VOL. 53 NO. 5, MAY 2000 THE JOURNAL OF ANTIBIOTICS pp. 516 — 524

Macrolide Esterase-producing *Escherichia coli* Clinically Isolated in Japan

AKIO NAKAMURA, KYOKO NAKAZAWA, IZUMI MIYAKOZAWA, SATOKO MIZUKOSHI, KAZUE TSURUBUCHI, MIYUKI NAKAGAWA, KOJI O'HARA* and TETSUO SAWAI

Department of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan

(Received for publication January 25, 2000)

Current Japanese clinical practice involves the usage of large amounts of new macrolides such as clarithromycin and roxithromycin for the treatment of diffuse panbronchiolitis, *Helicobacter pylori* and *Mycobacterium avium* complex infections. In this study, the phenotypes, genotypes, and macrolide resistance mechanisms of macrolide-inactivating *Escherichia coli* recovered in Japan from 1996 to 1997, were investigated.

The isolation rate of erythromycin A highly-resistant *E. coli* (MIC $\geq$1600 μg/ml) in Japan slightly increased from 0.5% in 1986 to 1.2% in 1997. In six macrolide-resistant strains, recovered from the strains collected for this study during 1996 to 1997, the inactivation of macrolide could be detected with or without added ATP in the assay system. The appearance of erythromycin A-inactivating enzyme independent of ATP was novel from Japanese isolates, and the ^{1}H NMR spectra of oleandomycin hydrolyzed by the three ATP-independent isolates were examined. It was clearly shown that the lactone ring at the position of C-13 was cleaved as 13-H signal in aglycon of oleandomycin upper shifted. These results suggested the first detection of macrolide-lactone ring-hydrolase from clinical isolates in Japan. These results suggested the first detection of an ATP-independent macrolide-hydrolyzing enzyme from Japanese clinical isolates. Substrate specificity of the macrolide-hydrolyzing enzyme was determined with twelve macrolides including the newer members of this group and it was found that not only erythromycin A but also the new macrolides, such as clarithromycin, roxithromycin, and azithromycin were inactivated. The NMR data, broad spectrum of activity, and independence of co-enzyme supported our naming of the enzyme as a macrolide esterase. PCR methodology was employed to detect an *ereB*-like gene from the 3 isolates producing macrolide esterase, and one of these was subsequently shown to contain both *ereB*-like and *ermB*-like genes. It was also clearly shown that the other three isolates, which inactivated macrolide in the presence of ATP, had an *mphA*-like gene.

Macrolide antibiotics are mainly active against Gram-positive bacteria such as *Staphylococcus aureus*. Acquired resistance to macrolide antibiotics has been extensively studied in these bacteria and is generally due to N^6-dimethylation of a specific adenine residue in 23S rRNA[1,2].

Members of the family Enterobacteriaceae, like most Gram-negative organisms, are intrinsically resistant to low levels of erythromycin A, probably by efflux pumps[3]. However, higher local antibiotic concentrations of 0.5 to 6 mg/g of feces are obtained in the lumen of the intestinal tract after oral absorption of usually recommended therapeutic doses. Enterobacteriaceae highly resistant to erythromycin A (MIC, >500 μg/ml) can be isolated, after oral administration of the drug[4].

Recently, pathogenic, opportunistic, normal flora bacteria, or an antibiotic-producing species resistant to erythromycin A were classified against rRNA methylase genes, efflux and inactivating genes[2]. Most of the strains were isolated from feces or blood cultures during selective digestive tract decontamination with erythromycin A in neutropenic patients. The strains inactivate the 14-membered macrolides by producing erythromycin esterases[5,6] or macrolide 2′-phosphotransferase I[7,8]. The 16-membered ring macrolides are efficiently utilized as substrates by only macrolide 2′-phosphotransferase II[9,10].

Macrolide 2′-phosphotransferase [MPH (2′)] I was

discovered in Japan[7], and erythromycin esterase I[5], erythromycin esterase II[6], and MPH (2′) II[9] were reported from clinical isolates in France. Until now lactone-ring hydrolases such as erythromycin esterase, had not yet been discovered in any other country[2].

In Europe, macrolide antibiotics are prescribed for the prevention of infection by Gram-negative bacteria such as the prophylaxis of septicemia in immuno-compromised patients and the prevention of traveler's diarrhea. It was reported that about 50% of highly macrolide-resistant strains recovered from patients have the *ermAM* gene, and about 25% of macrolide-resistant strains have the *ereA* or *ereB* gene[11].

In Japan, large amounts of 14-membered ring macrolides such as erythromycin A, clarithromycin, and roxithromycin are currently used for the treatment of diffuse panbronchiolitis, *Helicobacter pylori* infection and atypical *Micobacterium* complex infections. For the treatment of diffuse panbronchiolitis, long-term chemotherapy (longer than 6 months) with erythromycin A, roxithromycin, or clarithromycin with doses of 400~600 mg/day can improve clinical manifestations and restore lung function in patients with chronic respiratory infections including *P. aeruginosa*[12,13].

