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Cloning of Multidrug Resistance Gene *pqrA* from *Proteus vulgaris*†

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The multiple antibiotic resistance gene *pqrA* was cloned from the chromosomal DNA of a clinical isolate of *Proteus vulgaris* 881051 into *Escherichia coli* KY2563. The MICs of quinolones tetracycline, cephalosporin, and chloramphenicol for transformant strain DNS7020 were from 8 to 32 times higher than those for the parent strain, KY2563. The level of expression of outer membrane protein F (OmpF) by DNS7020 was lower than that of KY2563 but not as low as that of an OmpF-deficient control strain. The 1.4-kb fragment containing the *pqrA* gene had an open reading frame encoding a polypeptide of 122 amino acid residues with a molecular weight of about 14,000, which was consistent with the experimental value identified by the Maxicell method. The putative PqrA polypeptide showed significant amino acid sequence similarity to the *E. coli* proteins SoxS and MarA. These polypeptides are strongly conserved in predicted helix-turn-helix DNA binding domains. The MarA protein, which is responsible for multiple antibiotic resistance in *E. coli*, also decreases OmpF expression. Moreover, the SoxS protein, which is characterized as a superoxide response regulon of *E. coli*, has also been shown to increase resistance to many structurally unrelated antibiotics. The *soxS* gene increases superoxide dismutase levels in addition to decreasing OmpF expression. The expression level of superoxide dismutase with DNS7020 was about 1.5 times higher than that with KY2563. These findings suggest that the *pqrA* gene in *P. vulgaris* confers multidrug resistance in a way similar to that of the *soxS* and *marA* genes in *E. coli*.

The recent increase in the clinical use of fluoroquinolones has been accompanied by a subsequent increase in the frequency of bacterial resistance to these antibacterial agents (1, 22, 27, 32). This may become a problem in the treatment of bacterial infections, since it may involve cross-resistance not only to quinolones but also to some of the more commonly used antibiotics such as the broad-spectrum cephalosporins tetracycline and chloramphenicol (10). We previously reported that *Proteus vulgaris* 881051 isolated from a patient with upper urinary tract infection in Japan was resistant to the quinolones tetracycline and chloramphenicol (21). We have already reported the quinolone resistance mechanisms of this strain, which involve an alteration of DNA gyrase, an alteration in outer membrane proteins, and an active efflux of drug. However, these results do not fully explain the multidrug resistance of this strain. Genetic characterization of bacterial resistance to quinolones has revealed three mechanisms of resistance. The first mechanism involves mutations in the *gyrA* gene encoding the A subunit of DNA gyrase (3, 30, 31). The second is mutations in the *nfxB* and *cfxB* genes to decrease expression of porin outer membrane protein OmpF (5, 11, 19). The third mechanism has been proposed to involve the presence of an active efflux system for quinolones across the inner membrane in *Staphylococcus aureus* (36, 37, 39) and *Bacillus subtilis* (28, 29) which is encoded by the *norA* gene and *bmr* gene, respectively. These mechanisms may confer resistance either alone or in combination (6, 16). Mechanism 1 is thought be specific to the quinolones, but mechanisms 2 and 3 may confer resistance to unrelated antibacterial agents.

To study the mechanism of multidrug resistance with *P. vulgaris*, we cloned the multidrug resistance gene of this strain.

MATERIALS AND METHODS

Bacterial strains and plasmid. *P. vulgaris* 881051, a strain highly resistant to fluoroquinolones, was isolated from a patient with an upper urinary tract infection. *Escherichia coli* KY2563 (*thi tsx malA*) and KY2201 (KY2563Δ*ompF*) were used as recipients for transformation, and CSR603 (Δ*uvrA6*) was used for the Maxicell method (34). Plasmids pUC19 (Amp^r) and pKK233-2 (*trc* promoter) were used as the vector and as the expression vector plasmid, respectively, to confirm the structure of the resistance gene.

