JOURNAL OF BACTERIOLOGY, June 2006, p. 4413–4423 Vol. 188, No. 12
0021-9193/06/$08.00+0 doi:10.1128/JB.01502-05

Role for Tandem Duplication and Lon Protease in AcrAB-TolC-Dependent Multiple Antibiotic Resistance (Mar) in an *Escherichia coli* Mutant without Mutations in *marRAB* or *acrRAB*

Hervé Nicoloff,[1,2] Vincent Perreten,[1,2]† Laura M. McMurry,[1,2] and Stuart B. Levy[1,2,3]*

Center for Adaptation Genetics and Drug Resistance[1] and the Departments of Molecular Biology and Microbiology[2] and Medicine,[3] Tufts University School of Medicine, Boston, Massachusetts 02111

Received 3 October 2005/Accepted 5 April 2006

A spontaneous mutant (M113) of *Escherichia coli* AG100 with an unstable multiple antibiotic resistance (Mar) phenotype was isolated in the presence of tetracycline. Two mutations were found: an insertion in the promoter of *lon* (*lon3*::IS*186*) that occurred first and a subsequent large tandem duplication, dupIS*186*, bearing the genes *acrAB* and extending from the *lon3*::IS*186* to another IS*186* present 149 kb away from *lon*. The decreased amount of Lon protease increased the amount of MarA by stabilization of the basal quantities of MarA produced, which in turn increased the amount of multidrug efflux pump AcrAB-TolC. However, in a mutant carrying only a *lon* mutation, the overproduced pump mediated little, if any, increased multidrug resistance, indicating that the Lon protease was required for the function of the pump. This requirement was only partial since resistance was mediated when amounts of AcrAB in a *lon* mutant were further increased by a second mutation. In M113, amplification of *acrAB* on the duplication led to increased amounts of AcrAB and multidrug resistance. Spontaneous gene duplication represents a new mechanism for mediating multidrug resistance in *E. coli* through AcrAB-TolC.

Emergence of antibiotic resistance among bacteria can be achieved by horizontal acquisition of resistance genes or by mutations of chromosomal loci. In the environment, most of the interactions between bacteria and antibacterial agents probably occur at low concentrations of drugs that favor the selection of chromosomal mutations which mediate low-level drug resistance.

In *Escherichia coli*, the *marRAB* operon codes for two transcriptional regulators: MarR represses the transcription of the operon, whereas MarA positively regulates *marRAB* (8, 19). In the presence of various inducers, some of which (such as 2,4-dinitrophenol [DNP] and sodium salicylate) directly interact with and inactivate MarR (1, 37), overexpression of *marRAB* occurs. Once produced, MarA can increase or decrease (directly or indirectly) the transcription of 60 to 80 genes of the *mar* regulon (6, 33), resulting in decreased amounts of OmpF porin and increased amounts of the AcrAB-TolC multidrug efflux pump. Reduction in membrane permeability and increase in drug export lead to resistance to multiple antibiotics and other antimicrobial compounds such as dyes, detergents, organic solvents, and oxidative stress agents (2, 26, 31, 44), referred to as the *m*ultiple *a*ntibiotic *r*esistance (Mar) phenotype. Overexpression of SoxS and Rob proteins also leads to increased antibiotic resistance by up-regulating *marRAB* and a set of genes that resembles the *mar* regulon (3, 7, 15, 25, 33). All mutations conferring a spontaneous Mar phenotype heretofore have been found within the *marRAB*, *soxRS*, and *acrRAB* loci among laboratory (8, 16, 25) and clinical (18, 30, 42, 43) isolates of *E. coli*.

We now describe an overproduction of MarA and a multidrug resistance phenotype resulting from novel mutations found in a spontaneous *E. coli* mutant selected in the presence of a low concentration of tetracycline.

MATERIALS AND METHODS

Plasmids, strains, and growth conditions. Bacteria were grown in liquid or on agar of Luria-Bertani (LB) medium at 37°C or 33°C as specified. Ethidium bromide was purchased from Amresco Inc., Solon, Ohio; ampicillin, chloramphenicol, kanamycin, nalidixic acid, tetracycline, and DNP were purchased from Sigma-Aldrich Co., St. Louis, Mo. Tetracycline was used at 12 μg/ml for selection of the Tn*10* marker. Kanamycin (30 μg/ml) was used for selection of the Tn*10*Kan and Kan markers. Plasmid pSP-*nfnB*1, with an *nfnB*-promoter::*luc* fusion, and plasmid pSP-*luc*+ used to construct pSP-*nfnB*1 (5), were selected with ampicillin (100 μg/ml).

The *E. coli* strains used during this work are presented in Table 1. M113 was selected as follows: a single colony of *E. coli* AG100 was grown at 37°C under vigorous shaking in LB broth up to the late logarithmic phase (A_{600} of 1.0). One hundred microliters of this culture (representing about 10^8 bacteria) was spread onto one LB agar plate containing 4 μg/ml of tetracycline. The tetracycline-resistant spontaneous mutant M113 appeared after 3 days of incubation at 33°C. M113 was purified two times on LB plates supplemented with tetracycline (4 μg/ml). One isolated colony was grown in LB broth and stored at −80°C in 20% glycerol. Subsequently, a colony of M113 isolated on LB medium (M113R) that was found to have spontaneously lost its resistance was isolated and purified two more times on LB plates. After growth in LB broth, M113R (shown to bear the *lon3*::IS*186* mutation) was stored frozen in 20% glycerol. The strains constructed were purified on LB plates after transduction, grown in LB broth, and stored frozen. Because of the presence of the *ppiD*::Tn*10* marker near *lon* and the junction of two amplified units, P1 transductions allowed construction of strains with different genotypes. For example, P1 transduction from the donor strain CAG12017 to the recipient M113 and selection for Tn*10* allowed the isolation of

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: stuart.levy@tufts.edu.

† Present address: Institute of Veterinary Bacteriology, University of Berne, Berne, Switzerland.

TABLE 1. *Escherichia coli* strains used in this study[a]

Name	Genotype	Description or construction	Reference
AG100	*argE3 thi-3 rpsL xyl mtl supE44*		11
AG112	AG100 *marR* (5-bp deletion near 5′ end)	Spontaneous AG100 mutant	29
AG100A	AG100 Δ*acrAB*::Kan		31
AG100B	AG100 Δ*acrR*::Kan		31
CAG12017	MG1655 *ppiD*::Tn*10*		39
M113	AG100 *lon3*::IS*186* dupIS*186*	Spontaneous AG100 mutant	This study
GJ1913	MG1655 *zba-911*::Tn*10*Kan		36
GJ1912	MG1655 *zba-911*::Tn*10*Kan *lon-103*::IS*186*	Isogenic to GJ1913	36
AB1157	Wild type		14
AB1899	AB1157 *lon1*::IS*186*, unknown	Spontaneous AB1157 mutant	14
Constructed strains			
M113R	AG100 *lon3*::IS*186*	Partial revertant of M113	This study
M113HN1	AG100 *ppiD*::Tn*10*	M113 × CAG12017	This study
M113HN12	AG100 *lon3*::IS*186 ppiD*::Tn*10*	M113 × CAG12017	This study
M113HN11	AG100 *dupIS186 ppiD*::Tn*10*	M113 × CAG12017	This study
M113HN2	AG100 *lon3*::IS*186* dupIS*186 ppiD*::Tn*10*	M113 × CAG12017	This study
AG100HN19	AG100 *ppiD*::Tn*10*	AG100 × M113HN12	This study
AG100HN17	AG100 *lon3*::IS*186 ppiD*::Tn*10*	AG100 × M113HN12	This study
AG100HN3	AG100 *dupIS186 ppiD*::Tn*10*	AG100 × M113HN2	This study
AG100HN50	AG100 *zba-911*::Tn*10*Kan	AG100 × GJ1912	This study
AG100HN55	AG100 *lon-103*::IS*186 zba-911*::Tn*10*Kan	AG100 × GJ1912	This study
AG100AHN2-21	AG100 Δ*acrAB*::Kan dupIS*186 ppiD*::Tn*10*	AG100A × M113HN2	This study
AG100AHN61	AG100 Δ*acrAB*::Kan *ppiD*::Tn*10*	AG100A × M113HN12	This study
AG100AHN60	AG100 Δ*acrAB*::Kan *lon3*::IS*186 ppiD*::Tn*10*	AG100A × M113HN12	This study
AG112HN36	AG100 *marR ppiD*::Tn*10*	AG112 × M113HN12	This study
AG112HN48	AG100 *marR lon3*::IS*186 ppiD*::Tn*10*	AG112 × M113HN12	This study
AG112HN2-74	AG100 *marR* dupIS*186 ppiD*::Tn*10*	AG112 × M113HN2	This study
AB1157HN69	AB1157 *ppiD*::Tn*10*	AB1157 × M113HN12	This study
AB1157HN70	AB1157 *lon3*::IS*186 ppiD*::Tn*10*	AB1157 × M113HN12	This study
AB1899HN67	AB1157 *ppiD*::Tn*10*, unknown	AB1899 × CAG12017	This study
AB1899HN68	AB1157 *lon1*::IS*186 ppiD*::Tn*10*, unknown	AB1899 × CAG12017	This study

[a] P1 transductions are noted as follows: recipient strain × donor strain. Construction of the different mutants and the conditions for isolation of M113 and M113R are described in Materials and Methods. The term "unknown" indicates that an additional unknown mutation is present. *ppiD*::Tn*10* is also called *zba-3054*::Tn*10* (27).