Such widespread usage threatened the clinical community with the development of macrolide-resistant strains of *Enterobacteriaceae* such as *Escherichia coli*, because intestinal bacteria were exposed to a significant concentration of macrolide over a long term.

The appearance of macrolide resistance among *E. coli* strains recently isolated in Japan and the phenotypes and genotypes of macrolide highly-resistant strains were investigated in this study.

Materials and Methods

Bacterial Strains

Five hundred clinical isolates of *E. coli* were collected at random from 1996 to 1997 in Japan. Three erythromycin A highly-resistant strains producing a macrolide-inactivating enzyme were used as the reference strains. These were MPH (2′) I-producing *E. coli* Tf481A[7,8,14,15], MPH(2′) II-producing *E. coli* BM2506[9,10,17], and erythromycin-esterase I-producing *E. coli* BM694/pAT63[5,18]. *E. coli* BM2506 and BM694/pAT63 were gifts of Dr. P. C. COURVALIN (Institut Pasteur, Paris, France).

Three erythromycin A-resistant Gram-positive strains were used as controls: *S. aureus* RN1389 which contains an *ermA* gene, *S. aureus* 01A1117 which contains an *ermB* gene, and *S. aureus* RN4220/pEP194 which contains an *ermC* gene. These were gifts from Dr. J. SUTCLIFFE (Central Research Division, Pfizer, Inc., Groton, USA).

E. coli K12W3110*rif* (rifampicin-resistant mutant), *E. coli* ML1410*nal* (nalidixic acid-resistant mutant), *P. aeruginosa* PAO2142Rp (rifampicin-resistant mutant), and *S. aureus* 209P[20] were used as standard strains susceptible to macrolides[7,8,15]. *E. coli* ML1410*nal*/RP4 and *P. aeruginosa* PAO2142Rp/RP4[21] were used as donor strains for the transconjugation of drug resistance. *Bacillus subtilis* ATCC6633 was used as the indicator organism for the determination of the potency of the macrolide antibiotics[7,8].

Chemicals

The macrolides used were erythromycin A, roxithromycin, oleandomycin, triacetyloleandomycin, spiramycin (Sigma Chemical Co., Ltd., St. Louis, MO, USA), leucomycin, josamycin, midecamycin, rokitamycin, and tylosin (Wako Pure Chemical Co., Ltd., Osaka, Japan). Clarithromycin was a gift of Taisho Pharm. Co. Ltd. Azithromycin was a gift from Pfizer Pharm. Co. Ltd., and miokamycin was donated from Meiji seika kaisha Co., Ltd. Other anti-microbial agents (tetracycline, carbenicillin, norfloxacin, kanamycin, and gentamicin) were purchased from Wako Pure Chemical Co., Ltd.

Determination of Minimum Inhibitory Concentrations (MICs)

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method. Hundred-fold dilutions of overnight culture in Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI, USA) were inoculated on MH agar plates containing serial two-fold dilutions of macrolides. The MICs were determined after incubation at 37°C for 18 hours[15,22].

Detection of Macrolide-inactivating Enzyme by Lysozyme-DNase-RNase (LDR) Assay method

For the detection of macrolide-inactivating enzymes, the LDR assay method was performed as follows[21,23]. Washed cells from 6 ml of overnight cultures were suspended in 330 μl of TMK buffer, 100 μl of co-enzyme solution (40 mM ATP, 2 mM acetyl CoA, and 40 mM UDPG, or 80 mM GSH), and 50 μl of antibiotic solution (125 μg/ml). To this suspension 20 μl of LDR solution [lysozyme (5 mg/ml), DNase (100 μg/ml), and RNase (100 μg/ml)] was added.

The reaction mixture was incubated at 37°C for 20 hours. During the reaction, a 20 μl aliquot was periodically

removed onto a paper disc (8 mm diameter, thin: Toyo Filter Co., Ltd., Tokyo Japan), and heated in a microwave oven (model NE-6200, Matsushita electric industrial Co., Ltd., Osaka, Japan) (2×15 sec) to stop the reaction and subsequently the residual activity was determined by microbioassay.

