbrial regulatory proteins are present not only in *E. coli*, and they are not mutually exclusive. For instance, the operon encoding fimbriae in *Salmonella typhimurium* includes proteins PefB and PefI, which are homologous to PapB and PapI, as well as ORF11, which belongs to the AraC family (22).

A third group of fimbrial regulators consists of two component systems as identified in *Pseudomonas aeruginosa* (27), *Bordetella pertussis* (43), and *V. cholerae* (17).

In a search for genes required in *trans* for *myfA* transcription, we identified *myfE* and *myfF*. As is generally the case for fimbrial regulators, they are situated immediately upstream from the gene encoding the fimbrial subunit (*myfA*). However, their products did not show similarity either to any of the fimbrial regulators cited above or to any known bacterial regulatory protein. A putative ORF which presents significant identity to MyfF is also present in the *psa* system, but its function has not been established. MyfF is an 18.5-kDa protein with no typical helix-turn-helix motif. T7 expression, osmotic shock, and Tn*phoA* fusion analyses carried out in *E. coli* suggest that it is a membrane protein which resides predominantly within the periplasm. These features strikingly resemble the characteristics of ToxS, a protein involved in regulation of Tcp pilus expression in *V. cholerae* (18). ToxS is a 19-kDa protein with a unique hydrophobic domain in the $NH_2$-terminal part. The protein remains associated with the membrane by means of this hydrophobic domain, and the hydrophilic part of the protein protrudes in the periplasm (18). In the current model for regulation of Tcp pilus expression, ToxS is part of a cascade including ToxR, a 32-kDa transmembrane protein, and ToxT, a DNA-binding protein that belongs to the AraC family. ToxS would interact in the periplasm with ToxR dimers, resulting in

an active form of ToxR. ToxR would in turn activate expression of *toxT*, and the *toxT* product would activate expression of *tcpA*, the gene encoding the Tcp subunit (17, 18, 45).

We could identify only part of the *myfE* gene. Its product presents significant identity to PsaE, a protein involved in regulation of the pH6 antigen in *Y. pestis*. According to Lindler et al. (37), pH6 expression is regulated normally in *psaE* mutants, but the level of expression is greatly reduced. In contrast, in our *Y. enterocolitica myfE* mutant, transcription of *myfA* was completely abolished, and this effect was not due to a polar effect on *myfF*. Our present data do not allow us to explain the differences observed between the effects of the *psaE* and *myfE* mutations.

The *myfE* stop codon overlaps the ATG start codon of *myfF*, suggesting that these two genes form an operon or the distal part of a longer operon. They are presumably only part of a complex regulatory system, because other chromosomal mutations, not yet analyzed, affect the expression of Myf (29).

By primer extension, we localized a putative promoter upstream from the transcription start of *myfA*. The -10 box is similar to that recognized by the vegetative sigma factor, but the -35 box is missing. Most surprisingly, the DNA sequence immediately upstream from the transcription start of *myfA* contains a nearly perfect $\sigma^{28}$ (RpoF) promoter (25). So far, no virulence genes from an animal pathogen have been shown to require this sigma factor for the initiation of their transcription. Our data do not allow us to conclude that RpoF is indeed involved. The answer to this question requires the cloning and mutagenesis of *rpoF*.

## ACKNOWLEDGMENTS

We thank Dominique Desnoeck for technical assistance. We are grateful to Ralf Schulte and Pierre Wattiau for a critical reading of the manuscript and to Abdelmounaaim Allaoui for discussion. We also thank the I.C.P. illustration service for the artwork.

This work was supported by the Belgian Fonds National de la Recherche Scientifique Médicale (convention 3.4627.93) and the Human Capital and Mobility Program from the European Community (contract ERBCHRXCT930171).