Chemicals. Ofloxacin, ciprofloxacin, and imipenem were synthesized at the Exploratory Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. Sodium ceftazidime (Nippon Glaxo Co., Ltd., Tokyo, Japan), sodium ampicillin (Meiji Seika Co., Ltd., Tokyo, Japan), tetracycline chloride (Nippon Lederle Co., Ltd., Tokyo, Japan), rifampin and chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), and gentamicin sulfate (Schering-Plough, Osaka, Japan) were obtained commercially. Restriction endonucleases *Xba*I (Toyobo, Osaka, Japan), *Hin*dIII, *Eco*RI, and *Pst*I; a sequencing kit (Takara, Kyoto, Japan); lysozyme (Seikagaku Kogyo, Osaka, Japan); RNase (Sigma Chemical Co.), and T4 DNA ligase (Takara) were also obtained commercially.

Preparation of chromosomal DNA. Chromosomal DNA was purified from the cells of *P. vulgaris* 881051 grown to the late logarithmic phase in 600 ml of Luria-Bertani broth as previously described (23).

Preparation of plasmid DNA and transformation. Purification of plasmid DNA and transformation were carried out as previously described (13, 33).

DNA sequencing. DNA sequences were determined by the dideoxynucleotide chain termination method (33) by using a DNA sequencing kit.

Measurement of MIC. The MIC of each agent was measured by the agar dilution method. Five microliters of a dilution on overnight culture (ca. 10^7 CFU/ml) with Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was inoculated onto Mueller-Hinton agar (Difco Laboratories) containing the drugs at concentrations from 0.003 to 100 μg/ml, and the plates were incubated at 37°C for 18 h. The MIC was defined as the lowest concentration which prevented the visible growth of organisms.

Identification of plasmid-encoded proteins. The plasmid-encoded proteins were prepared by the Maxicell method (34) unless otherwise cited. *E. coli* CSR603 and its plasmid pHI2-carrying derivatives were grown in M9 medium supplemented with 1% Casamino Acids (Difco Laboratories)–10 μg of thiamine

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† This paper is dedicated to the memory of Yasuaki Osada.

‡ Deceased 15 April 1993.

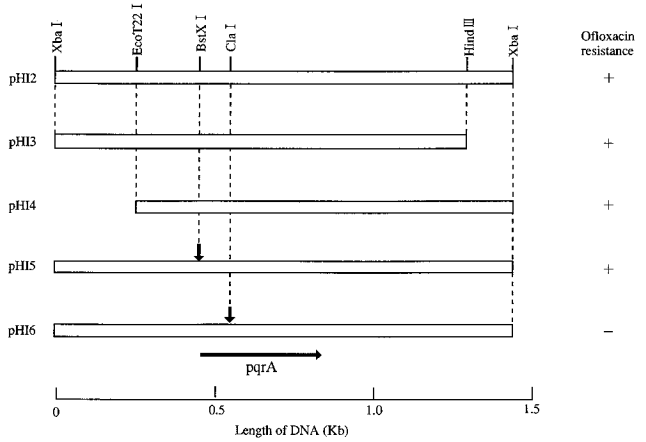

FIG. 1. Restriction map of the cloned 1.4-kb *Xba*I fragment containing the *pqrA* gene. Subclones are indicated below the map, and the presence (+) or the absence (−) of ofloxacin resistance is shown at the right. The approximate extent of the *pqrA* region is indicated by a thick arrow.

per ml–0.2% glucose to an optical density of 0.2 measured at 660 nm. Twenty-microliter samples were loaded onto a 15% polyacrylamide–sodium dodecyl sulfate (SDS) gel. ^{14}C-methylated carbonic anhydrase (30,000), trypsin inhibitor (21,500), cytochrome *c* (12,500), aprotinin (6,500), and insulin (5,740) were used as molecular weight standards.

