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A Promoter for the First Nine Genes of the *Escherichia coli mra* Cluster of Cell Division and Cell Envelope Biosynthesis Genes, Including *ftsI* and *ftsW*

HIROSHI HARA,[1]* SEIICHI YASUDA,[1] KENSUKE HORIUCHI,[1]† AND JAMES T. PARK[2]

National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan,[1] and Department of Molecular Biology and Microbiology, Tufts University Health Sciences Campus, Boston, Massachusetts 02111[2]

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We constructed a null allele of the *ftsI* gene encoding penicillin-binding protein 3 of *Escherichia coli*. It caused blockage of septation and loss of viability when expression of an extrachromosomal copy of *ftsI* was repressed, providing a final proof that *ftsI* is an essential cell division gene. In order to complement this null allele, the *ftsI* gene cloned on a single-copy mini-F plasmid required a region 1.9 kb upstream, which was found to contain a promoter sequence that could direct expression of a promoterless *lacZ* gene on a mini-F plasmid. This promoter sequence lies at the beginning of the *mra* cluster in the 2 min region of the *E. coli* chromosome, a cluster of 16 genes which, except for the first 2, are known to be involved in cell division and cell envelope biosynthesis. Disruption of this promoter, named the *mra* promoter, on the chromosome by inserting the *lac* promoter led to cell lysis in the absence of a *lac* inducer. The defect was complemented by a plasmid carrying a chromosomal fragment ranging from the *mra* promoter to *ftsW*, the fifth gene downstream of *ftsI*, but not by a plasmid lacking *ftsW*. Although several potential promoter sequences in this region of the *mra* cluster have been reported, we conclude that the promoter identified in this study is required for the first nine genes of the cluster to be fully expressed.

Many of the genes involved in the cell division process in *Escherichia coli* are found localized in a few clusters on the chromosome. The best characterized of the clusters is located at 2 min. It has been designated the *mra* (for murein A) cluster (11, 36, 41). It contains at least six cell division genes (*fts* genes), together with seven murein biosynthetic genes (*mur* genes, *mraY*, and *ddl*) and a lipopolysaccharide biosynthetic gene (*envA*) (see Fig. 1). Among them is the *ftsI* (also named *pbpB*) gene, which codes for penicillin-binding protein 3 (PBP 3). PBP 3 is a cytoplasmic membrane protein that functions as DD-transpeptidase for the formation of a septum of the murein sacculus and is a principal lethal target of β-lactam antibiotics (1, 16, 26). Analyses of thermosensitive cell division mutants showing thermolabile penicillin binding of PBP 3 and their revertants indicated that PBP 3 is an essential cell division component (48), although addition of sucrose suppressed the division defect but not the PBP 3 thermolability. The transpeptidase activity is carried by the C-terminal penicillin-binding domain of PBP 3, while the function of the N-terminal domain is still controversial (16, 26, 50, 57).

We initiated an attempt to create a new class of *ftsI* mutants in the hope of understanding the function of the N-terminal domain of PBP 3, especially in terms of its interaction with other cell division proteins, and realized that a null allele of the *ftsI* gene on the chromosome would be very useful to characterize mutant alleles newly created on plasmids. To eliminate a possible effect of copy number, we decided to clone the mutant alleles on single-copy mini-F vectors rather than on multicopy vectors. In this study, we constructed a deletion-insertion allele of *ftsI* and tried to complement it with wild-type *ftsI* on a mini-F plasmid. Such a study led to identification of a promoter sequence 1.9 kb upstream required for *ftsI* expression. This is much farther upstream than previously presumed. We show that this promoter is necessary not only for the genes up to *ftsI* but also for five more genes downstream of *ftsI*, up to *ftsW*, to be fully expressed.

(A preliminary report on part of this work was presented at a Federation of European Microbiological Societies Symposium on Bacterial Growth and Lysis [21].)

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used were derivatives of *Escherichia coli* K-12 and are described in Table 1. They were grown in buffered L broth-glucose-thymine (BLGT) medium (22) (buffered salt solution [100-fold concentrated] was further modified to include Na_2SO_4 [14.2 g], $NH_4H_2PO_4$ [115 g], $Na_2HPO_4 \cdot 12H_2O$ [179 g], and K_2HPO_4 [365 g] per liter), in L broth containing 10 mM glucose, or in M9 medium (40). The following antibiotics were used at the indicated concentrations (in micrograms per milliliter) for multicopy and single-copy resistance genes, respectively: ampicillin, 100 and 20; chloramphenicol, 50 and 10; kanamycin, 50 and 20; and tetracycline, 12.5 and 5. Cell growth was monitored with a Klett-Summerson colorimeter equipped with a no. 54 filter.

Plasmids. Most of the plasmids constructed in this study are illustrated in Fig. 1, 4, and 5. Mini-F plasmid vectors used were pMF3 (34), pSY396, pHR277, and pFZY1ΔH. The vector pSY396 was constructed by replacing a 5.4-kb *Eco*RI-*Bam*HI fragment of pMF3 that contains a staphylococcal *blaZ* fragment and a part of F DNA with a 1.9-kb *Eco*RI-*Bam*HI fragment of pKC7 (44) that contains the kanamycin resistance (*neo*) gene. In this vector there seems to be no transcription of significant strength flowing into the *Eco*RI-*Hin*dIII cloning region, whereas pMF3 contains the promoter of the truncated *cadA* gene in the *blaZ* fragment that drives transcription proceeding beyond the *Eco*RI cloning site (see Discussion). pHR277 carries a 1.2-kb *Pst*I-*Kpn*I *lacI*^{q} fragment of pMJR1560 (52) cloned into the *Bam*HI site of pSY396 with the pUC19 multiple cloning sites (MCSs) as linkers. pFZY1ΔH is a derivative of pFZY1 (31) with its unique *Hin*dIII site destroyed by the filling reaction of T4 DNA polymerase.

The *ftsI*-containing chromosomal fragments cloned in this work (see Fig. 1) were obtained from pLC26-6 (54), pMS316 (24), and pGY100 (a gift from Guy Dufford). In pMS316, a 2.6-kb *Pvu*II fragment containing *ftsI* was cloned into the *Bam*HI site of pACYC184, with the *Bam*HI site filled in before ligation, thus regenerating a *Bam*HI site on either side of *ftsI*. In constructing pGY100, an *Xba*I site was created a little upstream of a putative ribosome-binding site for *ftsI* by modifying nucleotides (nt) 599 to 602 (see Fig. 4 of reference 42, for nucle-

* Corresponding author. Mailing address: National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan. Phone: 81-559-81-6758. Fax: 81-559-81-6762. E-mail: hhara@lab.nig.ac.jp.

† Present address: The Rockefeller University, New York, NY 10021.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype and/or phenotype	Source or reference
DH5	*recA1 endA1 hsdR17*	Laboratory collection
JM109	*recA1 endA1 hsdR17*/F′ *lacI*^{q}	Laboratory collection
P4X8	HfrP4X	Laboratory collection
N1126	*polA12*(Ts) *lacZ str*	Laboratory collection
JE7931	HfrP4X *polA12*(Ts) Sm^s	P4X8 × N1126
BW7261	*leu-63*::Tn*10*	61
W3110	Wild type	Laboratory collection
JE7936	W3110 *leu-63*::Tn*10*	P1(BW7261) × W3110
JE7941[b]	W3110 Δ*ftsI*::*cat*[a]	Leu$^+$ Δ*ftsI*::*cat*[a] transductant of JE7936; see text
JE7942[c]	Wild type	Transductant isogenic to JE7941; see text
MM146	MC4100 *srl*::Tn*10 recA1*	32
JE7943[b]	W3110 Δ*ftsI*::*cat*[a] *srl*::Tn*10 recA1*	P1(MM146) × JE7941
JE7944[c]	W3110 *srl*::Tn*10 recA1*	P1(MM146) × JE7942
BW5660	*srlC300*::Tn*10*	61
JE7845[b]	W3110 Δ*ftsI*::*cat*[a] *srlC300*::Tn*10*	P1(BW5660) × JE7941
JE7946[c]	W3110 *srlC300*::Tn*10*	P1(BW5660) × JE7942
LC248	*recA1*	Laboratory collection
JE7947[b]	W3110 Δ*ftsI*::*cat*[a] *recA1*	P1(LC248) × JE7945
JE7948[c]	W3110 *recA1*	P1(LC248) × JE7946
MC1061-5	Δ*lacX74*	31
JE7611	*ftsI730*(Ts) *recA1*	42
JE7966	HfrP4X *polA12*(Ts) *leu-63*::Tn*10* Sm^s	P1(BW7261) × JE7931
JE7967[d]	W3110 P_{mra}::P_{lac}[e] *leu-63*::Tn*10*	Tc^r P_{mra}::P_{lac}[e] transductant of W3110; see text
JE7968[d]	W3110 P_{mra}::P_{lac}[e]	P1(W3110) × JE7967
JE7969[d]	W3110 P_{mra}::P_{lac}[e] *srlC300*::Tn*10*	P1(BW5660) × JE7968
JE7970[d]	W3110 P_{mra}::P_{lac}[e] *recA1*	P1(LC248) × JE7969