## REFERENCES

1. **Allaoui, A., S. Woestyn, C. Sluiters, and G. R. Cornelis.** 1994. YscU, a *Yersinia enterocolitica* inner membrane protein involved in Yop secretion. J. Bacteriol. **176:**4534–4542.
2. **Amirmozafari, N., and D. C. Robertson.** 1993. Nutritional requirements for synthesis of heat-stable enterotoxin by *Yersinia enterocolitica*. Appl. Environ. Microbiol. **59:**3314–3320.
3. **Baga, M., M. Göransson, S. Normark, and B. E. Uhlin.** 1985. Transcriptional activation of a Pap pilus virulence operon from uropathogenic *Escherichia coli*. EMBO J. **4:**3887–3893.
4. **Ben-Efraim, S., M. Aronson, and L. Bichowsky-Slomnicki.** 1961. New antigenic component of *Pasteurella pestis* formed under specified conditions of pH and temperature. J. Bacteriol. **81:**704–714.
5. **Bilge, S. S., J. M. Apostol, Jr., K. J. Fullner, and S. L. Moseley.** 1993. Transcriptional organization of the F1845 fimbrial adhesin determinant of *Escherichia coli*. Mol. Microbiol. **7:**993–1006.
6. **Blomfield, I. C., P. J. Calie, K. J. Eberhardt, M. S. McClain, and B. I. Eisenstein.** 1993. Lrp stimulates phase variation of type 1 fimbriation in *Escherichia coli* K-12. J. Bacteriol. **175:**27–36.
7. **Boyce, J. M., D. J. Evans, Jr., D. G. Evans, and H. L. DuPont.** 1979. Production of heat-stable, methanol-soluble enterotoxin by *Yersinia enterocolitica*. Infect. Immun. **25:**532–537.
8. **Braaten, B. A., L. B. Blyn, B. S. Skinner, and D. A. Low.** 1991. Evidence for a methylation-blocking factor (*mbf*) locus involved in *pap* pilus expression and phase variation in *Escherichia coli*. J. Bacteriol. **173:**1789–1800.
9. **Braaten, B. A., J. V. Platko, M. W. van der Woude, B. H. Simons, F. K. de Graaf, J. M. Calvo, and D. A. Low.** 1992. Leucine-responsive regulatory protein controls the expression of both the *pap* and *fan* pili operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**4250–4254.
10. **Brubaker, R. R.** 1991. Factors promoting acute and chronic diseases caused by *Yersinia*. Clin. Microbiol. Rev. **4:**309–324.
11. **Caron, J., L. M. Coffield, and J. R. Scott.** 1989. A plasmid-encoded regulatory gene, *rns*, required for expression of the CS1 and CS2 adhesins of enterotoxigenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**963–967.
12. **Cornelis, G. R.** 1992. Yersiniae, finely tuned pathogens. Symp. Soc. Gen. Microbiol. **49:**231–265.
13. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
14. **Currie, M. G., K. F. Fok, J. Kato, R. J. Moore, F. K. Hamra, K. L. Duffin, and C. E. Smith.** 1992. Guanylin: an endogenous activator of intestinal guanylate cyclase. Proc. Natl. Acad. Sci. USA **89:**947–951.
15. **Delor, I., and G. R. Cornelis.** 1992. Role of *Yersinia enterocolitica* Yst toxin in experimental infection of young rabbits. Infect. Immun. **60:**4269–4277.
16. **Delor, I., A. Kaeckenbeeck, G. Wauters, and G. R. Cornelis.** 1990. Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and nonpathogenic yersiniae. Infect. Immun. **58:**2983–2988.
17. **DiRita, V. J.** 1992. Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol. Microbiol. **6:**451–458.
18. **DiRita, V. J., and J. J. Mekalanos.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxS and ToxR, results in signal transduction and transcriptional activation. Cell **64:**29–37.
19. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179:**125–142.
20. **Finkelstein, A. V.** 1975. Dokl. Acad. Nauk SSSR **223:**744–747.
21. **Fordtran, J. S.** 1973. Diarrhea, p. 291–301. *In* M. H. Sleisenger and J. S. Fordtran (ed.), Gastrointestinal disease. Saunders Co., Philadelphia.
22. **Friedrich, M. J., N. E. Kinsey, J. Vila, and R. J. Kadner.** 1993. Nucleotide sequence of a 13,9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. Mol. Microbiol. **8:**543–558.
23. **Göransson, M., K. Forsman, and B. E. Uhlin.** 1988. Functional and structural homology among regulatory cistrons of pili-adhesin determinants in *Escherichia coli*. Mol. Gen. Genet. **212:**412–417.
24. **Hal Jones, C., F. Jacob-Dubuisson, K. Dodson, M. Kuehn, L. Slonim, R. Striker, and S. J. Hultgren.** 1992. Adhesin presentation in bacteria requires molecular chaperones and ushers. Infect. Immun. **60:**4445–4451.
25. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. **5:**2875–2882.
26. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili and *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168:**1487–1492.
27. **Hobbs, M., E. S. R. Collie, P. D. Free, S. P. Livingston, and J. S. Mattick.** 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. **7:**669–682.
28. **Huisman, T. T., D. Bakker, P. Klaasen, and F. K. de Graaf.** 1994. Leucine-responsive regulatory protein, IS*1* insertions, and the negative regulator FaeA control the expression of the *fae* (K88) operon in *Escherichia coli*. Mol. Microbiol. **11:**525–536.
29. **Iriarte, M., and G. R. Cornelis.** Unpublished data.
30. **Iriarte, M., J.-C. Vanooteghem, I. Delor, R. Diaz, S. Knutton, and G. R. Cornelis.** 1993. The Myf fibrillae of *Yersinia enterocolitica*. Mol. Microbiol. **9:**507–520.
31. **Jordi, B. J. A. M., B. Dagberg, L. A. M. de Haan, A. M. Hamers, B. A. M. van der Zeijst, W. Gaastra, and B. E. Uhlin.** 1992. The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/I fimbrial operon of *Escherichia coli*. EMBO J. **11:**2627–2632.
32. **Klaasen, P., and F. K. de Graaf.** 1990. Characterization of FapR, a positive regulator of expression of the 987P operon in enterotoxigenic *Escherichia coli*. Mol. Microbiol. **4:**1779–1783.
33. **Krogfelt, K. A.** 1991. Bacterial adhesion: genetics, biogenesis, and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **13:**721–735.
34. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157:**105–132.
35. **Lambert de Rouvroit, C., C. Sluiters, and G. R. Cornelis.** 1992. Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol. Microbiol. **6:**395–409.
36. **Levine, M. M., P. Ristaino, G. Marley, C. Smyth, S. Knutton, E. Boedeker, R. Black, C. Young, M. L. Clements, C. Cheney, and R. Patnaik.** 1984. Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. Infect. Immun. **44:**409–420.
37. **Lindler, L. E., M. S. Klempner, and S. C. Straley.** 1990. *Yersinia pestis* pH 6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague. Infect. Immun. **58:**2569–2577.
38. **Lindler, L. E., and B. D. Tall.** 1993. *Yersinia pestis* pH 6 antigen forms fimbriae and is induced by intracellular association with macrophages. Mol. Microbiol. **8:**311–324.
39. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein

