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Integron-Mediated Rifampin Resistance in *Pseudomonas aeruginosa*

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A new rifampin resistance gene, *arr-2*, has been found in *Pseudomonas aeruginosa*. The ARR-2 protein shows 54% amino acid identity to the rifampin ADP-ribosylating transferase encoded by the *arr* gene from *Mycobacterium smegmatis*. This *arr-2* gene is located on a gene cassette within a class I integron.

Rifampin has been used in the treatment of tuberculosis and leprosy for several decades, and the length of antibiotic treatment may be 6 months or more. This creates selective pressure favoring bacteria that acquire resistance by mutation or gene transfer. Rifampin is derived from rifamycin, a product of *Amycolatopsis mediterranei* (11) that has been in natural environments for a long time. Resistance genes may have evolved in response to rifampins in natural environments, as well as in clinical situations.

Rifampin resistance in clinical isolates of *Mycobacterium tuberculosis* is predominantly due to missense mutations in the *rpoB* gene which decrease binding of RNA polymerase to rifampin. However, a considerable percentage of resistant strains do not contain mutations in *rpoB* (16). In nonclinical isolates, rifampin resistance of bacteria such as *Nocardia*, *Bacillus*, and *Pseudomonas* spp. and nontubercle species of mycobacteria is not usually due to mutations in the *rpoB* gene. Several other resistance mechanisms have been identified, including a rifampin efflux gene that has been identified in *Pseudomonas fluorescens* (4); inactivation of rifampin by decomposition (6), glucosylation (13, 17), phosphorylation (18), and ribosylation (5, 8) has also been reported.

Integrons are specialized genetic elements that allow genes to move by site-specific recombination (for reviews, see references 7, 9, and 10). An integron consists of an integrase gene with adjacent gene cassettes that commonly contain antibiotic resistance genes. The cassettes are bounded by integrase recombination core sites and have conserved features at the 3′ ends of the cassettes with an inverse core site and a 59-base element. The core site sequence is GTTRRRY (R is purine, and Y is pyrimidine). Integrons have been found in a variety of gram-negative species and are common in *Pseudomonas aeruginosa* (20).

Here we report a multiply resistant strain of *P. aeruginosa*, PaTh2, isolated from a patient in Bangkok, Thailand. It is highly resistant to many antibiotics, including rifampin, expanded-

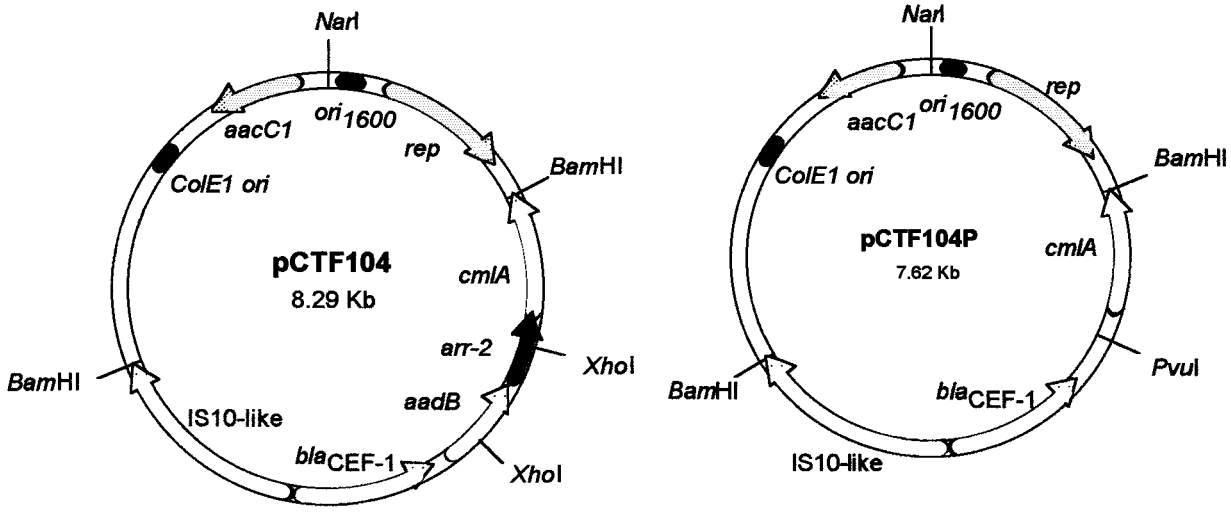

FIG. 1. Plasmid maps of two resistance constructs with intact *arr-2* and disrupted *arr-2* and *aadB*. pCTF104 is a recombinant plasmid created by insertion of a *Bam*HI fragment from the total DNA of PaTh2 into the *Bam*HI site of pUCP24. ori_{1600} is the origin of replication used by *Pseudomonas*, *rep* is the gene coding for replication protein, *ColE1 ori* is the origin of replication used by *E. coli*, and *aacC1* is a gentamicin resistance gene in pUCP24. IS*10*-like is an insertion element, bla_{CEF-1} is a CEF-1 β-lactamase gene, *aadB* is a gentamicin resistance gene, *arr-2* is a rifampin resistance gene, and *cmlA* is a chloramphenicol resistance gene in an insert from PaTh2. pCTF104P is a recombinant plasmid derived from pCTF104.