(i) a strain which reverted one of the *lon*-mutated loci, but not the duplication (strain M113HN11); (ii) a strain which reverted the duplication, but not the *lon3*::IS*186* mutation (strain M113HN12); (iii) a strain with no change (strain M113HN2); and (iv) a strain that reverted both the duplication and the *lon3*::IS*186* mutation (strain M113HN1).

Drug susceptibility testing. MICs were determined by two different methods: LB agar plating or Etests (AB Biodisk, Solna, Sweden). Strains freshly grown in LB broth up to mid-logarithmic phase ($A_{600} = 0.5$) were diluted in saline solution (0.9% NaCl) to an A_{600} of 0.05. Alternatively, 5-μl drops of suspensions of isolated colonies, each in 130 μl of saline solution, were put on LB agar plates supplemented with increasing amounts of nalidixic acid or chloramphenicol (0, 2, 3, 4, 6, 8, 10, 12, and 16 μg/ml), and incubated at 37°C for 24 h. The increased susceptibility to DNP was determined on LB agar plates supplemented with 0.6 mM DNP after 24 h of incubation at 37°C. Etests were used as recommended by the manufacturer. When strains with the large tandem duplication dupIS*186* were analyzed, bacteria were usually grown in the presence of nalidixic acid (5 μg/ml) to maintain the duplication.

Protein extractions. Whole-cell proteins were analyzed from *E. coli* cultures freshly grown in LB broth to an A_{600} of 0.5 as described previously (42). Membranes and cytosol proteins were separated as follows. Ten milliliters of bacteria freshly grown in LB broth up to an A_{600} of 1.0 was harvested by centrifugation, resuspended in 200 μl of a mixture of cold Tris-HCl, pH 8.0 (20 mM), $MgCl_2$ (2 mM), EDTA (1 mM), and lysozyme (50 μg/ml), and incubated for 30 min on ice. After addition of 3 μl of protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, Mo.), bacteria were disrupted by sonication for 1 min (output, 2; duty cycle, 15%; Branson Sonifier 250). Unbroken cells were removed by centrifugation for 5 min at 5,000 × *g* at 4°C, and the membranes were harvested from the supernatant by centrifugation for 45 min at 100,000 × *g* at 4°C. Cytosolic proteins from the supernatant were precipitated by addition of deoxycholate at a final concentration of 0.002%, incubation 30 min on ice, addition of trichloroacetic acid at a final concentration of 10%, and incubation overnight at 4°C. Precipitated proteins were harvested by centrifugation for 15 min at 13,000 × *g*, washed with cold acetone, dried, and finally resuspended at room temperature in 200 μl of 1% sodium dodecyl sufate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride. Fifty microliters of the same solution was used to solubilize the membranes for 30 min at room temperature.

Detection of MarA, AcrA, and TolC by Western blot hybridization. Proteins were loaded on a 15% (MarA) or 10% (AcrA and TolC) sodium dodecyl sulfate-polyacrylamide gel and separated by electrophoresis (17). The proteins from the gel were transferred electrophoretically for 1 h at 25 V (Trans-Blot SD semidry transfer cell; Bio-Rad, Richmond, Calif.) to a polyvinylidene difluoride membrane (Polyscreen PVDF transfer membrane; NEN Life Science Products, Inc., Boston, Mass.). The membrane was blocked overnight at room temperature in Tris-buffered saline (TBS; 17.1 mM NaCl, 5.1 mM KCl, 2.5 mM Tris-base, pH 7.4) containing 1% bovine serum albumin, washed three times in wash buffer (0.045% Tween 80 in TBS), and hybridized for 1 h at room temperature with polyclonal antibodies diluted 1:10,000 (anti-AcrA), 1:3,000 (anti-MarA) (22), or 1:5,000 (anti-TolC) in buffer A (0.5% Triton X-100, 0.2% sodium dodecyl sulfate, 0.1% bovine serum albumin in TBS). After three washes in wash buffer, the membrane was hybridized at room temperature with horseradish peroxidase conjugated to anti-rabbit antibodies (Cell Signaling Technology, Inc., Beverly, Mass.) diluted 1:2,000 in buffer A. After three washes in wash buffer, the blots were developed with the Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, Mass.). Quantification of the band intensities was done with ImageQuant software (Amersham Biosciences Inc., Piscataway, N.J.).

Luciferase assays. Luciferase was quantified with the Steady-Glow luciferase assay system (Promega, Madison, Wis.) and a Wallac Victor^3 V microplate reader (Perkin-Elmer, Boston, Mass.) as described previously (5).

P1 transduction. Fifty microliters of an overnight culture of the donor strain was diluted into 5 ml of LB broth supplemented with 5 mM $CaCl_2$. After 1.5 h of incubation at 37°C under vigorous shaking, the culture was inoculated with 100 μl of bacteriophage P1*vir* lysate of AG100. After complete lysis of the donor cells (about 3 h of incubation at 37°C), 200 μl of chloroform was added and the culture was vortexed and centrifuged (15 min at 1,500 × *g*). Chlo-

roform was added to the supernatant, which was vortexed and stored at 4°C. Two milliliters of an overnight culture of the recipient strain was centrifuged for 10 min at 9,000 × *g*. Pellets were resuspended in 1 ml of a solution of 10 mM $MgSO_4$ and 5 mM $CaCl_2$ and incubated for 20 min at 37°C. P1*vir* lysate (1, 10, or 100 μl) was added to 100 μl of recipient cells (controls of cells or lysate only were included). After 25 min of incubation at 37°C without shaking, 200 μl of LB broth supplemented with 50 mM sodium citrate was added. After 1 h of incubation at 37°C under vigorous shaking, tubes were centrifuged (10 min at 9,000 × *g*), and the pellets were resuspended and plated on LB selective medium supplemented with 10 mM sodium citrate and the appropriate antibiotic and incubated overnight at 37°C.

Nucleic acid extraction. For high-quality DNA, extraction from an overnight culture of bacteria in LB broth was performed with the DNeasy tissue kit (QIAGEN, Inc.). For noncomparative PCR-grade DNA template, one colony was suspended in 130 μl of sterile water. Alternatively, 100 μl of bacterial culture was centrifuged for 10 min at 9,000 × *g*, and the pellet was suspended in 50 μl of sterile water. Twenty-five microliters of those suspensions was heated for 10 min at 98°C and then centrifuged for 5 min at 9,000 × *g*, and a maximum of 3 μl of the supernatant was used as template DNA for PCR.

Total RNA was extracted from cultures in exponential phase (A_{600} of 0.6) with the RNeasy minikit (QIAGEN, Inc.), and treated twice with DNase (QIAGEN, Inc.). RNA quality was verified after electrophoresis in 1% agarose gel containing 0.25 μg/ml of ethidium bromide. Comparison of RNA concentrations in each extraction was assessed by quantification of the rRNA bands with the Gel doc 1000 digital camera system and the Molecular Analyst software (both by Bio-Rad).

Reverse transcription. About 200 ng of total RNA was used for cDNA synthesis by reverse transcription using the Superscript III First-Strand synthesis system for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, Calif.) and the random hexamer primers, following the conditions recommended by the manufacturer. Control reactions without reverse transcriptase were also performed.

