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Mechanisms of disease

Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*

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Summary

Background Bacteria develop resistance to aminoglycosides by producing aminoglycoside-modifying enzymes such as acetyltransferase, phosphorylase, and adenyltransferase. These enzymes, however, cannot confer consistent resistance to various aminoglycosides because of their substrate specificity. Notwithstanding, a *Pseudomonas aeruginosa* strain AR-2 showing high-level resistance (minimum inhibitory concentration >1024 mg/L) to various aminoglycosides was isolated clinically. We aimed to clone and characterise the genetic determinant of this resistance.

Methods We used conventional methods for DNA manipulation, susceptibility testing, and gene analyses to clone and characterise the genetic determinant of the resistance seen. PCR detection of the gene was also done on a stock of *P aeruginosa* strains that were isolated clinically since 1997.

Findings An aminoglycoside-resistance gene, designated *rmtA*, was identified in *P aeruginosa* AR-2. The *Escherichia coli* transformant and transconjugant harbouring the *rmtA* gene showed very high-level resistance to various aminoglycosides, including amikacin, tobramycin, isepamicin, arbekacin, kanamycin, and gentamicin. The 756-bp nucleotide *rmtA* gene encoded a protein, RmtA. This protein showed considerable similarity to the 16S rRNA methylases of aminoglycoside-producing actinomycetes, which protect bacterial 16S rRNA from intrinsic aminoglycosides by methylation. Incorporation of radiolabelled methyl groups into the 30S ribosome was detected in the presence of RmtA. Of 1113 clinically isolated *P aeruginosa* strains, nine carried the *rmtA* gene, as shown by PCR analyses.

Interpretation Our findings strongly suggest intergeneric lateral gene transfer of 16S rRNA methylase gene from some aminoglycoside-producing microorganisms to *P aeruginosa*. Further dissemination of the *rmtA* gene in nosocomial bacteria could be a matter of concern in the future.

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Introduction

Acquisition of multidrug resistance in nosocomial pathogens such as *Pseudomonas aeruginosa* has become a global concern.[1] For the treatment of infectious diseases caused by *P aeruginosa*, fluoroquinolones, broad-spectrum β lactams including carbapenems, and aminoglycosides such as the anti-pseudomonal drug amikacin, are the drugs of last resort. In Japan, however, about 20% of clinically isolated *P aeruginosa* have acquired resistance to imipenem or ciprofloxacin, while about 5% of clinical isolates also show resistance to amikacin.[2] Therefore, continuing amplification of resistance rates and levels, and expansion of resistance profiles to aminoglycosides in *P aeruginosa*, is becoming a general and genuine threat in clinical settings.[3]

Various aminoglycosides—such as gentamicin, kanamycin, amikacin, tobramycin, and isepamicin—have been developed and used for chemotherapy since the 1950s.[4] These drugs have high affinities for 16S rRNA of the bacterial 30S ribosome, and they block protein synthesis.[5] Over the past few decades, results of many studies on the mechanisms of resistance to aminoglycosides have shown self-modification of drugs to be the most typical mechanism; impermeability caused by upregulation of the active multidrug efflux system MexXY-OprM also confers broad but low-level resistance to aminoglycosides.[6] Several aminoglycoside-modifying enzymes—such as acetyltransferase, phosphorylase, and adenyltransferase—that catalyse covalent modification of specific amino or hydroxyl groups have been identified.[7] These enzymes have been noted in various nosocomial bacteria and are generally associated with transposable elements mediated by transferable R-plasmids. To overcome these modifying enzymes, a novel semisynthetic aminoglycoside, arbekacin, a derivative of kanamycin, was developed in Japan; this drug shows strong activity against various bacterial species and is rarely inactivated by single 6′ acetylation or 2″-phosphorylation.[8] Arbekacin showed effective antibacterial activity against various gram-positive and gram-negative bacteria by inhibition of 16S rRNA in bacterial 30S ribosome.[9,10]

Arbekacin has been used in Japan since 1990,[11] although this drug was approved only for control of infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) for prudent antibiotic use. However, several arbekacin-resistant MRSA strains have emerged in Japan, which produce the bifunctional enzyme, aminoglycoside-6′-*N*-acetyltransferase-2″-*O*-phosphotransferase, which mediates both 6′-acetylation and 2″-phosphorylation; this type of modification, however, confers only low-level drug resistance (minimum inhibitory concentration [MIC] between 4 and 32 mg/L).[12]

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In this study, we aimed to characterise the genetic determinant for multiple and high-level aminoglycoside resistance in a clinically isolated P aeruginosa strain showing consistent and very high-level resistance to all clinically useful aminoglycosides, including amikacin and arbekacin. We also aimed to characterise the prevalence of the molecular mechanism of very high-level resistance to arbekacin found in P aeruginosa strain AR-2 among clinically isolated P aeruginosa strains.

Methods

Procedures

DNA manipulation, susceptibility testing, and gene analyses

We isolated P aeruginosa strain AR-2 from a clinical sample (sputum) taken in 1997. We used E coli strain XL1-Blue (Stratagene, La Jolla, CA, USA) as the

transformation host and for propagation of plasmids. We used P aeruginosa strain 105 (ciprofloxacin-resistant, arbekacin-sensitive, amikacin-sensitive) as recipient in a CONJUGATION experiment. The plasmid pBC-SK+ (Stratagene) was used as the cloning vector, and pTO001—an E coli-P aeruginosa shuttle-cloning vector—was also used. P aeruginosa PAO1 served as the host for subcloning experiments. Unless noted otherwise, we grew cultures at 37°C in Luria-Bertani broth. We established MICs of aminoglycosides by an agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA), according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines M7-A5.[13]

DNA prepared from P aeruginosa AR-2 was digested with HindIII and ligated into the HindIII site of pBC-SK+ with T4 DNA ligase (Nippon Gene, Tokyo, Japan); the resulting recombinant plasmid was named pBCH9, and the deleted plasmid was named pBCH9-13 (figure 1). We selected E coli strain XL1-Blue transformants carrying a roughly 8-kb insert on Luria-Bertani agar plates containing both arbekacin (2 mg/L) and chloramphenicol (30 mg/L). We assayed MICs on both the parent strain and transformants, according to the guidelines of the NCCLS. We established the nucleotide sequence by the dideoxy-chain termination method with a model 3100 DNA sequencer (Applied Biosystems Japan, Tokyo, Japan). We did nucleotide and aminoacid sequence homology searches with the internet program FASTA (National Institute of Genetics, Mishima, Japan).[14] We aligned nucleotide and aminoacid sequences with GENETYX-MAC software, version 10.1.1 (Software Development, Tokyo, Japan).

To ascertain the transferability of the rmtA gene for arbekacin resistance, we did conjugation experiments with P aeruginosa strain 105 as a recipient. TRANSCONJUGANTS were selected on Mueller-Hinton agar

	P aeruginosa AR-2	E coli XL1-blue			P aeruginosa PAO1		P aeruginosa	
		pBCH9	pBCH9-13	pBC-SK+	pTORmtA	pTO001	Transconjugant	105*
4,6-substituted deoxystreptamine antimicrobials								
Kanamycin groups								
Arbekacin	>1024	>1024	>1024	0·5	>1024	1	>1024	4
Amikacin	>1024	>1024	>1024	1	>1024	8	>1024	4
Kanamycin	>1024	>1024	>1024	2	>1024	128	>1024	>1024
Tobramycin	>1024	>1024	512	1	>1024	1	>1024	256
Gentamicin groups								
Gentamicin	>1024	>1024	1024	0·5	>1024	256	>1024	>1024
Sisomicin	>1024	512	>1024	0·5	>1024	256	>1024	>1024
Isepamicin	>1024	>1024	>1024	0·5	>1024	4	>1024	8
4,5-substituted deoxystreptamine antimicrobials								
Neomycin	>1024	4	>1024	4	512	16	>1024	>1024
Other aminoglycosides								
Streptomycin	128	4	2	4	32	32	>1024	>1024
Hygromycin B	1024	64	2	32	512	512	1024	512
Others								
Ceftazidime	2	0·5	0·25	0·25	ND	ND	128	32
Imipenem	1	0·25	0·25	0·125	ND	ND	16	16
Ciprofloxacin	0·25	0·125	0·125	0·125	ND	ND	64	64

ND=not determined. pBCH9, pBCH9-13, pBC-SK+, pTORmtA, and pTO001 are the plasmids harboured by each transformant. pBC-SK+, pTO00=cloning vector, expression vector. pBCH9=pBC-SK+ + 8 kb insert fragment. pBCH9-13=pBC-SK+ + 1–2 kb insert fragment. pTORmtA=pTO001+rmtA.*105 was recipient for the conjugation study.

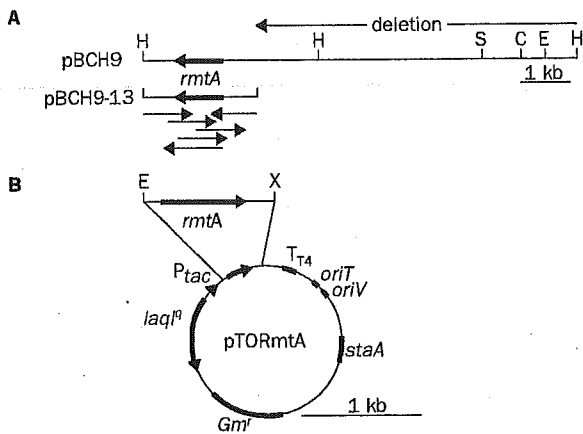**MICs (mg/L) for parental strain, transformants, and transconjugant**

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Figure 1: **Sequencing strategy (A) and restriction map (B) of the DNA insert**
Black bar represents the coding region of the *rmtA* gene and the arrow the direction of transcription. Horizontal thin arrows show the sequencing strategy. H=HindIII; C=ClaI; S=SacI; E=EcoRI; X=XbaI.

containing arbekacin (64 mg/L) and ciprofloxacin (5 mg/L). We did genomic DNA analysis of the parent, recipient, and transconjugants with pulsed-field gel electrophoresis. SpeI-digested genomic DNA fragments were separated for 22 h at 6 V/cm and 14°C with a CHEF-DR system (BioRad Laboratories, Tokyo, Japan). We did electrophoresis in two ramps as follows: pulse times were linearly increased from 4 s to 8 s for 12 h during the first ramp and from 8 s to 50 s for 10 h during the second ramp.

