Selected Amplification of the Cell Division Genes *ftsQ-ftsA-ftsZ* in *Escherichia coli*

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ABSTRACT

Rapidly growing *Escherichia coli* is unable to divide in the presence of the antibiotic mecillinam, whose direct target is penicillin-binding protein 2 (PBP2), responsible for the elongation of the cylindrical portion of the cell wall. Division can be restored in the absence of PBP2 activity by increasing the concentration of the cell division proteins FtsQ, FtsA, and FtsZ. We tried to identify regulators of the *ftsQ-ftsA-ftsZ* operon among mecillinam-resistant mutants, which include strains overexpressing these genes. By insertional mutagenesis with mini-Tn*10* elements, we selected for insertions that conferred mecillinam resistance. Among 15 such mutants, 7 suppressed the thermosensitivity of the *ftsZ84*(Ts) mutant, strongly suggesting that they had increased FtsZ activity. In all 7 cases, however, the mutants resulted from a duplication of the *ftsQAZ* region. These duplications seemed to result from multiple events, suggesting that no simple insertional inactivation can result in a mutant with sufficiently amplified *ftsQAZ* expression to confer mecillinam resistance. The structure of the duplications suggests a general method for constructing directed duplications of precise sequences.

GROWTH of rod-shaped bacteria such as *Escherichia coli* takes place in two different modes, lateral extension of the cylindrical portion of the rod and formation, at midcell, of a septum, or cross wall, which becomes the new pole of each daughter cell. The elongation phase of growth distinguishes bacilli from cocci, which grow and divide by pure septation. In *E. coli*, elongation specifically requires penicillin-binding protein 2 (PBP2; Spratt 1975). During rapid growth, however, a block of the elongation process brought about by inactivation of PBP2 also results in a specific arrest of cell division (Vinella *et al.* 1993) and consequent cell death (Matsuhashi *et al.* 1974; James *et al.* 1975). Division in the absence of PBP2 activity can be restored if the cell division proteins FtsQ, FtsA, and FtsZ are all overexpressed (Vinella *et al.* 1993; Joseleau-Petit *et al.* 1994). These proteins thus seem to participate in a sort of cell division potential that has to be increased to allow cell division in spherical *E. coli* cells growing in rich medium.

The *ftsQ*, *ftsA*, and *ftsZ* genes are adjacent to each other on the chromosome (Robinson *et al.* 1984, 1986; Yi *et al.* 1985), within the 16-gene cluster of cell wall and cell division genes located at 2 min. These genes, governed by multiple promoters, are known to respond to several transcriptional regulators—σ^S, SdiA, and RcsB (for review, see Joseleau-Petit *et al.* 1999). In the hope of identifying additional transcriptional regulators of the *ftsQ-ftsA-ftsZ* operon, we selected mutants in which the expression of these three genes was increased enough to permit cell division in the absence of PBP2 activity.

Inactivation of PBP2 can be brought about either genetically, by mutations in the structural gene *pbpA*, or by use of the highly specific β-lactam mecillinam (Lund and Tybring 1972; Spratt and Pardee 1975; Spratt 1977). *E. coli* can be made resistant to mecillinam not only by overproduction of the division proteins FtsQ, FtsA, and FtsZ (Vinella *et al.* 1993; Joseleau-Petit *et al.* 1994), as mentioned above, but also by a moderate increase in the concentration of ppGpp (Vinella *et al.* 1992; Joseleau-Petit *et al.* 1994). This nucleotide is the effector of the stringent response (see Cashel *et al.* 1996 for review) observed when *relA*$^+$ cells are starved for amino acids (Cashel 1969; Cashel and Gallant 1969). Under these conditions, the RelA protein, bound to the ribosomes, responds whenever an uncharged cognate tRNA is presented by synthesizing a compound derived from GTP, called pppGpp or "magic spot," which is rapidly hydrolyzed to ppGpp, the probable effector of the stringent response. This nucleotide binds to RNA polymerase (Chatterji *et al.* 1998) and alters its specificity; during amino acid starvation, the high level of ppGpp produced rapidly blocks the transcription of ribosomal operons, thus arresting ribosome formation. *E. coli* has two ppGpp synthetases, as shown by the fact that *relA* mutants still have almost normal pools of the nucleotide (Lazzarini *et al.* 1971; Ryals *et al.* 1982; Metzger *et al.* 1989). The identity of the second ppGpp synthetase was unknown until the observation that Δ*relA* Δ*spoT* strains are devoid of detectable ppGpp

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(Hernandez and Bremer 1991; Xiao *et al.* 1991), strongly suggesting that the SpoT protein is the second synthetase, although no *in vitro* synthetase activity has been detected so far.

We selected mutants that had become resistant to mecillinam through insertion of a mini-Tn*10*. Initially, we expected two classes of mutants, those with increased levels of FtsQ, FtsA, and FtsZ and those with increased ppGpp levels. Of 12 independent mutants isolated and characterized, 5 remained mecillinam-resistant in the complete absence of ppGpp (*i.e.*, in a Δ*relA* Δ*spoT* genetic background). They were thus good candidates for affecting the level of expression of the *ftsQ-ftsA-ftsZ* operon. We present here a detailed analysis of these 5 mutants and 2 similar mutants previously selected. We show that their mecillinam resistance results from gene duplication via a novel mechanism that in principle could be used for specific, directed amplification of virtually any region of the chromosome.

MATERIAL AND METHODS

Bacterial strains and phages: All strains used in this work are *E. coli* K12 derivatives; the genotypes of the principal strains are given in Table 1. The strains carrying various Tn*10* transposons, kindly provided by D. Touati, are from the Singer collection (Singer *et al.* 1989). Strain CF6301 was constructed as follows: the $P1_{rrnB}$*::lacZ* fusion was first introduced from strain VH270 (Hernandez and Bremer 1990) into a Δ*lacZ argA::*Tn*10* derivative of MG1655, selecting for kanamycin resistance (which is associated with the fusion), then Δ*relA-251::kan* was introduced by cotransduction with Arg^+, donor strain CF1651, selecting on minimal glucose plates and screening for transductants sensitive to serine, methionine, and glycine, a sensitivity associated with *relA* mutants (Uzan and Danchin 1976). Strain DV250 [Δ*relA251::kan lacIpoZ*Δ(*Mlu*) $P1_{rrnB}$*::lacZ spoT::kan*] was constructed by transducing a *pyrE zib-563::*Tn*10* derivative of strain CF6301 with a P1 stock grown on a *relA1 spoT206::kan* $pyrE^+$ strain and selecting Pyr^+ transductants on minimal glucose medium supplemented with Casamino acids; strain DV250 is a Pyr^+ Tc^s clone that cannot grow on minimal glucose plates lacking amino acids, indicating the absence of ppGpp (Xiao *et al.* 1991). The *ftsZ84*(Ts) mutation was brought into a *leu::*Tn*10* derivative of CF1742, selecting Leu^+ transductants on minimal glucose plates; strain DV262 is a Leu^+ clone unable to grow at 42° on Luria broth (LB) plates lacking NaCl. Phage λNK1324 (Kleckner *et al.* 1991) was used for random insertional mutagenesis; it carries the mini-Tn*10* (Cm^r) transposable element and the *Ptac-ats1 ats2* gene, which provides transposase *in trans*. The selection of insertional mutants was carried out as described previously (Vinella *et al.* 1996), except that kanamycin was replaced by chloramphenicol in this work.

