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Multiple Antibiotic Susceptibility Associated with Inactivation of the *prc* Gene

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A Tn*5* insertion which led to increased susceptibility to multiple drugs, including tetracycline, chloramphenicol, nalidixic acid, erythromycin, spectinomycin, norfloxacin, and novobiocin, was identified in *Escherichia coli*. Cloning and sequence studies showed that the insertion was in the previously identified *prc* gene at min 40.4. The *prc* product is known to function as a protease linked to processing of penicillin-binding protein 3 and λ repressor and when absent to allow some leakage of periplasmic constituents. Complementation studies with the *prc* gene on plasmids showed complete recovery of parental levels of susceptibility to all drugs except chloramphenicol, with which only partial reversion to wild-type levels was observed.

Resistance to multiple different antibiotics can be associated with a mutation in the *mar* operon on the *Escherichia coli* chromosome (3, 7). This locus consists of three putative genes expressed from a single transcript (3, 8) which appear to mediate resistance to multiple drugs by action at other chromosomal loci. The Mar phenotype has a multilocus genetic basis (6, 7). As part of our efforts to identify other genes involved in the Mar phenotype, we introduced random Tn*5* insertions into the chromosomes of Mar mutants with selection for loss of resistance. In the course of these studies, a locus which affects drug susceptibility but appears unrelated to Mar was identified. In the present work, we show this locus to be identical to *prc*, which has previously been linked to C-terminal processing of penicillin-binding protein 3 in vivo (9) and to λ repressor degradation in vitro (17).

Bacterial strains, phage, and culture media. Bacterial strains were all *E. coli* K-12 derivatives (Table 1). KL14-10T was a Mar derivative of KL14 selected first on 5 and then on 10 μg of tetracycline per ml on MacConkey agar; insertion of *marA*::Tn*5* (7) inactivated its multiple resistances. λ*b*221 *c*I857 *O*am29 *P*am80 *rex*::Tn*5* (λ::Tn*5*) was obtained from Andrew Wright of the Tufts University School of Medicine.

Bacteria were grown in LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, all per liter). LB plates contained 1.5% agar. Salt-free 1/2 L agar medium contained Bacto-Tryptone and yeast extract reduced by half and no NaCl.

All antibiotics used for selective media were purchased from Sigma Chemical Co. (St. Louis, Mo.) except norfloxacin (Merck and Co., Rahway, N.J.).

General DNA and RNA techniques. Plasmid DNA isolation, plasmid analysis, and transformation were performed as previously described (11, 15). Chromosomal DNA was isolated by the method described by Beji et al. (2). Restriction enzymes and T4 DNA ligase, purchased from New England BioLabs (Beverly, Mass.), were used according to the manufacturer's specifications. DNA labeling, transfer, and hybridization and RNA isolation, analysis, and hybridization were done as described previously (8).

Cloning of a Tn*5* insertion which increased antibiotic susceptibility. To identify genes which reduced drug resistance in Mar mutants, random Tn*5* mutagenesis (7) with λ::Tn*5* was employed. Cells of Mar mutant KL14-10T were infected with λ::Tn*5* at a multiplicity of 0.2 and plated on kanamycin at 42°C; 980 kanamycin-resistant colonies were picked onto master plates in a grid pattern and replica plated onto 3 and 7 μg of tetracycline per ml and 4 and 7 μg of nalidixic acid per ml. One multiply susceptible insertional mutant, bearing Tn*5*-49, was found. Tn*5*-49 was transduced by bacteriophage P1 (13) from strain KL14-10T into wild-type AG100, forming strain LM274. The susceptibility of AG100 and LM274 to a variety of antibiotics was examined by a gradient plate method (5) after 40 h of incubation at 30°C. The concentration of antibiotics in plates was chosen to allow growth across ≥10% and <100% of the length of the gradient plates. The LM274 recipient showed an approximately 30% increase in susceptibility to tetracycline as well as to chloramphenicol, nalidixic acid, erythromycin, spectinomycin, norfloxacin, and novobiocin (Table 2). (On at least one occasion, one P1 transductant of the Tn*5*-49 insertion was found to have lost the susceptibility phenotype, Tn*5* having moved to an incorrect chromosomal position; therefore, transductants were monitored when necessary for the susceptibility phenotype.) There was no difference in growth rate between AG100 and LM274 in LB medium at 30 or 37°C.

