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Mecillinam Resistance in *Escherichia coli* Is Conferred by Loss of a Second Activity of the AroK Protein

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Mecillinam, a β-lactam antibiotic specific to penicillin-binding protein 2 (PBP 2) in *Escherichia coli*, blocks cell wall elongation and, indirectly, cell division, but its lethality can be overcome by increased levels of ppGpp, the nucleotide effector of the stringent response. We have subjected an *E. coli* K-12 strain to random insertional mutagenesis with a mini-Tn*10* element. One insertion, which was found to confer resistance to mecillinam in *relA*$^+$ and *relA* strains, was mapped at 75.5 min on the *E. coli* map and was located between the promoters and the coding sequence of the *aroK* gene, which codes for shikimate kinase I, one of two *E. coli* shikimate kinases, both of which are involved in aromatic amino acid biosynthesis. The mecillinam resistance conferred by the insertion was abolished in a Δ*relA* Δ*spoT* strain completely lacking ppGpp, and it thus depends on the presence of ppGpp. Furthermore, the insertion increased the ppGpp pool approximately twofold in a *relA*$^+$ strain. However, this increase was not observed in *relA* strains, although the insertion still conferred mecillinam resistance in these backgrounds, showing that mecillinam resistance is not due to an increased ppGpp pool. The resistance was also abolished in an *ftsZ84*(Ts) strain under semipermissive conditions, and the *aroK*::mini-Tn*10* allele partially suppressed *ftsZ84*(Ts); however, it did not increase the concentration of the FtsZ cell division protein. The insertion greatly decreased or abolished the shikimate kinase activity of AroK in vivo and in vitro. The two shikimate kinases of *E. coli* are not equivalent; the loss of AroK confers mecillinam resistance, whereas the loss of AroL does not. Furthermore, the ability of the *aroK* mutation to confer mecillinam resistance is shown to be independent of polar effects on operon expression and of effects on the availability of aromatic amino acids or shikimic acid. Instead, we conclude that the AroK protein has a second activity, possibly related to cell division regulation, which confers mecillinam sensitivity. We were able to separate the AroK activities mutationally with an *aroK* mutant allele lacking shikimate kinase activity but still able to confer mecillinam sensitivity.

Escherichia coli owes its rod shape to the rigidity of its peptidoglycan (also called cell wall or murein). The polymerization of this highly cross-linked macromolecule is catalyzed by inner membrane enzymes called penicillin-binding proteins (PBPs) (42). Peptidoglycan growth during the cell cycle includes an elongation phase and a septation phase, processes which require particular peptidoglycan synthesis. PBP 2, coded for by the *pbpA* gene, is essential for elongation, as shown by the observation that when it is inactivated, the cells lose their rod shape and become spherical (53). Moreover, mecillinam (34), a β-lactam which specifically inactivates PBP 2 (54, 55), causes wild-type cells to become spherical and die, indicating that elongation is essential to cell survival (24, 37). This conclusion is further supported by the inability of wild-type strains to tolerate a deletion of the *pbpA* gene (44).

We attributed the lethality of PBP 2 inactivation to cell division inhibition (64). However, PBP 2 is not an essential element of the septation apparatus, since mecillinam-resistant mutants can be isolated which survive as spheres and tolerate a *pbpA* deletion. One class consists of mutants which remain rod shaped in the absence of mecillinam but grow as spheres in its presence. These include aminoacyl-tRNA synthetase mutants, partially defective in aminoacylation (7, 62), and *cya* and *crp* mutants (2, 23), defective for the cyclic AMP (cAMP)-catabolite gene activator protein (CAP) complex, a transcriptional regulator (58). A second class consists of mutants which grow as spheres even in the absence of mecillinam; they are mutated at the *mre* locus (65–67).

Cell division, or septation, is an essential event in the *E. coli* cell cycle which is tightly controlled during balanced growth and is coupled to cell mass and protein synthesis (61). The FtsZ protein has attracted much attention, since it is essential for septation (15), it acts early (3), and it may be limiting for the process (71) (see also reference 57), and it is the target of all known endogenous cell division inhibitors (35). Using immunoelectron microscopy, Bi and Lutkenhaus (4) observed that FtsZ, which is dispersed in the cytoplasm of nondividing cells, condenses to form a membrane-associated ring at the center of the cell at about the same time that a constriction appears. The formation of the FtsZ ring is the earliest known septation event and appears to be essential since it is always observed in dividing cells but is not seen when cell division inhibitors are induced (5). FtsZ is able to bind and hydrolyze GTP (16, 40, 47) and to form tubulin-like filaments in vitro (9, 20, 41), which suggests that it plays a structural role in septation, possibly constricting the cell envelope. We found that the lethality of PBP 2 inactivation is suppressed by overproduction of the FtsZ, FtsA, and FtsQ proteins (64). This led us to

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suggest that the division block observed when PBP 2 is inactivated results from an insufficient division capacity in spherical cells, whose diameters are larger than those of rods. Thus, elongation might be essential simply because it keeps the cell diameter constant as the cell mass increases.

The nucleotide ppGpp was discovered through the dramatic increase in its concentration following amino acid starvation of RelA$^+$ strains (11, 13). This increase induces the stringent response (14), whose major effect is a strong decrease in the transcription rate of stable RNA operons, probably via a direct interaction of ppGpp with RNA polymerase. This leads to a decrease in ribosome synthesis; ppGpp is thus an indicator of ribosome activity, and it adjusts the rate of ribosome formation to the availability of aminoacyl-tRNA. The ribosome-bound RelA protein is responsible for the increase in the ppGpp pool whenever the ratio of uncharged tRNA to charged tRNA increases, such as during amino acid starvation (49). The presence of ppGpp in Δ*relA* strains (38) indicates that *E. coli* has a second ppGpp synthetase, and the complete absence of the nucleotide in Δ*relA* Δ*spoT* strains strongly suggests that the second enzyme is SpoT (22, 73), making this protein bifunctional since it is also the major ppGpp hydrolase (1, 29). Δ*relA* Δ*spoT* strains display heterogeneous cell morphology, including filaments (22, 73). This suggests that the presence of ppGpp during balanced growth promotes cell division, possibly by stimulating the transcription of cell division genes.

We have shown that the mecillinam resistance of aminoacyl-tRNA synthetase mutants requires RelA activity and that wild-type cells can be made mecillinam resistant simply by increasing the ppGpp pool via the overproduction of RelA activity (8, 26, 62). Furthermore, the *ftsZ84*(Ts) mutant is partially suppressed by overexpression of RelA activity (reported in reference 45). These observations led us to suggest (64) that ppGpp is a positive regulator of FtsZ activity, possibly stimulating transcription of the *ftsQAZ* operon. The nucleotide thus might be one of the elements involved in coupling septation to cell mass and protein synthesis.

We speculated that the selection of mecillinam-resistant mutants in a *relA* genetic background might allow the identification of regulators of FtsZ or SpoT activity. We report here the selection and characterization of one mutant of this type, obtained by random insertional mutagenesis. We show that the insertion causes a deficiency in the expression of the *aroK* gene, which in turn causes mecillinam resistance by decreasing an AroK activity distinct from shikimate kinase, possibly related to the modulation of cell division capacity.

MATERIALS AND METHODS

Bacterial strains and phages. All of the strains used in this work are *E. coli* K-12 derivatives; the principal strains are described in Table 1. During the course of this study, the parental strain GC3782 (60) was found to carry a presumed *rpoS* mutation which causes a delay in bubbling in the catalase test (30) and cotransduces with the *relA251* mutation. Although this mutation does not affect the mecillinam resistance or sensitivity of any of the strains tested (data not shown), we constructed strain DV4 (*rpoS*$^+$) for further analysis. To do this, strain DV1 was transduced to Δ*relA251* Km^r *rpoS*$^+$ (detected by the bubbling test), and this strain was transduced to *argA*::Tn*10* *relA*$^+$ Km^s, and subsequently to Arg$^+$, to produce DV4. Strain DV15 is an Arg$^+$ *relA1* transductant obtained in a similar manner (donor strain, DV2); the presence of the *relA* mutation was detected by the inability of the organism to grow on minimal glucose plates supplemented with serine, methionine, and glycine (59). The Δ*relA251* Km^r and Δ*spoT207* Cm^r mutations are from strain CF1693 (73). To construct strain DV20, which has two kanamycin resistance markers, we introduced the Δ*relA251* allele into an *argA*::Tn*10* *aroK3*::*kan* strain by cotransduction with Arg$^+$. The *ftsZ84*(Ts) mutation from strain GC3443 (48) was introduced into strain DV23 by cotransduction with *leu*::Tn*10*, screening for thermosensitive growth at 42°C on Luria-Bertani (LB) plates lacking NaCl; one such clone was transduced to Leu$^+$.