Assay of gene product
For thin layer chromatography analyses, we harvested bacterial cells grown in Luria-Bertani broth at the middle of the logarithmic phase. Cells were washed and resuspended with 0·1 mol/L phosphate buffer (pH 7·0). We disrupted the cell suspension by a French press (Ohtake, Tokyo, Japan), and then centrifuged it at 7700 g for 10 min at 4°C. The supernatant was ultracentrifuged at 100 000 g for 3 h at 4°C with a 65 Ti rotor (Beckman Instruments, Fullerton, CA, USA), and we stored the resulting cell-free extract at −20°C before use. We did acetylation or phosphorylation under the following conditions: 0·5 mmol/L arbekacin, 0·1 mol/L phosphate buffer (pH 7·0), 10% (volume/volume) cell-free extract, and 4 mmol/L acetylCoA or ATP in a 50 µL reaction mixture. After incubation for 3 h at 37°C, we monitored every reaction mixture by thin layer chromatography, which we did with a silica gel plate (Merck silica gel 60 F254; Merck, Darmstadt, Germany) developed with 5% KH_2PO_4 and stained with ninhydrin reagent.
Preparation of ribosomes, ribosomal subunits, and post-ribosomal supernatant containing material removed from 70S ribosomes by high salt washing (S100) was done as

described by Skeggs and others.[15] For assay of methylase activity, the extract (S100) from a *P aeruginosa* clone that harbours a recombinant plasmid, pTORmtA, carrying the *rmtA* genes was used as a source of methylase together with S-adenosyl-L-methionine as cofactor. Radiolabelled methyl groups were incorporated into 16S rRNA at 35°C in reaction mixtures (100 µL total volume) made up in a buffer containing 50 mmol/L Hepes-KOH (pH 7·5 at 20°C), 7·5 mmol/L $MgCl_2$, 37·5 mmol/L NH_4Cl, and 3 mmol/L 2-mercaptoethanol. Such mixtures contained 20 pmol of *P aeruginosa* PAO1 (pTO001) 30S ribosomal subunits (substrates for methylation) together with 50 µL S100 from the clone of *P aeruginosa* (controls received S100 from *P aeruginosa* PAO1 [pTO001]) plus 9·25×10⁴ Bq S-adenosyl-[methyl-³H]-L-methionine (18·5 GBq/mmol). We removed samples (10 µL) at intervals (0, 10, 30, and 50 min) and allowed them to permeate a DEAE (diethylaminoethyl) filtermat (glass fibre filter, with DEAE active groups; Wallac, Turku, Finland), and the filtermat was washed three times with ice-cold 50 mmol/L glycine hydrochloride (pH 4·5) and a further two times with ice-cold ethanol. The filtermat was dried and soaked in a scintillator, MeltiLexTM (Wallac), and then radioreactivity was counted by MicroBeta plus (Wallac).

PCR screening of mtA gene harbouring strains
We screened a bacterial stock of 1113 clinically isolated *P aeruginosa* strains for the *rmtA* gene. PCR analyses with the primers shown in the panel, which amplify a 635-bp fragment within the *rmtA* gene, were done on strains showing a degree of resistance to gentamicin, amikacin, and arbekacin (MICs ⩾32 mg/L) .
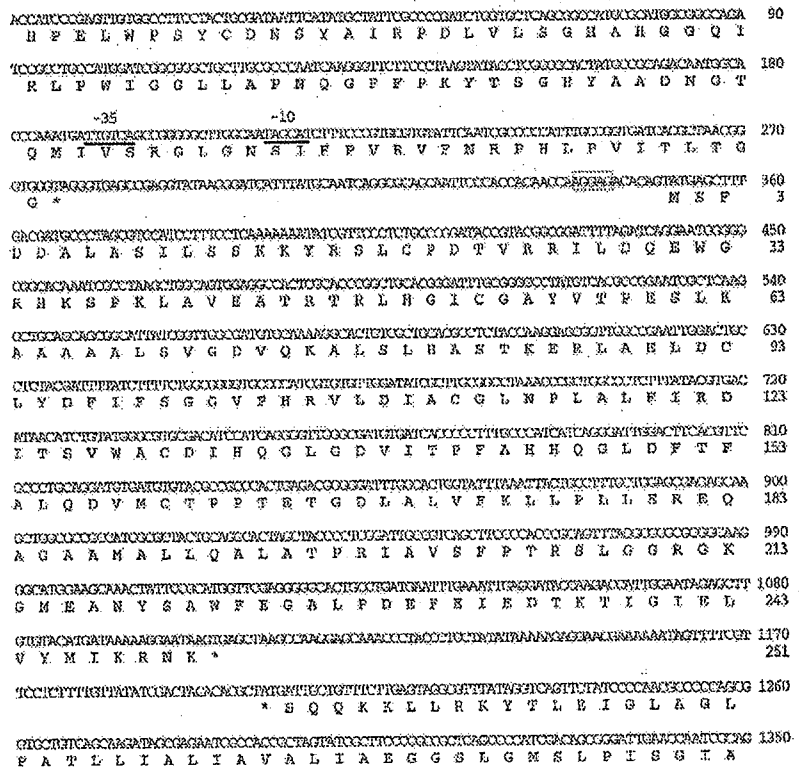

Figure 2: **Nucleotide and deduced aminoacid sequences of *rmtA* gene and RmtA protein**
The 1350 bp sequence of the 1662 bp area determined is shown. Stop codons are indicated by asterisks. A putative SHINE-DALGARNO SEQUENCE is boxed. The putative promoter −35 region is marked by a bold line at positions 190–195, and the possible −10 region is indicated by a bold line below the nucleotide sequence at positions 212–217.

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Figure 3: **Comparison of aminoacid sequences of known 16S rRNA methylases with P aeruginosa AR-2 RmtA**

Proteins in comparison: GrmB, Micromonospora rosea; Sgm, M zionensis; NbrB, Streptoalloteichus hindustanus; Kmr, Streptomyces kanamyceticus; and KgmB, Streptomyces tenebrarius. Identical aminoacid residues among all six enzymes are indicated by asterisks, and aminoacids with similar properties are indicated by dots. Dashes represent gaps introduced to improve alignment.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

P aeruginosa strain AR-2 showed very high-level resistance to various aminoglycosides (table). Arbekacin resistance was transferred from AR-2 to *P aeruginosa* PAO1 by conjugation, and the *E coli* clone (XL1-Blue) and transconjugant of *P aeruginosa* strain 105 showed similar resistance profiles to AR-2 against various aminoglycosides, as shown in the table. The pattern on pulsed-field gel electrophoresis of SpeI-digested genomic DNA fragments of the transconjugant was closely similar to that of the
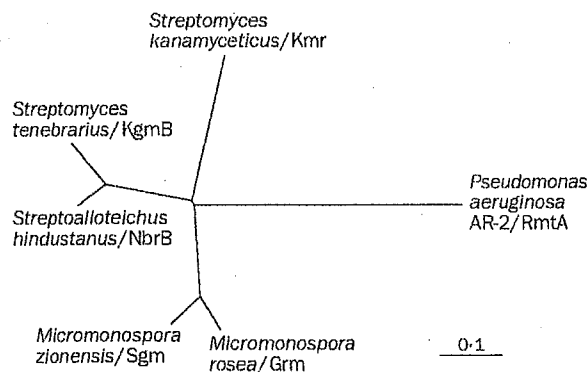
Figure 4: **Dendrogram of 16S rRNA methylases**
Units for bar are genetic units calculated with the CLUSTAL W program, which reflects the number of aminoacid exchanges.

P aeruginosa recipient strain 105 (not shown). This finding suggested that the transconjugant was not a ciprofloxacin-resistant mutant of the donor strain *P aeruginosa* AR-2. By thin layer chromatography, however, no detectable conversion was noted in the rate of flow value of arbekacin after in-vitro acetylation or phosphorylation reactions (data not shown). Therefore, the mechanism underlying the wide range of resistance to various aminoglycosides is difficult to establish, since it is not merely production of known amino-glycoside-modifying enzymes. These findings suggest that in strain AR-2, novel molecular mechanisms determine multiple aminoglycoside resistance.

By sequencing of the plasmid carrying the *rmtA* gene, we determined a 1662-bp nucleotide sequence carrying arbekacin resistance (figure 1). An open reading frame of 756 bp was noted, with the initiation codon ATG at position 352 and the stop codon TGA at position 1105. The G+C content of the open reading frame was 55%. By part sequencing of the flanking region of the *rmtA* gene, the gene was suggested to be carried by Tn5041, which mediates Hg^+-resistance in *Pseudomonas* spp. The nucleotide sequence has been submitted to the EMBL, GenBank, and DDBJ databases and assigned accession number AB083212.

The open reading frame encoded a putative protein, RmtA, with 251 aminoacids (molecular weight 27 430; figure 2). The predicted aminoacid sequence of RmtA showed considerable similarity to the 16S rRNA methylases produced by aminoglycoside-producing actinomycetes (figure 3).[16,17] The deduced 251 aminoacid sequence of RmtA was closely similar to GrmB and Sgm methylases found in sisomicin-producing
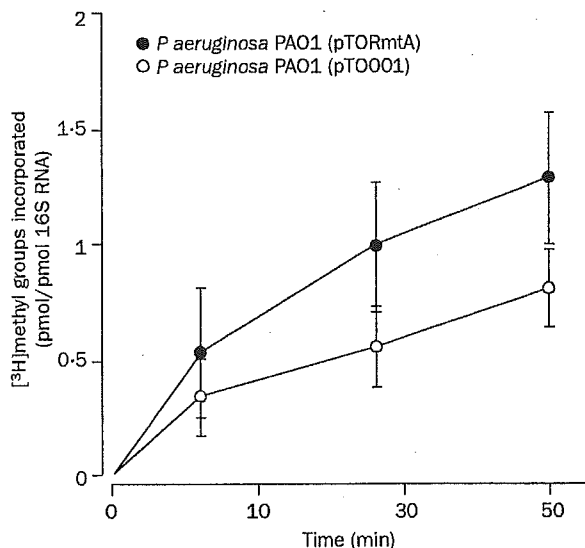
Figure 5: **Methylation of 30S ribosomal subunit by RmtA**
Error bars indicate SD.

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Micromonospora rosea (35%; SWISS-PROT accession number P24619) and *M zionensis* (34%; EMBL accession number JG0018), respectively. RmtA also showed similarities to the NbrB methylase of the nebramycin-complex-producing *Streptoalloteichus hindustanus* (33%; EMBL accession number AF038408), to the Kmr methylase from the kanamycin-producing *Streptomyces kanamyceticus* (30%; EMBL accession number CAA75800), and to the KgmB methylase from the nebramycin complex-producing *Streptomyces tenebrarius* (31%; EMBL accession number AAB20100). The dendrogram in figure 4 suggests the evolutionary relation between the 16S rRNA methylases and RmtA, implying a potential intergeneric transfer of the gene from some aminoglycoside-producing actinomycetes to *P aeruginosa*. The dendrogram was calculated with the CLUSTAL W program.[14]

Incorporation of a radiolabelled methyl group into the 30S ribosomal subunits prepared from *P aeruginosa* PAO1 (pTO001) was seen (figure 5).

Of 1113 clinically isolated *P aeruginosa* strains that have been isolated from Japanese hospitals and stocked in our laboratory since 1997, nine strains were shown to carry the *rmtA* gene by PCR. These strains were isolated from seven separate hospitals in five prefectures in Japan.

Discussion

We have reported a completely new mechanism for multiple aminoglycoside resistance—that is, enzymatic methylation of the 16S rRNA found in gram-negative bacteria.

Although intergeneric lateral gene transfer has been regarded as a method of acquisition of new phenotypes for bacteria to survive in hazardous environments,[18,19] its rate and background are not well known. The *rmtA* gene product, RmtA, showed considerable similarity to 16S rRNA methylases that protect 16S rRNA in aminoglycoside-producing actinomycetes such as *Streptomyces* spp and *Micromonospora* spp. In fact, a cell-free cytosolic fraction of *P aeruginosa* PAO1 (pTORmtA) containing RmtA accelerated uptake of the ³H-labelled methyl group into the 30S ribosome of *P aeruginosa* PAO1. Moreover, the *rmtA* gene was suggested to be carried by the transposon Tn5041, which mediates mercury resistance. These results suggest that traces of the 16S rRNA methylase gene have moved by intergeneric lateral gene transfer from some aminoglycoside-producing bacteria into *P aeruginosa* because of the increasingly heavy clinical use of arbekacin, which is rarely inactivated by ordinary aminoglycoside-modifying enzymes generally found in gram-negative bacteria.