Primers and routine PCR. Primers used for PCR amplifications (Table 2) were synthesized by the Tufts University Core Facility. PCRs in a final volume of 25 μl were performed in the Gene Amp PCR Systems 2700 and 9700 apparatus (Applied Biosystem, Foster City, Calif.) using *Taq* polymerase enzyme (Invitrogen) and the conditions recommended by the manufacturer. Amplified products were separated by electrophoresis with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, 0.114% glacial acetic acid) in 1% agarose gels containing ethidium bromide at a final concentration of 0.25 μg/ml and visualized under UV light, and their size was determined by comparison with the 1-kb DNA ladder (Invitrogen).

Comparative PCR protocols. The comparative PCR protocols were derived essentially from references 13 and 9 and were done in thin-walled 0.2-ml PCR tubes (USA Scientific, Inc.). DNA at a concentration of 1 ng/μl was diluted 1:40 in sterile water. From this solution, eight twofold dilutions were performed and used as template DNA (10 μl) in different PCR amplifications of 30 cycles using the same mixture of water, enzyme, deoxynucleoside triphosphates (dNTPs), and primers hybridizing to *marA* (Table 2). Amplified products were separated by electrophoresis in a 1.2% agarose gel in TAE buffer. The gel was stained for 30 min in TAE buffer supplemented with 0.5 μg/ml of ethidium bromide, followed by 30 min of destaining in TAE buffer solution. Amplified bands were visualized and quantified under UV light (see RNA extraction protocol). PCR amplifications stopped in their log phase (faint bands with proportionality between the quantity of DNA amplified and the quantity of DNA used as template) were used to compare the quality and concentration of DNA in each of the extractions. Amplifications of *ybaO* were done following a similar protocol and with the same dilutions of template DNA, but the intensities of the amplified bands were normalized according to the results obtained with the standard *marA*. The final result is a comparison of the ratio of the quantity of gene *ybaO* detected per gene *marA* present in the extracted DNA.

Comparative RT-PCR was achieved using a similar approach using template cDNA obtained from 200 ng of total RNA (see above). The sequence of reference (16S rRNA) was amplified from 10 μl of template cDNA diluted 130,000 to 4,160,000 times. *acrA*, *marA*, and *lon* were amplified from 10 μl of cDNA diluted 8 to 2,048 times. Absence of DNA in the RNA extractions used for cDNA synthesis was checked by 16S rRNA gene PCR amplification using the reverse transcription controls as template DNA.

DNA sequencing. PCR-amplified DNA fragments purified with the QIAquick PCR purification kit (QIAGEN) were quantified by measure of their A_{260} and then sequenced at the Tufts University Core Facility.

RESULTS

Isolation and characterization of a spontaneous multidrug-resistant *E. coli* mutant. A spontaneous tetracycline-resistant mutant of *E. coli* AG100 was isolated after 3 days of incubation at 33°C on an LB agar plate containing 4 μg/ml of tetracycline (see Materials and Methods). This mutant, M113, took only 1 day to form visible colonies when replated on similar medium. When compared with the parental strain AG100 and a *marR* mutant, AG112, M113 presented a typical multiple antibiotic resistance (Mar) phenotype, as seen by its increased resistance to compounds belonging to different families of antibiotics (Table 3). M113 was also serendipitously found to have an unexpected increase in DNP sensitivity (DNP^s) (MIC, 0.6 mM) compared to AG100 and AG112 (MIC, 1.2 mM). When M113 from the −80°C stock was spread on an LB plate and the phenotype of five isolated colonies was checked, two had lost their Mar phenotype. Therefore, M113 inoculations, made from the frozen stock, were grown overnight in LB broth in the presence of nalidixic acid (5 μg/ml) to maintain the resistance phenotype.

A Western blot analysis with anti-MarA antibodies showed at least 9 times more MarA in M113 than in the parental strain, AG100 (Fig. 1A). M113 produced 55% ± 12% of the amount of MarA detected in AG112 (mutant *marR*). Comparative RT-PCR showed no difference in *marA* transcription between AG100 and M113, while an increase of ~15-fold was seen for AG112 (Fig. 2A). Therefore, unlike in previously described Mar mutants, MarA overproduction in M113 was not linked to increased *marA* transcription. In accord with the RT-PCR findings, no mutation predicted to affect *marA* transcription was found by sequencing *marR* and the *marRAB* promoter (data not shown).

Mar phenotype linked to chromosomal duplication in M113. In the course of evaluating (by P1 transduction) the cause of the multidrug resistance, we noted that a mutation affecting the Mar phenotype seemed to map near, but not in, the *acrAB* locus, although the instability of the phenotype impaired precise mapping of the mutation. No mutation was found by sequencing the *acrAB* promoter or *acrR* coding for the transcriptional repressor of the *acrAB* operon. We then transferred by P1 transduction a *ppiD*::Tn*10* marker (also called *zba-3054*::Tn*10* (27) and located 18 kb to the left of *acrB*) from the donor strain, CAG12017, to M113. PCR amplification with primers that detected either the wild-type or the mutated *ppiD* locus (Table 2) unexpectedly showed both the wild-type and the mutant loci in some single transductants (Fig. 1B), indicating a duplication of the *ppiD* locus in M113. When the same P1*vir* lysate was used with the recipient strain AG100, only the *ppiD*::Tn*10* locus was detected in the selected tetracycline-resistant transductants (data not shown), indicating that the duplication detected in M113 was neither carried by the donor strain CAG12017 nor present in the parental strain AG100.

To analyze the linkage between the MarA overproduction, the Mar resistance phenotype, the DNP sensitivity and the presence of the duplication in M113, additional P1 transductions of M113 with P1 grown on the donor strain CAG12017 were performed. Twenty-three tetracycline-resistant transductants (bearing *ppiD*::Tn*10*) were examined, and four different types of transductants were encountered (Fig. 1C). We con-

TABLE 2. Primers and PCR conditions

Locus or mutation	Use	Temp[a]	Primer name	Sequence (5′→3′)	Coordinate (orientation)[b]
acrAB	Transcription analysis	60	acrAR1	CCGCAGCATTCGCCTGTTGC	484375 (>)
			acrA1	GGTCGTTCTGATGCTCTCAGG	484811 (<)
	Detection of *acrB* wild type	56	acrB2R	TGACATCAACCGGCGTTAGC	483018 (>)
			acrB1	TGGGTGATCGCCATTATCATC	483591 (<)
	Detection of Δ*acrAB*::Kan	56	acrB1R	AGTCCTTCAAGGAAACGAACG	481728 (>)
			Tn9031R	TTTCCAGACTTGTTCAACAGG	Kan (<)
acrR	PCR and sequencing	55	acrFR1	CTAACGCCTGTAAATTCACG	484960 (>)
			acrRV1	CCAGGAAAAATCCTGGAGTCAG	485662 (<)
dupIS*186*	Detection by PCR and sequencing	60	hokE1	ACATGCTGACGAAATATGCCC	607057 (>)
			lonR1	ATGCGTTCAGAACGCTCAGG	458137 (<)
lon	PCR and sequencing	60	clpX1	GGTTATGTGGGTGAAGACGT	457103 (>)
			lonR2	TTTTGACCTTGCTACGCGC	459042 (<)
	Additional sequencing		clpX3	TTTATGGCAAGCCGGAAGCG	457884 (>)
			lon1	ACGTACATGTTAATAGATGGCG	458033 (>)
			lonR1	ATGCGTTCAGAACGCTCAGG	458137 (<)
	Transcription analysis	58	lon4	AGGTTGAGAAACGCATTCGC	458734 (>)
			lonR2	TTTTGACCTTGCTACGCGC	459042 (<)
marOR	PCR and sequencing	54	orab2	GCTAGCCTTGCATCGCAT	1617010 (>)
			or1R	GAATGGTAATAGCGTCAG	1617631 (<)
marA	Transcription and comparative PCR analysis	58	mar8826	ACTGACGCTATTACCATTCATAG	1617613 (>)
			mar9150	CCTGCATATTGGTCATCCGGT	1617937 (<)
ppiD	Detection of *ppiD* wild type	56	ppiDF2	CGAAGTTCAAGGGCGCATC	460972 (>)
			ppiDR2	AGTGGAATCACCTTAACGGC	463136 (<)
	Detection of *ppiD*::Tn*10*	56	Tn10-4	CGAGTTCGCACATCTTGTTGTCTG	Tn*10* (>)
			ppiDR2	AGTGGAATCACCTTAACGGC	463136 (<)
ybaO	Comparative PCR analysis	58	ybaO2760	CGCACAGAATAAATTGTCGTG	467533 (>)
			ybaO3218	GCACGCTGTTTACCAGACGC	467991 (<)
16S	Transcription analysis	58	Eco16S	GCGGCCCCCTGGACGAA	4206900 (>)[c]
			Eco16SR	GACCGCTGGCAACAAAGGATAAG	4207305 (<)[c]