Strains ALO939, ALO863 (32), and ANL11 (36), kindly provided by E. Boye, were the donors of the *aroK*::*cat*, *urf74.3*::mini-Tn*10* (Tc^r), and *urf74.3*::*kan* insertions, respectively. The donor of the *dam-13*::Tn*9* insertion was described previously (63). The *aroL*::Tn*10* and *aroA*::Tn*10* insertions were from strains JP3123 and LCB273, respectively, kindly provided by B. Bachmann. We introduced the *aroK3*::*kan* mutation (which is Aro$^+$ Km^r) into the *aroB* strain POP1010 (68); an *aroK3*::*kan aroB* transductant was used as the donor to construct strain DV14, which in turn was used as the donor to construct the *aroK3*::*kan* and *aroK3*::*kan aroB* derivatives of strains *dam-13*::Tn*9*, *urf74.3*::mini-Tn*10*, and *urf74.3*::mini-Tn*10* *dam-13*::Tn*9*. In all cases, we selected for kanamycin resistance and tested the transductants on minimal glucose plates; auxotrophic clones carried the *aroB* mutation. Strain DV7 (*ompR101*::TN*10* *aroK*$^+$ *aroB*) is an auxotrophic Tc^r Km^s transductant of strain DV14, with the donor being an *ompR101*::Tn*10* derivative of MC4100; DV7 was the recipient for the construction of the *ompR*::Tn*10* *aroB dam-13*::Tn*9*, *ompR*::Tn*10* *aroB urf74.3*::*kan*, and *ompR*::Tn*10* *aroB dam-13*::Tn*9* *urf74.3*::*kan* strains.

TABLE 1. Bacterial strains

Strain	Relevant genotype	Origin or reference
DV1	*rpoS*	GC3782 (60)
DV2	DV1 *relA1*	GC3784 (60)
DV3	DV1 *lacIpoZ*Δ(*Mlu*)*malB*::Tn*9* *relA1*	This work
DV4	DV1 *rpoS*$^+$	This work
DV5	DV4 *aroK3*::*kan*	This work
DV6	DV4 *aroK*::*cat*	This work
DV7	DV4 *ompR*::Tn*10* *aroB*	This work
DV8	DV4 *aroA*::Tn*10*	This work
DV9	DV4 *aroL*::Tn*10*	This work
DV10	DV4 *aroK3*::*kan aroL*::Tn*10*	This work
DV11	DV4 *aroK*::*cat aroL*::Tn*10*	This work
DV12	DV4 *aroK3*::*kan aroA*::Tn*10*	This work
DV13	DV4 *aroK*::*cat aroA*::Tn*10*	This work
DV14	DV4 *aroK3*::*kan aroB*	This work
DV15	DV4 *relA1*	This work
DV17	DV4 *relA1 aroK3*::*kan*	This work
DV19	DV4 Δ*relA251* (Km^r)	This work
DV20	DV4 *aroK3*::*kan* Δ*relA251*	This work
DV21	DV4 Δ*relA251* Δ*spoT207* (Cm^r)	This work
DV22	DV4 *aroK3*::*kan* Δ*relA251* Δ*spoT207*	This work
DV23	DV4 *ftsZ84*(Ts)	This work
DV24	DV4 *ftsZ84*(Ts) *aroK3*::*kan*	This work
DV25	DV1 *aroK3*::*kan*	This work
DV26	DV2 *argS201* Δ(*rodA-pbpA*)::*kan*	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*	10

The strains carrying various Tn*10* transposons used for genetic mapping were from the Singer collection (52); the Hfr strains were described by Wanner (70). P1 *vir* transduction and Hfr crosses were carried out as described by Miller (39).

Phage λNK1205 (27) was used as the mini-Tn*10* donor. For the selection of insertional mutants, 3.5×10^{10} cells of an exponential culture of strain DV3 were infected with 5×10^8, 1×10^9, or 1.5×10^9 λNK1205 phage. After 30 min of preadsorption at 37°C, the cells were washed and resuspended in LB broth for 1 h and then plated on LB plates supplemented with kanamycin, mecillinam (1 or 10 μg/ml), or kanamycin plus mecillinam (1 or 10 μg/ml). Km^r Mec^r mutants were purified after 48 h of incubation at 37°C. The mean frequency of transposition from λNK1205 onto the chromosome, calculated as Km^r clones per infecting phage, was 6×10^{-5}. The plating efficiency of the uninfected culture was 5×10^{-5} on medium with 1 μg of mecillinam per ml and 7×10^{-6} on medium with 10 μg of mecillinam per ml. The frequency of Mec^r mutants among Km^r clones was 4×10^{-3} with 5×10^8 phage and 6.7×10^{-4} with 1×10^9 or 1.5×10^9 phage.

Media and growth conditions. The rich and minimal media used in this work were, respectively, LB broth (containing 10 g of NaCl per liter unless otherwise indicated) and M9 containing 0.2% glucose (39). For ppGpp assays, we used MOPS (morpholinepropanesulfonic acid) medium (43) as modified by Bochner and Ames (6), except that the phosphate concentration was 0.4 mM and the NaCl concentration was 10 g/liter. Shikimic acid (100 μg/ml) and amino acids (100 μg/ml) were added to minimal glucose medium when needed. Solid media contained 1.5% agar. Antibiotics were used at the following concentrations: chloramphenicol (Cm), 30 μg/ml; kanamycin (Km), 50 μg/ml; mecillinam (Mec), 1 or 10 μg/ml; tetracycline (Tc), 40 μg/ml; spectinomycin (Spc), 50 μg/ml; and ampicillin (Amp), 100 μg/ml.

The Lac$^-$ phenotype was tested on MacConkey lactose plates (39).

DNA techniques and plasmids. Plasmids were extracted and transformation was carried out as described by Sambrook et al. (50). Sequence determinations