Since arbekacin resistance of strain AR-2 can be easily transferred to *P aeruginosa* strain 105 by conjugation (10^{-4}–10^{-5}), the *rmtA* gene could be contained on a self-transmissible large plasmid, though more precise characterisation is now underway. This finding indicates that further widespread dissemination of the *rmtA* gene in gram-negative bacteria is possible as an important ecological result of heavy antibiotic use in clinical settings.[20] In this study, nine *P aeruginosa* strains that carry the *rmtA* gene were isolated from seven separate hospitals located in five prefectures across Japan. This finding indicates that in Japanese clinical settings there has been stealthy multifocal proliferation of *P aeruginosa* strains that have acquired consistent and very high-level resistance to various clinically important aminoglycosides through production of the newly identified 16S rRNA methylase. Since resistance to fluoroquinolones and carbapenems has already developed in gram-negative bacteria including

P aeruginosa,[2,21] emergence of multidrug resistant superbug strains through further acquisition of the *rmtA* gene threatens to become a serious clinical problem. Further global transmission of the *rmtA* gene in gram-negative bacteria could become a matter of grave concern in the future. Like vancomycin-resistant *S aureus* strains[22] and plasmid-mediated quinolone-resistant *Klebsiella pneumoniae*,[23] bacteria readily cope with hazardous environments by accepting any genes, even those from hereditarily distant microorganisms.[24,25]

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Clinical picture

Peritoneal dialysis and an inguinal hernia

Michel Tintillier, Emmanuel Coche, Jacques Malaise, Eric Goffin

A 59-year-old man with diabetes started chronic ambulatory peritoneal dialysis (CAPD) in January, 2002, with 1500 mL exchanges four times daily. After 3 weeks, he developed massive scrotal oedema. We found a left inguino-scrotal hernia, which was surgically repaired, and the patient was switched to haemodialysis. CAPD was resumed after 4 weeks. Massive scrotal oedema recurred 10 days later. Abdominal multislice helical CT with intraperitoneal injection of 100 mL contrast medium showed a dialysate diffusion through a right inguinal hernia (figure, scout view), but absence of contralateral leakage, indicating an efficient surgical repair. Surgical repair was then done on the right side. 4 weeks later, we resumed CAPD without any further complications. Clinical examination had failed to detect any hernias before starting CAPD. The increased intraperitoneal pressure due to the dialysate was the probable cause.
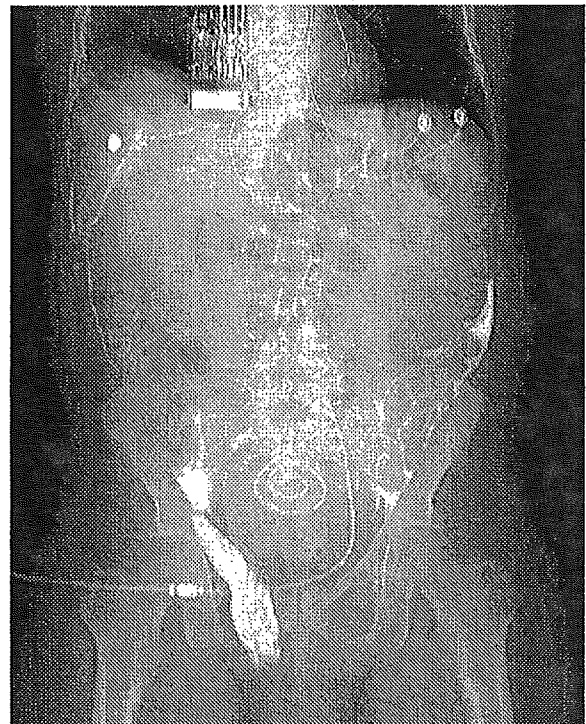
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Highly Conjugative pMG1-Like Plasmids Carrying Tn*1546*-Like Transposons That Encode Vancomycin Resistance in *Enterococcus faecium*

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A total of 12 VanA-type vancomycin-resistant enterococci, consisting of 10 *Enterococcus faecium* isolates and two *Enterococcus avium* isolates, were examined in detail. The vancomycin resistance conjugative plasmids pHTα (65.9 kbp), pHTβ (63.7 kbp), and pHTγ (66.5 kbp) were isolated from each of three different *E. faecium* strains. The plasmids transferred highly efficiently between enterococcus strains during broth mating and were homologous with pMG1 (Gmʳ; 65.1 kb).

Gene transfer systems are an essential requirement for the spread of drug resistance in microorganisms. In general, the systems of efficient plasmid transfer have not been well characterized for the gram-positive bacteria. However, enterococci possess potent and unique capabilities of transferring plasmids among themselves and to other genera (4, 5, 21, 35). One type of enterococcal plasmid consists of the group of narrow-host-range and pheromone-responsive plasmids (4, 5, 9). The other type consists of the broad-host-range pAMβ1 and pIP501 plasmids, which were originally isolated from *Enterococcus faecalis* (8, 24) and *Streptococcus agalactiae* (13, 18), respectively, and transfer on a solid surface at low frequency (8, 13, 18, 24, 27, 40).

We have described the isolation of the pheromone-independent gentamicin resistance conjugative plasmid pMG1 (Gmʳ; 65.1 kb) from an *Enterococcus faecium* clinical isolate in Japan (20). pMG1 transfers efficiently among enterococcus strains during broth mating. pMG1-like plasmids are widely disseminated in vancomycin-resistant *E. faecium* clinical isolates obtained from a hospital in the United States (39).

In this report, we show that the VanA resistance encoded on a Tn*1546*-like transposon was mediated by a pMG1-like plasmid and that this vancomycin resistance pMG1-like plasmid was capable of highly efficient transfer among the enterococci.

Drug resistance of VRE isolates and isolation of vancomycin resistance conjugative plasmids. The laboratory strains and plasmids used in this study are listed in Table 1. A total of 12 isolates of vancomycin-resistant enterococci (VRE) were used in this study (Table 2). The vancomycin resistance of each strain transferred to *E. faecium* BM4105RF at a frequency of about 10^{-5} per donor cell by mating in broth for 4 h at 37°C.

The transconjugants of each strain acquired only vancomycin and teicoplanin resistance, indicating that the glycopeptide resistance was transferred during broth mating.

Analysis of agarose gel electrophoresis of restriction fragments of plasmid DNAs of each strain showed many DNA bands, indicating that each of the strains harbored several plasmids (Fig. 1, A1). The conjugative vancomycin resistance plasmid pHTα was identified from the transconjugant of *E. faecium* FH1 by repeated transfer experiments between *E. faecium* BM4105 strains. The plasmids isolated from each of the strains were classified into three types, α, β, and γ, with respect to the restriction profiles that hybridized to the type α plasmid pHTα (Fig. 1, A2) (Table 2). The pHTβ and pHTγ plasmids, which were type β and γ plasmids, respectively, were identified from the transconjugants of strains FH4 and FH7, respectively (Fig. 1, B1) (Table 2). Each type of plasmid DNA encoded the VanA gene by PCR analysis with the vanA-specific primer (data not shown) (11, 12, 29). pHTα DNA hybridized to all *Nde*I and *Eco*RI fragments of each type of plasmid DNA (Fig. 1, B2). DNA from the conjugative plasmid pMG1 (Gmʳ; 65.1 kbp) hybridized to specific *Nde*I or *Eco*RI fragments (data not shown). Each type of plasmid transferred at a frequency of around 10^{-3} to 10^{-5} per donor cell between *E. faecium* BM4105 or around 10^{-6} to 10^{-7} per donor cell between *E. faecalis* JH2 strains during broth mating.

The restriction maps of the vancomycin resistance plasmids. The restriction maps of pHTα (65.9 kbp), pHTβ (63.7 kbp), and pHTγ (66.5 kbp) were constructed (Fig. 2). The molecular sizes of the *Nde*I A fragment of pHTα and the *Nde*I B fragment of pHTγ were 18.2 and 13.3 kbp, respectively, which were 2.2 and 2.8 kbp larger than the *Nde*I A fragments (16 kbp) and *Nde*I B fragments (10.5 kbp) of pHTβ, respectively.

The nucleotide sequences showed that the 2.2-kbp (2,156-bp) fragment of pHTα contained two open reading frames of 1,236 bp (412 amino acids) and 759 bp (253 amino acids), which were homologous with the IS*232*-mediating transposase

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Description; source or reference
Strains		
E. faecium BM4147 (pIP816 Van^r)	van	25
E. faecalis FA2-2	rif fus	Derivative of JH2; 7
E. faecalis JH2SS	str spc	Derivative of JH2; 37
E. faecium BM4105RF	rif fus	Derivative of plasmid-free E. faecium BM4105; 3
E. faecium BM4105SS	str spc	Derivative of plasmid-free E. faecium BM4105; 3
Plasmids		
pMG1	Gm^r	65.1-kb conjugative plasmid from E. faecium strain; 20
pG200	Gm^r	pMG1-like conjugative plasmid from VRE; 39
pG445	Gm^r	pMG1-like conjugative plasmid from VRE; 39
pG566	Gm^r	pMG1-like conjugative plasmid from VRE; 39
pG700	Gm^r	pMG1-like conjugative plasmid from VRE; 39
pG120	Gm^r	pMG1-like conjugative plasmid from VRE; 39
pAD1	hly/bac uvr	59.6-kb pheromone-responsive conjugative plasmid from DS16; 7, 19, 37
pPD1	bac	59-kb pheromone-responsive conjugative plasmid from E. faecalis 39–5; 15, 38, 41
pAM373	tet	36-kb pheromone-responsive conjugative plasmid; 6
pAMβ1	erm	26.5-kb broad-host-range conjugative plasmid from DS5; 8, 24
pIP501	erm cat	39.2-kb broad-host-range conjugative plasmid; 2, 13, 18

and the transposition helper protein, respectively (28). The nucleotide sequence of the 2.8-kb (2,748-bp) fragment of the pHTγ plasmid was homologous with that of the group II intron that encodes a reverse transcriptase consisting of 638 amino acids (22, 23, 30, 31). The nucleotide sequences around the 2.2-kbp fragment of the NdeI A fragment of pHTα were completely identical to the nucleotide sequence of the NdeI A fragment of the pHTβ plasmid. Likewise, the nucleotide sequences around the 2.8-kbp fragment of NdeI

B framgent of pHTγ were completely identical to that of the NdeI B fragment of the pHTβ plasmid. These results indicated that pHTβ might be the original or wild-type plasmid, and the 2.2-kbp fragment and the 2.8-kbp fragment were inserted into the NdeI A and NdeI B fragments of the pHTβ plasmid, respectively.