Preparation of Crude Extract and Enzymatic Inactivation of Antibiotics

A crude extract suspended in TMK buffer (0.01 M $MgCl_2$, 0.06 M KCl, and 0.006 M 2-mercaptoethanol in 0.1 M Tris-HCl buffer, pH 7.8) was prepared according to the method of O'HARA *et al.*[8]. The protein concentration was measured by the method of LOWRY *et al.*[24], with bovine serum albumin as the standard. Enzymatic inactivation by the crude extract of antibiotics was performed according to the method of O'HARA *et al.*[7]. Aliquots of 50 μl of 40 mM ATP, 50 μl of each antibiotic (125 μg/ml), and 400 μl of the crude extract diluted with TMK buffer were mixed and allowed to react at 37°C for 0, 0.5, 1, 2, 4, and 18 hours. Over the course of the reaction, a 20 μl aliquot was periodically removed onto a paper disc, heated to stop the reaction, and the residual activity was determined by microbioassay.

Release of Enzyme from *E. coli* Cells by Osmotic Shock

The release of the enzyme from *E. coli* cells by osmotic shock was performed by the modified method[8] of NEU and CHOU[25] with 250 ml of culture. Detection of β-lactamase, a reference for the release of enzymes in the periplasmic space, was performed by UV spectrometry[25] with penicillin G as the substrate.

NMR Spectrometry of Esterase

The NMR spectrometric measurement of the inactivation of macrolide antibiotics by esterase was prepared as follows: 0.1 ml of a suspension of the solution of crude enzyme (600 μg of protein), 1 mg of oleandomycin and 0.4 ml of 0.1 M phosphate buffer (pH 7.0) were mixed in a micro centrifuge tube and incubated at 37°C for 0, 1, 2, and 4 hours. The reaction was stopped by heating the mixture at 100°C for 3 minutes. The supernatant was lyophilized after centrifugation of the reaction mixture at 9,000×*g*, and then dissolved in 0.5 ml of deuterium oxide (D_2O; Merck, Germany) in an NMR tube (5 mm internal dia.).

The ^{1}H-NMR spectra were recorded with a high-resolution 500 MHz Fourier Transform (FT) NMR spectrometer JNM-A500α (JAOL, Tokyo, Japan). The residual 13-H (at 5.34 ppm) signal relating to the extent of hydrolysis of the lactone ring of aglycone was integrated. The intensity of the signal was compared with that of the 1″-H signal at 5.07 ppm of L-oleandrose. The homogenated decoupling mode of the NMR spectrometer was used for suppression of the HOD-signal at 4.78 ppm[17~19,26].

Transfer of Drug Resistance

Transfer of drug resistance was done by mixed cultivation of donor and recipient, either in nutrient broth or on a membrane filter (Type HA, 0.45 μm, Nihon Millipore Kogyo K.K., Tokyo, Japan). For the filter mating method, exponentially growing cultures of donor (0.4 ml) and recipient (0.4 ml) were mixed, and the mixed culture was filtered through the membrane filter[28].

After overnight culture at 37°C on nutrient agar plates containing both 50 μg/ml of rifampicin and 200 μg/ml of erythromycin A, colonies were counted, and the transfer frequency of drug resistance was determined.

Detection of the Macrolide-inactivating Enzyme Gene by PCR Methodology

Primers for *ereA*, *ereB*, *mphA*, *ermA*, *ermB* and *ermC* were used to provide specific PCR products of 420, 546, 837, 645, 639 and 642 bp respectively. The detection method was equivalent to that described by SUTCLIFFE J. *et al*[29]. For these experiments, the *ereA* primers were 5′-AACACCCTGAACCCAAGGGACG-3′ and 5′-CTTCAC-ATCCGGATTCGCTCGA-3′, the *ereB* primers were 5′-AGAAATGGAGGTTCATACTTACCA -3′ and 5′-CATA-TAATCATCACCAATGGCA-3′, the *mphA* primers were 5′-AACTGTACGCACTTGC-3′ and 5′-GGTACTCTTC-GTTACC-3′. the *ermA* primers were 5′-TCTAAAAA-GCATGTAAAAGAA-3′ and 5′-CTTCGATAGTTTATTA-ATATTAGT-3′, the *ermB* primers were 5′-GAAAAG-GTACTCAACCAAATA -3′ and 5′-AGTAACGGTACT-TAAATTGTTTAC-3′, the *ermC* primers were 5′-TCA-AAACATAATATAGATAAA-3′ and 5′-GCTAATATTGTT-TAAATCGTCAAT-3′, respectively. Primers for *mphB*[10,23] were specifically designed for this study. The *mphB* primers were 5′-GCGATAGAATTCAAGGAGAAATAATATGAC-CGTAGTCACGACCGCCGAT-3′ (first 14 bases are the restriction enzyme site, next 12 bases represent the ribosome binding site for cloning, and the final 24 bases are from the structural gene) and 5′-GTTTTCCCAGTCA-CGACGTTGT-3′. The Takara LA PCR kit was used as recommended by Takara Syuzo Co., Ltd. Kyoto, Japan, employing 4 mM $MgCl_2$ for each primer of the *ereA*, *ereB*, *mphA*, and *mphB* genes and 2 mM $MgCl_2$ for each primer of the *ermA*, *ermB*, and *ermC* genes. PCR products were detected and distinguished by electrophoresis on 1% agarose gels in 40 mM Tris acetate-2 mM EDTA buffer.