[a] Hybridizing temperature of the PCR.
[b] Coordinates of the 5′ nucleotides are given according to the *E. coli* K-12 genome (accession no. U00096), except for primers Tn9031R and Tn10-4; >, forward primer; <, reverse primer.
[c] One of the several 16S rRNA loci of *E. coli*.

cluded from this analysis that (i) the MarA overproduction and the Mar phenotype were separable and resulted from different mutations (mutant types 2 and 4 in Fig. 1C); (ii) the MarA overproduction and the DNP^s phenotype were always found together (mutant types 3 and 4) and thus possibly resulted from the same mutation; reversion of this DNP^s mutation was independent of the presence of the duplication (mutant types 1 and 2); and (iii) the Mar resistance phenotype was tightly linked to the presence of the duplication (mutant types 2 and 3), but not the MarA overproduction (mutant types 3 and 4).

Presence of a *lon*::IS*186* mutation in M113. Using P1 transduction from the donor strain CAG12017 and DNP^s as a possible marker for MarA overproduction, we mapped the mutation responsible for the apparent MarA overproduction. The DNP^s of M113 was reverted in 90 out of 102 *ppiD*::Tn*10* transductants analyzed. This 88.2% frequency of cotransduction with *ppiD*::Tn*10* revealed that the mutation causing DNP^s was ~3.7 kb away from *ppiD*. No mutation was found in the 10 kb to the right of *ppiD*::Tn*10* (primers used for the PCR and sequencing not shown). PCR amplification of the *lon* promoter region located 3.6 kb to the left of *ppiD*::Tn*10* showed an additional 1.3-kb-long sequence in M113 compared to its parental strain, AG100. This additional sequence was found in all DNP^s transductants examined, but was absent from the DNP^r ones (data not shown). Sequencing revealed a 1,306-bp IS*186* inserted in the promoter of the *lon* gene of M113 (Fig. 3). We named this mutation *lon3*::IS*186*.

Using comparative RT-PCR, we found a decrease of about 85% in *lon* transcription in M113 compared to AG100 after growth in LB broth up to mid-logarithmic phase. The stability of DNP^s was examined in the strain M113R (bearing only a *lon3*::IS*186* mutation). After seven sequential overnight

TABLE 3. Phenotypes of different strains

Strain	Relevant genotype[a]	MIC (μg/ml)[b]					DNP[s/rc]	MarA[d]	AcrA[e]
		Chl	Nal	Amp	Nor	Tet			
AG100	Wild type	6	6	4	0.047	3	R	−	+
AG112	*marR*	24	12	12	0.38	8	R	++	++
M113	*lon3*::IS*186* dupIS*186*	24	12	6	0.064	6	S	+	+++
M113R	*lon3*::IS*186*	8	4	4	0.047	3	S	+	++
AG100A	Δ*acrAB*	1.5	1.5	0.75	0.023	NT	R	NT	−
AG100B	Δ*acrR*	24	12	6	0.25	6	NT	NT	+++
Constructed strains									
M113HN1	Wild-type *lon*	6	6	3	0.047	NT	R	−	NT
M113HN12	*lon3*::IS*186*	8	4	4	0.047	NT	S	+	NT
M113HN11	dupIS*186* wild-type *lon*	12	12	4	0.064	NT	R	−	NT
AG100HN19	Wild-type *lon*	6	6	3	0.047	NT	R	−	NT
AG100HN17	*lon3*::IS*186*	8	4	4	0.047	NT	S	+	NT
AG100HN3	dupIS*186* wild-type *lon*	16	12	4	0.064	NT	R	−	NT
AG112HN36	*marR* wild-type *lon*	24	12	8	0.38	NT	R	++	NT
AG112HN48	*marR lon3*::IS*186*	32	12	12	0.38	NT	S	+++	+++
AG112HN2-74[f]	*marR* dupIS*186* wild-type *lon*	96	48	12	2	NT	R	NT	NT
AG112HN2-74[g]	*marR* dupIS*186* wild-type *lon*	128	192	12	3	NT	R	NT	++++
AB1157	Wild-type *lon*	6	6	6	0.064	1.5	R	−	NT
AB1899	*lon1*::IS*186*, unknown	8	8	8	0.19	3	S	+	NT
AB1899HN67	Wild-type *lon*, unknown	6	8	8	0.19	NT	NT	NT	NT
AB1899HN68	*lon1*::IS*186*, unknown	8	8	12	0.19	NT	NT	NT	NT
AB1157HN69	Wild-type *lon*	6	6	6	0.094	NT	NT	NT	NT
AB1157HN70[h]	*lon3*::IS*186*	8	3	6	0.064	NT	NT	NT	NT
GJ1913	Wild-type *lon*	***6***	***6***	NT	NT	NT	R	−	NT
GJ1912	*lon-103*::IS*186*	***10***	***6***	NT	NT	NT	R	+	NT
AG100HN50	Wild-type *lon*	***2***	***6***	NT	NT	NT	R	−	NT
AG100HN55	*lon-103*::IS*186*	***3***	***6***	NT	NT	NT	S	+	NT
AG100AHN2-21	Δ*acrAB* dupIS*186* wild-type *lon*	1.5	1.5	0.5	0.023	NT	NT	NT	−
AG100AHN61	Δ*acrAB*	1.5	1.5	0.75	0.023	NT	R	NT	−
AG100AHN60	Δ*acrAB lon3*::IS*186*	1.5	0.75	0.75	<0.016	NT	S	NT	−

[a] For a complete genotype, see Table 1.

[b] MICs as determined by Etests, except for strains GJ1912, GJ1913, AG100HN50, and AG100HN55, which were determined by the LB agar plating technique (data in italics and boldface). Chl, chloramphenicol; Nal, nalidixic acid; Amp, ampicillin; Nor, norfloxacin; Tet, tetracycline; NT, not tested (strains may contain *ppiD*::Tn*10* conferring tetracycline resistance).

[c] DNP[r/s], resistance (r) or sensitivity (s) to 2,4-dinitrophenol at 0.6 mM.

[d] − ≪ + < ++ < +++, increased MarA overproduction as observed by Western blot hybridization with anti-MarA antibodies (for MarA overproduction quantifications, see text and Fig. 2A).

[e] + < ++ < +++ < ++++, increased AcrA overproduction as observed by Western blot hybridization with anti-AcrA antibodies (for AcrA overproduction quantifications, see text and Fig. 2C). −, the AcrAB pump is absent from the strain.

[f] The strain was always grown in LB.

[g] The strain was grown overnight in LB plus nalidixic acid (10 μg/ml) followed by exponential growth in the presence of nalidixic acid (5 μg/ml).

[h] To partially compensate for the slower growth of this mutant on LB plates, the phenotype was checked after 2 days of growth instead of 1.

growths on LB plates at 37°C, no loss of the DNP[s] was found among 10 isolated colonies from the seventh plate.

***lon* mutations caused DNP sensitivity.** *lon* mutants of strains AG100 and AB1157 presented an increased sensitivity to DNP (Table 3). As shown above, the increased sensitivity for M113 compared to AG100 was twofold. Although no DNP[s] was seen for the *lon* mutant GJ1912 (Table 3), when its *lon-103*::IS*186* mutation was transduced into AG100, it did confer the DNP[s] (strain AG100HN55 in Table 3). Therefore, the DNP[s] phenotype associated with a *lon* mutation appears to be strain-dependent.

MarA overproduction linked to *lon3*::IS*186* and other *lon* mutations in additional *E. coli* strains. We examined MarA overproduction by Western blot hybridization in the constructed strains M113R, M113HN12, AG100HN17 (all harboring a *lon3*::IS*186* mutation), and AG100HN55 (*lon-103*::IS*186*) (see Materials and Methods) and in the spontaneous mutants AB1899 (*lon1*::IS*186*) isogenic to AB1157 (14) and GJ1912 (*lon-103*::IS*186*) isogenic to GJ1913 (36). The different *lon* mutations in different *E. coli* strains all resulted in increased amounts of MarA as compared to the wild-type parent strains (Table 3).