Analysis of the pMG1 traA gene. The traA gene of pMG1, which encodes a 287-amino-acid protein, is involved in the tra gene system for conjugation and is specific to pMG1 (36). Each

TABLE 2. Characterizations of vancomycin-resistant enterococci[a]

Species	Strain no.	Patient[b]	Antimicrobial drug resistance level (MIC, μg/ml)[c]								Drug resistance pattern	Van^r plasmid type harbored in strain
			Apc	Erm	Gen	Kan	Str	Tet	Tei	Van		
E. faecium	FH1	A	256	>128	2	>1,024	1,024	>256	32	256	Apc Erm Kan Str Tet Tei Van	α
E. faecium	FH2	B	256	>128	2	>1,024	512	>256	64	256	Apc Erm Kan Str Tet Tei Van	β
E. faecium	FH3	C	2	4	2	64	64	1	64	512	Tei Van	β
E. faecium	FH4	D	128	>128	2	>1,024	256	>256	64	256	Apc Erm Kan Tet Tei Van	β
E. faecium	FH5	E	64	>128	1	>1,024	1,024	>256	128	512	Apc Erm Kan Str Tet Tei Van	β
E. faecium	FH6	F	64	>128	2	>1,024	1,024	>256	64	256	Apc Erm Kan Str Tet Tei Van	β
E. faecium	FH7	G	128	>128	>256	>1,024	1,024	32	128	512	Apc Erm Gen Kan Str Tet Tei Van	γ
E. faecium	FH8	H	128	>128	>256	>1,024	>1,024	2	128	512	Apc Erm Gen Kan Str Tei Van	γ
E. faecium	FH9	I	128	>128	>256	>1,024	>1,024	128	128	512	Apc Erm Gen Kan Str Tet Tei Van	γ
E. faecium	FH10	J	128	>128	>256	>1,024	>1,024	128	128	>1,024	Apc Erm Gen Kan Str Tet Tei Van	γ
E. avium	FH11	C	16	0.25	2	128	64	16	16	256	Apc Tet Tei Van	β
E. avium	FH12	E	16	0.25	1	64	64	256	16	512	Apc Tet Tei Van	β

[a] The isolation of VanA-type VRE from clinical sources is still rare in Japan (i.e., fewer than 30 cases) (16, 33; N. Fujita, M. Yoshimura, T. Komori, K. Tanimoto, and Y. Ike, Letter, Antimicrob. Agents Chemother. 42 :2150, 1998; Y. Ike, K. Tanimoto, Y. Ozawa, T. Nomura, S. Fujimoto, and H. Tomita, Letter, Lancet 353:1854, 1999). The presence of VanA VRE was examined in the feces of a total of 1,699 inpatients obtained by the microbiology division of the clinical microbiology of the university hospital of Fujita Health University School of Medicine, Aichi, Japan, between 1 August 1999 and 31 March 2001.

[b] All strains were isolated from feces of patients. E. faecium FH3 and E. avium FH11, and E. faecium FH5 and E. avium FH12, were isolated from patient C and patient E, respectively. Each of the other strains was isolated from a different patient.

[c] Abbreviations: Apc, ampicillin resistance; Gen, gentamicin resistance; Kan, kanamycin resistance; Str, streptomycin resistance; Tet, tetracycline resistance; Tei, teicoplanin resistance; Van, vancomycin resistance. The drug resistance levels of ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, teicoplanin, and vancomycin were equal to or greater than 16, 64, 1,024, 512, 8, 16, and 64 μg/ml, respectively. Enterococcus strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) throughout this study. Mueller-Hinton broth and Mueller-Hinton agar for the sensitivity disk agar-N (Nissui, Tokyo, Japan) assay were used to test the MICs of antimicrobials. The MICs of the antimicrobials were determined according to the criteria of the National Committee for Clinical Laboratory Standards using Mueller-Hinton agar (32).
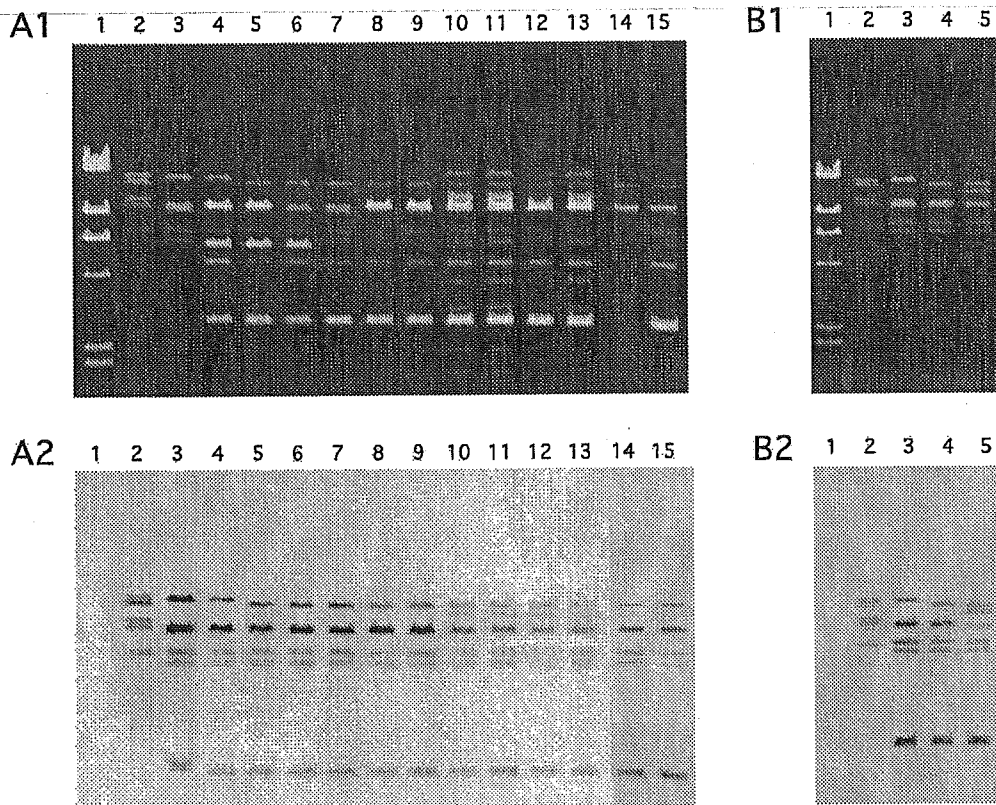
FIG. 1. Agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs and hybridization with the pHTα probe. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols (34). (A1) Agarose gel electrophoresis of NdeI-digested plasmid DNAs isolated from vancomycin-resistant *E. faecium* or *E. avium* (VRE) isolates. (A2) The gel was Southern blotted and hybridized to pHTα. Lanes of panels A1 and A2: 1, HindIII-digested lambda DNA; 2, NdeI-digested pMG1; 3, NdeI-digested pHTα; 4 to 15, NdeI-digested plasmid DNAs from the strains FH1, FH2, FH3, FH4, FH5, FH6, FH7, FH8, FH9, FH10, FH11 and FH12, respectively. (B1) Agarose gel electrophoresis of NdeI-digested pHTα, pHTβ, and pHTγ plasmid DNA isolated from each transconjugant of FH1, FH4, and FH7, respectively. (B2) The gel was Southern blotted and hybridized to the pHTα probe. Lanes of panels B1 and B2: 1, HindIII-digested lambda DNA; 2, pMG1; 3, pHTα; 4, pHTβ; 5, pHTγ.

plasmid was examined to determine whether *traA* was conserved in each of these plasmids by sequence analysis of the PCR product for *traA*.

The nucleotide sequence and the deduced amino acid sequence of the open reading frame in 945-bp PCR products analyzed in pHTα, pHTβ, and pHTγ were completely identical to those of *traA* of pMG1, with the exception of eight nucleotide substitutions and six amino acid substitutions (i.e., V19F, S23N, R26S, V84M, A102V, and K237E). The nucleotide sequence and the deduced amino acid sequence of the gentamicin resistance pMG1-like plasmids (39) pG200, pG445, pG560, pG700, and pG120 were completely identical to those of pMG1 *traA*.

Based on the differences observed in the nucleotide sequence of *traA*, these results indicated that the *traA* gene of pMG1 was conserved in pMG1-like plasmids and that there was no direct connection between the gentamicin resistance pMG1 plasmid (including pMG1-like plasmids) and the vancomycin resistance pHT plasmids.

Incompatibility of vancomycin resistance plasmids and pMG1 and Southern analysis with other reported plasmids.

The transfer frequency of each of the vancomycin resistance plasmids to the recipient cell carrying pMG1 was lower than that when the recipient was plasmid free (Table 3). All transconjugants were vancomycin resistant (conferred by the incoming plasmid), but they had lost gentamicin resistance (encoded by the resident plasmid). These results indicate that each of the vancomycin resistance plasmids and pMG1 were incompatible. Southern analysis showed that the pHTβ plasmid did not contain any sequence homologous with those of the pheromone-responsive plasmids (Table 1) (4–7, 10, 15, 19, 38, 41) and the broad-host-range plasmids (Table 1) (2, 8, 13, 18) (data not shown).

Gentamicin and kanamycin resistance determinants on pMG1. pMG1 was examined to determine whether the gentamicin and kanamycin resistance determinants also reside on a transposon. The nucleotide sequence revealed that the *Eco*RI B fragment of pMG1 encoded a Tn*4001*-like transposon (4,523 bp) (17, 26). The composite transposon Tn*4001* (4,566 bp) carries the gentamicin and kanamycin resistance gene *aacA-aphD*, which is flanked by two 1,324-bp inverted repeats, IS*256*L and IS*256*R (26). The nucleotide sequence of the
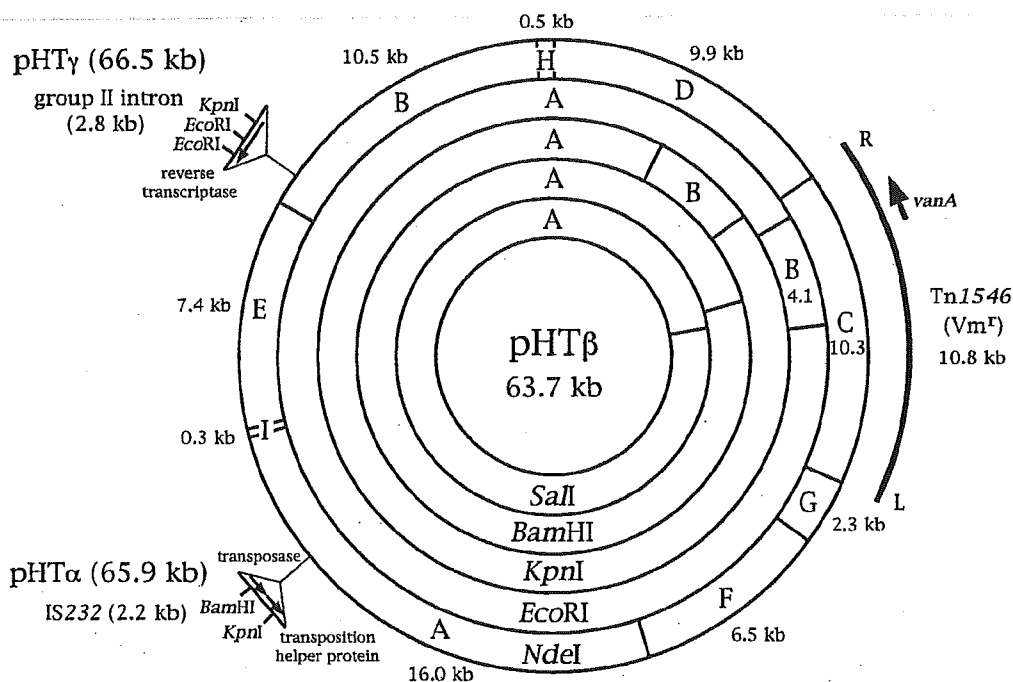
FIG. 2. Physical map of the vancomycin resistance conjugative plasmid pHTβ (63.7 kb) and its relation to pHTα (65.9 kb) or pHTγ (66.5 kb). To determine the DNA sequence of the 2.2-kbp fragment of pHTα and the 2.8-kbp fragment of pHTγ and to confirm that these fragments had inserted into the *NdeI* A and *NdeI* B fragments of pHTβ, respectively, random fragments of the region of the 2.2-kb fragment or of the 2.8-kb fragment were cloned and sequenced as previously described (38). pHTα resulted from the insertion of the 2.2-kb fragment of IS*232* into the region of *NdeI* fragment A of pHTβ. pHTγ resulted from the insertion of the 2.8-kb fragment of the group II intron into the region of *NdeI* fragment B of pHTβ. DNA sequence and PCR analysis were carried out to analyze the VanA determinant as described previously (1, 11, 16). The VanA-type determinant of pHTβ was encoded on the transposon Tn*1546* or a closely related transposon. The location of the VanA determinant of each plasmid was determined by Southern analysis, PCR, and comparison of the restriction map covering the region of the VanA determinant with that of Tn*1546*.