[a] The internal *Mlu*I-*Nar*I fragment within the *ftsI* gene was displaced with the *cat* gene of the same orientation (Fig. 1) (see text).
[b] This Δ*ftsI*::*cat* strain exists only when it has an additional copy of *ftsI*. During the construction of *recA1* derivatives, the mini-F plasmid pHR296 (Ap^r) (Fig. 1) was used. Other mini-F plasmids (Km^r) were introduced in place of pHR296 by transformation.
[c] This strain carried the same plasmids during construction as its isogenic Δ*ftsI*::*cat* strain did.
[d] This strain requires a *lac* inducer for growth.
[e] The *mra* promoter was disrupted by inserting the *lac* promoter, which was preceded by *cat*, *rrnB* transcriptional terminators, and *lacI*^{q} (Fig. 5) (see text).

otide numbering) by oligonucleotide-directed mutagenesis, and the *Xba*I-*Pvu*II *ftsI* fragment was then cloned into the *Xba*I-*Bam*HI region (with a *Bam*HI site regenerated as in pMS316) of a pIN-III vector (35).

For construction of pHR377 (see Fig. 1) and for promoter activity assays (see Fig. 4), a DNA fragment containing the promoter region flanked by the appropriate restriction sites was chemically synthesized in both strands. The *ftsI* gene and the pKC7 *neo* gene that were sandwiched by linkers derived from pUC18 and pUC19 MCSs were inserted into the synthetic promoter and cloned on pSY396 (pHR377) and pFZY1ΔH (pHR351 and pHR352), respectively.

Chromosomal fragments of the 2-min region cloned in this work (see Fig. 5) were obtained from cosmid 83-2 (a gift from Teru Ogura), which carries an ~47-kb chromosomal fragment (genomic address of about kb 75.5 to 122.5 according to Rudd [45]) on vector pHSG439 (5). A derivative of pSY396 that has synthetic polylinker cloning sites (*Hin*dIII-*Aat*II-*Pst*I-*Not*I-*Bsu*36I-*Eco*RI) in place of the *Hin*dIII-*Eco*RI fragment was a vector in pHR416, pHR426, pHR427, and pHR439. In pHR432, a 4.9-kb *Eco*RI fragment containing *murD*, *ftsW*, *murG*, and a 5′ part of *murC* was inserted into the *Eco*RI site of pHR311 (see Fig. 1). The internal fragments of this *Eco*RI fragment were displaced by an Ω-Tc interposon (14) and inserted into pHR311 to construct pHR477, pHR478, and pHR479; in these plasmids, *murC* is 3′-terminally truncated and separated from the *mra* promoter-driven transcription by a terminator in the Ω interposon. In pHR485, the *ftsW* gene was cloned downstream of the spectinomycin-streptomycin resistance (*aadA*) gene on pGB2 (7) so that it was transcribed together with *aadA*.

Construction of a null allele of *ftsI* and replacement of the *mra* promoter with a *lac* promoter on the chromosome. The construction of a null allele and replacement of the *mra* promoter with a *lac* promoter were done by integration into and resolution from the chromosome of a ColE1-type plasmid in *polA*(Ts) strains. A deletion-insertion mutation of *ftsI* (Δ*ftsI*::*cat*) was first constructed on pHR4 (see Fig. 1), a pBR322 derivative (Ap^r) that carries *ftsI* and its flanking region of about 4 kb on either side. In place of the *Mlu*I-*Nar*I fragment (corresponding to amino acids 43 to 543 out of 588 residues of the precursor PBP 3) within *ftsI*, a 1.3-kb *Hae*II fragment of pACYC184 containing the chloramphenicol resistance (*cat*) gene was inserted, with all the cut sites having been filled in before being ligated. The *cat* gene was placed in the same orientation as *ftsI*. JE7931 [Hfr *polA*(Ts)] was transformed with the resulting plasmid at 30°C and then grown at 42°C in the presence of ampicillin and chloramphenicol, selecting for integration of the plasmid into the chromosome by homologous recombination around *ftsI*. The integrated plasmid was transferred to N1126 [F$^-$ *polA*(Ts) *str*] at 42°C by conjugation with selection for Ap^r Cm^r Sm^r, and the exconjugant was subsequently grown at 30°C to promote excision, which should result in Δ*ftsI*::*cat* on the chromosome being complemented by the wild-type *ftsI* on the plasmid or vice versa, depending on the site of the recombination in the excision event. Phage P1 lysate was prepared and used to transduce JE7936 (*leu*::Tn*10*) harboring pHR223 (see Fig. 1), an *ftsI*-carrying pACYC177 derivative (Km^r), to Leu$^+$. The transductants were tested for drug resistance, and one of the Tc^s Cm^r Ap^s Km^r transductants, which should have received the chromosomal Δ*ftsI*::*cat*, was used for further experiments.

The *mra* promoter identified in the present study was replaced by the *lac* promoter (P_{mra}::P_{lac}) (see Fig. 5), first on a plasmid that carries an 8.5-kb chromosomal fragment between the *Aat*II site 1.2 kb upstream of the *mra* promoter and the *Eco*RI site within *mraY*. The vector was a pBR322 derivative that had the 1.2-kb *Cla*I-*Nar*I fragment deleted. Into the *Hin*dIII site within the *mra* promoter, a DNA fragment containing the *lac* promoter was inserted. The 3-kb *Hin*dIII fragment was composed of the *cat* gene followed by two transcriptional terminators of the *rrnB* operon, the *lacI*^{q} gene (placed in the orientation opposite to the following *lac* promoter), and the *lac* promoter. The 5′ part of *cat* was derived from pBR325 (2), with the 3′ part followed by the terminators from pKK232-8 (6), *lacI*^{q} from pMJR1560, and the *lac* promoter from pUC19. The *Hin*dIII site at the upstream end of the fragment was created by inserting a synthetic, palindromic oligonucleotide into the *Aat*II site of pBR325. P_{mra}::P_{lac} was then crossed into the chromosome essentially in the same way as for Δ*ftsI*::*cat* except that isopropyl-thio-β-D-galactoside (IPTG) was included in media throughout the procedure. The plasmid carrying P_{mra}::P_{lac} was integrated into the chromosome of JE7966 [Hfr *polA*(Ts) *leu*::Tn*10*] and transferred to N1126 together with *leu*::Tn*10*. Chromosomally integrated P_{mra}::P_{lac} was P1 transduced to W3110 by selecting for Tc^r and then testing for Cm^r.

Genetical and recombinant DNA procedures. The genetical and recombinant DNA procedures used were essentially based on the methods of Miller (40) and Sambrook et al. (46). In transductions, P1*kc* was used except for *recA1*, which was transduced by P1*vir* that had gone through MM146 for generations and could produce high-titer lysates of *recA* strains (a gift from Carol Kumamoto). Oligonucleotides were synthesized with a Cyclone Plus DNA Synthesizer (Millipore). The β-galactosidase (β-Gal) assay method and the unit definition used were as described by Koop et al. (31). Computer analyses of nucleotide sequences were performed by using the GENETYX program (Software Development Co.).