Relationship of the *lon3*::IS*186* mutation to the Mar phenotype. Although the *lon* mutation increased the amounts of MarA (Fig. 2A), it was, surprisingly, not responsible for the multidrug resistance phenotype of M113 (see M113R in Table 3). Introduction of the *lon3*::IS*186* mutation into a *marR* mutant caused a fivefold increase in MarA (Fig. 2A) but had only a small effect on drug resistance (compare strains AG112HN36 and AG112HN48 in Table 3) (Fig. 2A). The results clearly showed that MarA overproduced in a *lon* mutant failed to confer Mar resistance. Still, the *lon* mutation did not seem to affect the MarA-dependent Mar phenotype in the *marR lon* double mutant (compare AG112HN36 and AG112HN48 in Table 3).

A small increased sensitivity to nalidixic acid was observed in M113R and other *lon* mutants of AG100, while no apparent difference was observed with norfloxacin, another quinolone

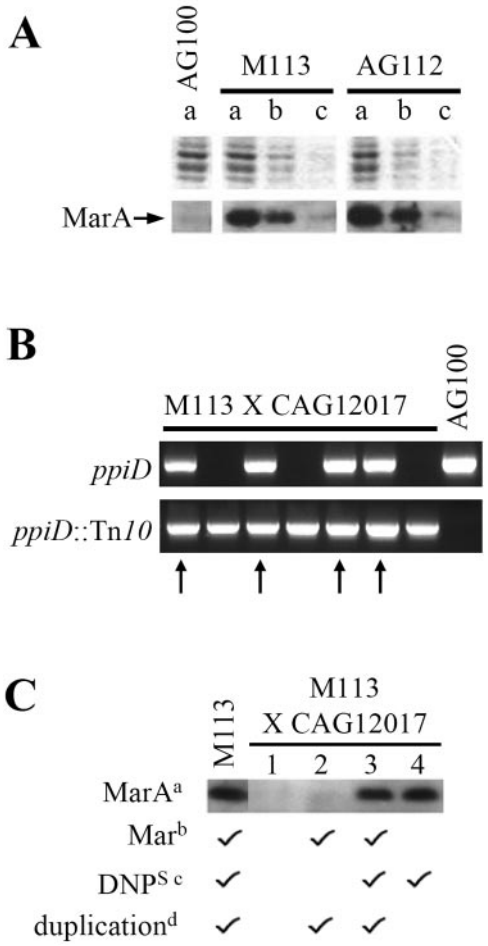

FIG. 1. Analysis of M113. (A) Western blot showing MarA production in AG100, M113, and AG112. A portion of the gel stained with Coomassie blue to show protein loading is presented above the MarA protein identified by Western blot hybridization with anti-MarA antibodies. The quantities of total proteins loaded are 30 μg (lanes a), 10 μg (lanes b), and 3.3 μg (lanes c). (B) Detection of the duplications by PCR. The recipient strain M113 grown in the presence of nalidixic acid (5 μg/ml) was P1 transduced with lysate from the donor strain CAG12017 (*ppiD*::Tn*10*). Random tetracycline-resistant transductants were tested by PCR for the presence of the wild-type *ppiD* and the *ppiD*::Tn*10* loci. Arrows indicate transductants in which both loci were detected, indicating the presence of a duplication. Wild-type *E. coli* AG100 was used as a control. (C) Relationship between MarA overproduction, Mar phenotype, DNP sensitivity, and the presence of the duplication. M113 was transduced with a P1 lysate from the donor strain CAG12017 (*ppiD*::Tn*10*). One example of each of the four different types of transductants obtained (lanes 1 to 4) is presented. a, MarA overproduction measured by Western blot hybridization with anti-MarA; b, persistence of the Mar phenotype detected by an increased resistance to nalidixic acid and chloramphenicol compared to AG100; c, sensitivity to 0.6 mM 2,4-dinitrophenol; d, duplications detected by PCR (as in panel B).

(Table 3). Because of their sensitivity to SOS system-inducing conditions (24), *lon* mutants usually present an increased sensitivity to quinolones (23, 38).

The spontaneous *lon1*::IS*186* mutant AB1899 has been reported to present increased resistance to multiple antibiotics compared to its parental strain, AB1157 (Table 3) (32). However, further analysis showed that AB1899 was not isogenic to its parent, AB1157, since reversion of the *lon* mutation of AB1899 to wild type by P1 transduction with the donor strain CAG12017 only minimally decreased the chloramphenicol resistance of AB1899 (compare strains AB1899HN67 and AB1899HN68 in Table 3). Evidently, at least one other mutation which causes most of the Mar phenotype is present in this strain. Similarly, P1 transduction of the *lon3*::IS*186* mutation from the strain M113HN12 to AB1157, producing the strain AB1157HN70, did not confer a Mar phenotype (Table 3). These findings confirm that the multidrug resistance phenotype of AB1899 results from another "unknown" mutation, not from *lon1*::IS*186*.

Endpoints of the large tandem duplication dupIS*186* responsible for multidrug resistance. *clpX* is the gene directly upstream of *lon* in the *E. coli* K-12 chromosome, and the *clpX*-IS*186-lon* locus is found in *lon3*::IS*186* mutants. When we transduced M113 with a P1*vir* lysate from the donor strain CAG12017, transductants that had a duplication of the *ppiD* locus (with one copy carrying the *ppiD*::Tn*10* marker) and a wild-type *lon* locus (and thus reverted the *lon3*::IS*186* mutation; verified by PCR amplification) also still had another IS*186-lon* locus, but no *clpX*-IS*186* locus. Therefore, the *lon* gene was part of the duplication, but *clpX* was not. We hypothesized that the IS*186* inserted in the *lon* promoter could be one of the two borders of the amplified unit, with the second border being another IS*186*. We searched the sequenced *E. coli* K-12 genome for the nearest IS*186* present to the right of *lon* and inserted in the same orientation as in *lon*. One such IS was present 149 kb to the right of *lon* at positions 607231 to 608537 according to GenBank sequence accession no. U00096. A forward primer, hokE1, that hybridized to the left of this IS*186* was designed and used along with the reverse primer lonR1 to detect by PCR the potential linking region of two amplified units (see Table 2 and Fig. 3A for primer sequences and hybridization sites). Sequencing of the PCR product amplified from M113 confirmed the order *hokE*-IS*186-lon* at the junction of two amplified units; therefore, the amplified unit was indeed bordered by the two IS*186*s. We called this duplication dupIS*186*. PCR amplification confirmed that the tandem duplication studied was present in M113 but not AG100 and was not induced by P1 transduction per se. Comparative PCR amplification of the *marA* and *ybaO* genes present outside and inside the 149-kb amplified unit, respectively, revealed an average of 2.5 copies of the amplified unit after overnight growth of M113 in the presence of nalidixic acid (5 μg/ml) (Fig. 4A). The duplication was maintained during growth of M113 in nalidixic acid, but it was rapidly lost along with the Mar phenotype when M113 was grown in its absence (Fig. 4B).