Tn*4001*-like transposon was completely identical to that of the original Tn*4001* transposon, except that the resistance gene *aacA-aphD* was flanked by two 1,324-bp (IS*256*) direct repeats and there was deletion of a 43-bp sequence upstream from the end of IS*256*R.

Conclusions. The pheromone-independent gentamicin resistance plasmid pMG1 and pMG1-like plasmids are found in

TABLE 3. Transfer frequencies of vancomycin resistance plasmids from donor strains to recipients carrying the pMG1 plasmid[a]

Plasmid from donor cells of *E. faecium* BM4105SS	Transfer frequency in broth mating with recipient *E. faecium* BM4105RF carrying:		Transfer frequency in filter mating with recipient *E. faecium* BM4105RF carrying:	
	pMG1	None	pMG1	None
pHTα	<1 × 10⁻⁷	1 × 10⁻⁴	2 × 10⁻³	>1 × 10⁰
pHTβ	<1 × 10⁻⁷	3 × 10⁻⁴	5 × 10⁻³	>1 × 10⁰
pHTγ	<1 × 10⁻⁷	2 × 10⁻⁴	3 × 10⁻³	>1 × 10⁰

[a] The mating experiments were carried out as previously described (14, 20). The mating times of broth mating and filter mating were 3 and 18 h, respectively. The transconjugants were examined after 48 h of incubation of the selective agar plates at 37°C. Throughout the mating experiments, the antibiotic concentration used for the selection of gentamicin- or vancomycin-resistant transconjugants was 100 or 12.5 µg/ml, respectively. The selection of rifampin- and fuscidic acid-resistant recipient strains was carried out at a concentration of 25 µg/ml each, while selection of streptomycin- and spectinomycin-resistant recipient strains was carried out at concentrations of 500 and 250 µg/ml, respectively.

E. faecium and are widely disseminated in vancomycin-resistant *E. faecium* isolates in the United States (39). The data shown in this report suggest that pMG1-like plasmids without any resistance gene or any other selectable determinant must be prevalent in *E. faecium*, and there is the possibility that a mobile genetic element encoding drug resistance or another determinant might insert onto them. As shown by this study, there is now evidence that in addition to gentamicin and kanamycin resistance transposon Tn*4001*-like elements, vancomycin resistance transposon Tn*1546*-like elements and other mobile genetic elements, such as IS*232* and the group II intron, are capable of insertion onto pMG1-type plasmids.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB091473, AB105542, and AB105543

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MexXY-OprM Efflux Pump Is Necessary for Adaptive Resistance of *Pseudomonas aeruginosa* to Aminoglycosides

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Exposure of *Pseudomonas aeruginosa* to aminoglycosides frequently selects for recalcitrant subpopulations exhibiting an unstable, "adaptive" resistance to these antibiotics. In this study, we investigated the implication in the phenomenon of MexXY-OprM, an active efflux system known to export aminoglycosides in *P. aeruginosa*. Immunoblotting experiments demonstrated that the transporter MexY, but not the outer membrane pore OprM, was overproduced during the post-drug exposure adaptation period in wild-type strain PAO1. Furthermore, MexY production was dependent upon the degree of bacterial exposure to gentamicin (drug concentration). In contrast to parental strain PAO1, mutants defective in MexXY or in OprM were unable to develop adaptive resistance. Altogether, these results indicate that the resistance process requires the rapid production of MexXY and the interaction of these proteins with the constitutively produced component OprM.

Aminoglycosides remain invaluable antibiotics in the treatment of severe infections caused by *Pseudomonas aeruginosa*. However, their in vivo efficacy may be compromised by the development of transiently resistant subpopulations (7, 8, 15, 34). Exposure of susceptible *P. aeruginosa* to an aminoglycoside classically results in an early and rapid drug concentration-dependent killing followed by a phase of bacterial refractoriness characterized by a slow drug concentration-independent killing (27). This so-called adaptive resistance, which is distinct from the postantibiotic effect and which disappears when the organism is no longer in contact with the aminoglycoside, has been observed in vitro (5, 11, 19), in animal models of infection (12, 40), and in patients with cystic fibrosis (6). Because of its ephemeral and reproducible nature, adaptive resistance is not believed to result from mutational events. Ribosomal alterations and drug inactivation are not considered plausible mechanisms, either, as they would result in specific patterns of susceptibility to aminoglycosides and not cross-resistance to these antibiotics (11). Reduced intracellular accumulation of aminoglycosides, which is concomitant to adaptive resistance, was first interpreted as the consequence of lower drug uptake across the bacterial envelopes (11, 19). Supporting this assumption, pleiotropic changes in the protein profiles of the cytoplasmic membrane were detected in drug-exposed bacteria by some investigators (19). However, clear evidence for a substantial decrease in aminoglycoside transport across the inner membrane could not be obtained. The membrane potential $\Delta\psi$ (the driving force for drug entry) appears to be marginally diminished in adaptively resistant bacteria (19), a finding which agrees well with the observation that surviving bacteria grow normally during the postexposure refractory phase (11, 19). These characteristics are opposite of those of another drug-recalcitrant subpopulation also selectable by aminoglycosides, composed of energy-deficient variants (also called small-colony variants) (7, 34).

Recently, several groups have almost simultaneously reported the identification in *P. aeruginosa* of a new multidrug efflux pump named MexXY (32, 35) or AmrAB (37). In addition to its ability to accommodate a wide range of antibiotics (e.g., tetracyclines, macrolides, quinolones, chloramphenicol, and β-lactams), the MexXY system has the distinctive property of being able to export aminoglycosides (29, 30, 35). This system is also involved in antagonism of aminoglycosides by divalent cations (28). MexXY proteins form a functional tripartite efflux machinery with outer membrane component OprM (32, 35) and contribute to the natural resistance of *P. aeruginosa* towards tetracyclines, macrolides, and aminoglycosides. Consideration of the inducible expression of MexXY by aminoglycosides (29) led us to examine the implication of the efflux system in adaptive resistance.

(A first account of this work was given at the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 17 to 20 September 2000 [W. Mao, M. Warren, A. Lee, A. Mistry, and O. Lomovskaya, abstr. 1498]).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *P. aeruginosa* PAO1 was used as the wild-type reference strain (B. W. Holloway). Mutant 11B is a *mexX::Tn501* (Hg^r [mercuric chloride-resistant]) insertion derivative of PAO1 showing hypersusceptibility to aminoglycosides, erythromycin, and tetracycline (35). PAO1T, an *oprM::ΩHg^r* interposon mutant of PAO1, was kindly provided by T. Köhler (31). MICs of antibiotics were determined by the standard microdilution method in Mueller-Hinton broth (MHB) with adjusted concentrations of Ca^{2+} and Mg^{2+} (BBL, Cockeysville, Md.) by using inocula of 2.5 × 10^5 bacteria/ml (3). The resistance levels of PAO1, 11B, and PAO1T to gentamicin were 1.6, 0.4, and 0.25 mg/liter, respectively; MICs of ticarcillin for these strains were 14, 13.5, and 4 mg/liter, respectively. *Escherichia coli* JM105 (41) was the host strain in all DNA cloning experiments, while *E. coli* M15(pREP4) (Qiagen, Courtaboeuf, France) was used to produce recombinant peptides. Bacteria were cultured in Luria-Bertani broth (2), in MHB, or on Mueller-Hinton agar plates (Bio-Rad, Ivry sur

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Seine, France). When necessary, growth media were rendered selective by the addition of the following agents (final concentrations in milligrams per liter): amoxicillin (100), kanamycin (25), and mercuric chloride (15). All bacterial cultures were incubated at 37°C.

DNA methodology. Chromosomal DNA suitable for PCR amplification was extracted and purified by following the procedure of Chen and Kuo (9). Plasmid DNA was prepared by the standard alkaline lysis method (2) or by using the Plasmid Midi Preps kit from Qiagen. Selected restriction fragments were purified from agarose electrophoresis gels with the JetSorb kit (Genomed Gmbh, Bad Oeynhauden, Germany). Other reagents for molecular biology were purchased from Invitrogen (Cergy Pontoise, France), Stratagene (La Jolla, Calif.), or Sigma-Aldrich (Saint Quentin, France). Transformation of strains of *E. coli* with plasmid DNA has been described in detail elsewhere (2).

Polyclonal MexY antiserum. The alignment of amino acid sequences of Mex pumps in *P. aeruginosa* indicated the second periplasmic loop of MexY (238 residues located between transmembrane segments TM7 and TM8) as a potentially interesting peptide for the generation of specific MexY antibodies, as it does not share significant sequence homologies with other Mex pumps. A His tag was first added to the N terminus of the peptide to facilitate its purification by affinity chromatography. The 713-bp sequence encoding the periplasmic loop was amplified by PCR from the pBlueScript II KS(+) recombinant plasmid pJR49 (35). The sense (5'-GGGGATCCGAAGGCACGCCGATG-3') and antisense (5'-GGAGATCTGTAGCGGGTCAGTTGCGG-3') DNA primers used in the PCR were designed to add BamHI and BglII restriction sites (in bold), respectively, at the ends of the amplicon in order to help subsequent subcloning experiments. The reaction mixture (50 μl) contained 100 ng of pJR49, 1 μM (each) primers, 100 μM (each) deoxynucleoside triphosphates, 6% (vol/vol) dimethyl sulfoxide, 2.5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Perkin Elmer, Foster City, Calif.) in 1X amplification buffer. The mixture was heated at 94°C for 5 min and then subjected to 30 thermal cycles, each consisting of 45 s at 94°C, 45 s at 62°C, and 1 min at 72°C, before a final step at 72°C for 7 min. Once digested with BamHI and BglII, the amplification product was ligated to BamHI-linearized plasmid pQE-30 (Amx^r) to yield pQY and introduced by transformation into competent cells of *E. coli* JM105. Plasmid pQY was subsequently transferred to *E. coli* M15(pREP4) (Km^r) for quantitative production of the selected peptide.