PBP assay. Assays for PBPs with benzyl[^{14}C]penicillin (Amersham) were performed as described previously (53). JE7611, an *ftsI730*(Ts) strain whose PBP 3

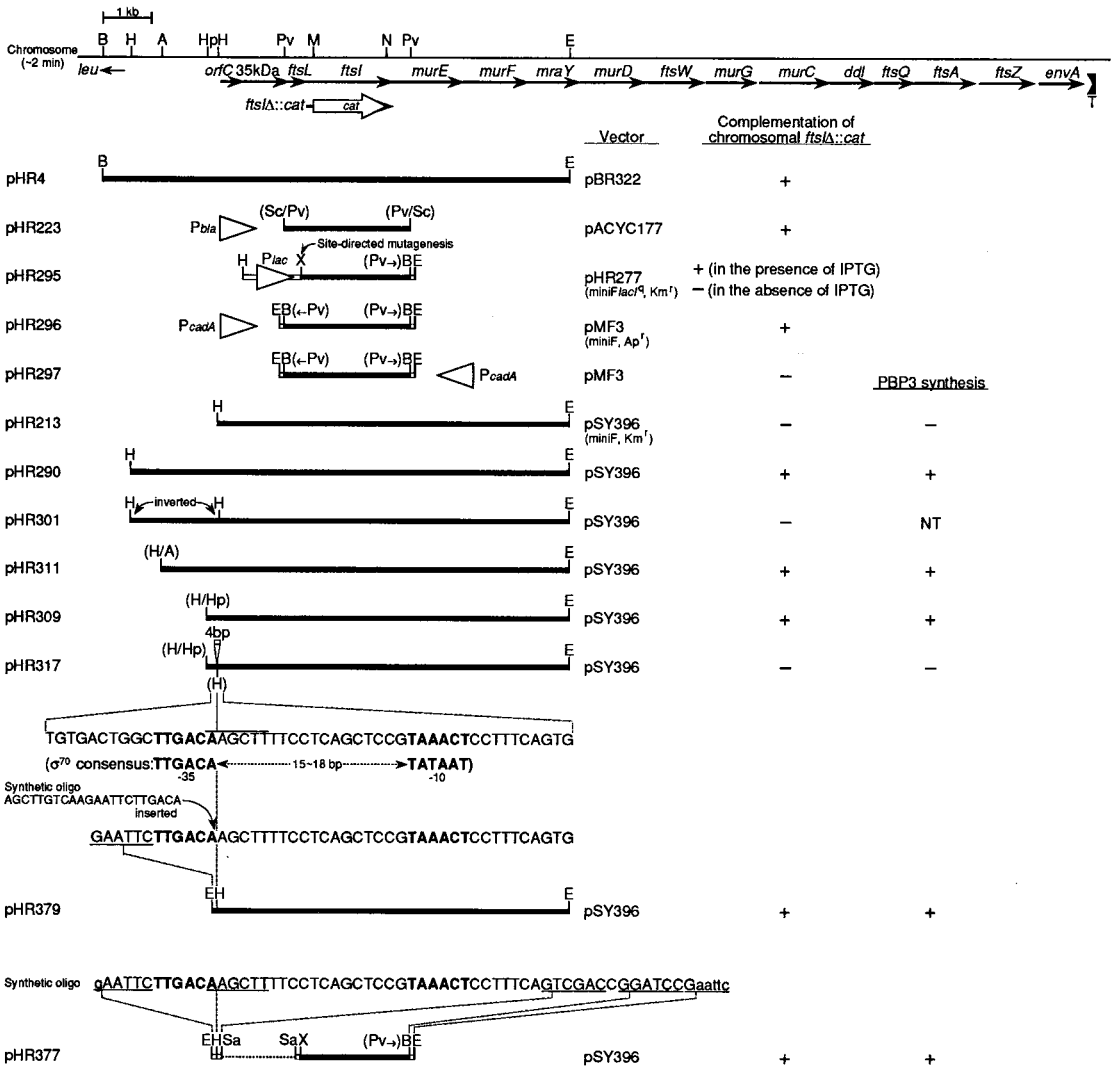

FIG. 1. Plasmids carrying the *ftsI* gene. Only the cloned chromosomal regions are shown (filled bars). The long open bar in pHR295 represents DNA derived from the pUC18 MCS and the *Pvu*II-*Xba*I region containing the *lac* promoter of a derivative of pUC19 with the *Hin*dIII, *Sph*I, and *Pst*I restriction sites deleted. A short, open bar represents a small insertion created by the filling reaction of T4 DNA polymerase (pHR317) and polylinkers derived from pUC18, pUC19, pUC-4K (59), or synthetic oligonucleotides (pHR295, pHR296, pHR297, and pHR377). Open arrowheads denote the direction of the promoters on the vectors (pHR223, pHR296, and pHR297) or of the *lac* promoter of a pUC19 origin joined to *ftsI* in the cloning procedure (pHR295). The orientation and approximate sizes of the *mra* cluster genes are shown at the top. The thick bracket with a T beneath it represents a ρ-independent transcriptional terminator. Parentheses surrounding enzyme pairs indicate the ligation of blunt or filled-in ends. (Pv→)B and B(←Pv) stand for *Pvu*II sites converted to *Bam*HI sites by ligating with the *Bam*HI-cut and filled-in ends. The maps are drawn to scale except for some regions crowded with restriction sites. The *cat* fragment in Δ*ftsI*::*cat* is drawn not in its own size but in the size of the fragment it displaced. Cloned fragments in pHR379 and pHR377 are in the same orientation as in other pSY396-based plasmids, with the proximal side of the *mra* cluster joined toward the *Hin*dIII site of the vector. A dotted line in pHR377 indicates that the synthetic *mra* promoter sequence and the *ftsI* gene were directly connected. Oligonucleotides used in the construction of pHR377 were synthesized in both strands and are shown only in the upper strand, in uppercase letters. Bases in lowercase letters are shown to indicate the *Eco*RI restriction sites joined to the vector. Complementation of chromosomal Δ*ftsI*::*cat* and PBP 3 synthesis were tested as described in the footnotes to Table 2 and 3 and the legend to Fig. 3, respectively. NT, not tested; oligo, oligonucleotide. Abbreviations for restriction sites (only relevant sites are shown): A, *Aat*II; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; M, *Mlu*I; N, *Nar*I; Pv, *Pvu*II; Sa, *Sal*I; Sc, *Sca*I; X, *Xba*I.

does not bind penicillin in vitro even at temperatures permissive for growth (53), was used as a host of plasmids. Radioactive protein bands in a sodium dodecyl sulfate-polyacrylamide gel were detected with the BAS2000 (Fuji Photo Film Co.) phosphorimaging system.

RESULTS

***ftsI* is essential for cell division and viability.** A null allele of *ftsI*, in which almost all of the coding region was deleted and instead the chloramphenicol acetyltransferase (*cat*) gene was inserted in the same orientation as *ftsI*, was constructed as described in Materials and Methods, first in a strain carrying additional copies of *ftsI* on a pACYC177 derivative (pHR223) (Fig. 1). Phage P1 grown on this Δ*ftsI*::*cat* strain was used to infect JE7936, a *leu*::Tn*10* strain harboring either no plasmid or pHR4, an *ftsI*-carrying pBR322 derivative (Fig. 1), and Leu^+ transductants were selected. The *ftsI* null allele (Cm^r) was cotransduced with *leu* to the pHR4-harboring recipient at a high frequency, as expected from the close linkage of Δ*ftsI*::*cat* and *leu*, but not to the recipient with no extrachromosomal *ftsI* (Table 2). One of the Δ*ftsI*::*cat* transductants harboring pHR4 was then used as a donor in a similar transduction experiment, in which the recipient was a strain condi-

TABLE 2. Complementation test of Δ*ftsI*::*cat* by transduction[a]

Expt no.	Plasmid	Cm^r transductants[d]
1	None	0/12
	pHR4	7/11
	pHR296	7/10
	pHR297	1[e]/12
2	pHR295[b]	11/12
	pHR295[c]	0/12
	pHR213	0/12
	pHR290	9/11
	pHR223	9/12

[a] JE7936 (*lev*::Tn*10*) harboring the plasmid (Ap^r and Km^r in experiments 1 and 2, respectively) to be tested was transduced to Leu$^+$ from a Δ*ftsI*::*cat* strain harboring pHR223 (Km^r) and pHR4 (Ap^r) in experiments 1 and 2, respectively. Results of typical experiments are shown.