Analysis of outer membrane proteins. Outer membrane proteins were prepared by the method reported previously (35). The protein preparations were analyzed electrophoretically with an SDS-polyacrylamide gel containing 8 M of urea by the modified method of Mizushima and Yamada (26). About 50 μl of 5-mg/ml outer membrane proteins was dissolved in 200 μl of 1.25% SDS–1.25% β-mercaptoethanol solution and heated at 100°C for 5 min. Sodium urea was then added to the solution to give a final concentration of 8 M, and then 1% bromophenol blue was added. Ten microliters of each sample was applied to a gel containing 8% acrylamide, 0.13% *N,N′*-ethylenebisacrylamide, 0.5% SDS, 8 M urea, 0.1 M sodium phosphate buffer (pH 7.2), and 0.6 μl of *N,N,N′,N′*-tetramethylethylenediamine per ml. Polymerization of the gel was initiated with ammonium persulfate (1.2 mg/ml). Electrophoresis was performed in 0.1 M sodium phosphate (pH 7.2)–0.1% SDS buffer.

Superoxide dismutase assay. Superoxide dismutase was assayed by the xanthine oxidase-cytochrome *c* method (24).

The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D13561.

RESULTS

Cloning of the *pqrA* gene. To identify genes whose product conferred multidrug resistance, we digested chromosomal DNA of the strain *P. vulgaris* 881051 with *Xba*I, *Hin*dIII, *Eco*RI, or *Pst*I; cloned the fragments into the plasmid pUC19; and transformed them to *E. coli* KY2563. Colonies were selected on agar plates containing both 0.4 μg of ofloxacin and 50 μg of ampicillin per ml. Consequently, two recombinant plasmids, one containing *Xba*I- and one containing *Hin*dIII-digested fragments, were obtained. These two independently cloned inserts contained overlapping DNA fragments, indicating that they were probably derived from the same chromosomal locus, which was confirmed by hybridization experiments (data not shown). They oriented in the opposite direction with respect to the vector *lac* promoter; this overlapping fragment contained the internal promoter for the expression of the resistance phenotype. For further study, we used the plasmid containing the 1.4-kb *Xba*I fragment, which we called plasmid pHI2. The *E. coli* KY2563 transformant of pHI2 was called *E. coli* DNS7020.

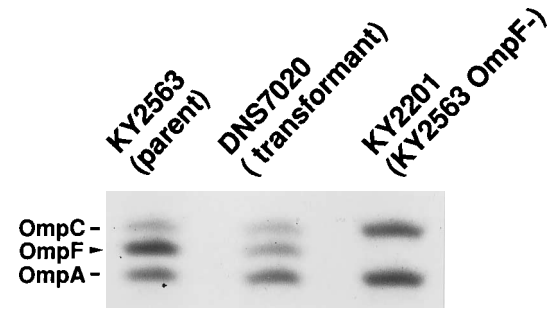

FIG. 2. Gels (8 M urea–SDS–polyacrylamide) of outer membrane proteins of strains KY2563, DNS7020, and KY2201 (KY2563 OmpF$^-$). The OmpF, OmpC, and OmpA regions are indicated on the left.

Localization of the minimal complementation unit. To get more detailed information about the 1.4-kb *Xba*I fragment, fragments derived from plasmid pHI2 were subcloned and analyzed for ofloxacin susceptibility (Fig. 1). A deletion of the sequence to the right of the *Hin*dIII site or the left of the *Eco*T22I site had no effect on the level of resistance. Frameshift at the *Bst*XI site, caused by digestion with *Bst*XI, followed by blunting of the end of the fragment and self-ligation, also had no effect on the level of resistance. However, a frameshift at the *Cla*I site caused by digestion with *Cla*I and treated with Klenow fragment and self-ligation conferred no resistance to ofloxacin at all. These results indicate that the region involved in the resistance phenotype was clearly localized inside the *Bst*XI-*Hin*dIII fragment.

Resistance levels of transformants. The MICs of ofloxacin, ciprofloxacin, ceftazidime, gentamicin, tetracycline, chloramphenicol, rifampin, and imipenem for the recipient strain, *E. coli* KY2563, and its transformant strain, DNS7020, are shown in Table 1. The MICs of ofloxacin, ciprofloxacin, tetracycline, chloramphenicol, and ceftazidime for DNS7020(pHI2) were 8 to 32 times higher than those for KY2563, whereas the MICs of imipenem, rifampin, and gentamicin were the same or approximately the same for both strains. The MICs for DNS7020 were 2 to 16 times greater than for KY2201, an OmpF-deficient mutant of KY2563.