Media and growth conditions: The rich and minimal media used in this work were, respectively, LB broth (containing 10 g/liter NaCl unless otherwise indicated) and M63 (Miller 1992). Glucose (0.4%) and amino acids (100 μg/ml) were added when required. Solid media contained 1.5% agar. Antibiotics were used at the following concentrations: 20 μg/ml chloramphenicol (Cm), 50 μg/ml kanamycin (Km), 1 or 10 μg/ml mecillinam (Mec), 20 μg/ml tetracycline (Tc), 50 μg/ml spectinomycin (Spc), and 100 μg/ml ampicillin (Amp).

DNA techniques and plasmids: Plasmids were extracted and transformations were carried out as described by Sambrook *et al.* (1989). Sequence determinations and PCR amplifications were carried out using a sequenase kit (United States Biochemical, Cleveland) and Taq polymerase (Perkin-Elmer, Norwalk, CT), respectively. The cloning vectors used in this work were pKS+ (high copy, Amp^r; Stratagene, La Jolla, CA) and pCL1920 (low copy, Spc^r; Lerner and Inouye 1990). To sequence the chromosomal DNA at the ends of the insertional element, we used the following synthetic primers (5′ to 3′): CmD, TTATTCTGCCTCCCAGAGCC and CmF, AACGGCAAAAGCACCGCCGG for the chloramphenicol-resistant mutants; and primers ISL, CTACCTTAACTTAATGA TTTTGATA and ISR, CCACCTTAACTTAATGATTTTTACC for the two kanamycin-resistant mutants.

Southern blots: Chromosomal DNA extracted from the parental strain MG1655 and its mutant derivatives was completely digested by *Eco*RI and, after agarose (0.9%) electrophoresis of ~5 μg, transferred onto hybond-N^+ nylon membranes (Amersham Life Science, Rockville, MD) using the VacuGene-XL vacuum blotting system (Pharmacia Biotech, Piscataway, NJ). After DNA fixation by UV irradiation, the membranes were probed in tubes with four different probes using ECL gold buffer (Amersham Life Science) following the exact protocol described in the manual (primary wash solution without urea at 55°, secondary wash solution containing 0.2% SSC). The primers used to generate the probes by PCR were (5′ to 3′) *fes*UP, GGTCTACATCACTGGTGTGA; *fes*DO, CGCAT GATCATCGGCTCGCG; *ftsZ*UP, GCGGTAAATACCGATGCA CAAGC; *ftsZ*DO, CATTCGGCGGGCCAGTTTAG; *yjjM*UP, GCAAGGGTGGCATCAAGGTC; *yjjM*DO, GACATGATTCGG CTCTCCAC; *ddlB*UP, TCGCGCTACACGGTCGCGGCGGTG; and *ddlB*DO, CGTACTACCAACTGCGAGAAGCTC. The probes for the *fes* (899 bp), *ftsZ* (1088 bp), *yjjM* (980 bp), and *ddlB*

TABLE 1

Bacterial strains

Strain	Genotype	Origin or reference
MG1655	Wild type	Bachmann (1996)
CF1651	As MG1655, Δ*relA251::kan*	Xiao *et al.* (1991)
CF1693	As MG1655, Δ*relA251::kan* Δ*spoT207::cat*	Xiao *et al.* (1991)
CF6301	As MG1655, Δ*relA251::kan lacIpoZ*Δ(*Mlu*) $P1_{rrnB}$*::lacZ*	This work
CF1742	As MG1655, *relA1*	M. Cashel, lab collection
DV250	As CF6301, *spoT206::kan*	This work
DV262	As MG1655, *relA1 ftsZ84*(Ts)	This work
XL1 Blue	*endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ*lac*/F′ *proAB*$^+$ *lacI*^{q} *lacZDM15* Tn*10*	Bullock *et al.* (1987)

(721 bp) genes were expected to hybridize with *Eco*RI-*Eco*RI fragments of 3048, 2232, 1812, and 838 bp, respectively. The probes were labeled with γ[^{32}P]ATP using T4 polynucleotide kinase (Sambrook *et al.* 1989); 1 pmol of each probe was used for one blot. The hybridized membranes were exposed in a storm 860 (Molecular Dynamics, Sunnyvale, CA) and the intensity of each band was measured using the Image Quant program. Using MG1655 DNA, the response was linear for each probe over the range 1.7–7.5 μg DNA.

RESULTS

Isolation of mini-Tn*10* insertions conferring mecillinam resistance: To avoid isolating mutants whose mecillinam resistance resulted from increased RelA-dependent synthesis of ppGpp, we carried out our selection in the Δ*relA* strain CF6301, derived from the wild-type strain MG1655. We made random mini-Tn*10* (Cm^r) inserts and selected simultaneously for resistance to chloramphenicol and mecillinam (at 1 or 10 μg/ml), as described previously (Vinella *et al.* 1996). Of 69 Cm^r Mec^r colonies, 52 grew on purification on the same medium, 30 with 1 μg/ml and 22 with 10 μg/ml mecillinam. The insertions from these clones were transduced back into strain CF6301, and the Cm^r transductants were tested for mecillinam resistance. We retained for further study only the 12 mutants for which mecillinam resistance was 100% cotransducible with chloramphenicol resistance (48 Cm^r transductants tested). They were provisionally named *mcr-4::cat* to *mcr-15::cat*. One Cm^r transductant of each mutant was chosen and used for further analysis.

Stability of the mutants: We tested the stability of the mini-Tn*10* insertions by growing the Cm^r transductants at 37° for at least 10 generations in LB in the absence of chloramphenicol. The strains with insertions *mcr-6*, *-7*, *-9*, *-10*, *-11*, *-13*, and *-14::cat* did not segregate Cm^s clones in these conditions (0/48), whereas the five mutants with alleles *mcr-4*, *-5*, *-8*, *-12*, and *-15::cat* did (15–71%), indicating that these insertions were unstable. Loss of chloramphenicol resistance was systematically accompanied by loss of mecillinam resistance.