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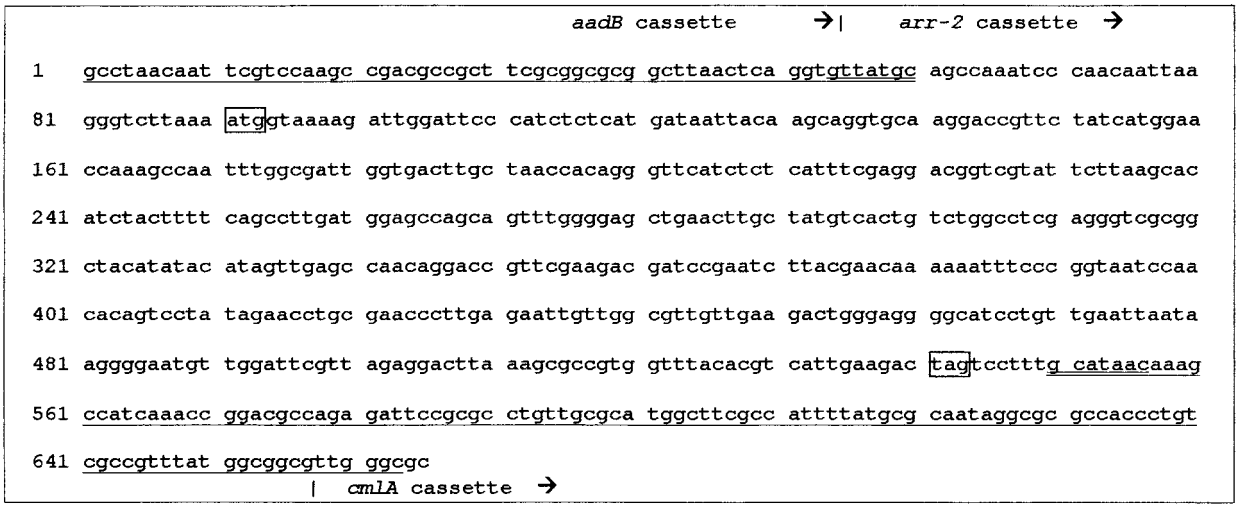

FIG. 2. DNA sequence of the *arr-2* cassette and its boundary. The underlined portions are 59-base elements. The double-underlined portions are the core site and the inverse core site of the *arr-2* cassette. The boxed codons are the start and stop codons of the *arr-2* gene. The vertical bars indicate the boundaries of the cassettes.

spectrum cephalosporins, many other β-lactam antibiotics, many aminoglycosides, chloramphenicol, tetracycline, and quinolone compounds. The MIC of rifampin for this strain is >256 μg/ml, while most *P. aeruginosa* strains are less resistant to rifampin and the MIC is around 32 μg/ml (19).

Cloning of *arr-2* and nearby genes. The DNA was isolated from PaTh2 by using the Sarkosyl-proteinase K method (12). One hundred micrograms of DNA was digested with *Bam*HI and ligated with 5 μg of pUCP24 (15) digested with *Bam*HI. The ligated library then was used to transform *Escherichia coli* XL1-Blue, and transformants were selected on Luria-Bertani (LB) agar plates containing 100 μg/ml ampicillin or ceftazidime (1). The clone pCTF104 was originally derived from an XL1-Blue transformant selected on LB with 100 μg/ml ampicillin. The DNA insert of this clone was sequenced with AmpliTaq DNA polymerase and dideoxynucleotide fluorescent terminators on an ABI Prism 377XL DNA sequencer (Cancer Research Center, University of Chicago). In order to inactivate what appears by sequence analysis to possibly be a rifampin resistance gene, pCTF104 was digested with *Xho*I, and then the recessed ends of the plasmid were filled in and religated to create pCTF104P, from which the 3′ end of the *aadB* gene and the 5′ end of the *arr-2* gene have been deleted (Fig. 1).