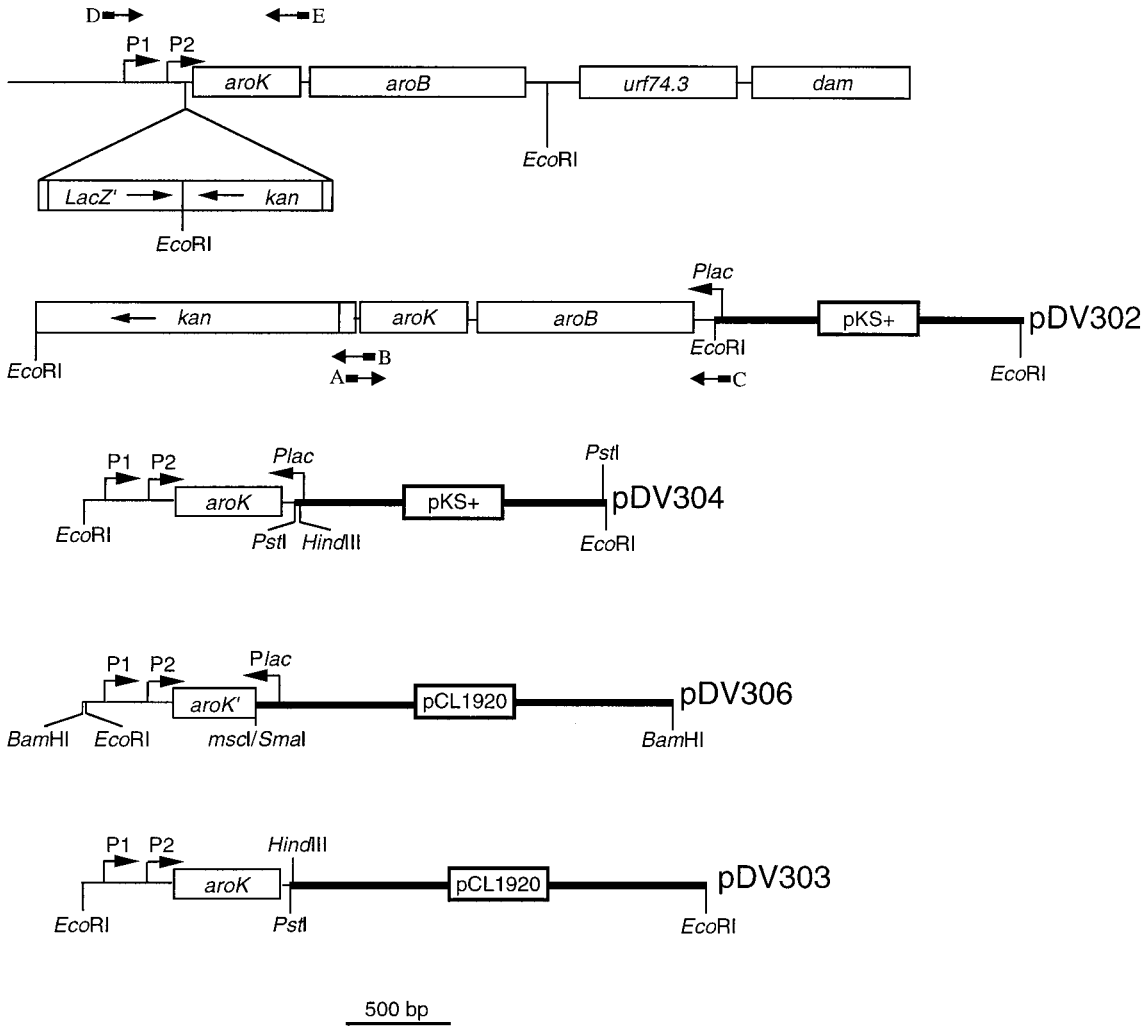


FIG. 1. Structure of the *aroK-dam* operon and relevant plasmids. The insertion site and orientation of the mini-Tn*10* in the *aroK3*::*kan* mutant are shown for the chromosomal *aroK-dam* operon (top). The four lower maps show plasmids constructed to contain either the *aroK* and *aroB* genes (pDV302) or the *aroK* region (pDV303, pDV304, and pDV306). Thick lines symbolize vector DNA of pKS+ or pCL1920, as indicated. Simple arrows indicate the direction of transcription. Primers A to E, which were used for amplification of the *aroK* gene and for determination of the insertion site, are represented by arrows with boxes at their origins.

were carried out with the Sequenase kit (U.S. Biochemicals). The inserts of plasmids pDV303 and pDV303-1 were sequenced on one strand by using suitable primers (Bioserve Biotechnologies); the point mutation in the *aroK* gene carried by pDV303-1 and the same region in pDV303 were sequenced on both strands. The low-copy-number vectors used in this work were pHSG576 (Cm^r) (56) and pCL1920 (Spc^r), plasmids that are present in about five copies per cell (31); pBluescript II KS+ (pKS+; Amp^r) is a high-copy-number vector (Stratagene). PCR amplification was carried out with *Taq* polymerase from Perkin-Elmer. Synthetic primers (5′ to 3′) were as follows: primer A, CTACCTTAACTTAAT GATTTTGATA; primer B, CCGGCACCCATAGGCCCAACC; primer C, AA CAGCTATGACCATG; primer D, GGGTGATCAGAATTCCGCGAGTTAG TGGTGTTTATCACGCC; and primer E, CCCCTGCAGCCACCTTAATTA CTGTACCCGC.

The *aroK* gene was amplified by PCR with primers D and E (Fig. 1), which create an *Eco*RI and a *Pst*I site at the 5′ and 3′ ends of the gene, respectively. The resulting band was digested with these two enzymes and ligated into pCL1920, yielding plasmids pDV303 and pDV303-1. In these plasmids, a *Hin*dIII site is located downstream of the *Pst*I site. Cloning of the *Eco*RI-*Hin*dIII insert of pDV303 into pKS+ yielded plasmid pDV304, in which the *aroK* gene is located between two *Pst*I sites. The *Pst*I-*Pst*I insert of pDV304 was cloned in pCL1920 to form plasmid pDV305. Digestion of pDV305 with *Msc*I and *Sma*I followed by ligation yielded plasmid pDV306.

Assay of ppGpp. The basal levels of ppGpp were assayed (12) by ^{32}P labeling of cells growing in MOPS medium containing glucose (0.2%), all 20 amino acids (40 μg/ml), and shikimic acid (100 μg/ml). Nucleotides were extracted by adding ice-cold formic acid to 7.5 M, and samples were frozen and thawed twice before the insoluble material was pelleted in a microcentrifuge for 5 min at 4°C. Samples of the supernatant were spotted on polyethyleneimine plates (Polygram CEL 300; Macherey-Nagel), and two-dimensional chromatography was carried out in 3.3 M ammonium formate–4.2% boric acid, pH 7, and then in 1.5 M KH_2PO_4 after the plates were washed with in methanol. Quantification was performed with the Ambis radioanalytic imaging system.

Immunoassay of FtsZ protein. Immunoblots were carried out with anti-FtsZ serum, generously provided by Miguel Vicente. Strains were grown in LB broth. Samples equivalent to 1 ml at an optical density at 600 nm (OD_{600}) of 1.0 were cooled on ice, centrifuged, resuspended in 60 μl of sodium dodecyl sulfate (SDS) sample buffer, heated at 100°C for 3 min, electrophoresed on SDS–10% polyacrylamide gels, and electrophoretically transferred to nitrocellulose. Blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris [pH 7.6], 0.1% Tween 20) and probed first with anti-FtsZ serum diluted 1:3,000 and then with ^{35}S-labeled anti-rabbit immunoglobulin G (Amersham) diluted 1:3,000. The radioactive bands were detected with a PhosphorImager (Molecular Dynamics) and quantified with the software Image Quant 1.33 (Molecular Dynamics).

Preparation of S100 extracts. Strains were grown in LB broth to an OD_{600} of 1; cells were harvested from 1 liter of medium and washed once with 300 ml of M9. The washed-cell pellets were resuspended in 3 ml of 50 mM Tris HCl buffer, pH 8, containing 1 mM EDTA, 2 mM dithiothreitol, 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride], and 5 μg of leupeptin per ml. They were sonicated for 15 s 10 times at 30-s intervals with a Branson Sonifer 250 sonicator at a setting of 5 and then centrifuged at 100,000 × *g* for 50 min at 4°C. The supernatants were aliquotted and stored at −70°C. No loss of shikimate

kinase activity was observed after 2 months of storage. The protein concentration was measured by the Bradford assay (Bio-Rad).

Test for shikimate kinase activity. Tests were carried out in 10 μl of 20 mM Tris (pH 7.5)–5 mM $MgCl_2$–100 mM NaCl. After 1 min of preincubation separately at 37°C, 5 μl of extract was added to 5 μl of twofold-concentrated reaction mixture, yielding final concentrations of unlabeled ATP and [γ-^{32}P]ATP as specified in Fig. 5 and of 5.7 mM shikimate. Samples (2 μl) were spotted directly on polyethyleneimine plates and chromatographed by ascending development in 1 M LiCl; lower concentrations of LiCl were less effective in separating shikimate phosphate from the origin.