The Tn*5* mutation from LM274 was transduced into a Mar mutant, AG102 (7), and into WY100, a strain with a deletion in the *mar* operon (19). All of the kanamycin-resistant transductants analyzed showed an approximately 30% increase in susceptibility to the seven antibiotics tested (Table 2). Therefore, the effect was the same in both wild-type and Mar strains.

By using a set of Hfr donor strains, each bearing Tn*10* near the origin of transfer (18), Tn*5*-49 was found to be between min 35.75 and 44.25 on the *E. coli* chromosome. Subsequent P1 transduction with a related set of defined Tn*10* insertions (18) showed Tn*5*-49 to be linked to both *eda51*::Tn*10* and *zeb-3190*::Tn*10* and therefore to be near min 41. To clone the Tn*5*-49 insertion, total chromosomal DNA from LM274 was digested with *Eco*RI and *Sal*I and separated by agarose gel electrophoresis. The fragments were transferred by blotting

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TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Description	Reference or source
E. coli strains		
AG100	*argE3 thi-1 rpsL xyl mtl supE44* Δ(*gal-uvrB*) λ⁻	7
AG102	Mar derivative of AG100	7
KL14	Hfr strain, *thi-1 relA1* λ⁻ *spoT1*	10
KL14-10T	Mar derivative of KL14	This study
KL14-10T::Tn*5*-49	KL14-10T bearing *prc*::Tn*5* (Tn*5*-49)	This study
WY100	Same as AG100 but contains a 1.2-kb *Bsp*HI deletion of the first two and the start of the third putative genes in the *mar* o . .ron (Δ*mar15*)	19
LM274	Same as AG100 but contains *prc*::Tn*5*	This study
ASS100	Same as WY100 but contains *prc*::Tn*5*	This study
ASS102	Same as AG102 but contains *prc*::Tn*5*	This study
Plasmids		
pUC18	Multicopy plasmid, Amp^r	20
pKan1	pUC18::(*marA*::Tn*5*/5.05 kb), Amp^r Km^r	8
pAS-1	pUC18::(*prc*::Tn*5*/3.8-kb *Eco*RI-*Sal*I fragment), Km^r	This study
pHR53	pACYC184::(*prc* region/2.9-kb *Eco*RI fragment), Tc^r	9
pHR61	pBR322::(*prc* region/12.2-kb *Pst*I fragment), Tc^r	9

to a nylon membrane and probed with ^{32}P-labeled 0.920-kb *Pst*I fragment of Tn*5* from plasmid pKan1 (8). A 3.8-kb *Eco*RI-*Sal*I chromosomal fragment hybridized with the probe. This fragment was cloned into the corresponding sites of pUC18 (20), forming plasmid pAS-1 (Fig. 1). This plasmid was selected with kanamycin and contained 2.3 kb from Tn*5*

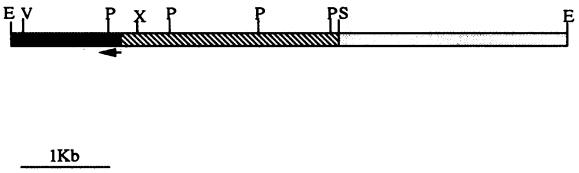

FIG. 1. Restriction enzyme map of pAS-1 carrying the Tn*5*-49 insertion in the *prc* gene. The arrow indicates the region and direction of junction sequencing. The solid bar indicates *E. coli* chromosomal DNA, the hatched bar indicates the Tn*5* insertion, and the shaded bar indicates pUC18. Abbreviations for restriction enzymes are as follows: E, *Eco*RI; P, *Pst*I; S, *Sal*I; V, *Eco*RV; X, *Xho*I.

(from the internal *Sal*I site through IS*50*L) plus 1.5 kb of *E. coli* chromosomal DNA flanking IS*50*L.