We previously described the isolation of three similar mecillinam-resistant mutants, selected in a *relA1* strain by insertion of mini-Tn*10* (Km^r). Two of these mutants, *mcr-1::kan* and *mcr-2::kan*, were similarly unstable, and their instability was shown to be RecA dependent (Vinella *et al.* 1996); the *recA1* mutation also stabilized the insertions *mcr-4*, *-5*, *-8*, *-12*, and *-15::cat*.

Mecillinam resistance and ppGpp: Our insertions, isolated in a *relA* mutant, also conferred mecillinam resistance on the wild-type strain MG1655 (Table 2). It was possible that their mecillinam resistance was due to a SpoT-dependent increase of the ppGpp pool. The Δ*relA* strain cannot grow on minimal medium supplemented with glucose in the presence of serine, methionine, and glycine (SMG^s; Uzan and Danchin 1976) or in the presence of aminotriazole (AT^s; Rudd *et al.* 1985), conditions in which the cells are starved for isoleucine and valine or for histidine, respectively. Both SMG^s and AT^s phenotypes can be suppressed by *spoT* alleles that decrease the ppGpp hydrolase activity of the protein, thereby increasing the ppGpp concentration (Sarubbi *et al.* 1988). None of the *mcr::cat* insertions suppressed the SMG^s or AT^s phenotypes of the Δ*relA* mutant CF6301, suggesting that they did not sufficiently increase the ppGpp pool. However, we could distinguish in the CF6301 strain two classes of insertions on the basis of the expression of the P1$_{rrnB}$*::lacZ* fusion, which is strongly repressed by ppGpp (Table 2): one class of mutations (*mcr-7*, *-9*, *-10*, and *-14*) strongly decreased the intensity of the blue coloration of the colonies obtained on LB plates containing X-gal, as if they increased the ppGpp pool, while none of the five unstable insertions (*mcr-4*, *-5*, *-8*, *-12*, and *-15::cat*) significantly affected the expression of the P1$_{rrnB}$*::lacZ* fusion, indicating that they did not affect the ppGpp level.

We transduced the insertions into a Δ*relA spoT-206::kan* derivative of MG1655 that is completely devoid of ppGpp and therefore auxotrophic for several amino acids (Hernandez and Bremer 1991; Xiao *et al.* 1991). We recovered Cm^r transductants in all cases but one, in which the *mcr-9::cat* mutant was donor; this insertion is apparently lethal in the absence of ppGpp. None of the Cm^r insertions tested restored prototrophy in the absence of ppGpp, indicating that they have not opened an alternative pathway of ppGpp synthesis. All 12 insertions conferred mecillinam resistance on the Δ*relA* strain (original selection) and also on transductants of the wild-type strain MG1655 (Table 2). In the ppGpp-deficient Δ*relA spoT206::kan* strain, however, only the 5 unstable insertions conferred mecillinam resistance, whereas none of the 6 stable, viable insertions tested did so (Table 2). We conclude that the mecillinam resistance due to the unstable class of insertions *mcr-4*, *-5*, *-8*, *-12*, and *-15::cat* does not require ppGpp.

In conclusion, we found three classes of insertional mutants: class 1, mutants that had an increased ppGpp pool and required the presence of the nucleotide to be mecillinam resistant; class 2, mutants that seemed to have a normal ppGpp pool but, nevertheless, required the nucleotide to be mecillinam resistant; and class 3, mutants for which mecillinam resistance did not require an increased ppGpp pool and, furthermore, did not require the nucleotide at all. We have previously reported the characterization of mutants of the first two classes. Aminoacyl-tRNA synthetase mutants belong to the first class: the mecillinam resistance of these mutants (*argS*, *alaS*, and *leuS*) is due to a RelA-dependent increase of the ppGpp pool (Vinella *et al.* 1992, 1993). The *aroK* mutant (*mcr-3::kan*) belongs to the second class: its mecillinam resistance requires SpoT-dependent ppGpp synthesis (Vinella *et al.* 1996). We showed that the AroK protein, one of two shikimate kinases in *E. coli*, has a second activity involved in mecillinam

TABLE 2

Three classes of mecillinam-resistant mutants

Strains	Mecillinam resistance[a]			Expression of P1$_{rrnB}$::*lacZ* in Δ*relA* (CF6301)[b]
	WT	Δ*relA*	Δ*relA spoT::kan*	
Parental	−	−	−	++++
Class 1				
mcr-7::cat	+	+	−	+
mcr-9::cat	+	+	(Lethal)	+
mcr-10::cat	+	+	−	+
mcr-14::cat	+	+	−	+
Class 2				
mcr-11::cat	+	+	−	++++
mcr-13::cat	+	+	−	++++
mcr-6::cat	+	+	−	++++
Class 3				
mcr-4::cat	+	+	+	++++
mcr-5::cat	+	+	+	++++
mcr-8::cat	+	+	+	++++
mcr-12::cat	+	+	+	++++
mcr-15::cat	+	+	+	++++

[a] Tested at 37° on LB plates containing 1 (*mcr-6* and *mcr-10*) or 10 μg/ml mecillinam. +, plating efficiency >50% compared to the count on LB plates; −, plating efficiency $<10^{-4}$. The strain backgrounds were MG1655, CF6301, and CF1693.

[b] Tested at 37° on LB plates containing X-gal (40 μg/ml). ++++, dark blue; +, very light blue.

sensitivity and we proposed that it might be the phosphorylation of protein(s) involved in cell division (Vinella *et al.* 1996). The third class, represented by the five unstable insertion mutants, is a new class of mecillinam-resistant mutants, possibly comprising overproducers of the division proteins FtsZ, FtsA, and FtsQ. We thus focused our work on the further characterization of this class, described below.

Mecillinam resistance and FtsZ activity: To test whether the mecillinam resistance conferred by the unstable insertions is associated with an increase in FtsZ activity, we first looked to see whether the insertions suppress the phenotype of an *ftsZ84*(Ts) mutant, since increased production of the mutant FtsZ84 protein is known to restore division in nonpermissive conditions (Wang *et al.* 1991). The insertions were transduced into the strain DV262 [*relA1 ftsZ84*(Ts)] and the plating efficiency of the mutant strains was measured on LB or LB lacking NaCl (LB*) plates at 30°, 37°, and 42°. The *relA1 ftsZ84* mutant, as previously reported (Wang *et al.* 1991), grew on LB plates at all three temperatures (plating efficiency >95%) but was unable to form colonies on LB* even at 30° (plating efficiency 1×10^{-2} at 30°, 2.5×10^{-4} at 37°, and $<1.3 \times 10^{-4}$ at 42°). All seven unstable insertions (five *mcr::cat* and two *mcr::kan*) suppressed the growth defect of the *ftsZ84*(Ts) mutant on LB* at 30° and 37° (plating efficiency >56%), suggesting that these mutations increase FtsZ activity, possibly by affecting regulators of FtsZ. These results are consistent with the hypothesis that the unstable class 3 insertions confer mecillinam resistance by increasing the concentration of the cell division proteins FtsZ, FtsA, and FtsQ.