RESULTS

Isolation of mini-Tn*10* insertions conferring mecillinam resistance. The plating efficiency of RelA$^+$ strains on LB plates containing 1 to 10 μg of mecillinam per ml is about 10^{-4}. This value is considerably lower in *relA1* strains (62), suggesting that the majority of mecillinam-resistant mutants of RelA$^+$ strains owe their phenotype to a RelA-dependent increase in the ppGpp pool, as is the case for aminoacyl-tRNA synthetase mutants (62). To isolate RelA-independent mecillinam-resistant mutants, we made random mini-Tn*10* (Km^r) insertions in the *relA1* strain DV3 and selected for mecillinam resistance (see Materials and Methods). Of 45 Km^r Mec^r mutants, 28 grew after purification on the same medium (5 when mecillinam was present at 10 μg/ml and 23 when the drug concentration was 1 μg/ml). The insertions of these clones were backcrossed into strain DV3, and the Km^r transductants were tested for mecillinam resistance. In 22 cases, mecillinam resistance was not cotransduced with kanamycin resistance (less than 2% cotransduction). In three cases, mecillinam resistance and kanamycin resistance were cotransducible, but not with 100% efficiency (50% for two insertions and 17% for the third). In the last three cases, kanamycin resistance and mecillinam resistance were 100% cotransducible (48 Km^r transductants tested), showing that the insertions were responsible for mecillinam resistance. Two of these insertions conferred resistance to mecillinam at 10 μg/ml and showed RecA-dependent genetic instability, as indicated by the spontaneous segregation of Km^s Mec^s clones when kanamycin was omitted from the culture medium of *recA*$^+$ (but not *recA1*) strains carrying these insertions (data not shown). The third insertion, *mcr-3*::Tn*10*, is stable and confers resistance to mecillinam at 1 μg/ml but not at 10 μg/ml. The characterization of this insertion, initially designated *mcr-3*::*kan*, is reported here.

Identification of the gene affected by the *mcr-3*::*kan* insertion. We roughly mapped the *mcr-3*::*kan* insertion by Hfr crosses, using the *mcr-3*::*kan* derivative of DV3, which is Cm^r (Table 1), as the recipient and different Hfr strains injecting early Tc^r markers. Exconjugants were purified on LB plates containing tetracycline and chloramphenicol and tested for kanamycin resistance. Km^s clones were recovered only with Hfr KL14 *ilv*::Tn*10* (33 Km^s; 48 Tc^r Cm^r), not with PK3 *argE*::Tn*10*, indicating that the *mcr-3*::*kan* insertion site lies between their origins of transfer, i.e., between 65 and 80 min on the *E. coli* genetic map.

We transduced the *mcr-3*::*kan* derivative of strain DV1 to Tc^r using strains carrying different Tn*10* transposons located between 65 and 80 min as donors to pinpoint the insertion sites more precisely. We obtained Km^s clones with *zhg-3086*::Tn*10*, located near 76.5 min (23 Km^s; 96 Tc^r). The *mcr-3*::*kan* insertion was later shown to be 50% cotransducible with *dam*::Tn*9* at 75.7 min.

The mini-Tn*10* used has no *Pst*I sites. We unsuccessfully attempted to clone the Km^r marker of strain DV3 *mcr-3*::*kan* in the low-copy-number Cm^r vector pHSG576 by using *Pst*I-digested chromosomal DNA. We were successful when the chromosomal DNA was digested with *Pst*I and *Eco*RI and cloned into pHSG576 that had been digested with these two enzymes. The Cm^r Km^r plasmids isolated in this case all carried a common *Eco*RI-*Eco*RI fragment of 3.6 kb and *Eco*RI-*Pst*I linker fragments of various sizes. The 3.6-kb *Eco*RI-*Eco*RI fragment carried the Km^r marker; it was subcloned in the high-copy-number vector pKS+ to produce plasmid pDV302 (Fig. 1).

Plasmid pDV302 was partially sequenced near one end of the mini-Tn*10* (Fig. 1) (see Materials and Methods) by using primer A to determine the exact point of insertion. The sequence obtained was identical to that of *aroK* (72). The sequence obtained with primer B indicated that the insertion occurred downstream of the two promoters of the *aroK* gene and upstream of its start codon (Fig. 1 and 2). We therefore renamed the *mcr-3*::*kan* insertion *aroK3*::*kan*. By sequencing with primer C, we found that pDV302 also carries the entire *aroB* coding sequence, downstream of *aroK* (Fig. 1). An *aroK* gene disruption by the insertion of a chloramphenicol resistance cassette reported previously (33) also conferred mecillinam resistance when transduced into strain DV4 (Fig. 2) (see also Table 4, strain DV6).

The *aroK* gene is the first gene in a complex operon containing the genes *aroK-aroB-urf74.3-dam-rpe-gph-trpS*, with a number of internal promoters (32, 36). The *aroB* gene codes for an enzyme required for the biosynthesis of aromatic amino acids and which acts before shikimic acid formation (46) (Fig. 3); *urf74.3* codes for a 46-kDa protein of unknown function (25); *dam* is the structural gene for DNA adenine methylase, which methylates the adenines in GATC sequences of double-stranded DNA (21, 28); *rpe* codes for ribose-5-phosphate epimerase; *gph* codes for glycolate-phosphate phosphatase (36); and *trpS* is the structural gene for tryptophanyl-tRNA synthetase (19). Since the mini-Tn*10* element is expected to have polarity effects on downstream genes, the *aroK3*::*kan* insert could reduce the expression of all the above-mentioned genes to varying degrees. We present below physiological, genetic, and biochemical characterizations of this mutant, showing that its mecillinam resistance results from the loss of a second activity of the *aroK* gene product.

Physiological characterization. Microscopic observation revealed that the *aroK3*::*kan* mutant had a normal rod shape in the absence of mecillinam and was spherical in its presence. This shows that it has an active PBP 2 which is still able to bind mecillinam and that it is not defective for MreB activity. The *aroK3*::*kan* mutant is Lac$^+$ on MacConkey lactose medium and therefore is not defective for the cAMP-CAP complex. The only phenotype conferred by the *aroK3*::*kan* mutation besides mecillinam resistance is a slightly slower growth rate than that of the parental strain, even in LB broth.

Mecillinam resistance in the *aroK3*::*kan* mutant and ppGpp. Partial inactivation of aminoacyl-tRNA synthetases confers mecillinam resistance via a RelA-dependent increase in the ppGpp pool (62). The *aroK3*::*kan* insertion could slightly decrease the expression of the tryptophanyl-tRNA synthetase, although this is unlikely to be the cause of its mecillinam resistance since it was selected in a *relA1* strain. However, the RelA1 protein retains low ppGpp synthetase activity (38), and *E. coli* possesses a second ppGpp synthetase, presumably the product of the *spoT* gene (22, 73). We therefore constructed the strains listed in Table 2 and tested their mecillinam resistance. In a *relA*$^+$ background, as in *relA1* and Δ*relA*, the *aroK3*::*kan* insertion conferred resistance to mecillinam at 1 μg/ml but not at 10 μg/ml. However, in the Δ*relA* Δ*spoT* background, in which ppGpp is completely absent, mecillinam resistance was totally lost (strain DV22), showing that it is ppGpp dependent.

In parallel, we determined the effect of the *aroK3*::*kan* mu-

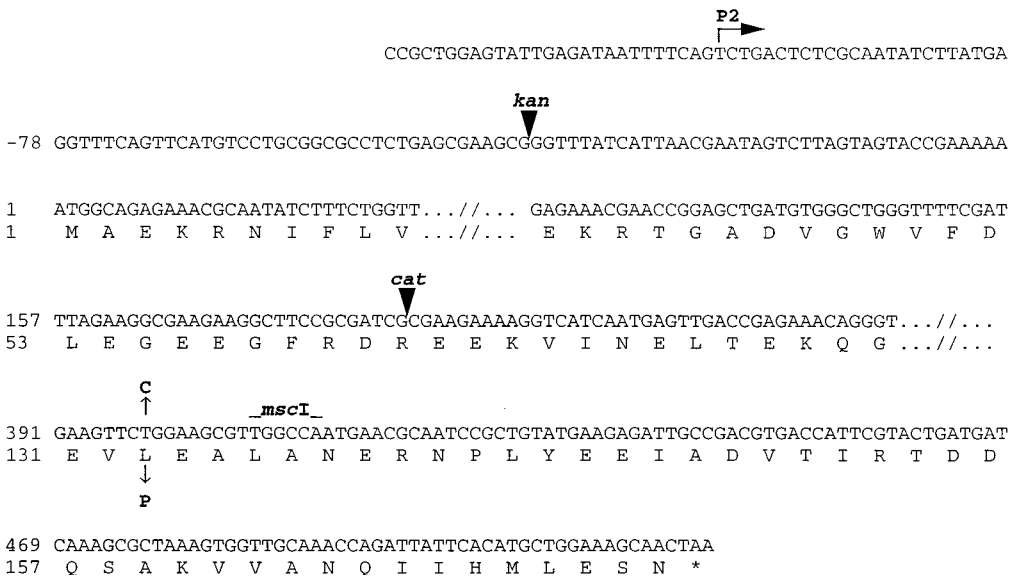


FIG. 2. Sequence of the *aroK* gene. The DNA sequence of the *aroK* gene obtained by sequencing plasmid pDV303 is shown with its translation. Vertical arrows indicate the changes observed in pDV303-1. The precise points of the two *aroK* insertions are indicated (*kan* for *aroK3*::*kan* and *cat* for *aroK*::*cat*), as is the location of the *Msc*I site used to construct plasmid pDV306 and the first nucleotide of the transcript initiated at promoter P2.