Determination of MICs for the transformants. We determined the MICs by using the agar dilution method. An inoculum of 10^4 CFU per spot was delivered onto LB plates containing antibiotics in twofold dilutions. The MIC was determined as the lowest concentration of an antibiotic at which no visible growth or the growth of two or fewer colonies was observed after 20 h of incubation at 37°C. We determined the MICs for both *E. coli* and *P. aeruginosa* strains carrying pCTF104, pCTF104P, or no plasmid.

We first obtained the rifampin resistance gene while cloning the nearby ceftazidime resistance gene. The results from the DNA sequencing showed several potential resistance genes, including genes for rifampin, aminoglycoside, chloramphenicol, and ceftazidime resistance (Fig. 1). The DNA sequence around the rifampin resistance gene revealed characteristic integron 5′ and 3′ elements (Fig. 2). We also found an integrase gene for a class I integron adjacent to the IS*10*-like element (data not shown). The *aadB* and the *cmlA* gene cassettes have already been identified (2, 3). The bla_{CEF-1} gene responsible for ceftazidime resistance was also on a gene cassette in this class I integron element. The *arr-2* gene putatively coded for a 150-amino-acid protein. We searched for the closest homologue in the GenBank database and found the ADP-ribosylating transferase encoded by the *arr* gene in *Mycobacterium smegmatis* (8). There is no other homologue in the databases. When we used the CLUSTAL W program (14), ARR-2 showed 54% identity, 68% similarity, and only two gaps in the alignment with ARR (Fig. 3). The mechanism of resistance from *arr* is inactivation of rifampin by ribosylation (5, 8).

The MICs of rifampin increased from 8 to >256 μg/ml for *E. coli* with pCTF104 and from 16 to >512 μg/ml for *P. aeruginosa* with pCTF104; the transformants with pCTF104P show

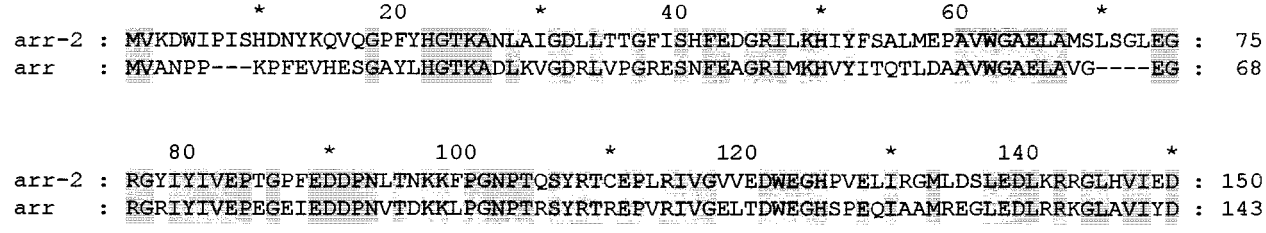

FIG. 3. CLUSTAL W protein alignment of a putative amino acid sequence of ARR-2 and ARR. The shaded areas indicate identical amino acids, and dashes indicate gaps inserted into the sequence to maximize the alignment.

TABLE 1. MICs for *E. coli* and *P. aeruginosa* strains with various cloned resistance genes

Strain[a]	MIC (μg/ml) of:			
	Ampicillin	Carbenicillin	Ceftazidime	Rifampin
WT *E. coli* DH5α	4	4	1	8
DH5α(pCTF104)	>2048	>2048	2048	>256
DH5α(pCTF104P)	>2048	>2048	2048	8
WT *P. aeruginosa* PAK	2048	64	8	16
PAK(pCTF104)	>2048	>2048	2048	>512
PAK(pCTF104P)	>2048	>2048	2048	16

[a] WT, wild type.

the same susceptibility to rifampin as the wild type. Ceftazidime resistance was not affected by deletion of a 665-bp *Xho*I fragment (Table 1). The finding that *arr-2* conferred rifampin resistance along with the homology between *arr* and *arr-2* strongly suggests that the protein from *arr-2* is an ADP-ribosylating transferase.

Our findings on *arr-2*, along with the findings on *arr* from *M. smegmatis*, raise the questions of how these two genes evolved and if they have been transferred between mycobacteria and pseudomonads. Both mycobacteria and pseudomonads are found in soil, and some species of each are also found in human lung infections. The *arr* genes could have arisen in response to rifampin in soil or to rifampin used in the treatment of infections. The available data do not allow us to select between these two possibilities. The second question is how these genes might have been transferred between mycobacteria and pseudomonads. One possibility is that the location of *arr-2* on an integron allows it to move around to various DNA molecules, such as plasmids or transposons, inside the cell, that could then be transferred to other bacterial species.

Nucleotide sequence accession number. The sequence in Fig. 2 has been deposited in GenBank under accession no. AF078527.

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