Genetic mapping of the unstable insertions: We first carried out Hfr mapping of the five *mcr::cat* unstable insertions. We transduced the *mcr::cat* alleles into Hfr strains injecting an early Tc^r marker (Wanner 1986) and crossed these with MG1655 Δ*relA::kan*, selecting either Tc^r Km^r or Cm^r Km^r exconjugants on appropriately supplemented LB plates. All five Cm^r markers cotransferred efficiently with the *thr::*Tn*10* marker (0 min) of strain BW6164 (HfrRa2), with >64% linkage between Cm^r and Tet^r, whereas no cotransfer occurred, for example, with the *ilv::*Tn*10* marker (85 min) of strain BW6159 (KL14; <2% linkage). The insertions thus all seemed to be near 0 min. The *mcr-1::kan* and *mcr-2::kan* insertions isolated previously were also found to be linked to *thr* (data not shown).

We next transduced the insertions into strains carrying Tn*10* markers regularly spaced (every 1–1.5 min) from 89.4 min (*argE::*Tn*10*) to 8.7 min (*aroL::*Tn*10*), selecting transductants on chloramphenicol- or kanamycin-containing LB plates. We never obtained Tc^s clones among at least 48 Cm^r or (in the case of *mcr-1* and *mcr-2*) 48 Km^r clones analyzed for each transduction.

Determination of the insertion sites: To determine the location of the unstable mini-Tn*10* insertions, we first cloned their Cm^r marker. Chromosomal DNA of strains carrying the unstable class 3 insertions was extracted, completely digested with the restriction enzyme *Pst*I, which does not cut within the transposable element, and cloned in the vector pCL1920 (Spc^r). These

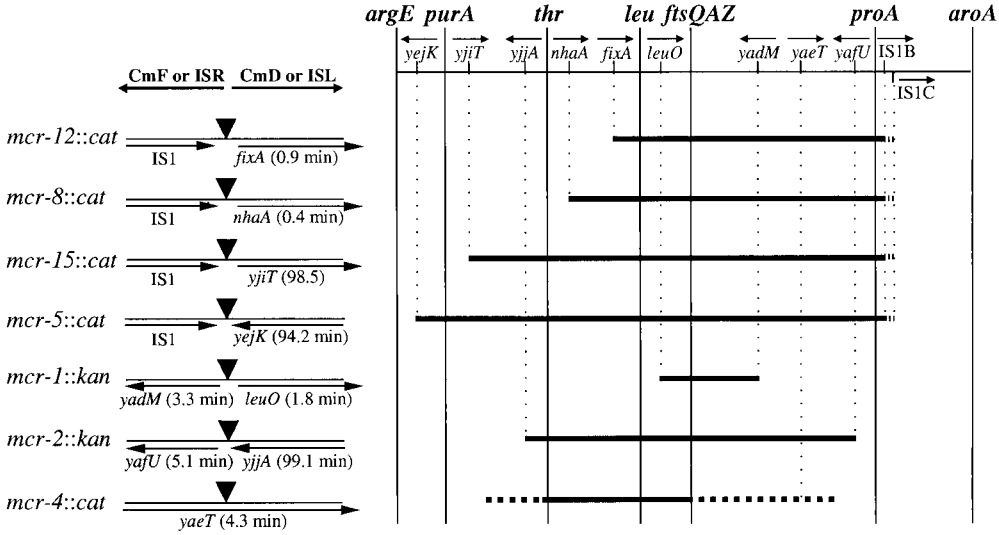

FIGURE 1.—Physical structure of the joints between the mini-Tn*10* inserts and flanking chromosomal sequences in the unstable *mcr::cat* and *mcr::kan* mutants and extent of diploidy. On the left are shown the genes flanking each mini-Tn*10* insert and their orientation. The solid triangles represent the mini-Tn*10*. On the right, the genetic map is shown (not drawn to scale), with the chromosomal order and orientation of these genes. The heavy lines indicate the extent of the duplication, as judged by diploidy for the markers in the top line and by the end points defined by the flanking regions shown on the left.

genomic libraries were used to transform strain XL1Blue, selecting on LB plates containing spectinomycin and chloramphenicol. Plasmid DNA was extracted from the Spc^r Cm^r clones and used to transform the same strain, XL1Blue, to verify that the plasmids carried both the Spc^r and the Cm^r markers. These plasmids were then partially sequenced using primers CmD and CmF to determine the joint between vector DNA and the cloned insert and to identify the adjacent chromosomal sequence. The sequences adjacent to the insertions, determined in this way, are shown on the left of Figure 1. At least four of the mutants appeared to result from an event more complex than simple insertion, involving an IS1 element.

The *mcr-1::kan* and *mcr-2::kan* inserts were similarly analyzed by cloning a fragment containing the *kan* determinant (Km^r) and adjacent chromosomal DNA in the vector pKS+. The right and left parts of the insertional element were then separately subcloned using the unique *Eco*RI restriction site present in the mini-Tn*10* element. The joint between the insertional element and chromosomal DNA, determined using primers ISR and ISL, indicated that the *mcr-1::kan* and *mcr-2::kan* mutants also had complex genomic rearrangements (Figure 1).

Transduction of the insertions creates duplications: In the course of strain constructions, we transduced the *leu::*Tn*10* marker (1.8 min) from the strain MG1655 *leu::*Tn*10* (phenotype Tc^r Leu$^-$) into the unstable *mcr::cat* derivatives of strain CF6301, selecting for Tc^r transductants. To our surprise, all Cm^r Tc^r transductants were able to grow on minimal glucose plates lacking leucine (Table 3), suggesting that the recipient strains carried a duplication of the *leu* operon. To get an idea of the extent of the putative duplications, we transduced the *mcr::cat* strains to *argE::*Tn*10* (89.4 min), *thr::*Tn*10* (0 min), *proA::*Tn*10* (5.7 min), and *aroA::*Tn*10* (20.7 min; Table 3). We also analyzed the two *mcr::kan* mutants for diploidy at these loci. The extent of the duplications, deduced from the results reported in Table 3, is shown in the right of Figure 1; all mutants except *mcr-1::kan* appeared to be diploid for at least two markers, and two displayed apparent duplication of three loci, from *thr* to *proA*, which are separated on the genetic map by 5.6 min (260 kb). Such long duplications cannot be transduced by P1, which encapsidates only ~2 min (95 kb) of DNA. The apparent transduction of these *mcr::cat* alleles is explained below.