tation on ppGpp levels. Since the mecillinam resistance of strain DV5 is abolished at low NaCl concentrations (data not shown), the assays were carried out in the presence of a higher NaCl concentration than is present in classical MOPS medium (10 g/liter instead of 2.9). On this medium, as on LB plates (10 g of NaCl per liter), the *aroK*$^+$ strains were all sensitive to mecillinam whereas the *aroK3*::*kan* (*spoT*$^+$) strains were resistant. Strains DV15 (*relA1*) and DV19 (Δ*relA*) had about twofold-lower ppGpp concentrations than strain DV4 (Table 2). The *aroK3*::*kan* mutation caused about a twofold increase in the basal ppGpp concentration in the *relA*$^+$ strain but not in the *relA1* or Δ*relA* backgrounds, showing that the increase is RelA dependent. Since the *relA1 aroK3*::*kan* and Δ*relA aroK3*::*kan* strains are mecillinam resistant but have less ppGpp than the parental strain DV4, which is mecillinam sensitive, we conclude that the mecillinam resistance conferred by *aroK3*::*kan* is not due to an increased ppGpp concentration.

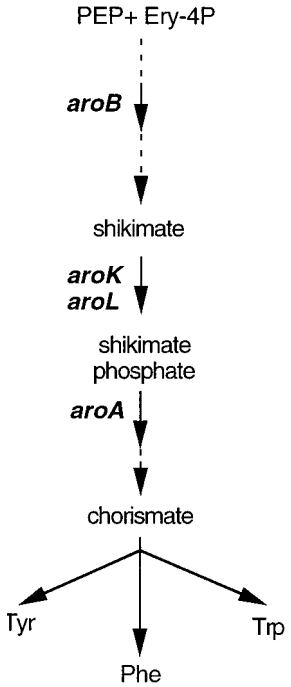


FIG. 3. The aromatic amino acid pathway. The pathway starts with phosphoenolpyruvate (PEP) and erythrose 4-phosphate (Ery-4P). AroL and AroK are the two shikimate kinases of *E. coli* which produce shikimate 3-phosphate (shikimate phosphate). Chorismate is the branch point from which tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe) are synthesized.

Mecillinam resistance of the *aroK3*::*kan* mutant is FtsZ dependent. Mecillinam resistance is conferred on wild-type strains by overproduction of the FtsZ, FtsA, and FtsQ proteins but not by overproduction of just FtsA and FtsQ (64). To see whether the mecillinam resistance conferred by *aroK* mutations involves FtsZ, we introduced the *aroK3*::*kan* allele into the *ftsZ84*(Ts) strain DV23 and tested the mecillinam resistance of the transductant (DV24) at different temperatures. On LB plates containing 10 g of NaCl per liter, neither this transductant nor the parents, DV23 and DV5, had reduced plating efficiency at 42°C (Table 3), showing that neither the *aroK3*::*kan* insertion nor the *ftsZ84*(Ts) mutation affects cell

TABLE 2. Requirement of ppGpp for mecillinam resistance in the *aroK3*::*kan* mutant

Strain	Plating efficiency on LB + Mec (1 μg/ml)[a]	ppGpp concn (pmol/OD_{600})
DV4 (wild type)	$\leq 10^{-4}$	13
DV15 (*relA1*)	$\leq 10^{-5}$	7
DV19 (Δ*relA*)	$\leq 10^{-5}$	6
DV21 (Δ*relA* Δ*spoT*)	$\leq 10^{-5}$	ND[b]
DV5 (*aroK3*::*kan*)	1	23
DV17 (*relA1 aroK3*::*kan*)	0.3	7
DV20 (Δ*relA aroK3*::*kan*)	0.5	9
DV22 (Δ*relA* Δ*spoT aroK3*::*kan*)	$\leq 10^{-5}$	ND

[a] Ratio at 37°C of titer on LB plates containing 1 μg of mecillinam per ml to that on LB plates lacking mecillinam.
[b] ND, not determined.

TABLE 3. FtsZ dependence of mecillinam resistance of the *aroK3*::*kan* mutant

Strain	Plating efficiency on LB + Mec at[a]:		
	30°C	37°C	42°C
DV4 (wild type)	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$
DV23 (*ftsZ*)	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$
DV6 (*aroK3*::*kan*)	1	1	0.5
DV24 (*ftsZ aroK3*::*kan*)	1	10^{-2}	$\leq 10^{-4}$

[a] Ratio of titer on LB plates (1% NaCl) containing 1 μg of mecillinam per ml to that on LB plates lacking mecillinam (at 30°C).

viability in this medium. However, on the same medium containing in addition 1 μg of mecillinam per ml, the *aroK3*::*kan ftsZ84*(Ts) strain DV24 could not grow at 37 or 42°C (Mec^s phenotype), although it was Mec^r at 25 and 30°C. The *ftsZ*$^+$ *aroK3*::*kan* parent DV23 remained Mec^r at all temperatures (Table 3). Thus, partial inactivation of the FtsZ84 protein abolishes the mecillinam resistance of the *aroK3*::*kan* mutant.

The *aroK3*::*kan* mutation partially suppresses *ftsZ84*(Ts). The phenotype conferred by the *ftsZ84*(Ts) mutation includes filamentation at high temperature and, on media of low osmolarity, loss of colony-forming ability; both defects are suppressed by overproduction of the altered protein FtsZ84 (69). The *aroK3*::*kan* mutation does not suppress the thermosensitivity of the *ftsZ84*(Ts) mutant in LB broth completely lacking NaCl, suggesting that it does not cause massive overproduction of FtsZ. However, under less stringent conditions, partial suppression of *ftsZ84*(Ts) was observed. When grown at 30°C in LB broth containing 5 g of NaCl per liter, the colony size for strain DV23 was larger than that for DV24, reflecting the slight growth defect due to the *aroK* mutation. At 37°C, although there was no significant decrease in plating efficiency, the colony size for strain DV23 was smaller than that for DV24, and DV23 generated faster-growing revertants at a high frequency. At 42°C, neither strain was able to form colonies on this medium, indicating that the suppression observed was only partial.

We next looked for suppression of filamentation in liquid culture. Overnight 30°C cultures of strains DV23 (*aroK*$^+$ *ftsZ84*) and DV24 (*aroK3*::*kan ftsZ84*) in LB broth containing 10 g of NaCl per liter were diluted 1,000-fold in LB broth containing 5 g of NaCl per liter and incubated at 30, 37, or 42°C. Samples of all cultures were taken at OD_{600} of 0.2 and 0.4 (about six and seven generations, respectively). Microscopic observation revealed that both strains had normal morphology at 30°C, with the cultures composed exclusively of small rod-shaped cells. At 37°C, the cells of strain DV23 were heterogeneous in size, with small and elongated cells and some long filaments, whereas DV24 cells remained morphologically normal. At 42°C, strain DV23 formed very long filaments while the DV24 cells were much shorter and more numerous at a given OD_{600} (data not shown).