Mar phenotype resulting from the dupIS*186* mutation. The strains M113HN11 and AG100HN3, each harboring dupIS*186* and a wild-type *lon* gene, were constructed by P1 transduction and analyzed (see Materials and Methods and Table 3). They did not overproduce MarA and were DNP^r (Table 3). dupIS*186* conferred increased resistance to chloramphenicol and nalidixic acid, as well as resistance to ampicillin and norfloxacin at a lower level (Table 3). The resistance to tetracycline could not be tested in these Tn*10*-bearing strains, but the original M113 strain presented increased resistance to tetracycline that was not caused by the *lon3*::IS*186* mutation and was hence presumably caused by the dupIS*186* mutation. When it

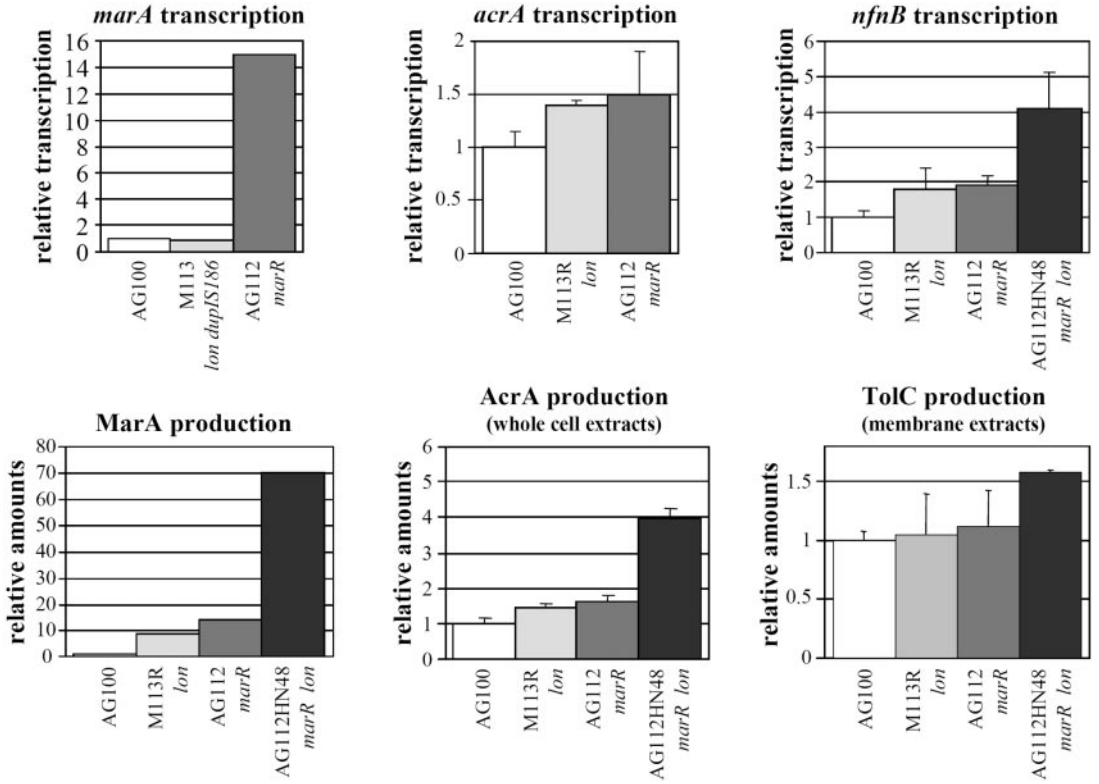

FIG. 2. Amounts of AcrA, MarA, and TolC proteins and induction of genes of the *mar* regulon in AG100 and mutants grown in LB broth. All results are presented in arbitrary units based on the value obtained with AG100 = 1. *marA* and *acrA* transcription was analyzed by comparative RT-PCR. MarA overproduction was quantified by Western blot hybridizations with anti-MarA antibodies using whole-cell extracts of bacteria. The quantifications were done in duplicate. Similar amounts of MarA were detected in M113R and M113 (data not shown). *nfnB* transcription was analyzed by luciferase dosage using bacteria transformed with the plasmid pSP-*nfnB1* carrying an *nfnB*-promoter::*luc* fusion. The amounts of AcrA and TolC proteins were quantified by Western blot hybridizations with anti-AcrA or anti-TolC antibodies using whole-cell or membrane protein extracts of bacteria. Experiments were done at least in duplicate.

was P1 transduced into the *marR* mutant AG112, the dupIS*186* mutation conferred high multidrug resistance (mutant AG112HN2-74 in Table 3).

Segregation of duplicated copies. M113 was transduced with a P1*vir* lysate from strain CAG12017, and one colony with a *ppiD*::Tn*10*-marked duplication was detected and purified twice on LB plates to give single colonies. One of these colonies that still harbored the duplication was then plated one more time on LB to give single colonies. Nine isolated colonies were then tested for the presence or loss of the duplication and for their Mar phenotype. Five colonies still had the duplication and the Mar phenotype, and four colonies had lost the duplication and the Mar phenotype. Of these four, three had lost the copy carrying the *ppiD*::Tn*10* locus and one had lost the copy with the wild-type *ppiD* gene. So, the Mar phenotype of M113 was not caused by a mutation carried by one of the amplified units, but resulted from the presence of several amplified units.

Role of *acrAB* in the multidrug resistance associated with dupIS*186*. The 149-kb-long amplified fragment harbored the multidrug resistance efflux locus *acrAB*. AcrAB is largely responsible for *mar*-mediated multiple antibiotic resistance in *E. coli* (31). Duplication of this locus would explain the Mar phenotype. To test this possibility, two approaches were investigated.

First, we deleted *acrAB* in M113 by P1 transduction from the donor strain AG100A (Δ*acrAB*::Kan). Of six M113 Δ*acrAB* transductants analyzed, one had lost the duplication, retained only the Δ*acrAB* locus, and had the phenotype of AG100A. Of the five others with a duplication, one was isolated on LB and lost the Mar phenotype but retained at least one copy of both wild-type *acrAB* and Δ*acrAB*. This transductant lost the Mar phenotype presumably because of the lower number of copies of the wild-type *acrAB* locus. When similar experiments were performed with a donor strain carrying a marker *ppiD*::Tn*10* and a wild-type *acrAB* locus, no colony with a detectable duplication ever lost its Mar phenotype. So, decreasing the number of copies of intact *acrAB* reverted the Mar phenotype in bacteria that still carried a dupIS*186*. This finding suggested a critical role for the *acrAB* locus as opposed to other loci in the duplication.

In a second approach, the dupIS*186* was transferred by P1 transduction from the donor strain M113HN2 to the recipient strain AG100A (Δ*acrAB*). Transductants were selected for their resistance to tetracycline (marker *ppiD*::Tn*10*). A transductant carrying a dupIS*186* but no wild-type *acrAB* locus (AG100AHN2-21) was detected by PCR. No increased resistance to the different antibiotics tested was found, revealing again that the presence of amplified *acrAB* was needed for dupIS*186*-dependent multidrug resistance (Table 3).

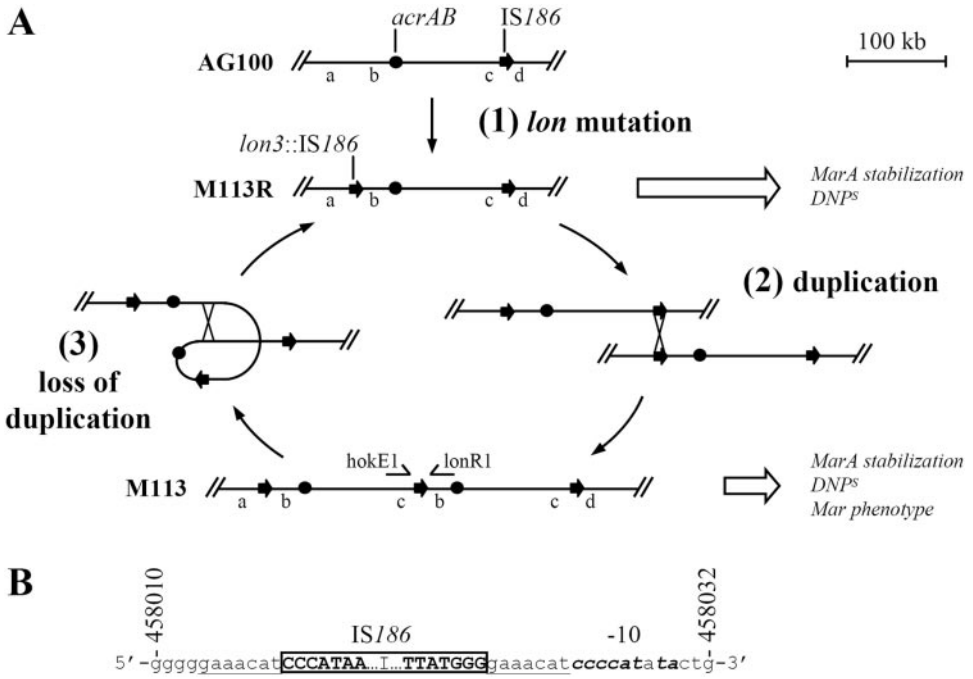

FIG. 3. Mutations in M113. (A) Sequence of the mutational events. The *acrAB* locus is shown as a black dot and the IS*186* insertion sequence as black arrow on the wild-type *E. coli* K-12 chromosome. The letters a, b, c, and d are used as visual references along the chromosome. Main phenotypes of M113R and M113 are presented in italic. The sequence of events is as follows. Event 1 is stable spontaneous IS*186* insertion in the *lon* promoter of AG100. Event 2 is spontaneous tandem duplication of the 149-kb-long unit. The event probably occurred by recombination between the IS*186* present on sister chromosomes (41) and allowed growth in the presence of the tetracycline used for M113 selection. Primers hokE1 and lonR1 were designed to specifically detect this dupIS*186* event. In event 3, frequent spontaneous loss of the duplication in the absence of selective pressure for the Mar phenotype allowed the selection of M113R. (B) Detail of the *lon3*::IS*186* mutation. Nucleotide coordinates are indicated according to *E. coli* K-12 genome sequence (GenBank accession no. U00096). The IS*186* sequence is partially presented and boxed. Its orientation (noted "I") is given according to reference 35. The 7-nucleotide-long duplication of the target site is underlined, and the −10 box of the σ^{32} promoter of *lon* is shown in boldface and italic.