When P1 was grown on MG1655 *leu::*Tn*10* (Tc^r Leu$^-$) and used to transduce derivatives of the wild-type (*relA*$^+$) strain MG1655 carrying the insertions (*mcr-2::kan*, *mcr-4*, *-5*, *-8*, *-12*, and *-15::cat*) to Tc^r, diploidy for the *leu* operon could again be demonstrated: all Tc^r Cm^r transductants remained prototrophic (Leu$^+$). This experiment was done with five transductants of MG1655 for each insertion. Our results indicate that the introduction of the five *mcr::cat* and the *mcr-2::kan* insertions by transduction was invariably correlated with the appearance of a duplication in the recipient strain. Moreover, we showed for

TABLE 3

The *mcr::cat* mutants have large DNA duplications

	Donor strain, Tn*10* in gene				
Recipient strain	*argE*	*thr*	*leu*	*proA*	*aroA*
mcr-1::kan	0/48	0/47	0/33	0/48	0/40
mcr-2::kan	0/48	39/39	34/34	0/41	0/40
mcr-4::cat	0/48	21/21	19/19	0/40	0/40
mcr-5::cat	0/48	26/26	33/33	23/23	0/40
mcr-8::cat	0/48	0/27	31/31	26/26	0/40
mcr-12::cat	0/48	0/34	26/26	26/26	0/40
mcr-15::cat	0/48	24/24	31/31	27/27	0/40

Number of Tc^r Cm^r transductants able to grow on minimal glucose medium. All tests were done in the presence of chloramphenicol or kanamycin. Recipient and donor strains are derivatives of MG1655 *relA1* and MG1655, respectively.

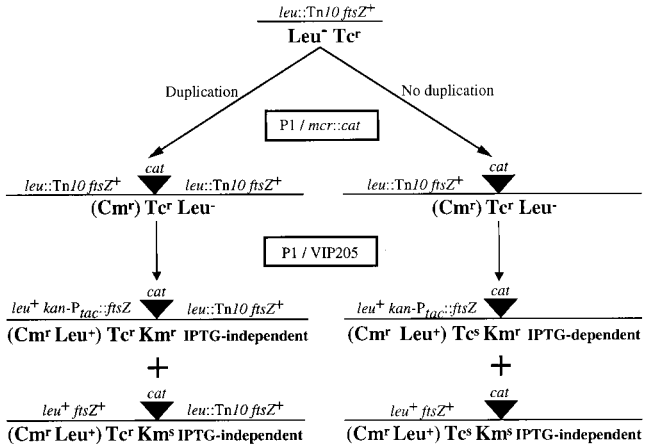

FIGURE 2.—Genetic demonstration of diploidy at the *leu* and *ftsZ* loci in *mcr::cat* strains. Donor strains for P1 transductions are shown in boxes. Selected phenotypes are in parentheses. The unselected phenotypes found correspond to the expectation for diploid strains (left).

at least one transductant of each insertion that the extent of the duplications was identical to that in the original mutant.

In reciprocal crosses, we transduced the *mcr::cat* and *mcr::kan* insertions from the CF6301 background into derivatives of the wild-type (*relA*$^+$) strain MG1655 carrying either a *thr::*Tn*10*, *leu::*Tn*10*, or *pro::*Tn*10* marker and phenotypically Thr$^-$, Leu$^-$, or Pro$^-$, respectively. As mentioned above, the Cm^r and Km^r transductants all remained tetracycline-resistant; we moreover observed that they also all remained auxotrophic (96 Cm^r or Km^r clones were tested for each transduction), indicating that the wild-type genes that were duplicated in the donor strains were not cotransduced with chloramphenicol or kanamycin resistance.

The fact that duplicated donor genes are not cotransduced with the *mcr::cat* markers presumably reflects the size of the duplications, which are longer than the 2 min that P1 can transduce. Nevertheless, introduction of the insertions seemed systematically to produce Cm^r transductants with duplications. We hypothesized that introduction of the insertions *induced* duplications of resident genes in the recipient strain. As just mentioned, when we transduced the *mcr::cat* insertions into strain MG1655 *leu::*Tn*10*, all Cm^r transductants remained Tc^r Leu$^-$. Our hypothesis would predict that they are in fact diploid for *leu::*Tn*10*. To test this, five such transductants, carrying the five *mcr::cat* insertions, were transduced to Leu$^+$ with P1 grown on a wild-type donor. Leu$^+$ Cm^r transductants were readily obtained with all five recipients, and indeed they remained Tc^r, confirming that these strains were diploid for the *leu* operon (*cf.* Figure 2).

The *mcr::cat* mutants are diploid for *ftsZ*: Since the *mcr::cat* strains are all diploid for the *leu* operon, which is closely linked to the *ftsQ-ftsA-ftsZ* operon, we wished to see whether they were also diploid for these cell division genes, potentially providing a mechanism for their mecillinam resistance (see Introduction). To test this, we took advantage of strain VIP205 (PALACIOS *et al.* 1996), in which the *ftsZ* gene has been cut off from its natural promoters by a Km^r insert that provides a *lac* promoter governing *ftsZ* expression. Since the FtsZ protein is absolutely required for division, this strain can grow only in the presence of an inducer of the *lac* operon. We introduced the 2-min region of VIP205 into the *mcr::cat* strains that were diploid for *leu::*Tn*10*, selecting transductants on minimal glucose plates lacking leucine and containing chloramphenicol and the *lac* operon inducer isopropyl thiogalactoside (IPTG) at 2×10^{-5} M. Leu$^+$ Cm^r transductants were readily obtained in each case. They were all Tc^r, and about half had also received the Km^r allele associated with the P_{tac}*::ftsZ* fusion. However, all these Tc^r Km^r Cm^r cotransductants were able to grow in the absence of IPTG, indicating the presence of a wild-type *ftsZ* allele in addition to the P_{tac}*::ftsZ* fusion (Figure 2).

These results show that the introduction of the *mcr::cat* insertions, in addition to duplicating the *leu::*Tn*10* allele, also duplicated the *ftsZ*$^+$ allele of the recipient strain. Since the *ftsQ* and *ftsA* genes lie between *leu* and *ftsZ*, they are presumably duplicated as well.