Overnight culture of the pQY-carrying M15 strain in Luria-Bertani medium supplemented with amoxicillin (100 mg/liter) and kanamycin (25 mg/liter) was diluted 1:59 into fresh medium and incubated at 37°C with shaking. Expression of the cloned sequence was induced by the addition of a 1 mM final concentration of isopropyl-β-thiogalactopyranoside (IPTG) to the exponentially growing cells (A_{650} of 0.5) and incubation for five additional hours at 37°C to reach peak levels. Bacteria were then collected by centrifugation (2,000 × g, 20 min, 4°C), resuspended in buffer A (50 mM Tris-HCl [pH 8], 100 mM NaCl, 0.2 mM β-mercaptoethanol, 0.05% [vol/vol] Tween 20) at 5 ml per g (wet weight) of pellet, and lysed by sonication (1 min, 20 W at 4°C) (Branson Ultrasonics, Danbury, Conn.). Inclusion bodies corresponding to aggregates of the recombinant peptide were recovered by centrifugation (8,000 × g, 10 min, 4°C), suspended in buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl [pH 8]), sonicated for 2 min, and left in the buffer for 1 h at room temperature under gentle shaking. The solution was cleared by another centrifugation (10,000 × g, 10 min, 4°C) and applied on a Ni-nitrilotriacetic acid column (1 by 10 cm; Qiagen S.A.) equilibrated with buffer B. Proteins bound to the column by nonspecific interactions were removed with buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl [pH 6.3], 10 mM imidazole), and the His-tagged peptide was finally eluted with buffer C in the presence of 250 mM imidazole. Fractions of interest were pooled and dialyzed at room temperature for 24 h against 5 liters of buffer consisting of 50 mM Tris-HCl (pH 6.3), 200 mM NaCl, and 0.1 mM EDTA. The purified peptide served to raise antibodies in two rabbits by multiple intradermal injections (36). Cross-reactivity of the MexY antiserum with other Mex pumps was checked by using mutants of PAO1 overproducing MexAB-OprM (strain 4098E), MexCD-OprJ (strain ERYR), and MexEF-OprN (strain PAO7H) efflux systems (21, 25, 31). No band in the 100- to 140-kDa range was detected by immunoblotting with the MexY antiserum in total membrane preparations from these strains (data not shown).

Membrane preparations. The protocol followed to extract the outer membrane or the total (inner and outer) membrane of *P. aeruginosa* was adapted from that described by Michéa Hamzehpour et al. (31). Briefly, drug-exposed bacteria in MHB were collected by centrifugation, resuspended in 10 mM HEPES (pH 7.2), and lysed by two passages through a French pressure cell (SLM AMINCO, Rochester, N.Y.). Unbroken cells were removed by centrifugation at 4°C. Total bacterial membranes were then harvested at 100,000 × g for 1 h at 4°C and resuspended in a 15 mM MOPS (morpholinepropanesulfonic acid)-100 mM
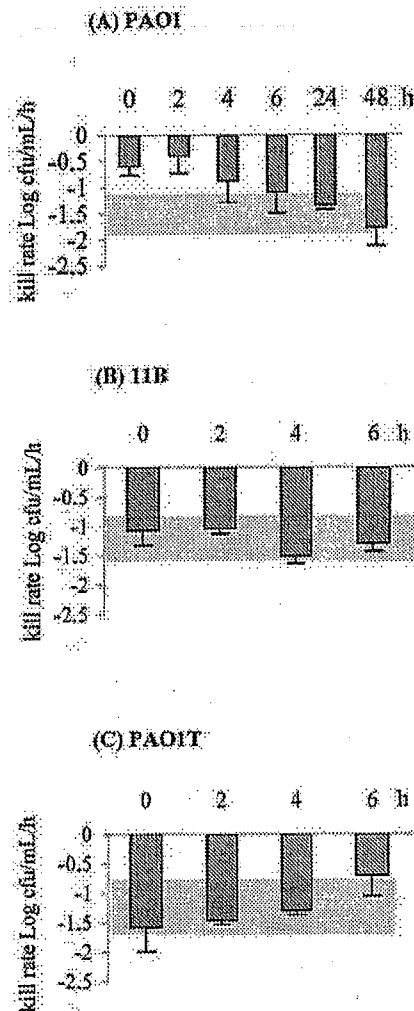

FIG. 1. Bactericidal effects on *P. aeruginosa* of a second exposure to the MIC of gentamicin for 2 h. (A) Wild-type PAO1. (B) MexXY-defective mutant 11B. (C) OprM null mutant PAO1T. Error bars show standard deviations. The test cultures were preexposed for 2 h to the MIC. The grey areas indicate the bactericidal rates in control cultures of a single exposure to gentamicin for 2 h at the MIC (-1.40 ± 0.30, -1.27 ± 0.38, and -1.25 ± 0.42 $\log_{10}$ CFU/ml/h for the PAO1, 11B, and PAO1T strains, respectively).

NaCl buffer (pH 8.0). The inner membrane components were subtracted from this preparation by differential solubilization in a 2% (wt/vol) final concentration of sodium *N*-lauroylsarcosinate. After a 30-min incubation at room temperature, insoluble materials corresponding to the outer membrane fraction were pelleted by centrifugation at 25,000 × g for 30 min at 4°C and resuspended in the same buffer. Proteins were quantified spectrophotometrically in each fraction by using the BCAprot reagent (Pierce Chemical, Rockford, Ill.) at 60°C and bovine serum albumin as a standard.

Immunoblotting experiments. Purified extracts of total membrane (20 μg of protein) and outer membrane (10 μg of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (with 15% [wt/vol] acrylamide in the running gel) according to the method of Laemmli (23) and transferred electrophoretically to nitrocellulose filters as previously described (43). These filters were subsequently blocked with 3% (wt/vol) gelatin and hybridized for 1 h with MexY, OprM (43), or MexB (17) antiserum diluted 1:20,000, 1:5,000, or 1:1,000, respectively, in phosphate-buffered saline. The development of membranes was carried out with alkaline phosphatase conjugated to an anti-rabbit secondary antibody by using the AP color reagent kit from Bio-Rad.
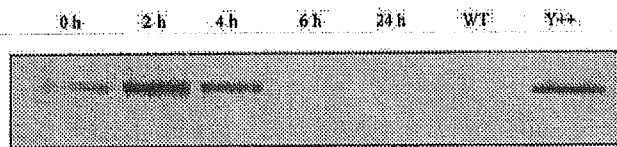
FIG. 2. Amounts of MexY produced by *P. aeruginosa* PAO1 immediately (0 h) and 2, 4, 6, or 24 h after a single gentamicin exposure of 2 h at the MIC. Total membranes were extracted from the exposed bacteria and electrophoresed (20 μg of protein per lane) in sodium dodecyl sulfate–15% polyacrylamide gels. Western blot analyses were performed with MexY-specific antiserum. MexXY-overproducing strain PAO1(pAGH97) was used as a positive control (Y++) for MexY detection, and PAO1(pAK1900) was used as a wild-type control (WT) (35).

Adaptive resistance in vitro. Adaptive resistance was induced as previously described (11). Briefly, an overnight culture was diluted 10-fold in prewarmed, cation-adjusted MHB and incubated with gentamicin at a fraction or multiple of the MIC for 2 h at 37°C (first exposure). In parallel, a sample of the same culture was simultaneously incubated in drug-free broth and used as a control. After 2 h, the bacteria were harvested by centrifugation at 1,350 × *g* for 10 min and washed twice with drug-free medium to remove cell-bound antibiotic. Previous studies have demonstrated that this procedure reduces the extracellular concentrations of aminoglycosides to inactive levels (about 1,000-fold) (20). The pellet was resuspended in drug-free broth to yield a postexposure suspension of 10^4 to 10^5 CFU/ml (determined in preliminary experiments) and then reincubated at 37°C. Aliquots of this culture were removed every 2 h for 6 h and reexposed to gentamicin (at the MIC for 2 h). Colony counts were determined in duplicates before and after the second exposure on Mueller-Hinton agar plates (supplemented with 15 ng of mercuric chloride/ml for strain PAO1T) with a Spiral Plater apparatus (AES Laboratoire, Combourg, France). Experiments were repeated at least twice to ensure the reproducibility of the data. Adaptive resistance was characterized by the reduced bactericidal effect of the second gentamicin exposure compared with that of the first exposure.

RESULTS AND DISCUSSION

Medical experience shows that the treatment of *P. aeruginosa* infections with antibiotics otherwise found efficacious by in vitro tests may be unsuccessful (10, 22). How drug-susceptible *P. aeruginosa* may survive and persist in patients under appropriate chemotherapy is a long-standing question. This complex, multifactorial phenomenon actually involves host-related and bacterium-related factors (8). In the present work, we attempted to identify some of the mechanisms by which initially susceptible bacteria may transiently develop resistance to aminoglycosides and evade chemotherapy.

Role of efflux system MexXY-OprM in adaptive resistance. The implication of MexXY-OprM was investigated in wild-type strain PAO1 and its *mexXY*- and *oprM*-defective mutants 11B and PAO1T, respectively. The conditions used in the pioneering work of Daikos et al. (11) to demonstrate and characterize adaptive resistance were reproduced. Figure 1A shows that a single exposure of strain PAO1 to the MIC of gentamicin (1.6 mg/liter) for 2 h resulted in significant bacterial killing averaging $-1.4 \pm 0.3 \log_{10}$ CFU/ml/h over the growth period tested. As observed elsewhere (11, 19), reexposure of PAO1 to gentamicin (at the MIC for 2 h) at different time intervals after the removal of the aminoglycoside demonstrated the appearance and recession of bacterial refractoriness to drug killing. This so-called adaptive resistance was maximal 2 h and reversed 6 h after the initial exposure. Interestingly, mutants 11B and PAO1T did not develop such a resistance when treated

under the same conditions, that is, with the MICs of gentamicin (0.4 and 0.25 mg/liter, respectively) (Fig. 1B and C). This finding strongly suggested that the MexXY-OprM system is, at least in part, responsible for the resistance process.

MexY is overproduced during the adaptation phase. To determine whether the development of adaptive resistance correlates with enhanced production of MexXY in gentamicin-exposed bacteria, total membrane extracts from strain PAO1 were analyzed by Western blotting with a MexY-specific antiserum (obtained as described in Materials and Methods). MexY was almost undetectable on immunoblots prepared from bacteria grown in antibiotic-free medium (Fig. 2, lane WT), a result that confirms the very low level of expression of *mexXY* efflux genes in the absence of aminoglycosides (29). On the other hand, the exposure of PAO1 cells to the MIC of gentamicin for 2 h triggered the rapid production of MexY, which reached a maximum 2 h and started to recess 4 h after drug removal (Fig. 2, lanes 0 h to 24 h). Thus, the increase in MexY amounts was concomitant to the adaptive period (compare with Fig. 1).

These data show quite conclusively that the efflux system MexXY is rapidly overproduced in a subpopulation of bacteria surviving the first contact with the aminoglycoside (less than 1% of the initial inoculum). Whether these bacteria have features distinctive from those of the rest of the population that could account for their stronger intrinsic resistance to drug killing is not known. In terms of survival, however, fast activation of MexXY production (within 2 h) is likely to be an advantage since it prevents intracellular accumulation of aminoglycosides, which are efficient inhibitors of protein synthesis. Consistent with this notion, adaptive resistance was reported to develop only in metabolically active, growing cells, to require a certain time to appear (1 to 4 h), and to be suppressed by DNA polymerase inhibitor rifampin (5, 11, 19, 38). De novo protein synthesis is therefore necessary to increase resistance to aminoglycosides during the adaptation period. The observation that the inactivation of MexXY or OprM abolishes the resistance process strongly supports the notion that adaptive resistance is due to aminoglycoside efflux rather than decreased drug uptake. As shown by Fig. 2, the activation of *mexXY* expression is turned down when *P. aeruginosa* is no longer in contact with an aminoglycoside. Recent data obtained in our laboratory with a plasmid-borne *mexX::lacZ* fusion have provided evidence that MexZ, a member of the TetR family, exerts a strong repression on efflux operon *mexXY* (C. Vogne, D. Hocquet, J. Ramos Aires, F. El Garch, P. Plésiat, Abstr.