[b] In the presence of IPTG.

[c] In the absence of IPTG.

[d] The ratios of Cm^r transductants to the total number of Leu$^+$ transductants that retained the plasmid of the recipient and did not receive the plasmid from the donor (e.g., Tc^s Km^r Ap^s in experiment 2) are shown.

[e] When this Cm^r transductant was used as a donor to transduce JE7936 harboring pHR290, Cm^r was not cotransduced with *leu* at all. Quadruple crossing over might have occurred, putting *leu*$^+$ and Δ*ftsI*::*cat* into the chromosome and the plasmid, respectively.

tionally expressing the extrachromosomal *ftsI* from mini-F plasmid pHR295 (Km^r), which carries *ftsI* under the control of a *lac* promoter and *lacI*^{q}. JE7936 harboring pHR295 could receive Δ*ftsI*::*cat* in the presence of the *lac* inducer IPTG but not in its absence (Table 2). In the presence of IPTG, an Δ*ftsI*::*cat recA* strain harboring pHR295, JE7943, grew normally and showed normal cellular morphology. When IPTG was removed from the culture medium, the viable cell count ceased increasing in about an hour and then started decreasing after incubation for another hour, even though cells were plated on an IPTG-containing medium (Fig. 2A). Meanwhile the culture turbidity continued to increase. The cells became filamentous, indicating that the strain was unable to divide (Fig. 2B). These results provided a final proof that *ftsI* is an essential gene for cell division and viability.

A 1.9-kb upstream region is required for a single copy of *ftsI* to complement the null allele. The *ftsI* gene is immediately preceded by a promoter-like sequence (42). Upstream is the

TABLE 3. Complementation test of Δ*ftsI*::*cat* by transformation[a]

Expt no.	Plasmid	No. of Km^r transformants	
		JE7947 (Δ*ftsI*::*cat*)	JE7948 (*ftsI*$^+$)
1	None	0	0
	pHR213	0	86
	pHR290	387	59
	pHR301	0	101
	pHR311	560	142
	pHR309	288	94
	pHR317	0	141
2	pHR213	0	123
	pHR290	82	128
	pHR379	109	62
	pHR377	131	193

[a] Strains harboring pHR296 (mini-F, Ap^r) were transformed with the mini-F plasmid (Km^r) to be tested. Results of typical experiments are shown. The same batches of competent cells and similar amounts of plasmid DNA were used for each experiment.

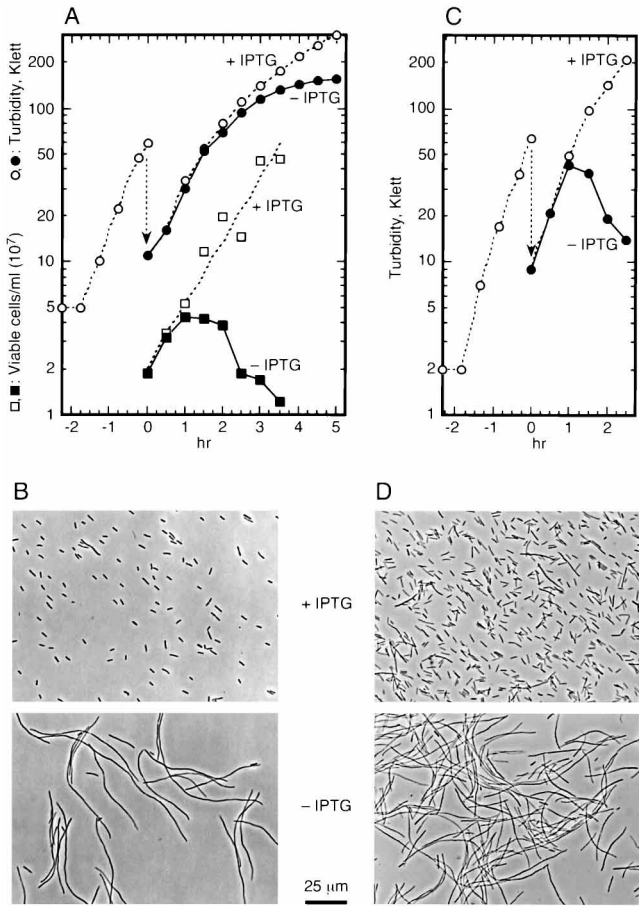

FIG. 2. (A) Effect of repression of the *ftsI* expression. Exponentially growing cells of a Δ*ftsI*::*cat srl*::Tn*10 recA* strain, JE7943, harboring a mini-F plasmid, pHR295, which carries *ftsI* under the control of the *lac* promoter and *lacI*^{q}, were incubated in the presence (open symbols) and the absence (closed symbols) of IPTG, with culture turbidities (circles) and viable cell numbers (squares) monitored. Viable cells were counted by plating diluted cultures onto Luria agar plates (about 30 ml) with soft Luria agar (0.5%) containing 15 μl of 1 M IPTG and incubating at 37°C overnight. (B) Micrographs of a Δ*ftsI*::*cat recA* strain, JE7947, harboring pHR295, that was incubated for 3 h in the presence (+) and the absence (−) of IPTG. (C) Lysis of a P_{mra}::P_{lac} strain, JE7968, upon the removal of IPTG from the medium. Exponentially growing cells were incubated in the presence (open circles) and the absence (closed circles) of IPTG. (D) Micrographs of a P_{mra}::P_{lac} *recA* strain, JE7970, harboring plasmid pHR478, which carries the chromosomal fragment from the *mra* promoter to *murD*, after incubation for 2 h in the presence and the absence of IPTG. In all of these experiments, exponential cultures grown to about 60 Klett units at 37°C in BLGT medium containing the appropriate antibiotics and 0.5 mM IPTG were washed and resuspended to about 10 Klett units in the same medium without IPTG at time zero and then incubated with and without 0.5 mM IPTG.

ftsL gene (also named *mraR*), which is also immediately preceded by another promoter-like sequence (19, 28, 56). These promoter-like sequences are included together with the whole *ftsI*-coding sequence in a 2.6-kb *Pvu*II fragment. When cloned on multicopy vectors, this fragment complemented the *ftsI*(Ts) mutation even if placed in the opposite orientation within the drug resistance genes of the vector. However, the same *Pvu*II fragment did not complement the *ftsI* null allele when cloned on a single-copy mini-F vector pMF3. The plasmid pHR296 carried the fragment, with the proximal end joined to the staphylococcal *blaZ* fragment of the vector, thus placing *ftsI* under the control of the *cadA* promoter (43), and the orientation was opposite in pHR297 (Fig. 1). JE7936 harboring

either of these plasmids was transduced to Leu$^+$ from an Δ*ftsI*::*cat* strain harboring pHR223, and it was found that pHR296 enabled the host to receive the null allele whereas pHR297 did not (Table 2). Complementation tests of mini-F plasmids were also tried by transforming JE7611 [*ftsI*(Ts)], but the results were not clear because of the thermosensitivity of the F replicon (51).