From these results, we conclude that the *aroK3*::*kan* mutation partially suppresses the phenotype conferred by the *ftsZ84*(Ts) mutation. This suggests that the *aroK3*::*kan* mutation increases FtsZ activity. However, quantitative immunoassaying of the FtsZ protein showed that its concentration is the same in strains DV1 (*aroK*$^+$) and DV25 (*aroK3*::*kan*) (Fig. 4), indicating that the suppression of the *ftsZ84*(Ts) mutation by *aroK3*::*kan* does not reflect increased synthesis of the FtsZ protein. It is possible that the absence of AroK causes a qualitative change in the FtsZ protein, increasing its activity (see Discussion).

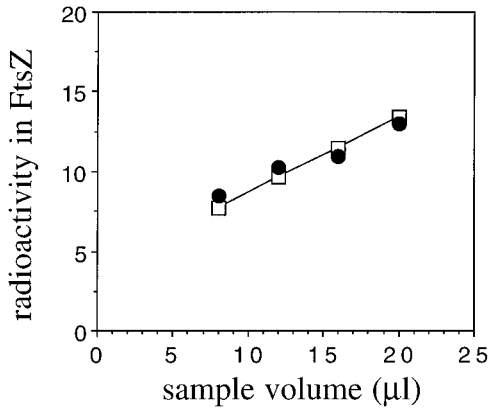


FIG. 4. FtsZ concentration in the *aroK* mutant. Strains DV1 (*aroK*$^+$) and DV25 (*aroK3*::*kan*) were grown in LB broth to OD_{600} readings of 0.18. Samples were then taken, and the concentration of FtsZ protein in each was quantified by immunoblotting, as described in Materials and Methods. Different volumes of the SDS extracts were deposited on the gel. The radioactivity values are in arbitrary units. Circles, DV1; squares, DV25.

Polarity of *aroK* insertions. The *aroK* gene codes for shikimate kinase I (33), one of the two shikimate kinases of *E. coli*; shikimate kinase II is the product of the unlinked *aroL* gene (17). Shikimate kinase activity is essential for the synthesis of aromatic amino acids. Either kinase alone is sufficient for growth in a minimal medium (17, 33), but *aroK aroL* double mutants have an aromatic requirement that cannot be satisfied by shikimate (33).

The previously described *aroK*::*cat* mutation has been shown to decrease the transcription of downstream genes; it causes aromatic-amino acid auxotrophy which can be satisfied by shikimate and therefore reflects a polarity effect on *aroB* rather than the loss of shikimate kinase activity (32, 33). We confirmed this observation (Table 4, strain DV6) but found that strain DV5 (*aroK3*::*kan*) can grow on minimal glucose plates, although the colonies are slower to appear than those of the parental strain. Since shikimate permits faster growth, we conclude that the *aroK3*::*kan* insertion decreases but, unlike *aroK*::*cat*, does not completely abolish expression of the *aroB* gene. However, either insertion, when combined with an *aroL*::Tn*10* mutation (inactivating shikimate kinase II), abolishes the ability to grow on glucose shikimate plates (strains DV10 and DV11). This suggests that the *aroK3*::*kan* insertion

TABLE 4. Specificity of mecillinam resistance to loss of AroK

Strain	Plating efficiency on LB + Mec at 37°C[a]	Growth at 37°C on:	
		Min glu[b]	Min glu + shikimate
DV4	$\leq 10^{-4}$	+	+
DV5 (*aroK3*::*kan*)	0.8	+	+
DV6 (*aroK*::*cat*)	0.9	−	+
DV9 (*aroL*)	$\leq 10^{-4}$	+	+
DV7 (*aroB*)	$\leq 10^{-4}$	−	+
DV8 (*aroA*)	$\leq 10^{-4}$	−	−
DV14 (*aroK3*::*kan aroB*)	0.8	−	+
DV10 (*aroK3*::*kan aroL*)	0.6	−	−
DV11 (*aroK*::*cat aroL*)	0.6	−	−
DV12 (*aroK3*::*kan aroA*)	1	−	−
DV13 (*aroK*::*cat aroA*)	0.6	−	−

[a] Ratio of titer on LB plates containing 1 μg of mecillinam per ml to that on LB plates lacking mecillinam.
[b] Min glu, M9 minimal medium plus glucose.

completely blocks the transcription of *aroK* but only partially blocks transcription of *aroB*. However, Løbner-Olesen et al. (32) did not find any promoters capable of driving the transcription of *aroB* other than P1 and P2, which are upstream of *aroK*. This apparent contradiction may result from a weak, previously undetected promoter within the *aroK* gene (32). Alternatively, it is possible that the transcripts initiated at P1 and P2 and continuing through *aroK3*::*kan*, although reduced in number, provide sufficient AroB to be Aro^+ but insufficient AroK to be Mec^s.

We inactivated *aroB*, *urf74.3*, and *dam* either singly, in pairs, or concomitantly to test whether the mecillinam resistance conferred by insertions in *aroK* is due to a polarity effect on downstream genes. All $aroK^+$ strains were sensitive to mecillinam (Table 4 and data not shown). Moreover, all of the *aroK3*::*kan* strains were resistant, indicating that none of these functions is required for the insertion to confer mecillinam resistance (Table 4). It is thus unlikely that this resistance is due to decreased expression of the genes following *aroK* in the transcription unit.

As final proof that the mecillinam resistance observed results simply from decreased expression of *aroK*, we amplified the P1-P2-*aroK* region of strain DV1 ($aroK^+$) by PCR and cloned it into the low-copy-number vector pCL1920 (see Materials and Methods). The resulting plasmid, pDV303 (Fig. 1), carries the minimal wild-type *aroK* gene, whose sequence was found to be identical to the one which was recently published (72). Plasmid pDV303 suppressed the mecillinam resistance of *aroK3*::*kan* and *aroK*::*cat* strains (data not shown) and also suppressed both the mecillinam resistance and the aromatic auxotrophy of the *aroK3*::*kan aroL*::Tn*10* double mutant (Table 4). This result proves that mecillinam resistance results from decreased expression of the *aroK* gene and not from polarity effects on expression of downstream genes in the operon.

***aroK* mutants can survive without PBP 2.** Although *aroK* mutants are completely resistant to mecillinam at 1-μg/ml, they do not form colonies on media containing 10 μg of the antibiotic per ml. To see whether this reflects an inability to survive in the complete absence of PBP 2, we tried to transduce a deletion of the PBP 2 structural gene, Δ(*rodA-pbpA*)::*kan*, into *aroK* strains by using a linked Tn*10* marker (donor strain DV26). In the *aroK*::*cat* strain DV6, of 44 Tc^r transductants, 38 had become Km^r and displayed a slower growth rate; the cells of Km^r transductants were spherical, confirming the lack of PBP 2. In the *aroK3*::*kan* strain DV5, which is already Km^r, of 40 Tc^r transductants, 31 grew more slowly and had acquired a spherical morphology. When, as a control, we transduced strain DV4, none of the 48 Tc^r transductants were Km^r. We conclude that *aroK* mutants have acquired the ability to grow and divide in the complete absence of PBP 2.

AroK possesses a second activity. The loss of shikimate kinase I activity does not confer auxotrophy since it is compensated for by shikimate kinase II. Nevertheless, it was possible that a slightly reduced efficiency of aromatic amino acid biosynthesis was the cause of mecillinam resistance in *aroK* mutants. We tested this hypothesis by blocking the pathway at other steps. A block before shikimate, in the *aroB* mutant, or after shikimate phosphate, in the *aroA*::Tn*10* mutant (Fig. 3), did not result in mecillinam resistance (Table 4). Furthermore, neither of these blocks prevented *aroK* insertions from conferring mecillinam resistance. The *aroB aroK3*::*kan* strain, which was presumed to be unable to carry out endogenous shikimate synthesis, remained mecillinam resistant even in the absence of exogenous shikimate, as seen on plates of M9 glucose supplemented with all 20 amino acids, *p*-hydroxybenzoic acid, and *p*-aminobenzoic acid (0.2 mM each). Similarly, the inactivation of shikimate kinase II by an *aroL*::Tn*10* mutation did not confer mecillinam resistance and did not prevent resistance in *aroK* mutants. This shows that the two shikimate kinases are not equivalent and indicates that the activity of the AroK protein which makes cells sensitive to mecillinam does not require the presence of shikimate (it is absent in the *aroB* and *aroK*::*cat* mutants). Therefore, AroK must possess a second activity, distinct from shikimate kinase and not involved in aromatic amino acid biosynthesis.