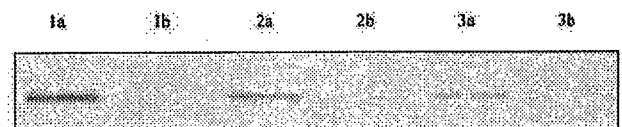FIG. 3. Amounts of MexY produced by strain PAO1 after a single gentamicin exposure at 1.6 mg/liter (MIC; lane 1a), 0.8 mg/liter (half the MIC; lane 2a), or 0.4 mg/liter (one-fourth the MIC; lane 3a) for 2 h. Western blots were prepared as described in the legend for Fig. 2 and developed with a MexY antiserum. In each case, exposed bacteria were subsequently cultured for 24 h in drug-free MHB (lanes 1b, 2b, and 3b).
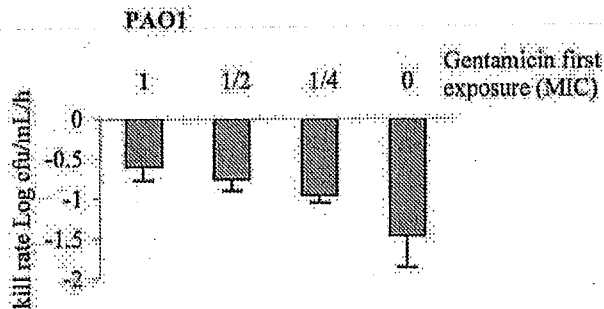
PAO1

FIG. 4. Bactericidal effects on *P. aeruginosa* PAO1 of a second exposure to gentamicin at the MIC for 2 h. The bacteria were preexposed for 2 h to no gentamicin (0), one-fourth or one-half the MIC, or the MIC (1) of gentamicin. Error bars show standard deviations.

42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-434, 2002).

Previous studies (5, 39) have established that bacterial refractoriness is greater in intensity (resistance of survivors to killing) and in duration (persistence of resistance after the original exposure) when *P. aeruginosa* cells are initially confronted with higher doses of aminoglycosides. We thus compared the inductive effects of different concentrations of gentamicin on MexY production. As expected, amounts of MexY (Fig. 3) and adaptive resistance (Fig. 4) proved to be greater in cells treated with higher concentrations of gentamicin, thereby demonstrating that the degree of adaptive resistance depends on MexXY levels.

Production of OprM and MexB. Since MexXY proteins are functional with outer membrane component OprM (32, 35), we investigated whether amounts of OprM and MexXY increase in parallel in adaptively resistant bacteria. OprM appears to be produced constitutively in *P. aeruginosa* cells grown under standard laboratory conditions (26). Its structural gene, *oprM*, has been found to be cotranscribed with *mexA* and *mexB* from two promoters located upstream of *mexA* (13). However, an expression of *oprM* independent of that of *mexAB* has also been demonstrated, involving a third promoter located within gene *mexB* (42). In agreement with other results (29), immunoblotting analysis of outer membrane preparations from strain PAO1 developed with an OprM-specific antiserum (43) failed to reveal an increased production of OprM in postexposure resistant cells (data not shown). The same result was obtained with strain 11B (defective in MexXY) examined 4 h after an initial exposure to the MIC of gentamicin (the time of peak of adaptive resistance in PAO1) (data not shown). This indicates that, while required by MexXY for aminoglycoside export in drug-challenged bacteria, OprM is not upregulated with MexXY. Alternatively, one could imagine that the expression of *mexAB* is repressed during the induction of *mexXY*, as some efflux pumps appear to be inversely coregulated in *P. aeruginosa* (24). Semiquantitative determination of MexB on total membrane immunoblots developed with a MexB-specific antiserum demonstrated that it is not the case (data not shown). Given that OprM forms a tripartite drug efflux complex with MexA-MexB and that MexB amounts do not decrease in adaptively resistant cells, it is reasonable to assume that MexXY competes with MexAB to bind OprM. If this

assumption is correct, resistance to aminoglycosides (substrates for MexXY but not for MexAB) will develop to the detriment of resistance to ticarcillin (a substrate for MexAB but not for MexXY) (30). If confirmed, such a competition between the two pump systems for OprM could, at least in part, account for the synergistic interactions frequently observed between aminoglycosides and β-lactams in *P. aeruginosa*.

Clinical aspects. It has long been known that the exposure of *P. aeruginosa* to inhibitory concentrations (near the MICs) of aminoglycosides easily selects for drug-resistant subpopulations (1, 33). Some of these bacteria, designated small-colony variants because of their reduced growth rate on solid media, are deficient in energy-dependent uptake of aminoglycosides (7, 14, 16). Over the past decade, evidence has accumulated that such variants may emerge in vivo, resulting in bad clinical response or failure of therapy (7, 15, 18, 34). A second recalcitrant subpopulation can also be selected by aminoglycosides. The resistance process developing in these bacteria does not alter their growth rates and has been qualified as adaptive resistance (5, 11, 19). There is now little doubt that the two aforementioned resistant populations reflect distinct phenotypic adaptations to the lethal action of aminoglycosides and that these populations may coexist at the infection site, such as the cystic fibrotic lung (6, 18). Adaptive resistance is one of the major arguments for once-daily aminoglycoside therapy (4). Interestingly, a MexXY-OprM efflux pump inhibitor enhancing the activity of aminoglycosides has recently been discovered (A. Lee, D. Lofland, D. Madsen, M. S. Warren, P. Plésiat, O. Lomovskaya, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-433, 2002). According to our results, such an inhibitor could prevent the manifestation of adaptive resistance and represent a valuable approach to improve the clinical efficacy of aminoglycoside antibiotics.

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Crystal Structure of Extended-Spectrum β-Lactamase Toho-1: Insights into the Molecular Mechanism for Catalytic Reaction and Substrate Specificity Expansion[†,‡]

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ABSTRACT: The crystallographic structure of the class A β-lactamase Toho-1, an extended-spectrum β-lactamase with potent activity against expanded-spectrum cephems, has been determined at 1.65 Å resolution. The result reveals that the Lys73 side chain can adopt two alternative conformations. The predominant conformation of Lys73 is different from that observed in the E166A mutant, indicating that removal of the Glu166 side chain changes the conformation of the Lys73 side chain and thus the interaction between Lys73 and Glu166. The Lys73 side chain would play an important role in proton relay, switching its conformation from one to the other depending on the circumstances. The electron density map also implies possible rotation of Ser237. Comparison of the Toho-1 structure with the structure of other class A β-lactamases shows that the hydroxyl group of Ser237 is likely to rotate through interaction with the carboxyl group of the substrate. Another peculiarity is the existence of three sulfate ions positioned in or near the substrate-binding cavity. One of these sulfate ions is tightly bound to the active center, while the other two are held by a region of positive charge formed by two arginine residues, Arg274 and Arg276. This positively charged region is speculated to represent a pseudo-binding site of the β-lactam antibiotics, presumably catching the methoxyimino group of the third-generation cephems prior to proper binding in the substrate-binding cleft for hydrolysis. This high-resolution structure, together with detailed kinetic analysis of Toho-1, provides a new hypothesis for the catalytic mechanism and substrate specificity of Toho-1.

The most common mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamases, enzymes that hydrolyze the amide group of the β-lactam ring to inactivate β-lactams. β-Lactamases are classified into four classes, A, B, C, and D, according to amino acid sequence and substrate specificity (*1, 2*). This classification separates serine β-lactamases (in which a hydroxyl group of a serine residue is acylated by β-lactams) into classes A, C, and D, while zinc metallo-β-lactamases are grouped into class B. Class A enzymes are most frequently encountered in clinical isolates, often being encoded by genes located on transferable plasmids and exhibiting diverse substrate profiles. These class

A enzymes were originally labeled as penicillinases on the basis of their substrate specificity; however, the number of class A enzymes with activity to hydrolyze expanded-spectrum cephalosporins has increased dramatically as clinical use of new β-lactam antibiotics increased (*1, 3*). These class A β-lactamases with expanded-spectrum substrate specificity are known as extended-spectrum β-lactamases (ESBLs)[1] and are further classified into two subgroups. The largest subgroup is that of non-ESBL derivatives, the extended-spectrum activity of which is the result of a few point mutations in penicillinases such as TEM-1 and SHV-1 (*4*). A large number of TEM and SHV variants have been identified, and their molecular evolution is a point of great interest (*5–8*). The other subgroup includes novel ESBLs distantly related to the previously identified class A β-lactamases. This subgroup includes a cluster of enzymes known as CTX-M-type β-lactamases, where the designation CTX refers to their powerful spectrum for hydrolysis of cefotaxime (*9*). These enzymes have unique amino acid sequences, with 70% or higher identity within this subgroup, yet exhibit only 40% or less identity with other class A β-lactamases, indicating that their activity to hydrolyze expanded-spectrum

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[1] Abbreviations: ESBL, extended-spectrum β-lactamase; PEG, poly(ethylene glycol); ND, not determined.

cephalosporins is an intrinsic enzymatic property of this subgroup and not the result of point mutations. The number of the reported CTX-M-type enzymes has been increasing, signifying their explosive spread over the world (*9–15*).

Toho-1 is an ESBL encoded by a plasmid and produced in *Escherichia coli* TUH12191 isolated from the urine of a patient treated with β-lactam antibiotics (*10*). Toho-1 is classified into the CTX-M group on the basis of its substrate profile and amino acid sequence and belongs to the 2be group according to the functional classification proposed by Bush et al. (*16*). Here we present the crystal structure of wild-type Toho-1 at 1.65 Å resolution. Although impeded by the strong tendency of the wild-type Toho-1 to form twinned crystals, structural analysis was finally achieved through successful preparation of single crystals suitable for X-ray analysis. The high-resolution structure and precise kinetic analysis provide insights into the catalytic mechanism and extended substrate profile of this enzyme.

MATERIALS AND METHODS

Enzyme Preparation. The gene for Toho-1 was cloned and sequenced by a method described previously (*10*). The DNA segment coding for the mature enzyme was inserted into the plasmid vector pET-9a (Novagen) to construct pET-bla for overexpression. *E. coli* BL21(DE3)pLysS [F⁻ *dcm ompT hsd*S$_B$ (r$_B$⁻m$_B$⁻) *gal* λ(DE3) pLysS(Cam^r)] transformed with the plasmid pET-bla was cultured in 2-TY broth at 30 °C for 8 h. Expression of the Toho-1 gene was induced with 0.1 mM isopropyl α-D-thiogalactopyranoside. Cells were harvested by centrifugation and then disrupted by sonication. The enzyme in the supernatant was purified by ion-exchange chromatography on a CM-Toyopearl column (Tosoh, Japan) in 20 mM MES buffer (pH 6.5) and eluted with a 0–0.15 M linear NaCl gradient. The purity of the enzyme was assessed to be more than 95% by Coomassie blue staining after SDS–polyacrylamide gel electrophoresis.