Table 1. MIC of macrolide antibiotic against *E. coli* producing a macrolide-inactivating enzyme.

Strain	14-membered					15-membered	16-membered					
	OL	TAO	EM	CAM	RXM	AZM	JM	LM	MDM	RKM	SPM	TS
M 26	>1600	>1600	>1600	800	>1600	200	>1600	>1600	>1600	>800	>1600	>1600
44	>1600	>1600	>1600	800	>1600	25	400	100	400	50	400	800
160	>1600	1600	>1600	100	400	25	800	200	800	100	800	1600
377	>1600	>1600	>1600	800	>1600	800	800	200	800	100	400	800
499	>1600	>1600	>1600	800	>1600	>1600	>1600	400	>1600	400	800	>1600
560	>1600	>1600	>1600	800	>1600	800	>1600	400	>1600	400	800	>1600
Tf481A[a]	>800[a]	>1600	800[a]	400[a]	800[a]	100	400[b]	200[b]	800[b]	200[b]	400[b]	800[b]
BM2506	>800[a]	>1600	800[a]	200[a]	800[a]	50	>400[b]	>800[b]	>1600[b]	>200[b]	>1600[b]	>1600[b]
BM694 /pAT63	>800[a]	>1600	>800[a]	> 800[a]	200[a]	50	800	400	800	400	800	800
W3110*rif*	200[a]	400	50[a]	25[a]	50[a]	1.6	200[b]	200[b]	200[b]	100[b]	200[b]	200[b]

[a] From reference 15.
[b] From reference 9.
OL: oleandomycin, TAO: triacetyloleandomycin, EM: erythromycin A, CAM: clarithromycin, RXM: roxithromycin, AZM: azithromycin, JM: josamycin, LM: leucomycin, MDM: midecamycin, MOM: miokamycin, RKM: rokitamycin, SPM: spiramycin, TS: tylosin.

Results and Discussion

From the 500 clinical isolates of *E. coli* which were recovered in Japan during 1996 and 1997, six strains (1.2%) were discovered on subculture to be capable of growth on plates containing 1,600 μg/ml of erythromycin A.

MIC values of various macrolides to these erythromycin A highly-resistant isolates were determined (Table 1). These six strains were highly resistant to all 14-membered ring macrolides. On the other hand, the resistance pattern to 15- and 16-membered ring macrolides was different and on this basis we classified the six strains into 3 groups consisting of strain M26, strains M44 and M160, and strains M377, M499 and M560 respectively.

As an example of the differences, we noted that strain M26 was highly resistant to azithromycin and 16-membered ring macrolides, whereas strains M44 and M160 showed low resistance to 16-membered ring macrolides.

The inactivation of macrolide antibiotics by the erythromycin A highly-resistant strains was investigated by the LDR method using intact cells, LDR solution and various co-enzymes (ATP, GTP, UDPG, NADP and GSH), upon which the reaction of some antibiotic-inactivating enzymes may be dependent as previously reported. It was found that three erythromycin A-resistant (MIC: >1,600 μg/ml) strains of *E. coli*, M26, M44 and M160, inactivated oleandomycin under the test conditions, independent of co-enzyme. These results suggested that strains M26, M44 and M160 had a macrolide-inactivating hydrolase, possibly an esterase. as esterases function independently of any co-enzymes.

The remaining three of the six highly erythromycin A-resistant strains (M377, M499 and M560), were found to inactivate macrolide in the presence of ATP. Oleandomycin, inactivated oleandomycin produced by erythromycin-esterase I from strain BM694/pAT63, and inactivated

Table 2. Liberation of macrolide-inactivating enzyme by osmotic shock method.

	Strains	Resistance marker [c]						
Donor strain	M26	EM	CBPC	TC	GM	KM	NFLX	
	M44	EM	CBPC	TC				
	M160	EM	CBPC	TC		KM	NFLX	
	ML1410*nal* / RP4		CBPC	TC		KM		
Recipient strain	W3110*rif*							RFP
Conjugant strain	W3110*rif* / M26 [a]	EM	CBPC	TC				RFP
	W3110*rif* / RP4 [b]		CBPC	TC		KM		RFP

[a] Selected by erythromycin A (200 μg/ml) and rifampicin (25 μg/ml).
[b] Selected by tetracycline (25 μg/ml) and rifampicin (25 μg/ml).
[c] EM; erythromycin A (200 μg/ml), CBPC; carbenicillin (400 μg/ml), TC; tetracycline (25 μg/ml), GM; gentamicin (25 μg/ml), KM; kanamycin (25 μg/ml), NFLX; norfloxacin (25 μg/ml), RFP; rifampicin (25 μg/ml).