Physical evidence for amplification of the *fts QAZ* region in the mutants: Cultures of all seven unstable mutants were grown in LB containing mecillinam; the control strain MG1655 was grown in LB. DNA was extracted from overnight cultures and analyzed by quantitative Southern blots (see MATERIALS AND METHODS), using four probes. From the genetic evidence, two of these, from genes *ftsZ* and *ddlB*, were expected to be amplified in all mutants. The third probe, from gene *yjjM* at 99.0 min, was expected to be amplified in mutants *mcr-5*, *mcr-15*, and possibly *mcr-4*. The fourth probe was from the *fes* gene at 13 min, which is not included in any of the duplications; it was used to normalize the data for each culture. The results (Figure 3 and Table 4) show that the *ddlB* and *ftsZ* genes are duplicated in all mutants and that the *yjjM* gene is duplicated only in *mcr-5*, *mcr-15*, and *mcr-4*. These data are in full agreement with the genetic data.

Is a duplication sufficient to confer mecillinam resistance? We have shown that overexpression of the FtsZ, FtsA, and FtsQ proteins confers mecillinam resistance (VINELLA *et al.* 1993; NAVARRO *et al.* 1998). Resistance was observed when the amount of FtsZ was increased sevenfold, but we did not determine the minimum amplification necessary for this phenotype. Since the *mcr::cat* insertions seemed to duplicate the *ftsZ* gene (and, presumably, the entire *ftsQAZ* operon), this could be the basis for their mecillinam resistance.

We tested whether duplication of the *ftsQAZ* operon conferred mecillinam resistance by introducing into MG1655 *leu::*Tn*10* the F′104 episome, which carries the

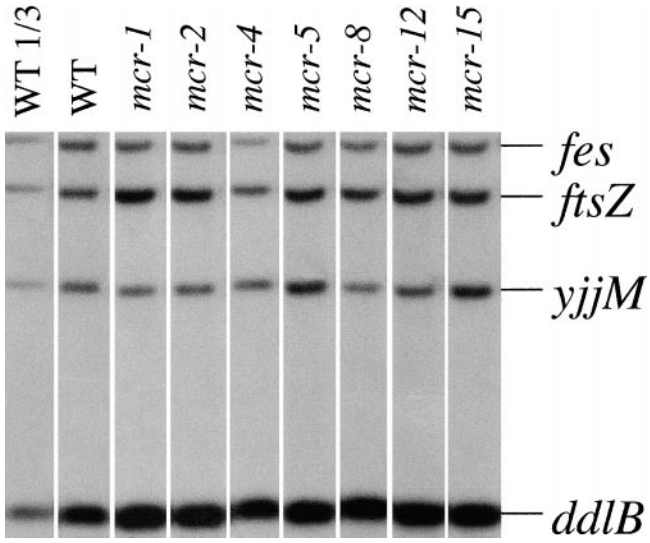

FIGURE 3.—Physical evidence for partial diploidy. The seven unstable *mcr* mutants were grown in LB containing mecillinam; the control strain MG1655 was grown in LB. DNA was extracted from overnight cultures and Southern blots were carried out with four probes, as described in MATERIALS AND METHODS.

chromosomal region between 97 min and 6 min and is present at about one copy per chromosome. Leu$^+$ Tc^r exconjugants were purified and tested for mecillinam resistance. They were sensitive, indicating that doubling the copy number of the *ftsQAZ* operon is not sufficient to confer mecillinam resistance.

Since the *mcr::cat* mutants all have duplications of the *ftsQAZ* operon, they might further amplify it by unequal crossing over (HAACK and ROTH 1995). To test this, we looked for evidence of triploidy of the *leu* operon. We transduced the *leu::*Tn*10* marker into the *mcr-2::kan* mutant; the resulting Tc^r Km^r strain remained Leu$^+$, as previously observed, indicating the presence of *leu::*Tn*10* and *leu*$^+$ alleles (Figure 2). We then transduced a *leu::cat* (Cm^r) marker into this strain, selecting the transductants on plates containing kanamycin and chloramphenicol. If the recipient strain had only two copies of the *leu* locus, we would expect to replace one of the resident alleles, giving Km^r Cm^r transductants that are either Tc^r Leu$^-$ or Tc^s Leu$^+$, according to whether the *leu*$^+$ or the *leu::*Tn*10* allele had been replaced, respectively. Indeed, we obtained these two types of transductants, but in addition we found Km^r Cm^r Tc^r Leu$^+$ clones, indicating that in the culture of the recipient strain, at least 50% of the cells had three or more *leu* operons. An explanation of these results is presented in Figure 4.

TABLE 4

Physical evidence for partial diploidy in the *mcr* mutants

Strain	*ftsZ:fes*	*ddlB:fes*	*yjjM:fes*
MG1655	≡1	≡1	≡1
mcr-1	1.54	1.77	1.23
mcr-2	1.97	2.06	1.18
mcr-4	1.79	2.70	2.50
mcr-5	2.08	2.01	2.43
mcr-8	2.02	2.48	1.15
mcr-12	1.97	2.07	1.25
mcr-15	1.78	1.79	1.96

The data from Figure 3 were quantified. The label in each band was normalized to that in the *fes* band of the same strain, and this ratio in turn was normalized to that in MG1655 for the corresponding probe.

Model: These duplications are all unstable, they are stabilized in *recA* strains, they are diploid for markers in the *ftsQAZ* region near 2 min on the genetic map and covering as much as 250 kb, transduction of the mini-Tn*10* inserts by P1 creates duplications of resident genes in the recipient strain, and the chromosomal sequences flanking the mini-Tn*10* inserts are from different genes on the left and right. To explain these results, we propose a model (Figure 5) in which the initial event was a double transposition event or a transposition associated with a homologous recombination event, creating a tandem duplication with the mini-Tn*10* located at the joint. When the mini-Tn*10* is transduced by P1, the surrounding DNA carries the end points of the duplication, with the right end to the left and the left end to the right (Figure 5). This DNA fragment can then recombine with two sister chromosomes in the recipient cell to recreate the same duplication as in the donor chromosome, except that it is the recipient genes that are duplicated. Further amplification of the duplicated region can then occur by unequal crossing over.

This model makes several predictions. First of all, the duplications created by transducing the mini-Tn*10* inserts should have exactly the same end points as those in the donor strains. We have shown above that for the *thr, leu*, and *proA* operons, the duplications in transductants have the same extent as in those in the donors. Second, on the basis of the sequences assumed to define the duplication end points, one predicts that the *mcr-5::cat* mutant should have a duplication of the *purA* gene while *mcr-15::cat* should not (Figure 1). When we transduced these mutants with a P1 stock grown on a *purA45 zjd::*Tn*10* donor strain, Tc^r Pur$^-$ cotransductants were never found when the recipient was *mcr-5::cat* (<1%) but readily recovered with *mcr-15::cat* (~30%).