Outer membrane proteins. The composition of the outer

TABLE 1. Antibacterial agent susceptibilities of strains used in this study[a]

E. coli strain no.	MIC (μg/ml)[b]								
	OFLX	CPFX	NFLX	CAZ	GM	TC	CP	RIF	IPM
KY2563 (parent)	0.05	0.006	0.025	0.10	0.20	1.56	6.25	12.5	0.20
DNS7020 (transformant)	0.39	0.10	0.39	3.13	0.39	12.5	100	12.5	0.39
KY2201 (OmpF$^-$)	0.10	0.013	0.10	0.20	0.20	3.13	12.5	6.25	0.20

[a] MICs were determined by the agar dilution method. Inoculum size, 5×10^4 CFU per spot.

[b] Abbreviations for antibiotics: OFLX, ofloxacin; CPFX, ciprofloxacin; NFLX, norfloxacin; CAZ, ceftazidime; GM, gentamicin; TC, tetracycline; CP, chloramphenicol; RIF, rifampin; IPM, imipenem.

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        BstX I
451 CCCACCATTTTTGTGGGAGGTTTATGGCTGAAAATGTCGTTAATGATATTCTAAAATGGTTAGAAACCCAGTTACAACGTAACGAAGGT
                  SD       MetAlaGluAsnValValAsnAspIleLeuLysTrpLeuGluThrGlnLeuGlnArgAsnGluGly
          Cla I
541 ATAAAAATCGATACTATTGCGAACAAAAGTGGTTATTCAAAATGGCACTTACAACGCATATTTAAAGATTTTAAAGGCTGCACATTAGGC
    IleLysIleAspThrIleAlaAsnLysSerGlyTyrSerLysTrpHisLeuGlnArgIlePheLysAspPheLysGlyCysThrLeuGly

631 GAATATGTCCGCAAACGTCGCTTATTAGAAGCGGCTAAATCATTACAAGAAAAAGATATGTCTATTTTAGACATCGCTTTAATGTATGGC
    GluTyrValArgLysArgArgLeuLeuGluAlaAlaLysSerLeuGlnGluLysAspMetSerIleLeuAspIleAlaLeuMetTyrGly

721 TTTAGCTCTCAAGCAACATTTACTCGTATTTTTAAAAAACATTTTAATACTACACCTGCTAAGTTTAGAGAAAATGGCACTATGCCAGAT
    PheSerSerGlnAlaThrPheThrArgIlePheLysLysHisPheAsnThrThrProAlaLysPheArgGluAsnGlyThrMetProAsp

811 ACACATTGCTTTATGTCATGTGAAACTCACTGATTAATTTAGCATTTATAAGATGTCATAAAAAAACCAGCTATCATCGAGCTGGTTTTT
    ThrHisCysPheMetSerCysGluThrHis***
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FIG. 3. Nucleotide sequence of the sense strand of the *pqrA* gene from the 5′ (left) to the 3′ (right) end is shown. It is numbered from the 5′ end of the 1.4-kb *Xba*I fragment. The deduced amino acid sequence of the *pqrA* gene product is given below the DNA sequence. The stars indicate the deduced transcription terminator. The Shine-Dalgarno sequence is underlined.

membrane proteins of strain DNS7020 was compared with that of the proteins of strains KY2563 and KY2201 by 8 M urea–SDS–polyacrylamide gel electrophoresis (Fig. 2). Strain KY2563 possessed three major protein bands, OmpC, OmpF, and OmpA. The expression of OmpF protein in strain DNS7020 was decreased in comparison with the parent strain KY2563, but not as low as in KY2201, an OmpF-defective mutant of strain KY2563 (25).