oleandomycin from strain M26 were analyzed by ^{1}H NMR. The 10-H signal of oleandomycin shifted from 3.41 ppm to 2.07 ppm, and the 13-H signal of oleandomycin shifted from 5.32 ppm to 4.22 ppm, in the NMR spectra of both enzymatically inactivated oleandomycin samples. These changes indicated the cleavage of the lactone ring of oleandomycin. Identical changes were seen in the inactivated oleandomycin produced by strains M44 and M160 and these results confirmed that the inactivated oleandomycin from the three strains (M26, M44, and M160) was the same as that from the erythromycin esterase I-producing strain BM694/pAT63. The test data indicated that, for the first time, strains producing the macrolide-hydrolyzing esterase enzyme had appeared in Japan.

The transfer frequency of the co-enzyme independent macrolide resistant phenotype was investigated by the membrane filter mating method. Macrolide resistance could only be transferred from *E. coli* M26 to recipient strain W3110rif, and the transconjugant obtained inactivated oleandomycin. Its transfer frequency was 1×10^{-4}, which is the same as the transferable plasmid RP4. Transfer was undetectable, being $<1\times10^{-8}$, for strains M44 and M160.

Strain M26 demonstrated multiple drug-resistance including tetracycline, carbenicillin, kanamycin, gentamicin and norfloxacin (Table 2). The drug resistant phenotype for tetracycline and carbenicillin transferred with the macrolide resistant phenotype to *E. coli* W3110rif. Macrolide resistance could not be transferred to *P. aeruginosa* PAO2142Rp. These results suggest that the gene encoding for the macrolide-hydrolyzing esterase is located on a transferable R-plasmid in strain M26.

The substrate specificity of the macrolide-hydrolyzing esterase to 12 macrolides was also determined. The relative rates (oleandomycin as 100) of the inactivation of oleandomycin, triacetyloleandmycin, erythromycin A, clarithromycin, roxithromycin, azithromycin, josamycin, leucomycin, midecamycin, miocamycin, spiramycin, and tylosin were 100, 100, 88, 73, 44, 15, <2, <2, <2, <2, <2, and <2, respectively (Fig. 1).

The macrolide-hydrolyzing esterase inactivated 14- and 15-membered-ring macrolide antibiotics, but not 16-membered-ring macrolides. It was clear that the enzyme's range of activity included not only older macrolides represented by erythromycin A, but also newer macrolides such as clarithromycin, roxithromycin, and azithromycin. From these results, it was thought that the macrolide-hydrolyzing enzyme should be termed a macrolide esterase, rather than an erythromycin esterase.

The location of the macrolide esterase in *E. coli* M26 cells was determined in fractions obtained by the osmotic

Fig. 1. Substrate specificity of macrolide-inactivating enzyme.