Ethidium bromide resistance conferred by other transporter gene(s) present on the amplified unit. The genes *cusAB*, *emrE*, *fsr*, and *mdlAB* code for transporters and are present, along with *acrAB*, on the amplified unit of dupIS*186*. When overproduced, *emrE* (positions 567538 to 567870 on the chromosome of *E. coli* K-12; GenBank accession no. U00096) confers resistance to ethidium bromide (28). Overproduction of *acrAB*, but not of *cusAB*, *fsr*, and *mdlAB* also confers an ethidium bromide resistance (28). The strain AG100AHN2-21 carrying a dupIS*186*, but no wild-type *acrAB* locus, showed an increased resistance to ethidium bromide (MIC of 18 μg/ml) compared to AG100A (MIC of 6 μg/ml). Therefore, the genetic amplification of other transporter genes—such as *emrE*—can also result in increased resistance to specific drugs.

Activity of MarA stabilized in a *lon* mutant. The finding that MarA overproduced in *lon* mutants did not confer the Mar phenotype suggested that the MarA stabilized in *lon* mutants might not be active. We therefore examined transcription of the MarA-regulated genes *acrAB*, *nfnB*, and *tolC* (6) and/or amounts of their products in the *lon* mutant M113R (Fig. 2) as an assay for MarA activity. The transcription of *acrA* and *nfnB* was increased in M113R (Fig. 2B). The amounts of AcrA found in a *lon* or *marR* mutant were both increased similarly compared to the wild-type strain (Fig. 2C). The *marR lon* double mutant showed increased amounts of TolC protein (Fig. 2C), suggesting that the stabilized MarA could also induce *tolC* transcription. Therefore, MarA stabilized in a *lon* mutant appeared to be active.

Effect of a *lon* mutation on the AcrAB-TolC pump function. Overexpression of AcrAB alone can confer the Mar phenotype (31). Therefore, the absence of a Mar phenotype in a *lon* mutant which overproduced AcrA (Fig. 2) was unexpected. One possibility was that a general increased sensitivity to antibiotics was caused by the *lon* mutation. If the pump was functional in a *lon* mutant but the *lon* mutation also increased the sensitivity of bacteria, a Δ*acrAB lon* double mutant (AG100AHN60) would show increased sensitivity to antibiotics such as chloramphenicol and ampicillin compared to the Δ*acrAB* mutant carrying a wild-type *lon* gene (AG100AHN61). This was not observed (Table 3), revealing that the absence of Mar phenotype in a *lon* mutant was not caused by a general increase in drug susceptibility.

The remaining possibility was that the AcrAB-TolC pump itself was present at a reduced amount or had a reduced activity in the absence of Lon protease. The AcrAB-TolC pump consists of two transmembrane proteins (AcrB in the inner membrane, TolC in the outer membrane) linked by periplasmic AcrA (40). Similar amounts of TolC were detected in the membrane protein extract (Fig. 2C), but TolC was not found in the cytosol fraction (data not shown) of AG100, AG112, and M113R. Therefore, the decreased activity of the AcrAB-TolC pump in a *lon* mutant was not the result of a decreased amount

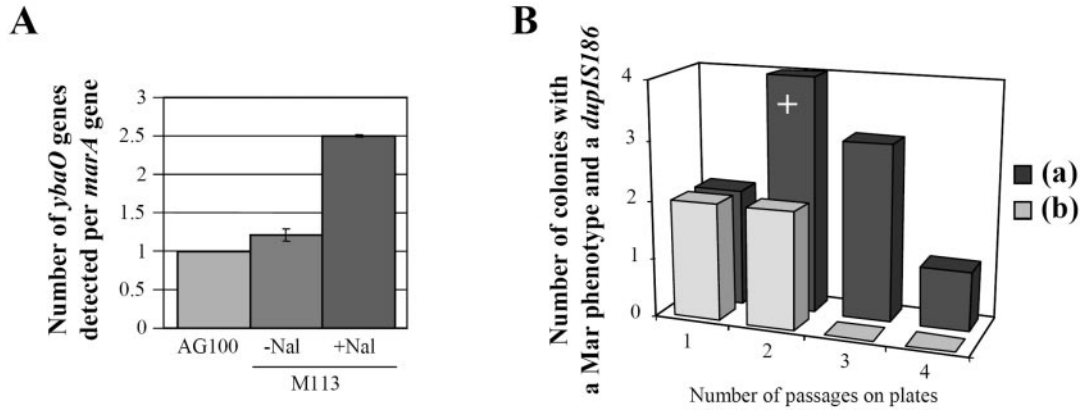

FIG. 4. Copy number and instability of the amplification. (A) Number of copies of the amplified unit. Three overnight growths of AG100 in LB broth and M113 in LB broth supplemented with (+Nal) or without (−Nal) nalidixic acid at 5 μg/ml were done. DNA was extracted, and a comparative PCR was performed as described in Materials and Methods to compare the number of copies of *ybaO* (present on the amplified unit) detected per copy of *marA* (present outside of the amplified unit) in each of the DNA extractions. (B) Instability of the Mar phenotype and the dupIS*186* mutation. Strain M113HN11 harboring dupIS*186* was passaged four times sequentially on agar plates in two independent experiments (a and b). In experiment a, the plate was supplemented with 5 μg/ml nalidixic acid at the second passage (+). For each experiment (a and b), after each passage on the plate, four isolated colonies were studied to determine the proportion of bacteria harboring a Mar phenotype. The detection of the dupIS*186* mutation in the isolated colonies was done by PCR as described in Fig. 1B. Only the bacteria harboring a Mar phenotype had the dupIS*186* mutation.

of the TolC protein present in the membrane fraction. Similarly, increased amounts of AcrA were found in whole-cell extracts of AG112 and M113R (Fig. 2C). AcrA was found in both the cytosol and the membrane fractions in AG100, AG112, and M113R (data not shown), presumably because of its interactions with AcrB and TolC. Thus, the *lon* mutation did not seem to affect the MarA-induced overproduction of AcrA or its compartmentalization. The effect of Lon protease on AcrAB-TolC function appears to occur via a mechanism other than an effect on production and compartmentalization of AcrA and TolC.

DISCUSSION

An unusual multiple-antibiotic-resistant spontaneous mutant of *E. coli* AG100 was isolated in the laboratory on LB medium supplemented with 4 μg/ml of tetracycline. This mutant, M113, presented an unstable Mar phenotype and increased amounts of MarA without increased transcription of the *marRAB* operon. Its analysis revealed the presence of two different mutations: one stable *lon3*::IS*186* mutation which occurred first and one unstable tandem amplification of a 149-kb-long unit (mutation dupIS*186*) with an endpoint at *lon3*::IS*186* and which contained the *acrAB* locus (Fig. 3). Although gene duplication as the basis for multidrug resistance is well documented in eukaryotes (10, 34), this is the first description of multidrug resistance in prokaryotes caused by a chromosomal amplification of the *acrAB* genes.