Nucleotide sequence and identification of the *pqrA* gene. The nucleotide sequence of both strands of the 1.4-kbp *Xba*I fragment was determined. The nucleotide sequence contained an open reading frame (nucleotides 475 to 840) (Fig. 3), long enough to encode a polypeptide of 122 amino acids, which we called the *pqrA* gene. No other open reading frame was found that could encode a polypeptide longer than 40 amino acids. To confirm that the *pqrA* gene was sufficient for resistance, we introduced an *Nco*I site just before the putative starting site by mutating the nucleotides at the position of 473 and 474 to cytosines. After that, we digested the predicted structure gene of PqrA with *Nco*I and *Hin*dIII and inserted it into the expression vector plasmid pKK233-2 (*trc* promoter). The resulting plasmid indicated multidrug resistance when it was transformed to KY2563 (data not shown). Thus, the *pqrA* gene really did play a role in the multidrug resistance.

Expression of protein PqrA by the Maxicell method. To confirm that the 1.4-kbp *Xba*I fragment contained only a single polypeptide, we investigated the expression of proteins by the Maxicell method. SDS-polyacrylamide gel electrophoresis and autoradiography of ^{35}S-labeled protein produced in the pHI2-carrying strain were compared with that for the parent strain (*E. coli* CSR603). Although there was no incorporation of label into the parent strain (data not shown), the strain carrying pHI2 showed two major protein bands with molecular weights of about 30,000 and 14,000 (Fig. 4). The former was the penicillinase expressed by the ampicillin-resistant gene derived from the vector plasmid, and the latter was thought to be PqrA. This value agreed with the molecular weight predicted to be produced by the open reading frame, *pqrA*.

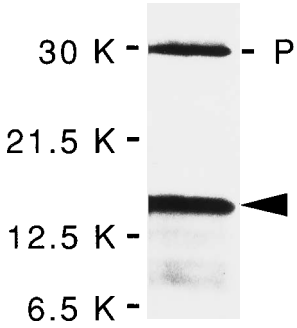

FIG. 4. SDS-polyacrylamide gel electrophoresis and fluorography of ^{35}S-labeled protein produced in the Maxicell harboring pHI2. The standard sizes (in kilobases) are shown at the left. The arrowhead on the right indicates the PqrA protein.

DISCUSSION

Our studies showed that the *pqrA* gene contributed to the multidrug resistance of *E. coli* KY2563. The MICs of fluoroquinolones tetracycline, ceftazidime, and chloramphenicol for the *E. coli* transformant DNS7020 were more than eight times higher than those for the recipient strain, KY2563. With regard to multidrug resistance, several resistant mutants have been recognized in *E. coli*. For example, *nfxB* (20) and *nfxC1* (17), *cfxB1* (18), and *norB* (15) mutations confer multidrug resistance. Moreover, *marA1* mutation selected with tetracycline (8) and *soxQ1* mutation selected with naphthoquinone menadione (14) also confer multidrug resistance, including fluoroquinolone resistance. The *cfxB1*, *marA1*, and *soxQ1* mutations are known to be mutant alleles of *marR* in the *marRAB* regulon (4). An important mechanism in multidrug resistance by the *marRAB* regulon is the reduction in the expression of OmpF due to increased expression of *micF*, which is an antisense RNA complementary to *ompF* mRNA (9).

In this study, the *pqrA* gene also had an effect on the composition of the outer membrane protein. The expression of OmpF protein in strain DNS7020 was decreased in comparison with that in the recipient strain. These findings indicate the possibility that a function of the *pqrA* gene is the same as that of other reported multidrug resistance genes.