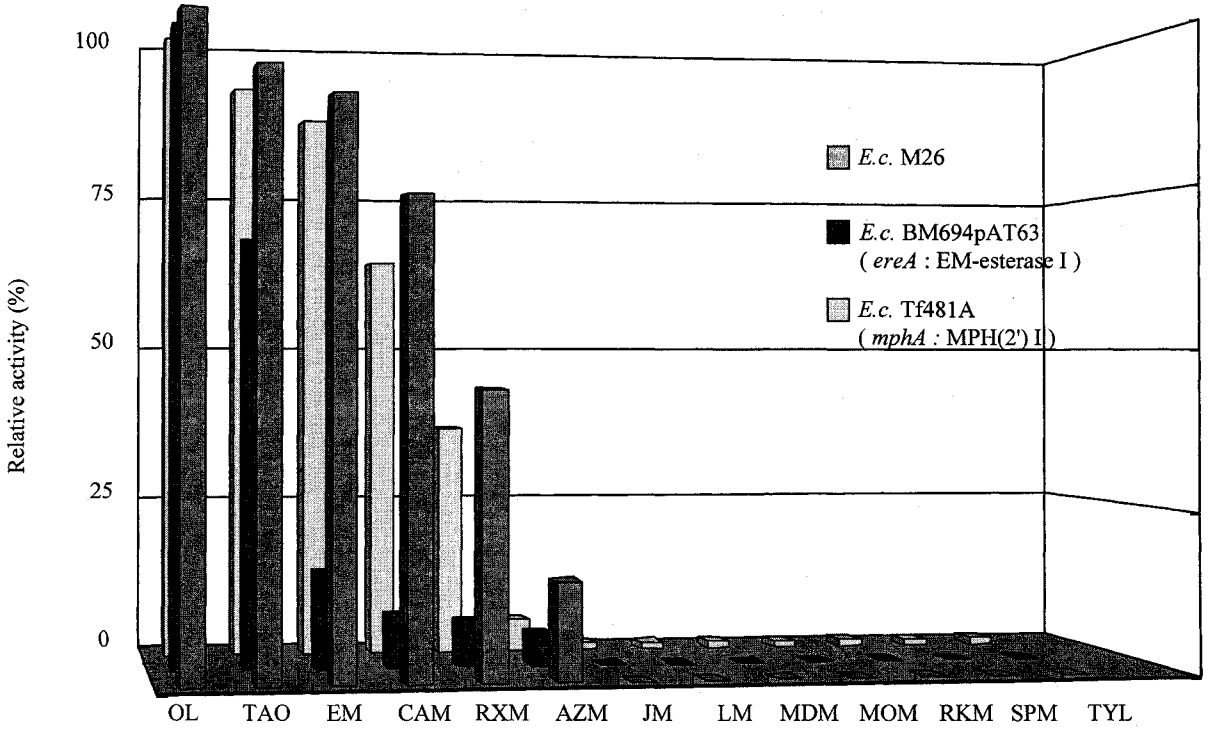


Table 3. Resistance marker of macrolide-resistant *E. coli* isolates.

Sample	Relative activity of macrolide esterase (%)	Relative activity of β-lactamase (%)
1. Supernatant of whole culture [a)]	0	0
2. 0.85% NaCl solution	0	0
3. Sucrose-Tris-EDTA solution	0	10
4. Cold water [b)]	98	90
5. Supernatant of sonicated cells [c)]	2	0
6. Cell debris	0	0

Macrolide (ML)-inactivating activity was measured by determining the amount of remaining oleandomycin in a bioassay. β-lactamase activity was measured as the inactivation of penicillin G determined by the UV spectrometry method.

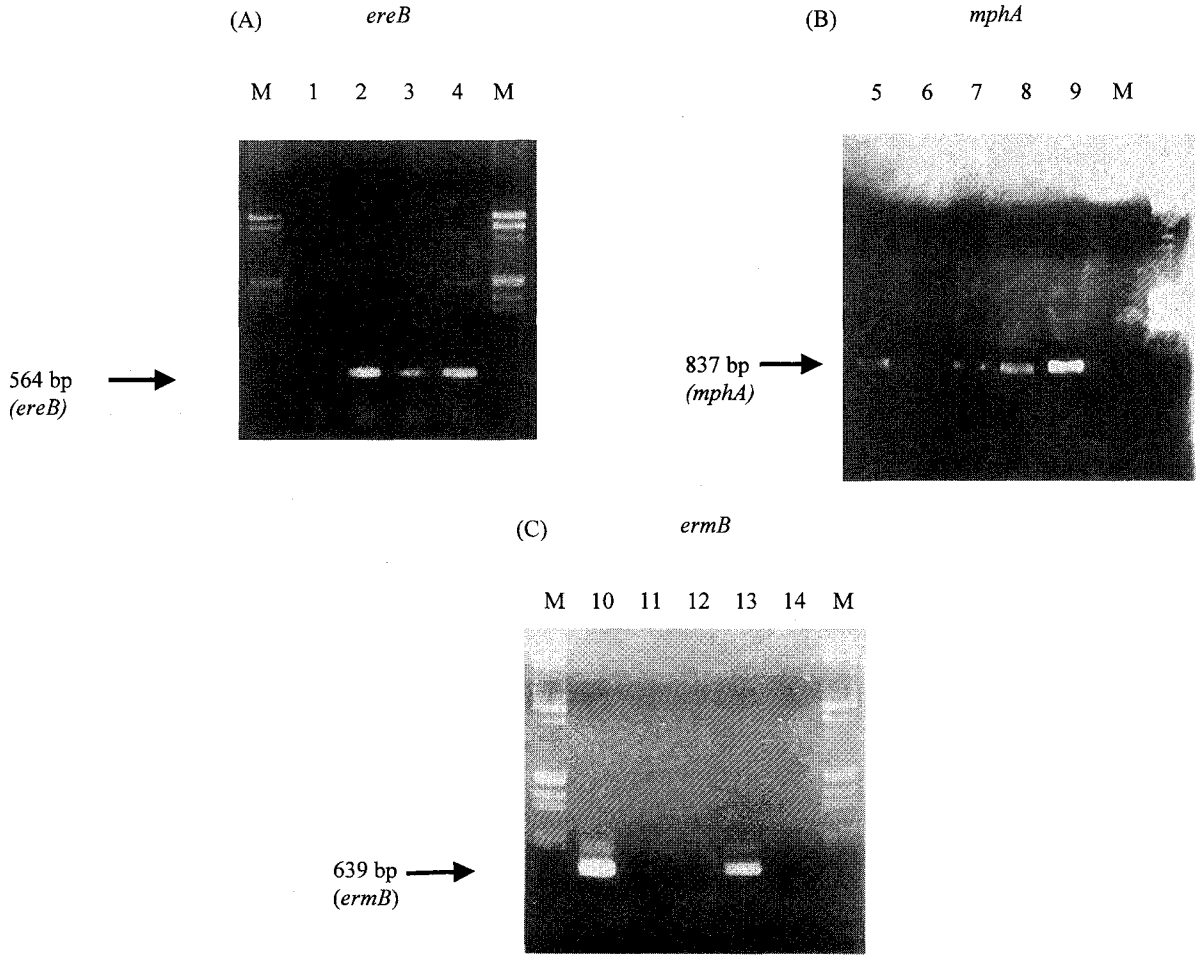
[a] Extracellular enzyme fraction.
[b] Periplasmic-space enzyme fraction.
[c] Intracellular enzyme fraction