export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
40. **Michiels, T., and G. R. Cornelis.** 1988. Nucleotide sequence and transcription analysis of *yop51* from *Yersinia enterocolitica* W22703. Microb. Pathog. **5:**449–459.
41. **Michiels, T., and G. R. Cornelis.** 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173:**1677–1685.
42. **Mikulskis, A. V., I. Delor, V. Ha Thi, and G. R. Cornelis.** Regulation of the *Yersinia enterocolitica* enterotoxin Yst gene. Influence of growth phase, temperature, osmolarity, pH and bacterial host factors. Mol. Microbiol., in press.
43. **Miller, J. F., C. R. Roy, and S. Falkow.** 1989. Analysis of *Bordetella pertussis* virulence gene regulation by use of transcriptional fusions in *Escherichia coli*. J. Bacteriol. **171:**6345–6348.
44. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
45. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48:**271–279.
46. **Newman, E. B., R. D'Ari, and R. T. Lin.** 1992. The leucine-Lrp regulon in *E. coli*. A global response in search of a raison d'être. Cell **68:**617–619.
47. **Pai, C. H., and V. Mors.** 1978. Production of enterotoxin by *Yersinia enterocolitica*. Infect. Immun. **19:**908–911.
48. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
49. **Pepe, J. C., J. L. Badger, and V. L. Miller.** 1994. Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica inv* gene. Mol. Microbiol. **11:**123–135.
50. **Ptitsyn, O. B., and A. V. Finkelstein.** 1983. Theory of protein secondary structure and algorithm of its prediction. Biopolymers **22:**15–25.
51. **Rex, J. H., B. D. Aronson, and R. L. Somerville.** 1991. The *tdh* and *serA* operons of *Escherichia coli*: mutational analysis of the regulatory elements of leucine-responsive genes. J. Bacteriol. **173:**5944–5953.
52. **Roosendaal, E., M. Boots, and F. K. de Graaf.** 1987. Two novel genes, *fanA* and *fanB*, involved in the biogenesis of K99 fimbriae. Nucleic Acids Res. **15:**5973–5984.
53. **Savelkoul, P. H. M., G. A. Willshaw, M. M. McConnell, H. R. Smith, A. M. Hamers, B. A. M. van der Zeijst, and W. Gaastra.** 1990. Expression of CFA/I fimbriae is positively regulated. Microb. Pathog. **8:**91–99.
54. **Skurnik, M., and P. Toivanen.** 1991. Intervening sequences (IVs) in the 23S ribosomal RNA genes of pathogenic *Yersinia enterocolitica* strains. The IVs in *Y. enterocolitica* and *Salmonella typhimurium* have a common origin. Mol. Microbiol. **5:**585–593.
55. **Smyth, C. J., M. Boylan, H. M. Matthews, and D. C. Coleman.** 1991. Fimbriae of human enterotoxigenic *Escherichia coli* and control of their expression, p. 37–53. *In* E. Z. Ron and S. Rottem (ed.), Microbial surface components and toxins in relation to pathogenesis. Plenum Press, New York.
56. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
57. **Takao, T., N. Tominaga, S. Yoshimura, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama.** 1985. Isolation, primary structure and synthesis of heat-stable enterotoxin produced by *Yersinia enterocolitica*. Eur. J. Biochem. **152:** 199–206.
58. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
59. **van der Woude, M. W., B. A. Braaten, and D. A. Low.** 1992. Evidence for global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: model building based on analysis of *pap*. Mol. Microbiol. **6:**2429–2435.
60. **van der Woude, M. W., and D. A. Low.** 1994. Leucine-responsive regulatory protein and deoxyadenosine methylase control the phase variation and expression of the *sfa* and *daa* pili operons in *Escherichia coli*. Mol. Microbiol. **11:**605–618.
61. **Van Noyen, R., R. Selderslaghs, J. Bekaert, and G. Wauters.** 1991. *Yersinia enterocolitica* and other enteric pathogen in patients with suspected appendicitis. Contrib. Microbiol. Immunol. **12:**265–271.
62. **Yim, H. H., and M. Villarejo.** 1992. *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. J. Bacteriol. **174:** 3637–3644.