The comparison of the amino acid sequence of the PqrA polypeptide with the translated DNA sequence in the GenBank and EMBL databases strengthened the possibility that *pqrA* functions in a manner similar to that of other multidrug resistance genes. The PqrA polypeptide had significant amino acid sequence similarities to *E. coli* proteins MarA and SoxS (Fig. 5). These polypeptides are similarly sized and are strongly conserved in the predicted helix-turn-helix motifs, the putative DNA binding domain (2, 12, 38). SoxS, which was reported to be strongly similar to the MarA protein (7), is known to constitute a two-component regulatory system (*soxRS* system) with SoxR in response to superoxide stress and seems to act as a structural regulator in the *soxRS* system. Activation of this system also causes increased resistance to many structurally unrelated antibiotics that are not implicated in redox stress (2, 10, 14). Furthermore, several oxidative stress genes such as *sodA*, *zwf*, and *nfo* are transcriptionally activated by both the *mar* and the *soxRS* system (38). Interestingly, the relative superoxide dismutase activity with *E. coli* DNS7020 (2.42 U/mg) was about 1.5 times higher than that with KY2563 (1.52 U/mg).

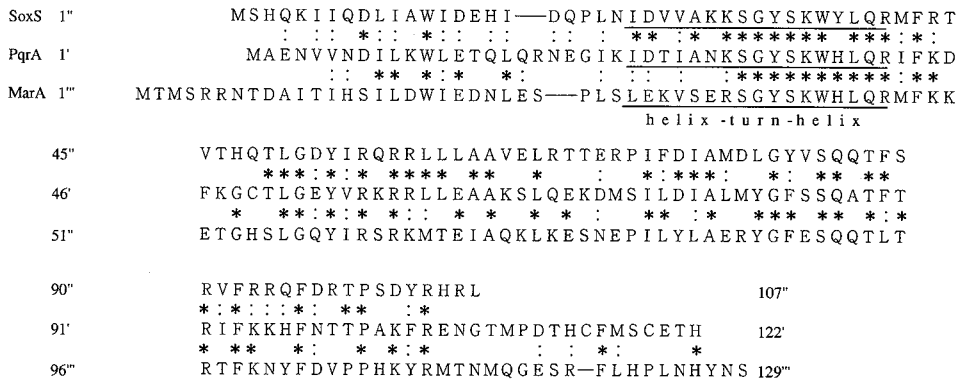

FIG. 5. Comparison of the amino acid sequences of the PqrA polypeptide and the MarA and SoxS proteins (7). Percent identity (*) and percent similarity (* and :) between PqrA and SoxS were 42.6 and 69.3%, respectively, and between PqrA and MarA were 38.5 and 58.1%, respectively. Similarity determinations were performed according to the Dayhoff table. The helix-turn-helix motif is underlined.

These results suggested that PqrA in *E. coli* may confer multidrug resistance by reduced expression of OmpF as a result of regulating some other genes, such as *micF* and *sodA*, in a way similar to that of *soxS* and *marA* in *E. coli.*

However, the OmpF-deficient mutant (KY2201) had lower MICs than the transformant strain (Table 1) in spite of the fact that the expression of OmpF in the transformant strain was not as low as in the OmpF-defective mutant. This phenomenon has also been reported by other researchers. Cohen et al. reported that not all quinolone resistance in the *nfxB*, *nfxC1*, *cfxB1*, and *marA1* mutation strains could be explained by reduced *ompF* expression alone (8). Despite substantial overlap in phenotype, there were some differences among strains with *soxQ1*, *cfxB1*, *nfxC1*, and *marA1* mutations. The level of resistance to norfloxacin conferred on mutants by *cfxB1* or *nfxC1* mutations was two times higher than resistance due to *marA1* and *soxQ1*. *soxQ1* and *cfxB1* differed in their effects on cellular levels of endonuclease IV and glucose-6-phosphate dehydrogenase (14). Ariza et al. reported that these were caused by mutational differences in the *marR* gene, which encodes a putative repressor of *mar* transcription (4). Thus, the multidrug resistance mechanism is thought to be complex. We conclude that we cloned a new multiple antibiotic resistance gene, *pqrA* from *P. vulgaris* 881051. PqrA in *P. vulgaris* may confer multidrug resistance by reduced expression of OmpF in a way similar to that of *soxS* and *marA* in *E. coli*. However, we have yet to confirm that an alteration in PqrA expression is directly linked with resistance in *P. vulgaris*. We are planning further studies to clarify the unknown mechanisms of multidrug resistance.

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