TABLE 5. Mecillinam resistance in a strain lacking a second AroK activity distinct from shikimate kinase

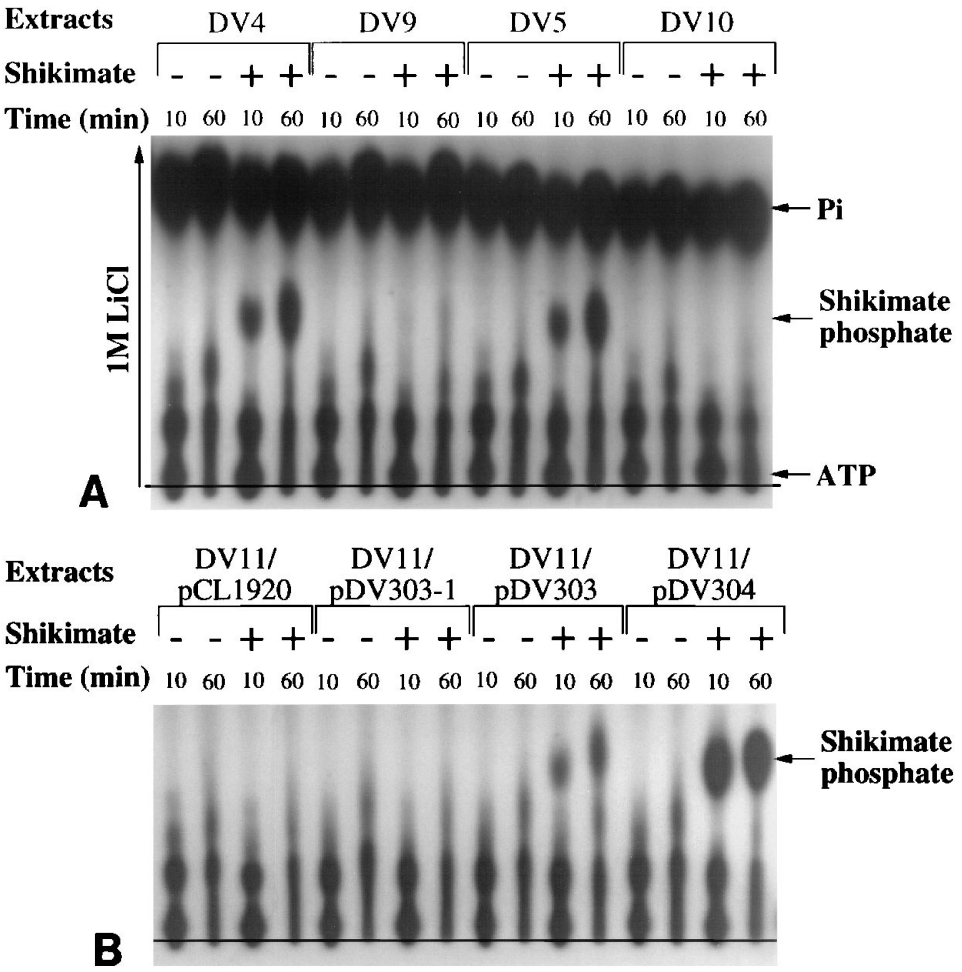
Strain[a]	Plating efficiency at 37°C on LB + Mec[b]	Growth at 37°C on:	
		Min glu[c]	Min glu + shikimate
DV10/pCL1920	1	−	−
DV10/pDV303	$\leq 8 \times 10^{-5}$	+	+
DV10/pDV303-1	4×10^{-4}	−	−
DV10/pDV306	1	−	−

[a] DV10 has the genotype *aroK3*::*kan aroL*::Tn*10*.
[b] Ratio of the titer on LB plates containing 1 μg of mecillinam per ml to that on LB plates lacking mecillinam.
[c] Min glu, M9 minimal medium plus glucose.

We separated these two activities mutationally. In our cloning of the *aroK* gene (see above), we fortuitously obtained plasmid pDV303-1 as a PCR-induced mutant which suppressed the mecillinam resistance of the *aroK3*::*kan aroL*::Tn*10* strain but did not suppress its auxotrophy for aromatic amino acids (Table 5). The *aroK* gene in pDV303-1 has a single sequence change, causing a Leu-133→Pro substitution (Fig. 2). To see whether a C-terminal deletion of the AroK protein had the same effect as the Leu-133→Pro mutation, we constructed plasmid pDV306 (Fig. 1); this plasmid corrected neither the mecillinam resistance nor the auxotrophy of the *aroK3*::*kan aroL*::Tn*10* strain. The properties of plasmid pDV303-1, together with the results presented above, show that the AroK protein has a second activity which can be disassociated from shikimate kinase activity and is involved in mecillinam sensitivity.

Shikimate kinase tests. We tested S100 extracts of various strains for shikimate kinase activity to verify deductions from growth requirements. The ability to phosphorylate shikimate was measured after resolving shikimate phosphate from both the [γ-^{32}P]ATP phosphate donor and its inorganic hydrolysis products, phosphate and pyrophosphate, by thin-layer chromatography in 1 M LiCl. A high shikimic acid concentration (5.7 mM) was used to allow the detection of either source of shikimate kinase activity, about 30-fold above the K_m for the *aroL*-encoded source of shikimate kinase and approaching the K_m value (≥20 mM) of the *aroK*-encoded shikimate kinase (18). In these crude extracts, shikimate kinase competed with other sources of ATPase; in the presence or absence of shikimic acid, release of labeled P_i from 2 mM [γ-^{32}P]ATP was linear with time during the first 10 min (data not shown) and hydrolysis of input ATP was complete by 60 min (Fig. 5).

One radioactive spot, which was presumed to be shikimate phosphate, appeared only when shikimic acid was added together with extracts of $aroL^+$ strains (Fig. 5A), regardless of the allelic state of *aroK*, i.e., for strains DV4 ($aroL^+$ $aroK^+$) and DV5 ($aroL^+$ *aroK3*::*kan*) but not for strain DV9 (*aroL*::Tn*10* $aroK^+$) or DV10 (*aroL*::Tn*10 aroK3*::*kan*). Quantification revealed that about 10% of the [γ-^{32}P]ATP substrate (2 mM, 25 μCi/μmol) was found associated with putative shiki-