Crystal Preparation and Data Collection. The purified protein was dialyzed against 5 mM Tris-HCl buffer (pH 7.0) and concentrated to 10 mg/mL for crystallization. The crystals were prepared by hanging drop or sitting drop vapor diffusion with a reservoir solution of 1.9–2.0 M ammonium sulfate (no other reagents) at 15–20 °C. Triangular pyramidal crystals suitable for X-ray analysis were obtained within about 1 month. As the crystals tended to twin during crystallization, we made efforts to obtain single crystals as follows: (i) the expression vector was modified to produce mature Toho-1 β-lactamase with no signal sequence, (ii) elution from the CM-Toyopearl column was performed with a slower gradient in the purification process, and (iii) finally at the time of data collection, many crystals were rigorously sorted to find single crystals that diffract to higher resolution. X-ray diffraction data were collected using the 6A beamline of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan) at 100 K with a Mar charge-coupled device (CCD) detector. The crystals were cryoprotected in a solution of 30% sucrose and 2.6 M ammonium sulfate and then flash-frozen in liquid nitrogen. The reflection was indexed, integrated, and scaled using the DPS/Mosflm software package (*17, 18*). The space group was determined to be P3$_2$21, with unit cell dimensions of a = b = 73.3 Å and c = 99.4 Å and one protein molecule per asymmetric unit. The statistics are summarized in Table 1.

Table 1: Data Collection and Processing

wavelength (Å)	1.0
temperature (K)	100.0
space group	P3$_2$21
unit cell (Å)	a = b = 73.3, c = 99.4
resolution range (Å)	23.8–1.65 (1.74–1.65)^a
observations	631678 (55893)
unique reflections	36271 (5246)
completeness (%)	100.0 (99.9)
average I/σ(I)	7.3 (6.0)
R$_{merge}$^{b} (%)	7.2 (10.2)

^{a} Values in parentheses refer to the highest resolution shell. ^{b} R$_{merge}$ = $\sum_i |I_{av} - I_i|/\sum_i |I_i|$, where I_{av} is the average of all individual observations I_i.

Structure Determination. The initial model for refinement was the structure of the Toho-1 E166A mutant (PDB entry 1BZA) (*19*). The model was subjected to rigid body refinement, simulated annealing protocol with an initial temperature of 2000 K, positional minimization, and individual B factor refinement using the CNS software package (*20*). Manual model building was performed with O (*21*). The stereochemical quality of the model was monitored periodically using the program Procheck (*22*). After modeling of the protein structure, water molecules were automatically picked out using CCP4 (*23*). Sulfate ions were modeled into the obvious tetrahedral-shaped electron density in the solvent.

Kinetic Assays. The following antibiotics and chemicals were used for kinetic assays. Benzylpenicillin (Δε$_{233}$ = −1140 M⁻¹ cm⁻¹), cephalothin (Δε$_{262}$ = −7660 M⁻¹ cm⁻¹), and cephaloridine (Δε$_{260}$ = −10200 M⁻¹ cm⁻¹) were purchased from Sigma Chemical Co. (St. Louis, MO); nitrocefin (Δε$_{482}$ = 10000 M⁻¹ cm⁻¹) was from Unipath Oxoid (Basingstoke, U.K.). Imipenem (Δε$_{278}$ = −5660 M⁻¹ cm⁻¹) and cefoxitin (Δε$_{270}$ = −8380 M⁻¹ cm⁻¹) were gifts from Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan); moxalactam (Δε$_{275}$ = −7960 M⁻¹ cm⁻¹), cefcapene (Δε$_{262}$ = −8500 M⁻¹ cm⁻¹), S1090 (Δε$_{296}$ = −7760 M⁻¹ cm⁻¹), and S4661 (Δε$_{298}$ = −9540 M⁻¹ cm⁻¹) were from Shionogi & Co. (Osaka, Japan); cefdinir (Δε$_{310}$ = −5390 M⁻¹ cm⁻¹) and ceftizoxime (Δε$_{257}$ = −7500 M⁻¹ cm⁻¹) were from Fujisawa Pharmaceutical Co. (Osaka, Japan); cefotaxime (Δε$_{264}$ = −7250 M⁻¹ cm⁻¹) was from Aventis Pharma (Tokyo, Japan); piperacillin (Δε$_{232}$ = −1640 M⁻¹ cm⁻¹) was from Toyama Chemical Co. (Tokyo, Japan); meropenem (Δε$_{298}$ = −9530 M⁻¹ cm⁻¹) was from Sumitomo Pharmaceutical Co. (Osaka, Japan); cefpodoxime (Δε$_{261}$ = −8500 M⁻¹ cm⁻¹) was from Sankyo Co. (Tokyo, Japan); faropenem (Δε$_{305}$ = −2950 M⁻¹ cm⁻¹) was from Suntory (Tokyo, Japan); ceftadizime (Δε$_{265}$ = −10300 M⁻¹ cm⁻¹) and clavulanic acid were from GlaxoSmithKline K.K. (Tokyo, Japan); cefepime (Δε$_{267}$ = −9120 M⁻¹ cm⁻¹) was from Bristol Pharmaceutical Co. (Tokyo, Japan); aztreonam (Δε$_{318}$ = −650 M⁻¹ cm⁻¹) was from Eisai Co. (Tokyo, Japan); sulbactam was from Pfizer Pharmaceutical Inc. (Tokyo, Japan); tazobactam was from Taiho Pharmaceutical Co. (Tokyo, Japan). The structures of several antibiotics are shown in Figure 6.

Hydrolysis of β-lactam antibiotics was detected by monitoring the variation in the absorbance of β-lactam solution in 50 mM phosphate buffer (pH 7.0). All measurements were made on a Uvikon 860 spectrophotometer linked to a personal computer. The reaction was performed in a total volume of 500 μL at 30 °C. For dilution of the enzyme,

Table 2: Refinement Statistics

resolution range (Å^2)	20−1.65 (1.73−1.65)^a
R factor^b (%)	18.2 (20.5)
R_{free} factor^c (%)	19.7 (23.2)
average B factors (Å^2)	
whole structure	11.1
main chain	8.0
side chain	11.2
solvent	21.0
rmsd from ideal values	
bonds (Å)	0.009
angles (deg)	1.40

^{a} Values in parentheses refer to the highest resolution shell. ^{b} $R = \Sigma|F_o - F_c|/\Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes. ^{c} $R_{free} = \Sigma|F_o - F_c|/\Sigma|F_o|$, calculated using a test data set of 10% of the total data randomly selected from the observed reflections.

BSA was added to the buffer in the final concentration of 20 μg/mL to prevent denaturation of the enzyme. The reaction is described by the model:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E-S^* \xrightarrow{k_{+3}} E + P$$
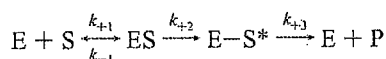
where E is the enzyme, S is the substrate, ES is the noncovalent Michaelis complex, E—S* is the acyl-enzyme intermediate, and P is the inactive degradation product of the substrate. The steady-state kinetic parameters were determined by analyzing the complete hydrolysis time courses as described by De Meester et al. (24) or using the Hanes linearization of the Michaelis—Menten equation. The characteristic steady-state parameters derived from the model are

$$k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}}$$

and

$$K_m = \frac{k_{+3}K}{k_{+2} + k_{+3}}$$

where

$$K = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

The progress curves were measured at least three times for each substrate, and reproducible results were obtained.

RESULTS

Structure Determination. The refined structure of the wild-type Toho-1 β-lactamase is well-defined except for the N-terminal methionine at position 26 inserted for overexpression. The amino acid numbering used in this study follows the consensus numbering of Ambler et al. (2). The secondary structural elements are labeled according to the notations used for the E166A mutant (19). The model includes 261 amino acid residues, 284 water molecules, and 8 sulfate ions. The final model is refined to an R factor of 18.2% and a R_{free} factor of 19.7% at 1.65 Å resolution. The refinement results are summarized in Table 2.

Overall Structure. The overall structure of wild-type Toho-1 consists of two domains: an α/β domain and an α domain, as seen for the other class A β-lactamases. The root-mean-square deviation (rmsd) values for Cα atoms of Toho-1 from other structure-solved enzymes are listed in Table 3. The structure is essentially the same as that of the Toho-1 E166A mutant (Figure 1). The rmsd value between wild-type Toho-1 and the E166A mutant is 0.274 Å for Cα atoms and 0.621 Å for all atoms. A difference is observed around residue 166, which is glutamate in the wild-type enzyme but alanine in the E166A mutant. In the wild-type enzyme, the Ω loop, which contains Glu166 and forms the bottom wall of the active site, moves outward slightly to widen the active site cavity. When the structures of the wild-type enzyme and the mutant are superimposed, the distance between Cα atoms is 0.54 Å at position 166, 0.79 Å at position 104, 0.75 Å at position 165, and 0.33 Å at position 170. The shift of the Ω loop is caused by steric constraint between Glu166 and the neighboring residues and by the existence of water molecules Wat41 and Wat185 near the carboxyl group of Glu166. In comparison with other structure-solved class A enzymes, the smallest Cα difference (rmsd of 0.706 Å) is observed between Toho-1 and *Proteus vulgaris* K1 β-lactamase, an ESBL that is highly homologous to Toho-1 with 70% identity in the amino acid sequence (25).

Alternative Conformations. Alternative conformations are assigned to the side chains of the residues at positions 73, 94, 146, 153, 197, 201, and 218. Lys73 is in the active site, being thoroughly conserved in all of the known class A enzymes (Figure 2a) (1, 2). Arg94, Asp146, Arg153, Lys197, and Glu201 are on the protein surface of the α domain, far from the active site. Ser218 is positioned on the top edge of the active site cleft.

Two alternative conformations of the Lys73 side chain are designated as conformation 1 and conformation 2 (Figure 3). In conformation 1, the side chain of Lys73 points toward Glu166, with the Nζ atom hydrogen-bonded to Glu166 Oε1, Ser70 Oγ, Asn132 Oδ1, and a water molecule Wat185. In conformation 2, the ammonium group of Lys73 points toward the hydroxyl group of Ser130, with the Nζ atom hydrogen-bonded to Ser130 Oγ, Ser70 Oγ, and Asn132 Oδ1. Conformation 1 is predominant in Toho-1, with an occupancy of 0.70. The B factors of Lys73 side-chain atoms are not high, and even lower than the average B factor for all side chains, suggesting that Lys73 prefers to take either one of the two conformations observed in this structure, rather than to be completely flexible. In the Toho-1 E166A mutant structure, the Lys73 conformation is largely analogous to conformation 2 (Figure 4). It indicates that the existence of the glutamate at position 166 strongly affects the conformation of Lys73, with considerable interaction between these two residues. Comparison of the Toho-1 structure with other class A enzymes shows that the Lys73 side chain takes a conformation similar to conformation 2 in most class A β-lactamases (Table 3). The exceptions are *Staphylococcus aureus* PC1 β-lactamase and *P. vulgaris* K1 β-lactamase, which have Lys73 side chains in a conformation resembling conformation 1 (Figure 4) (25, 26). In the case of *Bacillus licheniformis* 749/C β-lactamase, two β-lactamase molecules in an asymmetric unit exhibit slightly different Lys73 conformations, and the Lys73 side chain of molecule B takes a form more similar to conformation 1 than that of molecule