Partial nucleotide sequence of the genetic locus associated with increased antibiotic susceptibility. The DNA sequence of the junctional chromosomal DNA from LM274 in plasmid pAS-1 (Fig. 1) was determined by the method of Sanger et al. (16) by using a Sequenase sequencing kit (U.S. Biochemical, Cleveland, Ohio) according to the supplier's protocol. The Tn*5* primer used was 5′-CGTTCAGGACGATCCTT, derived from the 5′ end of IS*50*L of Tn*5* (1). We sequenced 231 bp (Fig. 2). We used the FASTA method of Pearson and Lipman (14) to search GenBank for nucleotide sequence homology but found no homology in the data base available. However, we discovered in the literature (9) a recently reported gene, *prc*, which mapped at min 40.4 and which had a sequence identical to that we had obtained. Tn*5*-49 was thereby unambiguously determined to have inserted next to alanine 401 of the *prc* gene (Fig. 2), downstream of the *Pst*I site in the middle of the gene. An opal (UGA) nonsense codon is created by the fusion junction (Fig. 2), interrupting the product of the *prc* gene, an 80-kDa protein.

Complementation of Tn*5*-49-inactivated strains by the intact *prc* gene. We examined the effect of the cloned *prc* gene of plasmids pHR53 and pHR61 (9; obtained from Hiroshi Hara

TABLE 2. Antibiotic susceptibility

Strain	MIC (μg/ml)[a] of antibiotic						
	Chloramphenicol	Tetracycline	Nalidixic acid	Erythromycin	Novobiocin	Norfloxacin	Spectinomycin
AG100 (*marA*⁺)	4.4	2.4	2.9	166	91	0.125	11.2
AG100/pHR53	4.4	—[b]	2.8	166	91	0.126	11.2
AG100/pHR61	4.5	—[b]	3.2	169	91	0.131	11.4
LM274 (*marA*⁺, Tn*5*-49)	2.9	1.8	2.1	118	63	0.087	7.4
LM274/pHR53	3.1	—	2.1	166	64	0.087	7.8
LM274/pHR61	3.6	—	3.2	173	93	0.136	11.8
WY100 (Δ*marA*)	4.1	2.2	2.8	155	79	0.124	10.3
WY100/pHR53	4.1	—	2.9	155	79	0.124	10.5
WY100/pHR61	4.3	—	3.1	161	83	0.130	10.7
ASS100 (Δ*marA*, Tn*5*-49)	2.7	1.6	2.1	111	56	0.086	6.8
ASS100/pHR53	2.8	—	2.2	158	56	0.087	7.3
ASS100/pHR61	3.2	—	2.9	166	85	0.134	10.6
AG102 (Mar)	17.5	9.7	10.5	275	352	0.325	16.6
AG102/pHR53	17.5	—	10.5	276	354	0.328	16.7
AG102/pHR61	17.7	—	10.7	281	362	0.351	16.8
ASS102 (Mar, Tn*5*-49)	12.2	7.1	7.5	193	250	0.225	10.8
ASS102/pHR53	12.4	—	7.6	276	254	0.227	11.2
ASS102/pHR61	15.5	—	10.8	288	364	0.363	16.8

[a] Average of three determinations.
[b] Plasmids pHR53 and pHR61 contain the class C *tet* resistance gene.

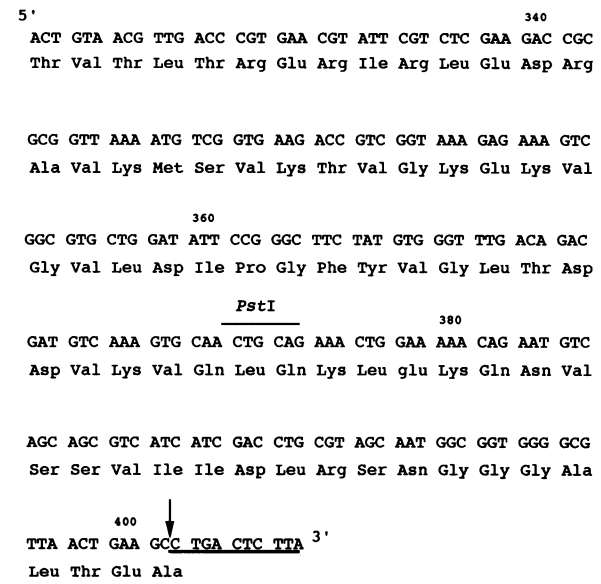

FIG. 2. Nucleotide sequence of the Tn*5*-49 insertion in the *prc* gene. The sequence corresponding to Tn*5* is underlined, and the insertion site of Tn*5* is indicated by an arrow. The *Pst*I site appearing in the chromosomal DNA of pAS-1 is indicated above the sequence. An opal (UGA) nonsense codon is created by the fusion junction. The amino acid residues are numbered according to reference 9.