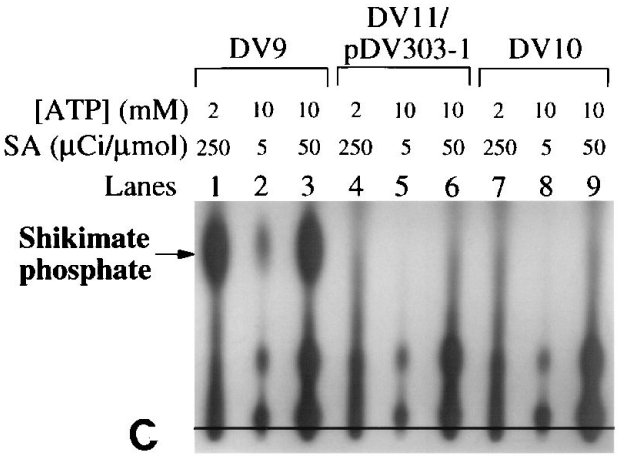


FIG. 5. Shikimate kinase assays with S100 extracts. Assays were carried out as described in Materials and Methods, with each 10-μl reaction mixture containing 12.5 μg of protein, 5.7 mM shikimic acid, and [γ-^{32}P]ATP as indicated below. Reactions were terminated by applying 2-μl aliquots at the origin, indicated by the horizontal thick line at the bottom of each polyethyleneimine-cellulose chromatogram, after 10 min (A), 60 min (B), or 2 h (C) of incubation. The chromatograms were subjected to ascending development with 1 M LiCl, dried, and autoradiographed for 17 h (A and B) or for 3 h (C). The position of what is presumed to be shikimate phosphate is indicated in all panels; in addition, the positions of ATP and P_i are shown in panel A. (A) Reaction mixtures containing extracts of strains DV4 (wild type), DV9 (*aroL*::Tn*10*), DV5 (*aroK3*::*kan*), and DV10 (*aroL*::Tn*10 aroK3*::*kan*) were incubated with 2 mM [γ-^{32}P]ATP (25 μCi/μmol). (B) Reactions were carried out as for panel A but with extracts of strain DV11 (*aroK*::*cat aroL*::Tn*10*) carrying either pCL1920 (low-copy-number vector control), pDV303-1 (low-copy-number *aroK* L133P mutant), pDV303 (low-copy-number *aroK*$^+$), or pDV304 (high-copy-number *aroK*$^+$). An extract of strain DV11 bearing the high-copy-number vector pKS+ behaved like the low-copy-number vector control, with no detectable shikimate kinase activity (data not shown). (C) Reactions were carried out in 5.7 mM shikimic acid with extracts of strain DV9, DV11/pDV303-1, and DV10, each under three different conditions of [γ-^{32}P]ATP abundance or specific activity.

mate phosphate after 60 min of incubation with *aroK*$^+$ extracts. Weaker shikimate kinase activity could be demonstrated in extracts of strain DV9 by increasing the reaction time and increasing the specific activity of [γ-^{32}P]ATP 10-fold (to 250 μCi/μmol) or by increasing the concentration of ATP 5-fold at a specific activity of either 5 or 50 μCi/μmol (Fig. 5C, lanes 1 to 3). Under these conditions, there was no detectable activity in extracts of the *aroL*::Tn*10 aroK3*::*kan* strain DV10 (Fig. 5C, lanes 7 to 9).

AroK-dependent shikimate kinase could be overexpressed to levels detected under the same assay conditions as described for Fig. 5A, i.e., 2 mM [γ-^{32}P]ATP at 25 μCi/μmol, by increasing the *aroK* gene copy number with *aroK* cloned in strain DV11, which is otherwise devoid of shikimate kinase because of *aroK*::*cat* and *aroL*::Tn*10* insertions (Fig. 5B). Extracts of strains carrying the *aroK* gene cloned in a low-copy-number pSC101 derivative (pDV303) and in a high-copy-number pKS+ plasmid (pDV304) gave 4.6 and 35%, respectively, of

the total input radioactivity associated with putative shikimate phosphate after 60 min of incubation (Fig. 5B). In each case, no activity was found in this region of the chromatogram either in the absence of added shikimic acid or when we used extracts from strain DV11 bearing the vector controls pCL1920 and pKS+ and assayed them in the presence of shikimate (Fig. 5B and data not shown). In addition, we did not detect shikimate kinase activity in extracts of cells bearing pDV303-1, the *aroK* (L133P) allele cloned in pCL1920, under these conditions or when the assay was performed under conditions that allowed detection of shikimate kinase expressed from a single copy of *aroK* on the chromosome (Fig. 5C, lanes 4 to 6).

In summary, the phosphorylated compound we believe to be shikimate phosphate could be detected in all extracts of Aro^+ strains ($aroL^+$ and/or $aroK^+$) but was never found in *aroL aroK* extracts, in reaction mixtures lacking added shikimic acid, or in the absence of extract. The shikimate kinase specific activity associated with AroL was higher than the specific activity associated with AroK, as expected (18), and the low activity associated with AroK increased with increasing gene copy number, eventually exceeding that present in extracts from single-copy $aroL^+$ strains. The in vitro assays further confirm that the L133P mutation in *aroK* carried by plasmid pDV303-1 inactivates the AroK shikimate kinase activity.

DISCUSSION

From a collection of random mini-Tn*10* insertions we selected one which confers resistance to mecillinam, a β-lactam which specifically inhibits PBP 2. Surprisingly, the insertion proved to lie between the promoters and the coding sequence of the *aroK* gene, which codes for shikimate kinase I, an enzyme of the aromatic pathway. This location, which was determined by sequencing the insert ends, is based on the revised *aroK* sequence (72), which we confirmed here. The insertion, called *aroK3*::*kan*, effectively abolishes shikimate kinase I activity, both in vivo and in vitro. An *aroK*::*cat* insertion, in which the chloramphenicol cassette lies within the *aroK* coding sequence, also confers mecillinam resistance. The *aroK* gene is promoter proximal in a complex operon which has several internal promoters. However, the mecillinam resistance clearly resulted from the loss of AroK, since plasmid pDV303, carrying only the $aroK^+$ gene, restored antibiotic sensitivity.

We have previously shown that an increase in the basal level of the nucleotide ppGpp makes *E. coli* mecillinam resistant (8, 26, 62). In a $relA^+$ background, the *aroK3*::*kan* mutation causes a doubling of the ppGpp concentration. This is sufficient to make cells mecillinam resistant (26) and therefore seemed to offer the key to understanding why *aroK* mutants can grow and divide in the absence of PBP 2. However, in a *relA1* or Δ*relA* context, the *aroK3*::*kan* allele had little effect on the ppGpp level, which in both strains was lower than that observed in the wild type, yet the *aroK relA* strains remained mecillinam resistant. The mecillinam resistance thus cannot be explained by an increased ppGpp concentration, although it does still seem to require either the nucleotide or the SpoT protein itself, since the *aroK* Δ*relA* Δ*spoT* triple mutant, which lacks ppGpp, is mecillinam sensitive.

We have also shown that *E. coli* can be made mecillinam resistant by increasing the concentration of the FtsZ, FtsA, and FtsQ proteins (but not by increasing FtsA and FtsQ alone), even in a *relA1* strain (64). This means that a higher division capacity is required for growth in the absence of PBP 2 (as a sphere) than for growth with functional PBP 2 (as a rod) (8, 62). This remains true in the *aroK3*::*kan* mutant, in which decreasing the FtsZ activity by cultivating a thermosensitive mutant under semipermissive conditions made the strain sensitive to PBP 2 inactivation by mecillinam. Furthermore, the *aroK3*::*kan* allele partially suppresses the *ftsZ84*(Ts) mutation, suggesting that it increases FtsZ activity. Direct measurement of the amount of FtsZ protein, however, revealed no difference between *aroK3*::*kan* and wild-type strains; our immunoassay should detect as little as a 15% increase. We previously suggested the possibility that the FtsZ protein must be activated to carry out septation and presented evidence that ppGpp does not itself activate FtsZ (64). It is possible that in *aroK* mutants, as in the presence of high ppGpp concentrations, FtsZ is more efficiently activated than in the wild type, providing a higher division capacity with the same amount of FtsZ protein. The mechanism of this hypothetical activation and the role played by AroK remain to be determined.

Although the *aroK* gene product has shikimate kinase activity (reference 33 and this work), it has been reported to have a K_m of greater than 20 mM for shikimic acid, compared with 0.2 mM for shikimate kinase II, suggesting that it may have another physiological function unrelated to the phosphorylation of shikimate (18). We have shown here that its function in making *E. coli* sensitive to mecillinam is indeed distinct from its shikimate kinase activity. First of all, *aroK* mutants are mecillinam resistant even in an *aroB* genetic background, in which there is presumably no endogenous synthesis of shikimic acid (see Fig. 3), and in a medium devoid of shikimic acid. Second, we isolated a mutant allele of *aroK* which had lost shikimate kinase activity, both in vivo and in vitro, but still restored mecillinam sensitivity to an *aroK* strain.

The nature of the second activity of the *aroK* gene product is not known. The results presented here suggest that AroK phosphorylates (or otherwise modifies) some substrate other than shikimate and that this product results in a lower division potential. One particularly appealing possibility is that AroK can act as a protein kinase, perhaps phosphorylating a division protein such as FtsZ, FtsA, or FtsQ, thereby lowering the cell's division potential. It is interesting to note, in this context, that FtsA is thought to be phosphorylated in vivo (51). Studies to determine the nature of the second reaction catalyzed by AroK are currently in progress.

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