Third, it should be possible to cotransduce donor markers that are very close to the mini-Tn*10*, and indeed we found this with our smallest duplication, *mcr-1::kan*. In one experiment, with a *leu::*Tn*10* recipient, we found one Tc^s clone among 125 Km^r transductants. With an *ftsI23*(Ts) recipient, we found 90% cotransduction of temperature resistance (*ftsI*$^+$) and Km^r. Note that the *leu* operon is not duplicated in *mcr-1::kan* and is ~1.6 min from the mini-Tn*10*, whereas the *ftsI* gene, between *leuO* and *ftsQ*, is covered by the duplication with the two copies located 0.2 and 1.3 min from the mini-Tn*10* (*cf.*

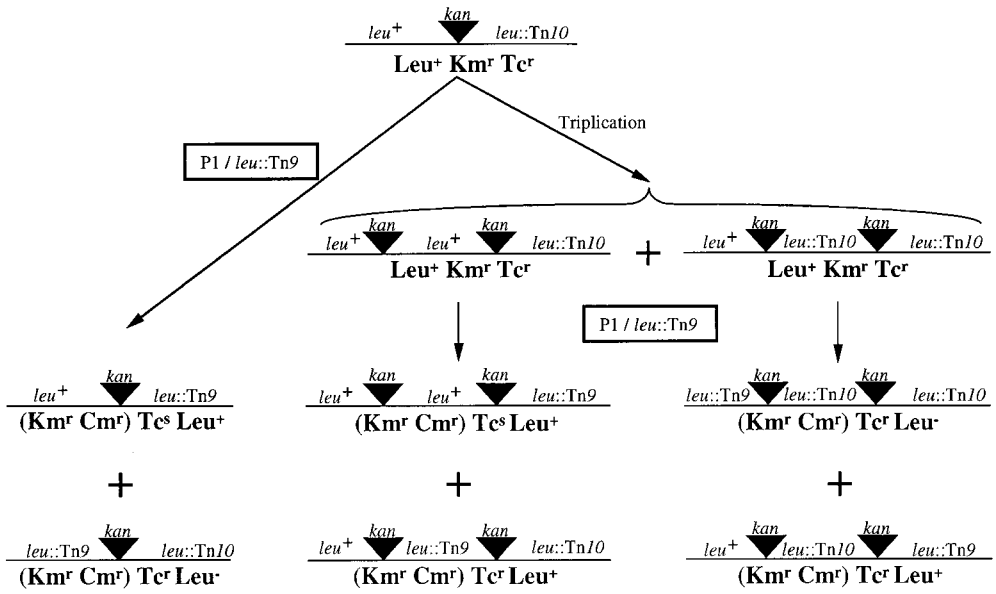

FIGURE 4.—Genetic demonstration of triploidy at the *leu* locus in the *mcr-2::kan* mutant. An *mcr-2::kan* strain heterozygous for *leu*$^+$/*leu::*Tn*10* (phenotype Leu$^+$ Tc^r) was transduced to chloramphenicol resistance from a *leu::*Tn*9* donor strain by selecting on LB plates containing chloramphenicol and kanamycin (to maintain the *mcr-2::kan* allele). The unselected phenotypes Leu$^{+/-}$ and Tc$^{r/s}$ were then tested. On the left are shown the possible phenotypes in the case of a diploid recipient; on the right are the expected phenotypes with a triploid recipient. Since all four possible phenotypes were found, at least part of the recipient population must be triploid at the *leu* locus.

Figure 1). We therefore expected the *ftsI*$^+$ cotransductants to be diploid and, for most of them, heterozygous. Indeed, when the donor strain was *ftsI23*(Ts) *mcr-1::kan* and the recipient wild type, only three temperature-sensitive clones were found among 144 Km^r transductants. These had presumably received both donor genes and were thus homozygous for the *ftsI23*(Ts) allele. Their temperature sensitivity suggests that further amplification of this allele by unequal crossing over does not restore temperature-resistant division, unlike the *ftsZ84*(Ts) allele.

The instability of these tandem duplications arises from RecA-dependent recombination between the duplicated sequences. As a fourth prediction of our model, one would expect heterozygous *leu*$^+$ *leu::*Tn*10* diploids to segregate two types of haploid, Tc^r Leu$^-$ and Tc^s Leu$^+$, according to where the crossover takes place. Heterozygous diploids of this sort, carrying the *mcr-2::kan* allele or any one of the five *mcr::cat* alleles, were grown and plated without antibiotics, and Km^s or Cm^s segregants were looked for. All six mutants gave rise to both types of segregants; the ratio of Tc^s Leu$^+$ to Tc^r Leu$^-$ colonies ranged from 0.08 to 27, according to the *mcr* allele.

Fifth, the mechanism proposed for recreation of the duplications by P1 transduction requires at least three crossover events, compared to a double crossover in the normal transduction process, so transduction of our mini-Tn*10* inserts would be expected to be less efficient than normal transduction. Indeed, we consistently found some 10-fold fewer transductants when selecting one of these mini-Tn*10* inserts as compared to the number of transductants obtained in straightforward selections for markers such as *leu::*Tn*10*.

A sixth prediction of our model concerns the presumed further amplification by unequal crossing over. As shown above, cultures of the *mcr-1::kan* mutant can be at least triploid for the *leu* operon. However, this amplification, which is absolutely necessary for mecillinam resistance, should require RecA-dependent recombination. We found that the *recA1* mutation, in addition to stabilizing the mini-Tn*10* element, also suppressed the mecillinam resistance of all seven unstable *mcr* mutants.

DISCUSSION

In this article, using insertional mutagenesis, we carried out a selection for mutants that overproduce the division proteins FtsQ, FtsA, and FtsZ, in the hope of identifying regulators of this complex operon. Of 12 new and 3 previously selected mecillinam-resistant mutants, 7 were of this type. However, in all cases the amplification was produced by increasing the gene copy number rather than by increasing the rate of transcription initiation. Since these duplications seemed to result from multiple events, it seems unlikely that a single insertional mutation can increase *ftsQAZ* transcription sufficiently to confer mecillinam resistance.

Gene amplification was brought about by creating a tandem duplication with the mini-Tn*10* at the joint between the repeated sequences (Figure 5). The duplications are too long to be encapsidated by the transducing phage P1, but they can be recreated by transduction. The mechanism proposed requires encapsidating the deletion end points and the adjacent mini-Tn*10*, which carries the selective marker (*cf.* Figure 5).