of the Tufts University School of Medicine) in AG100, AG102, and WY100 cells bearing the Tn*5*-49 insertion on the chromosome. pHR53 is a medium-copy-number plasmid bearing a 2.9-kb *Eco*RI chromosomal fragment containing the intact *prc* gene cloned into pACYC184; pHR61 is a recombinant plasmid carrying a 12.2-kb *Pst*I fragment of chromosomal DNA containing the intact *prc* gene cloned into pBR322 (9). In cells bearing pHR61, *prc* expression is higher than in those bearing pHR53: the *prc* product of pHR61, but not of pHR53, can be seen in maxicell and in vitro systems (9). We found that the parental levels of drug susceptibility were restored in AG100, AG102, and WY100 Tn*5*-49 cells bearing pHR61 (Table 2). However, there was only partial reversal of chloramphenicol susceptibility. Tn*5*-49 cells bearing pHR53 did not recover wild-type susceptibility except that for erythromycin (Table 2). Complementation studies could not be carried out for tetracycline because pHR53 and pHR61 have the class C *tet* genes from pACYC184 and pBR322, respectively.

prc mutants grow at 30°C but not at 42°C on a salt-free L agar plate (9). We found this property to be true of LM274 and other Tn*5*-49 transductants but not of nontransductants. Tn*5*-49 transductants transformed with the pHR53 or pHR61 plasmid were no longer thermosensitive. This result showed that the amount of *prc* gene product made by both pHR53 and pHR61 was sufficient to counteract the thermosensitivity of Tn*5*-49 mutants. Restoration of antibiotic resistance may require more *prc* product than does thermoresistance.

A deletion including the entire *prc* gene in strain JE7925 results in the leakage of periplasmic proteins (9). This deletion strain, compared with the isogenic wild-type strain JE7924, showed the same increase in susceptibility to antibiotics that was found with the Tn*5*-49 insertion (data not shown). The leakage of periplasmic proteins has been associated with defects in outer membrane components in the case of *envA1* mutants (21). Hypersensitivity to hydrophobic antibiotics and large-size (≥600 MW) hydrophilic antibiotics, neither of which use porins, was reported for *envA1* strains (21). The Tn*5* insertion in the *prc* gene differs from the *envA1* mutation in affecting cell susceptibility to the small hydrophilic antibiotics, such as norfloxacin, spectinomycin, and tetracycline, which use porins, as well as to hydrophobic antibiotics such as novobiocin, chloramphenicol, nalidixic acid, and erythromycin.

Further studies suggested that the *prc* gene was not regulated by the *mar* operon. First, the loss of *prc* function had the same effect on antibiotic susceptibility in both Mar and wild-type strains. Second, susceptibility of the Tn*5*-49 transductants to antibiotics was about the same at 30 and 37°C, whereas resistance due to the *mar* operon was higher at 30 than at 37°C (data not shown; growth during resistance determinations at 37°C was for 18 h, giving approximately the same number of generations as that for 40 h at 30°C). Finally, there was no difference in expression of the *prc* gene in wild-type strains and in Mar strains as judged by hybridization of RNA with an *Eco*RI-*Xho*I probe containing the *prc* region of pAS-1 even in the presence of salicylic acid, a strong inducer of the *mar* operon (4) (data not shown).

The *prc* gene has been implicated in proteolytic processing of penicillin-binding protein 3 in in vivo studies (9). Furthermore, the *prc* product is identical with a protease designated Tsp (tail-specific protease) (17) which specifically degrades a variant of the N-terminal domain of λ repressor in vitro.

This is the second non-Mar locus in *E. coli* identified by random Tn*5* insertions into Mar mutants. The first such insertion was in the *pnp* gene (12). The *marA* locus itself, of course, was originally defined by a Tn*5* insertion (7).

Our studies indicate that, directly or indirectly, the *prc* gene product affects sensitivity to multiple, structurally different antibiotics, possibly by influencing cell permeability.

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