The Lon protease, one of five ATP-dependent proteases found in *E. coli*, is important for the turnover of numerous proteins. It plays a central role in the heat shock response by degrading misfolded proteins. It also helps in the recovery from SOS system inductions by degrading the SOS-induced SulA protein that binds FtsZ and inhibits cellular division. This role confers a sensitivity of Lon-defective *E. coli* cells to conditions that induce the SOS system (24). The *lon* promoter corresponds to a hot spot insertion site for IS*186* in *E. coli*, and similar mutations were found in strains such as the radiation-sensitive natural isolate *E. coli* B/r (35). SaiSree and colleagues studied IS*186* transposition events in *E. coli* and proposed the 5′-$(G)_{\geq 4}(N)_{3-6}(C)_{\geq 4}$-3′ sequence as the consensus target site for IS*186* transposition (35). As seen in Fig. 2B, the *lon3*::IS*186* of M113 was found in such a site.

A *lon* mutation stabilized MarA. The *lon*::IS*186* mutations resulted in a ≥9-fold increase in MarA protein (Fig. 1A and 2A), without any increase in *marA* transcription. During the time of our investigation, Griffith and coworkers showed that the Lon protease, along with at least one other still unknown protease, was the only *E. coli* ATP-dependent protease (of those presently known) involved in a rapid turnover of MarA, and a *lon-510* mutation resulted in a ≥24-fold decrease in the breakdown rate of MarA (12). Our data agree with their result; the apparent overproduction of MarA observed in *lon* mutants presumably resulted from the stabilization of the basal quantities of MarA produced. In contrast to these investigators, we were able to detect increased quantities of MarA expressed from the wild-type chromosomal *marRAB* locus in *lon* mutants using our anti-MarA antiserum.

Lon indirectly regulates the *mar* regulon via MarA. M113R (*lon3*::IS*186*) overproduced about half of the amount of MarA found in the *marR* mutant AG112 (Fig. 2A), yet the levels of induction of the MarA-regulated *acrAB* and *nfnB* transcription and the production of AcrA were apparently similar in both strains (Fig. 2). This lack of proportionality was not caused by a saturation effect of MarA, because *nfnB* transcription and AcrA and TolC production were further increased in a *marR lon* double mutant in which increased amounts of MarA were found (Fig. 2A and B). A possibility would be that SoxS, which is also partially stabilized by a *lon* mutation (12) and which also induces the transcription of those genes (33), could contribute to the induction of the genes of the *mar* regulon in a *lon* mutant.

Unlike MarA, overproduced SoxS was described as inducing *marRAB* transcription in the presence of a wild-type MarR repressor (25). Since we did not observe any induction of *marRAB* transcription in a *lon* mutant (Fig. 2A), the contribution, if any, of SoxS to the increased transcription of the *mar* regulon in a *lon* mutant seems minimal.

The Lon protease is required for an efficient AcrAB-TolC-dependent Mar phenotype. The amount of MarA in M113R was at least 9 times that of the wild-type strain AG100. As seen in uropathogenic *E. coli* strains, such MarA overproduction should confer a Mar phenotype (4). Furthermore, quantities of AcrA and TolC proteins found in M113R were not less than that found in AG112 (Fig. 2C), where overproduction of the AcrAB-TolC pump causes a Mar phenotype (31) (Table 3). The lack of multidrug resistance in *lon* mutants appears linked to an inactive AcrAB-TolC pump. This dependence on Lon was only partial, since the *marR lon* mutant AG112HN48 and the dupIS*186 lon* mutant M113, which respectively overproduced 2.3 and 1.6 to 2.4 times the amount of AcrA found in AG112 (Fig. 2C) (data not shown for M113), did have a multidrug resistance, with almost the same phenotype as AG112 (Table 3). An increased multidrug resistance was also observed with a mutant carrying a deletion of the *lon* gene and a duplication including *acrAB* (data not shown). Therefore, the partial activity of AcrAB-TolC observed in *lon3*::IS*186* mutants was not explained by the remaining 15% of *lon* transcription detected, but rather by an intrinsic partial activity of the pump in the absence of Lon. Using these data, we estimated that the absence of Lon protease reduced by ~60% the ability of AcrAB-TolC to confer a Mar phenotype.

The reason for inactivity in the absence of Lon protease did not appear to be altered compartmentalization of AcrA and TolC proteins. A possible clue to the effect of Lon on AcrAB-TolC comes from the observation that the *acrR* mutant AG100B, which overproduced larger amounts of AcrA than a *marR* mutant (~2.5 times the amounts found in AG100 versus ~1.7 times the amount for AG112; Fig. 2C) (data not shown; see also references 42 and 43), did not have a higher multidrug resistance than AG112 (Table 3). This finding was unexpected because increased amounts of AcrAB in a *marR* dupIS*186* double mutant (~5.5 times the amount of AcrA found in AG100; data not shown) caused a much higher multidrug resistance than that of AG112 (Table 3). Therefore, in *marR* mutants carrying a wild-type *lon* gene, efficient up- or down-regulation of other genes by MarA might somehow increase the AcrAB-TolC-dependent multidrug resistance, while in a *lon* mutant, or in an *acrR* mutant which does not overproduce MarA, those genes might not be efficiently regulated. Current efforts are focused on identifying the effect of Lon protease on AcrAB-TolC function.

Unstable multidrug resistance phenotypes caused by the dupIS*186* mutation. Despite the lack of effect on the Mar phenotype of the overproduced AcrAB-TolC pump in the *lon* mutant, multidrug resistance was observed in *lon* mutants with an amount of AcrAB further increased. The latter was achieved by the amplification of the *acrAB* genes present on the large genetic duplication dupIS*186* (mutant M113 overproducing 2.7 to 3.8 times more AcrA than AG100; data not shown) or by a *marR* mutation in AG112HN48 (Fig. 2C; Table 3). We found that 2 to 3 copies of the amplified unit of *dupIS186* were maintained per chromosome after an overnight growth of M113 in the presence of 5 μg/ml of nalidixic acid (Fig. 4A). Because of the numerous chemically unrelated substrates that AcrAB expels (28), this transporter likely confers most of the resistance phenotype associated with dupIS*186*.

Mutations conferring low multidrug resistance seem to serve as intermediate steps to reach higher antibiotic resistances (see reference 11, for example), and mutations in *marR*, *soxR*, or *acrR* were found in numerous highly antibiotic-resistant isolates of *E. coli* (21, 30, 43). We found that, combined with a *marR* mutation, the dupIS*186* mutation caused high multidrug resistance (resistance to 128 and 192 μg/ml of chloramphenicol and nalidixic acid respectively; mutant AG112HN2-74 in Table 3). Up to 5.5 times more AcrA proteins were found in a *marR* dupIS*186* mutant compared to the wild-type strain (data not shown). Such high quantities of AcrA proteins were also found in highly antibiotic-resistant *E. coli* mutants in which the mutations causing the increased amounts of AcrAB were not characterized (21).

Because it required the presence of a preexisting *lon3*::IS*186* mutation, the dupIS*186* mutation studied here might not be a frequent spontaneous mutation. However, if other genetic amplifications of *acrAB* that do not require the presence of a first mutation were found, multidrug resistance caused by genetic amplification of *acrAB* could be an important mechanism of resistance among antibiotic-resistant isolates of *E. coli*. Unstable duplications could represent the mechanism for loss of resistance in some clinical strains upon subculturing in the laboratory, a phenomenon previously attributed to plasmid loss.

Other implications of tandem amplifications. Because of their instability, tandem genetic amplifications might represent an efficient mechanism for prokaryotes to temporarily respond to external toxic threats, such as antibiotics, without alteration of their genome with a stable mutation. As observed by Matthews and Stewart in some spontaneous methicillin-resistant *Staphylococcus aureus* mutants having an amplification causing the resistance, loss of the amplification but not loss of the resistance can also be observed after prolonged storage on high concentration of antibiotics (20). So, persistent selection for the resistance phenotype would ultimately favor the growth of mutants in which a new stable mutation conferring resistance allowed the loss of the amplification. Genetic amplification could then represent an early mechanism for selection of stable mutations conferring resistance to antibiotics. Our work also shows that transporter genes can be important determinants for the selection of large tandem amplifications under specific environmental conditions.

ACKNOWLEDGMENTS

We thank J. Gowishankar for kindly providing the strains GJ1912, GJ1913, AB1157, and AB1899; C. A. Gross for strain CAG12017; and H. Zgurskaya and J. Fralick for the anti-AcrA and anti-TolC polyclonal antisera. We thank P. E. Jeric for initial studies on the instability of strain M113 and AB BIODISK for the generous gift of the Etest strips used during this study.

This work was supported by PHS grant AI56021.

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