Since the promoter-like sequences immediately upstream of *ftsI* in the 2.6-kb *Pvu*II fragment did not allow complementation of the null mutation, we next tested pHR213 (Fig. 1), which carries a 7.3-kb *Hin*dIII-*Eco*RI fragment containing *ftsI* on the mini-F vector pSY396. The fragment includes 1.9 kb of DNA upstream of *ftsI*, yet Δ*ftsI*::*cat* could not be cotransduced with *leu* to a pHR213-harboring strain from a pHR4-harboring strain (Table 2). The chromosomal insert of pHR213 did, however, complement the *ftsI*(Ts) mutation when recloned onto pBR322. We then cloned into pSY396 a longer chromosomal fragment, starting at another *Hin*dIII site that is farther upstream by 1.8 kb (pHR290) (Fig. 1). A transduction experiment showed that pHR290 complemented the disrupted *ftsI* (Table 2). The complementation ability of Km^r mini-F plasmids was also tested by transformation of JE7947 (Δ*ftsI*::*cat recA*) harboring pHR296 (Ap^r). Since mini-F plasmids are incompatible with each other, pHR296 should have been displaced in Km^r transformants. As shown in Table 3, pHR290 could displace pHR296 in a Δ*ftsI*::*cat* strain, whereas pHR213 could not; both could displace pHR296 in JE7948, an isogenic *ftsI*$^+$ strain. Hence, the region further upstream of the 1.9-kb-upstream *Hin*dIII site is necessary for a single-copy *ftsI* to complement the null allele.

The chromosomal fragment cloned into pHR290 was shortened from the upstream *Hin*dIII site by 0.7 kb (to the *Aat*II site [pHR311]) and by 1.6 kb (to the *Hpa*I site [pHR309]) (Fig. 1). These mini-F plasmids complemented Δ*ftsI*::*cat*, as shown by transformation experiments (Table 3). Thus, the region necessary for the complementation extends at most up to the *Hpa*I site 2.1 kb upstream of *ftsI*. Since the upstream region is intact on the chromosome in the Δ*ftsI*::*cat* strain, there seems to be a sequence required in *cis*. The inversion of the upstream 1.8-kb *Hin*dIII fragment in pHR290 (pHR301) (Fig. 1) resulted in the loss of complementation ability (Table 3), indicating that the orientation of the upstream region or the contiguity at the *Hin*dIII site at 1.9 kb upstream or both were essential. We disrupted this *Hin*dIII site in pHR309 by performing the filling reaction of T4 DNA polymerase followed by ligation, thus introducing a 4-bp insertion, which was confirmed by creation of a new *Nhe*I site. A test by transformation (Table 3) showed that the resultant mini-F plasmid pHR317 did not complement Δ*ftsI*::*cat* (Table 3). Therefore, this *Hin*dIII site seems to be within the essential sequence.

These mini-F plasmids were tested for their ability to produce PBP 3 in PBP assays using radioactive penicillin (Fig. 3). Whereas no PBP 3 was detectable in the JE7611 cells harboring pHR213, which carries two promoter-like sequences immediately upstream of *ftsI*, a normal level of PBP 3 was found in those with the complementing plasmids pHR290, pHR311, or pHR309. The pHR317-harboring cells showed a very faint band, indicating that disruption of the *Hin*dIII site led to a great decrease in PBP 3 production.

A promoter sequence 1.9 kb upstream of *ftsI*. The DNA sequence around the *Hin*dIII site 1.9 kb upstream of *ftsI* contains a sequence very similar to the consensus promoter sequences recognized by σ^{70}-containing RNA polymerase: TTGACAN$_{18}$TAAACT (underlined bases correspond to the consensus [Fig. 1]). The *Hin*dIII site is between the −35 and −10 regions. The recognition sequence overlaps the −35 sequence by 1 bp. The disruption of this *Hin*dIII site in pHR317 should have increased the spacing between the −35 and −10 regions to 22 nt (the consensus is 15 to 18 nt). To test whether this sequence is sufficient for a single-copy *ftsI* to complement the *ftsI* deletion-insertion, we first created an *Eco*RI site on its 5′ side by inserting an oligonucleotide and cloned a fragment from this site to the *Eco*RI site within *mraY* on mini-F vector pSY396. The resultant plasmid (pHR379) (Fig. 1) complemented Δ*ftsI*::*cat* (Table 3). Next, this promoter-like sequence plus several base pairs downstream was chemically synthesized with additional restriction sites on each end. This fragment was joined to the *ftsI* gene at a site a little upstream of its putative ribosome-binding site and cloned into pSY396. This plasmid (pHR377) (Fig. 1) also complemented Δ*ftsI*::*cat* (Table 3). JE7611 cells harboring these plasmids were assayed for PBPs and found to contain a normal level of PBP 3 (Fig. 3). These results indicate that the promoter identified 1.9 kb upstream is necessary and sufficient to express the *ftsI* gene at a normal level from a single copy. The sequences immediately upstream of *ftsI* and of *ftsL* that were reported to resemble the consensus σ^{70} promoter sequence did not allow production of PBP 3 as found for pHR213 (Fig. 3). These sequences were not included in pHR377 and hence were shown to be unnecessary for the *ftsI* expression.

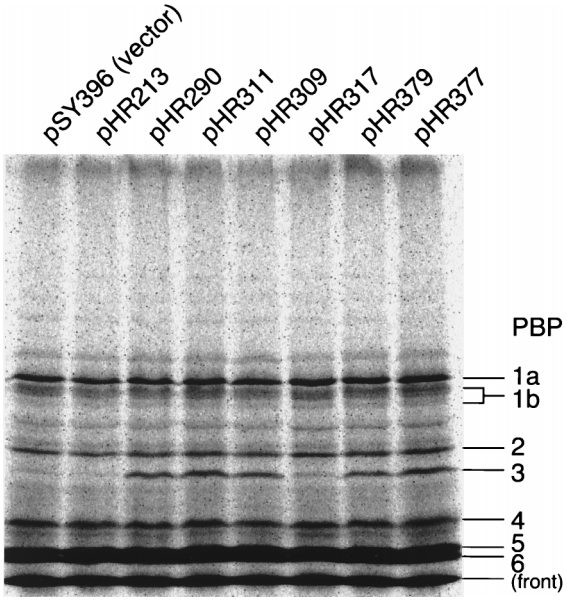

FIG. 3. PBP assay of an *ftsI730*(Ts) *recA* strain, JE7611, harboring mini-F plasmids that carry *ftsI* with and without the *mra* promoter.

The 1.9-kb upstream sequence was shown to actually enhance the expression of promoterless *lacZ* on a mini-F plasmid (Fig. 4). The synthetic DNA used for the construction of pHR377, with or without extra insertions, was cloned into a promoter analysis vector, pFZY1ΔH, at the polylinker upstream of the promoterless *galK′-′lacZ* fusion gene, which encodes the hybrid protein with functional β-Gal activity. A Δ*lac* strain, MC1061-5, was transformed with the resultant plasmids and assayed for β-Gal activity. When the intact sequence was placed in the proper orientation (pHR356 and pHR360) (Fig. 4), high β-Gal activity was observed. In the inverted orientation (pHR357), only a background level of activity was observed. Disruption of the *Hin*dIII site in the sequence by insertion of the *neo* gene in the opposite orientation (pHR351