shock method (Table 3). β-Lactamase in strain M26 was used as a reference periplasmic enzyme. The macrolide esterase of *E. coli* M26 was detected in the cold-water fraction (fraction 4), the same as the periplasmic β-lactamase. Thus, it appeared that macrolide esterase was a periplasmic enzyme.

The macrolide esterase gene was detected by PCR carried out with primers of each of the erythromycin esterase I- and erythromycin esterase II-producing genes (*ereA* and *ereB*, respectively). PCR products from the *ereB*-

Fig. 2. PCR analysis of erythromycin-resistant determinants.

(a) PCR products observed using primer *ereB*. (b) PCR products observed using primer *mphA*. (c) PCR products observed using primer *ermB*.



Source of DNA for each lane: lanes 2 and 10, *E. coli* M26, lanes 3 and 11, *E. coli* M44, lanes 4 and 12, *E. coli* M160, lane 5, *E. coli* Tf481A, lane 7, *E. coli* M377, lane 8, *E. coli* M499, lane 9, *E. coli* M560, lanes 1, 6 and 14, *E. coli* W3110*rif*, lane 13, *S. aureus* 01A1117, M, DNA makers.

primer were detected in strains M26, M44 and M160, respectively (Fig. 2), showing that these macrolide esterase genes had a strong similarity to the *ereB*-gene. The results supported the conclusion that, for the first time, an *ereB*-like gene had been detected in Japane and that it had been recovered at a rate of 0.6% from the 500 clinical isolates studied.

In addition, an *ermB*-specific PCR product was detected from strain M26, but not from the others (M44, M160, M377, M499, and M560) (Fig. 2). Our results indicated that the *ere* gene and *erm* gene were present in the same transferable plasmid in strain M26, and one might speculate that both genes may have been transported from Europe[11] to Japan.

PCR was also done using probes for the detection of the *mphA* or *mphB* genes. The *mphA* gene was detected from those three strains, M377, M499 and M560, which were co-enzyme dependent and which could inactivate macrolide only in the presence of ATP (Fig. 2).

In conclusion, macrolide resistant strains showed a slight increase from 0.5%[7] to 1.2% of clinical isolates over a period of about 10 years in Japan. And we detected the appearance of the macrolide esterase enzyme in clinical isolates of *E. coli* in Japan recovered in 1996 and 1997. The enzyme was characterized by its ability to inactivate the new macrolides such as clarithromycin, roxithromycin and

azithromycin in the absence of co-enzyme. The *ereB* and *ermB* genes were detected in these strains. In addition, the *mphA* gene was detected from three strains of *E. coli*, which elaborated a co-enzyme dependent resistance.

Acknowledgments

This study was supported by a grant from the Ministry of Health and Welfare, Japan, 1998, for molecular characterization of antibiotic resistance and development of methods for rapid detection of drug-resistant bacteria.

We thank Mr. PETER MACKLON for assistance in the preparation of the manuscript.