It is striking that four of the seven duplications described here have one end point in an IS1 element. On examining the sequence of the mini-Tn*10*, we noted that it has a 58-bp stretch that is homologous to one end of IS1 (one difference compared with IS1B and

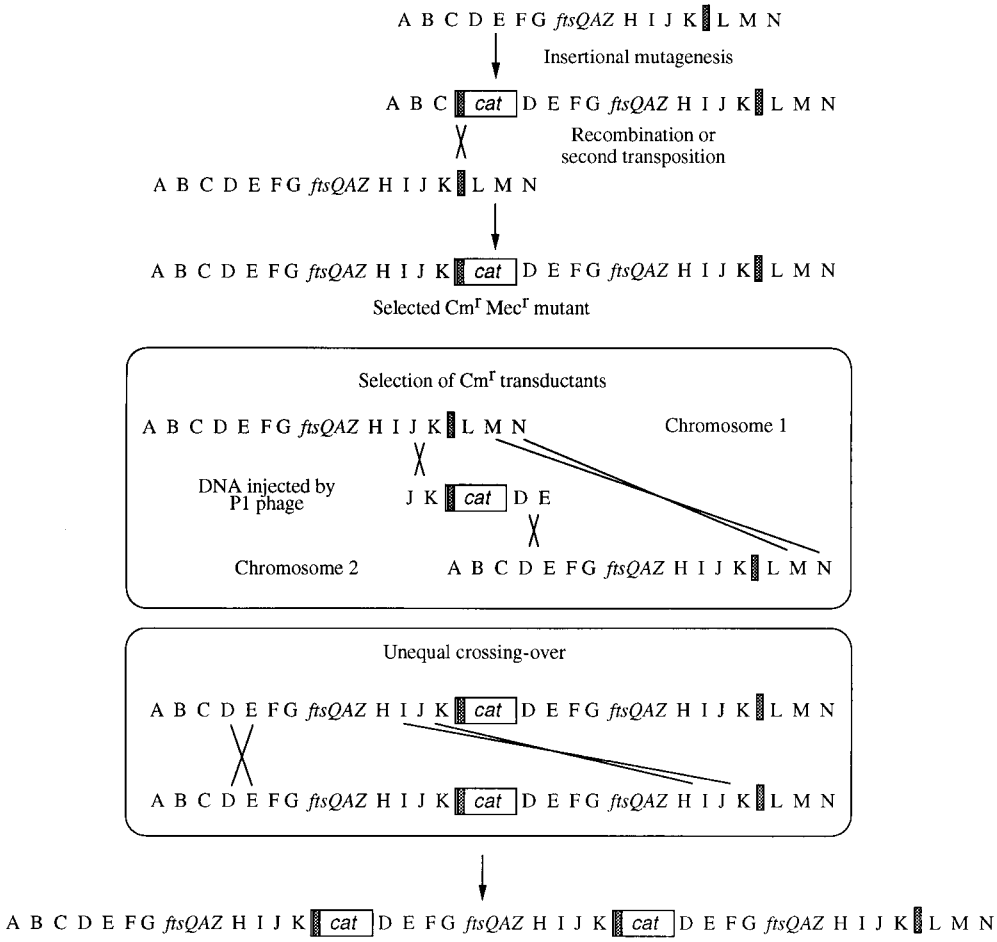

FIGURE 5.—Model for the formation and transduction of duplications. In the diagram, the mini-Tn*10* element, called *cat*, first transposes to a position between genes C and D. This chromosome then recombines with a sister chromosome in the region of homology between the mini-Tn*10* and an IS1 sequence between genes K and L (the homology is represented by a cross-hatched bar), resulting in a duplication covering the genes from D to K, including the *ftsQAZ* operon. If this strain is used as a transductional donor, the phage can pick up the mini-Tn*10* and flanking sequences (top box) and, by homologous recombination with two sister chromosomes, recreate the same duplication as in the donor strain. This duplication, by unequal crossing over between sister chromosomes (bottom box), can generate a triploid chromosome, which in turn can further amplify the number of copies of the duplicated region.

IS1C). Thus the initial formation of the duplications in these cases seems to have involved a transposition event, determining one duplication end point, and a homologous recombination event between the mini-Tn*10* and an IS1, determining the other end point. In *Salmonella typhimurium*, duplications have been shown to form between separate IS200 elements, presumably by unequal crossing over (HAACK and ROTH 1995). In the *mcr-4::cat* mutant, the sequences flanking the mini-Tn*10* are both from the open reading frame *yaeT*; however, the strain is diploid for *thr* and *leu*, showing that the structure, more complex than a simple insertion in *yaeT*, includes a duplication in the 2-min region, as in the other mutants.

Our model for duplication formation suggests a general method for constructing strains with a precise tandem duplication of virtually any sequence on the chromosome. To do this, the deletion end points should first be cloned in a plasmid vector, in the proper orientation, next to a selective marker (Tn, for example). If one wants to duplicate, say, the sequence DEFGHIJK, the right end point JK should be cloned to the left of the Tn marker and the left end point DE should be cloned to the right, taking care to maintain the normal chromosomal orientation of each sequence. The cloned fragment JK-Tn-DE can then be separated from plasmid replication functions and used in linear transformation, where, by the mechanism shown in Figure 5, it will create a tandem duplication having the structure AB CDEFGHIJK-Tn-DEFGHIJKLM.

In our system, a mere doubling of the number of *ftsQAZ* operons is not enough to confer mecillinam resistance. Nevertheless, essentially all cells carrying a tandem duplication of the operon succeed in forming a colony on mecillinam plates, apparently through further amplification by unequal crossing over. A selection of this sort can be included in the construction of a designed duplication. For this, one need only include, together with the selectable Tn element, genes whose further amplification can be selected for. The *ftsQAZ* genes would be one possibility; growth in the presence of mecillinam would then produce a population with more than two copies of the designed duplication. Other drug resistance markers could also be used, as well as metabolic functions with insufficient expression.

Our selection produced the phenotype we expected: 7 of our 15 mecillinam-resistant mutants overproduce FtsQ, FtsA, and FtsZ (Table 2, class 3), and at least 4 of the others seem to have a higher than normal ppGpp pool (Table 2, class 1). The fact that our class 3 overproducers did not define regulators of the *ftsQAZ* operon

illustrates once again that there are many ways to skin a cat, and *E. coli* is more clever than we are by eluding our attempt to define regulators by insertion mutagenesis. We are currently completing the characterization of the mutants of classes 1 and 2 and selecting new mutants that become mecillinam resistant via overproduction of specific gene products.

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