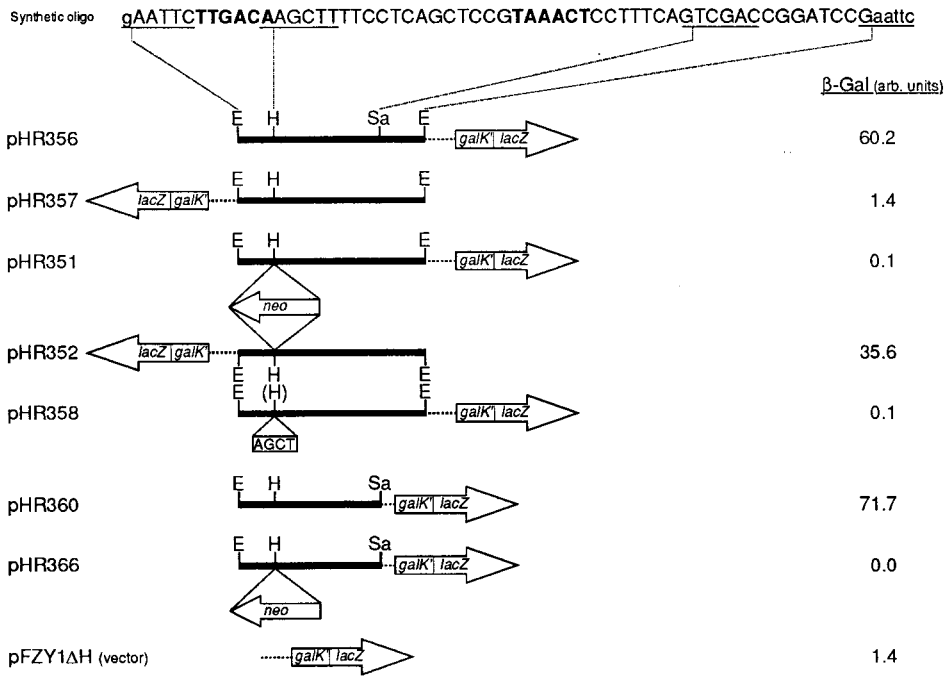

FIG. 4. β-Gal assays of *mra* promoter activity. The synthetic oligonucleotides used were the same as those used in the construction of pHR377 (Fig. 1). Open arrows with *neo* and the open bar with AGCT indicate the *neo* fragment inserted into the *Hin*dIII site in the orientation opposite to the *mra* promoter and a small insertion created by the filling reaction of T4 DNA polymerase, respectively. Abbreviations for restriction site are as given in the legend to Fig. 1. β-Gal activities (shown in arbitrary units [arb. units]) are averages of the measurements from three transformants.

and pHR366) or of 4 bp created by filling and religating as in pHR317 (pHR358) abolished the β-Gal activity.

The promoter sequence we have identified here forms an operon of *ftsI* together with at least the three open reading frames (ORFs) upstream, that is, a 456-base ORF which immediately follows the promoter and is temporarily named *orfC* in the features section of EMBL, GenBank, and DDBJ nucleotide sequence database entry EC2MIN, a gene coding for a 35-kDa protein (18), and *ftsL* (Fig. 1). Hereafter in this work, the promoter will be referred to as the *mra* promoter because it is located at the beginning of the *mra* cluster.

The *mra* promoter is responsible for expression of the five next genes downstream of *ftsI*, i.e., *murE*, *murF*, *mraY*, *murD*, and *ftsW*. The question naturally arises as to whether the *mra* promoter drives more of the 16 contiguous genes that exist in the *mra* cluster without a recognizable transcriptional terminator. In an experiment done in parallel to the construction of a Δ*ftsI*::*cat* allele on the chromosome, we attempted to replace the chromosomal *ftsI* with another deletion-insertion mutant (Δ*ftsI*::cat_{opp}), in which *cat* was substituted for the same internal segment of *ftsI* as in Δ*ftsI*::*cat* but was inserted in the opposite orientation to *ftsI*. A pHR4 derivative carrying Δ*ftsI*::cat_{opp} underwent integration into and resolution from the chromosome in the *polA*(Ts) background (see Materials and Methods), and P1 transduction to a *leu*::Tn*10* strain harboring pHR290, selecting for Leu^+, was tried. Among 16 transductants examined, none was Cm^r. In a similar transduction experiment, Δ*ftsI*::*cat* having *cat* in the same orientation as *ftsI* was found in 4 out of 15 Leu^+ transductants, indicating that, after integration and resolution of the Δ*ftsI*::*cat*-carrying plasmid, nearly half of the *polA*(Ts) cells had retained the disrupted gene on the chromosome. Such a discrepancy between Δ*ftsI*::*cat* and Δ*ftsI*::cat_{opp} can be explained if the *cat* insertion into *ftsI* in the opposite orientation gives a polar effect on essential genes beyond *murF* that are not covered by pHR4 in the transductional donor or by pHR290 in the recipient. We suspected that transcription originating from the *mra* promoter might proceed along the entire cluster to the only known terminator just downstream of *envA* (3) and be at least partly responsible for expression of all the genes in the cluster (see Discussion).

To examine how far into the *mra* cluster the function of the promoter at its beginning was required, we disrupted the promoter on the chromosome at the *Hin*dIII site and inserted the *lac* promoter (Fig. 5) as described in Materials and Methods. The inserted DNA fragment was composed of the *cat* gene followed by the *rrnB* transcriptional terminators, the $lacI^q$ gene in the opposite orientation, and the *lac* promoter in the same orientation. In the resultant P_{mra}::P_{lac} strain, the genes normally dependent on the *mra* promoter for their expression were expected to depend on the *lac* promoter. The P_{mra}::P_{lac} strain JE7968 did not grow at all in the absence of IPTG. When the cells grown in the presence of IPTG were deprived of the *lac* inducer, they started to lyse after about an hour (Fig. 2C). The loss of PBP 3 (Fig. 2A and B) or FtsL (19) would cause cell filamentation, not lysis. The lysis was probably due to repressed expression of one or more of the downstream murein biosynthetic genes. Two upstream genes, the gene coding for the 35-kDa protein and *orfC*, would also be repressed, but they have been found not to be essential for growth and viability (20).

The growth defect in the absence of IPTG of P_{mra}::P_{lac} *recA* strain JE7970 was not corrected by pHR311 (Fig. 5), which covers as much of the cluster as do pHR4 and pHR290. These plasmids did not support construction of chromosomal Δ*ftsI*::cat_{opp} as described above. When the 21-kb *Aat*II fragment containing the entire cluster was cloned into a mini-F vector, pSY396 (pHR416) (Fig. 5), and introduced into

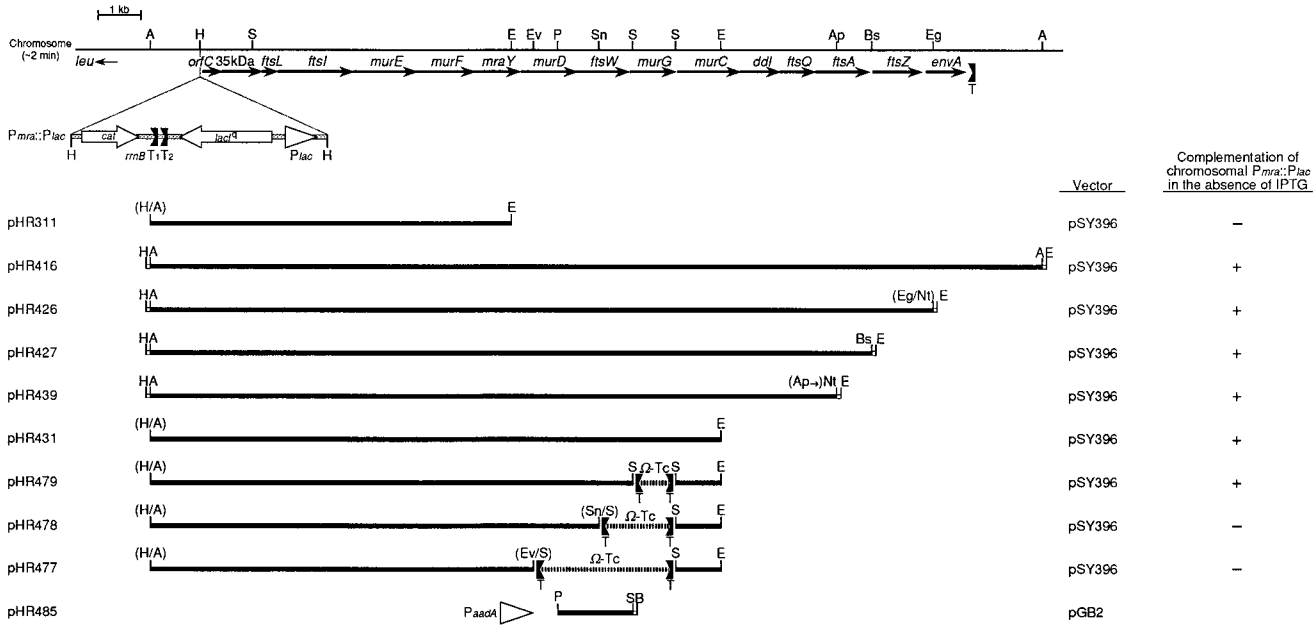

FIG. 5. Plasmids carrying chromosomal fragments of the 2-min region. Only the cloned chromosomal regions are shown (filled bars). Short, open bars represent polylinkers derived from synthetic oligonucleotides (pHR416, pHR426, pHR427, and pHR439) and from the Ω-Tc interposon (pHR485). The open arrowhead in pHR485 denotes the direction of the promoter on the vector. The orientation and approximate sizes of the *mra* cluster genes and the structure of P_{mra}::P_{lac} are shown at the top. The thick brackets with T's beneath them represent ρ-independent transcriptional terminators. Parentheses surrounding enzyme pairs indicate the ligation of compatible cohesive ends [(Eg/Nt)] or of blunt or filled-in ends. (Ap→)Nt stands for the *Apa*LI-cut end that was first ligated to a *Not*I linker (5′-dGCGGCCGC-3′) and then joined to the *Not*I site. The maps are drawn to scale except for the polylinkers and P_{mra}::P_{lac}. Ω-Tc interposons (broken bars) are drawn not in their own sizes but in the sizes of the respective fragments they displaced. Complementation of chromosomal P_{mra}::P_{lac} was tested by cross-brushing a P_{mra}::P_{lac} *recA* strain, JE7970, transformed with the respective plasmids against a brush of 200 mM IPTG on Luria agar plates and incubating at 37°C overnight. Abbreviations for restriction sites (only relevant sites are shown): A, *Aat*II; Ap, *Apa*LI; B, *Bam*HI; Bs, *Bsu*36I; E, *Eco*RI; Eg, *Eag*I; Ev, *Eco*RV; H, *Hin*dIII; Nt, *Not*I; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Sn, *Sna*BI.

JE7970, the transformant grew normally in the absence of IPTG. The chromosomal fragment on pHR416 was shortened from the cluster's distal end so that *envA*, *ftsZ*, and *ftsA* genes were deleted one by one (pHR426, pHR427, and pHR439) (Fig. 5). Although a long transcript covering the entire cluster was suspected at first, these distal genes proved to be unnecessary to complement the growth defect of JE7970. Next, the 13-kb chromosomal fragment ranging from the *Aat*II site upstream of the *mra* promoter to the *Eco*RI site within *murC*, thus covering up to *murG*, was cloned into pSY396 (pHR431) (Fig. 5) and was also found to correct the IPTG dependence of JE7970. When a 1.0-kb *Sma*I fragment within *murG* was deleted from pHR431 and replaced with an Ω-Tc interposon (14), the resultant plasmid pHR479 (Fig. 5) still corrected the IPTG dependence. The *murG* gene codes for *N*-acetylglucosaminyl transferase, essential for murein synthesis (39). Finally, the complementation ability was abolished when the deletion was extended upward to within *ftsW* (pHR478) or to within *murD* (pHR477) (Fig. 5). When deprived of IPTG, JE7970 harboring pHR478 did not lyse but grew into long, filamentous cells (Fig. 2D), indicative of the repressed expression of *ftsW*, whose defect is known to cause cell division block (28, 29). This division defect was complemented by additional introduction of pHR485 (Fig. 5), a plasmid expressing *ftsW* under the promoter for the aminoglycoside 3′-adenyltransferase (*aadA*) gene of the vector. Therefore, extrachromosomal expression of genes up to *ftsW*, the fifth gene downstream of *ftsI*, was necessary to complement the defect due to disruption of the chromosomal *mra* promoter. We conclude that the *mra* promoter is required for these first nine genes of the cluster to be fully expressed.

The growth defect of JE7970 harboring pHR477 was not corrected by pHR485, and the cells harboring these two plasmids lysed upon removal of IPTG. Neither of the plasmids carried *murD*, and its repression seemed responsible for the lysis. Although no mutation in the *murD* gene has been previously described, a lysis phenotype is very likely, since the *murD* gene product, the D-glutamic acid-adding enzyme (38), serves an essential function in the murein biosynthetic pathway.

DISCUSSION

We identified a promoter at the beginning of the *mra* cluster, a cluster of cell division and cell envelope biosynthesis genes in the 2-min region of the *E. coli* chromosome. Displacement of this *mra* promoter with the *lac* promoter (P_{mra}::P_{lac}) resulted in cell lysis in the absence of a *lac* inducer, and the defect was complemented by plasmids containing chromosomal fragments from the *mra* promoter to at least *ftsW*, the ninth gene of the cluster. With a plasmid containing up to the eighth gene but not the intact *ftsW*, the P_{mra}::P_{lac} strain showed a cell division defect, which was corrected by the addition of a plasmid expressing *ftsW*. We thus conclude that the *mra* promoter was required for full expression of the first nine genes, up to *ftsW*, of the cluster. Although the length of the transcript from the *mra* promoter has not been determined, it seems probable that transcription proceeds for more than 10 kb, to *ftsW* and beyond. All the genes from *orfC* through *envA* are tightly clustered in the same orientation without a transcriptional terminator.

Dai and Lutkenhaus (8) reported that a null allele of *ftsZ* was not complemented by a λ transducing phage that carried 6 kb of DNA upstream of *ftsZ*, including promoters located within *ftsA* and *ddl* (65), but was complemented by F′104,

which carried a much larger chromosomal insert, including the whole of the *mra* cluster. They suggested that a portion of *ftsZ* expression was due to a promoter(s) located more than 6 kb upstream. We first suspected that the *mra* promoter might be the upstream promoter that they had postulated to be required for full expression of *ftsZ*. However, the P_{mra}::P_{lac} strain carrying the *mra* promoter-*ftsW* region on a plasmid grew normally in the absence of IPTG, without extrachromosomal expression of genes further downstream, including *ftsZ*, indicating that these genes could be expressed independently of the *mra* promoter. There might be basal-level transcription from the *lac* promoter even in the absence of an inducer, but such transcription was unlikely to be sufficient for expression of the downstream genes, considering the existence of $lacI^q$ in the P_{mra}::P_{lac} strain and inclusion of glucose in media used in the experiments, both leading to tighter repression (4). Actually, the uninduced *lac* promoter in the P_{mra}::P_{lac} strain could not support sufficient expression of the proximal *ftsL*, *ftsI*, and *ftsW* genes, whose products have been shown to be very minor components of the cell (13, 19, 29, 49).

The *murG* gene coding for *N*-acetylglucosaminyl transferase is essential, as indicated by an *murG*(Am) *supF*(Ts) strain which lyses at the restrictive temperature (39). Coding frames of *ftsW* and *murG* are overlapped by 1 base or separated by 23 bases, depending on the two possible initiation codons of *murG*, and a promoter(s) for the *murG* gene should be somewhere in the *orfC-ftsW* region, possibly in the just-preceding *ftsW* gene. Analysis of the DNA sequence reveals a promoter-like sequence TTGGCGN$_{17}$TACGCT (nt 15203 to 15231 of nucleotide sequence database entry EC2MIN [underlined bases correspond to the consensus]) towards the 5′ end of *ftsW* (nt 14933 to 16174). The upstream endpoint of the cloned fragment in λ16-2, a transducing phage that contained *ftsZ* but failed to complement an *ftsZ* null allele, was in the midst of *ftsW* (8), and therefore a promoter(s) responsible for at least a portion of expression of genes from *murG* to *ftsZ* seemed to lie in the 5′ half of *ftsW* or further upstream.

There appear to be many potential promoter sequences in the *mra* cluster. The *envA* gene has its own promoter located in the intergenic space between *ftsZ* and *envA*, which seems sufficient for its expression (3, 8). The ability of the promoters in the *ddl-ftsA* region to regulate *ftsQAZ* expression has been studied (e.g., see references 15 and 47). Mengin-Lecreulx et al. (38) suggested the existence of several promoters in the *ftsI-murD* region. Although we have shown in the present study that the *mra* promoter is necessary for expression of genes up to *ftsW*, it is not known if the promoter alone is sufficient for expression of all of these genes. Distal genes might be partly dependent on the potential promoters in the *ftsI-murD* region, whose activities are yet to be examined. These promoters, if really active, possibly contribute to expression of *murG* and its downstream genes, too. Several promoter-like sequences have been described for the *orfC-ftsL* region (19, 28, 42) (also described in the features section of nucleotide sequence database entry EC2MIN). However, mini-F plasmid pHR213, which contains all of these sequences, did not lead to detectable production of PBP 3. Thus, these sequences seemed to have little if any promoter activity, at least under the conditions used, although the possibility was not excluded that they might play important roles in subtle adjustments under conditions not tested in the present study. There have been interesting reports for the 35-kDa protein gene-*ftsL* region, a sequence that has high homology with the LexA repressor-binding sequence (28) and a negative control by *mreB* of the *ftsI* expression (60). It is not known whether there is any effect of the SOS response on expression of the *mra* cluster genes or whether the negative control by *mreB* is transcriptional or posttranscriptional. Between the *mra* promoter and the *shl* gene upstream (33) is a 0.6-kb silent region that contains no ORF of significant length. It is interesting if there is any regulatory element affecting the *mra* cluster expression. However, no characteristic sequence has yet been identified in this silent region. As for the amount of PBP 3, no fluctuation was observed during the cell division cycle (63), whereas a downregulation under the direct or indirect influence of *rpoS* was reported at the entry into stationary phase (12). Further detailed analyses are necessary to elucidate the regulatory mechanism of the complex multipromoter system in the *mra* cluster.

PBP 3 and PBP 2 are members of class B high-molecular-mass PBPs (16). PBP 3 is thought to function in conjunction with FtsW for septal murein synthesis, which is analogous to PBP 2's function in cell elongation and maintenance of the rod shape in conjunction with RodA (27), a protein that is structurally very similar to FtsW (25). Under the control of the *mra* promoter, *ftsI* and *ftsW* are in a single transcriptional unit, as are *pbpA*, the structural gene coding for PBP 2, and *rodA* (37). This may be convenient for the bacterium to coordinate expression of these pairs of cooperating genes. In *Bacillus subtilis*, however, *spoVD* (9) and *spoVE* (25), which code for the homologs of *E. coli* PBP 3 and FtsW, respectively, required for spore cortex synthesis, seem to be transcribed separately (55), although they are within a gene cluster at 133 to 135° on the chromosome that is homologous to the *E. coli mra* cluster. The *pbpB* gene coding for PBP 2B, another homolog of *E. coli* PBP 3 that functions during vegetative growth and asymmetric spore septum formation (64) is also within the cluster at 133 to 135° but is transcribed separately from *spoVD* and *spoVE* (10). An ORF named *ipa-42d*, whose predicted product is highly similar to FtsW and RodA (17) and possibly functions in conjunction with PBP 2B, is well separated from the cluster at 133 to 135°.

In *B. subtilis* and also in *Haemophilus influenzae*, the genes homologous to the *E. coli mra* cluster genes are found clustered on their chromosomes, and the order of homologous genes is highly conserved among these three species. This is striking, considering that the conservation of the order of more than two homologous genes is very rare (62). Such exceptional conservation may not be explained simply by assuming constraints on the transcriptional combinations of the functionally homologous genes during evolution.

All the genes with known functions in this cluster are essential for cell division and/or envelope synthesis. Although no *E. coli* mutant defective in *murD* has been isolated, it was shown in the present study that repression of *murD* in the P_{mra}::P_{lac} strain with the other *mra* promoter-dependent genes expressed from plasmids resulted in cell lysis, indicating that the gene is indispensable. Functions are not known for the first two genes of the cluster, *orfC* and the gene coding for a 35-kDa protein, except for the existence of an *S*-adenosylmethionine-binding motif in the latter (30). These genes are well conserved in the homologous clusters of *B. subtilis* and *H. influenzae*. They are conserved even on the chromosome of *Mycoplasma genitalium* together with *ftsZ*, while the other genes of the cluster are absent from this murein-free organism. Hence, the first two genes have been assumed to play some important roles related to cell division. However, a *Pvu*II fragment that covers the most parts of these genes could be deleted from the *E. coli* chromosome and displaced by an antibiotic resistance gene of the same orientation with no apparent defect in growth or division (20). The *B. subtilis* homologs of these two genes have recently been shown to be nonessential (10).

Multicopy plasmids that carry *ftsI* and its upstream region

but not the *mra* promoter in the opposite orientation within drug resistance genes complemented the *ftsI*(Ts) mutation. This could be a cumulative effect of very weak promoters at high copy numbers but is more likely due to transcriptional readthrough from another promoter(s) on the vector. By using single-copy mini-F vectors, one can eliminate a possible effect of high copy numbers. The mini-F vector pMF3 (34) has a promoter, originally for the *cadA* gene (43), in its antibiotic resistance gene (*blaZ*) fragment. The transcription from it flows into the *Eco*RI cloning site. When cloned into this *Eco*RI site, the *Pvu*II fragment containing *ftsI* but not the *mra* promoter did not complement the *ftsI* null allele unless placed in the same orientation under the control of the *cadA* promoter. Only in this orientation was complementation observed also for the *prc* gene (23). Thus, only negligible if any transcription seems to extend out of the F replicon fragment beyond the *Eco*RI site. In pSY396, another mini-F vector used in this study (see Materials and Methods), the antibiotic resistance gene (*neo*) fragment does not seem to contain a promoter that directs transcription flowing into the *Eco*RI-*Hin*dIII cloning region, because pHR213 did not lead to production of a detectable amount of PBP 3; in pHR213, the *ftsI*-containing *Hin*dIII-*Eco*RI fragment was cloned with the *Hin*dIII site within the *mra* promoter sequence joined to the *Hin*dIII site of the *neo* fragment of pSY396. As described above for pMF3, no transcription proceeds into the *Eco*RI-*Hin*dIII region from the F replicon fragment. Thus, a gene(s) cloned in this region of pSY396 is not subjected to transcriptional read-through from either side. This is useful in examining promoter function at a single copy in vivo.

We discovered the existence of the *mra* promoter by testing the ability of *ftsI*-containing fragments cloned on pSY396 to complement a chromosomal null allele of *ftsI* (Δ*ftsI*::*cat*) in which a large internal fragment of the gene was deleted and the antibiotic resistance gene (*cat*) fragment was inserted in the same orientation. The deletion-insertion mutation with the *cat* fragment in the opposite orientation (Δ*ftsI*::cat_{opp}) could not be crossed into the chromosome even in the presence of plasmids that allowed the crossing into the chromosome of Δ*ftsI*::*cat*. This is probably because transcription of the *cat* gene in Δ*ftsI*::cat_{opp} counteracts transcription from the *mra* promoter and diminishes expression of the downstream essential genes that were not covered by the plasmids used in the experiment. One should be aware that such polar effects can occur in gene disruption experiments.

The Δ*ftsI*::*cat* strain carrying an extrachromosomal copy of *ftsI* under the control of the *lac* promoter was dependent on a *lac* inducer for cell division and viability, confirming the essential function of PBP 3. When incubated for 2 h or longer after removal of a *lac* inducer from the medium, cells were still growing into longer and longer filaments, but most cells became unable to form colonies on an IPTG-containing agar plate. This seems to indicate that they could not restore the ability to divide even if *ftsI* expression was resumed, although there is another possibility, namely, that filamentous cells were easily killed during the plating procedure. In normally dividing cells, PBP 3 is thought to interact with many other proteins involved in cell division to form a multimolecular complex. Depletion of PBP 3 might lead to formation of abortive complexes lacking this essential protein, which might be incapable of reactivation by incorporating PBP 3 afterwards. This could cause unrepairable damage to the cell. Genetic analyses have suggested interaction of PBP 3 with other cell division proteins, including FtsA and FtsZ, direct interaction with the latter being just recently demonstrated by new methodologies (58). For a thorough understanding of the bacterial cell division process, elucidation of interactions of division proteins as well as of transcriptional regulations of their genes will be required.

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