References

1) WEISBLUM, B.: Inducible resistance to macrolides, lincosamides, and streptogramin type B antibiotics: the resistance phenotype, its biological diversity, and structural elements that regulate expression-a review. J. Antimicrob. Chemother. 16 (Suppl. A): 63~90, 1985
2) ROBERTS, M. C.; J. SUTCLIFFE, P. COURVALIN, L. B. JENSEN, J. ROOD & H. SEPPALA: Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. 41: 2823~2830, 1999
3) NIKAIDO, H.: Multidrug efflux pumps of Gram-negative bacteria, J. Bacteriol. 178: 5853~5859, 1996
4) SABATH, L. D.; V. LORIAN, D. GERSTEIN, P. B. LODER & M. FINLAND: Enhancing effect on alkalinisation of the medium on the activity of erythromycin against Gram-negative bacteria. Appl. Microbiol. 16: 1288~1292, 1968.
5) BARTHELEMY, P.; D. AUTISSER, G. GERBAUD & P. COURVALIN: Enzymatic hydrolysis of erythromycin by *Escherichia coli*. A new mechanism of resistance. J. Antibiotics 37: 1692~1696, 1984
6) ARTHUR, M.; D. AUTISSIER & P. COURVALIN: Analysis of the nucleotide sequence of the *ereB* encoding the erythromycin esterase type II. Nucleic Acids Research 14: 4987~4999, 1986
7) O'HARA, K.; T. KANDA & M. KONO: Structure of a phosphorylated derivative of oleandomycin, obtained by reaction of oleandomycin with an extract of an erythromycin-resistant strain of *Escherichia coli*. J. Antibiotics 41: 823~827, 1988
8) O'HARA, K.; T. KANDA, K. OHMIYA, T. EBISU & M. KONO: Purification and characterization of macrolide 2′-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob. Agents Chemother. 33: 1354~1357, 1989
9) KONO, M.; K. O'HARA & T. EBISU: Purification and characterization of macrolide 2′-phoshotransferase type II from a strain of *Escherichia coli* highly resistant to macrolide antibiotics. FEMS Microb. Lett. 97: 89~94, 1992
10) TANIGUCHI, K.; A. NAKAMURA, K. TSURUBUCHI, A. ISHII, K. O'HARA & T. SAWAI: Identification of functional amino acid in the macrolide 2′-phosphotransferase II. Antimicrob. Agents Chemother. 43: 2063~2065, 1999
11) ARTHUR, M.; A. ANDREMONT & P. COURVALIN: Distribution of erythromycin esterase and rRNA methylase genes in member of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. 31: 404~409, 1987
12) HOMMA, H.; A. YAMANAKA, S. TANIMOTO, M. TAMURA, Y. CHIJIMATSU, S. KIRA & T. IZUMI: Diffuse panbronchiolitis: a disease of the transitional zone of the lung. Chest 83: 63~69, 1983
13) JAFFE, A.; J. FRANCIS, M. ROSENTHAL & A. BUSH: Long-term azithromycin may improve lung function in children with cyctic fibrosis. Lancet 351: 420, 1999
14) NOGUCHI, N.; A. EMURA, H. MATSUYAMA, K. O'HARA, M. SASATSU & M. KONO: Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase I in *Escherichia coli*. Antimicrob. Agents Chemother. 39: 2359~2363, 1995
15) O'HARA, K. & K. YAMAMOTO: Reaction of roxithromycin and clarithromycin with macrolide-inactivating enzymes from highly erythromycin-resistant *Escherichia coli*. Antimicrob. Agents Chemother. 40: 1036~1038, 1996
16) O'HARA K.: 1993. Reaction mechanism of macrolide 2′-phosphotransferase from *Escherichia coli* to the 2′-modified macrolide antibiotics. Jpn. J. Antibiotics 46: 818~825, 1993
17) NOGUCHI, N.; J. KATAYAMA & K. O'HARA: Cloning and nucleotide sequence of the *mphB* gene for macrolide 2′-phosphotransferase II in *Escherichia coli*. FEMS Microb. Lett. 144; 197~202, 1996
18) O'HARA, K.: Application of nuclear magnetic resonance spectrometry to measure the activity of bacterial macrolide esterase. Microbios. 79: 231~239, 1994
19) O'HARA, K.; T. KANDA, H. MATSUYAMA & N. NOGUCHI: Determination of ^{1}H- or ^{13}C-NMR spectra of oleandomycin (OL), the esterolitic cleavage compound of OL and OL 2′-phosphate using two-dimensional methods in D_2O solution. Jpn. J. Antibiotics 48: 437~440, 1995
20) NAKAMURA, A.; K. TANIGUCHI, K. TSURUBUCHI, K. O'HARA & T. SAWAI: Resistance to macrolide antibiotics found in methicillin-resistant Japanese clinical isolates of *Staphylococcus aureus* in 1996. Jpn. J. Antibiotics 51: 494~500, 1998
21) O'HARA, K.; T. KAWABE, K. TANIGUCHI, M. OHNUMA, M. NAKAGAWA, Y. NAITOU & T. SAWAI: A Simple assay for determining aminoglycoside inactivation in intact cells of Pseudomonas aeruginosa. Microbios, 90: 177~186, 1997
22) Japan Society of Chemotherapy: Method of MIC determination. Chemotherapy (Tokyo) 29: 76~79, 1981
23) TANIGUCHI, K.; A. NAKAMURA, K. TSURUBUCHI, K. O'HARA & T. SAWAI: Identification of *Escherichia coli* clinical isolates producing macrolide 2′-phosphotransferase by a highly sensitive detection method. FEMS Microb. Lett. 167: 191~195, 1998
24) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
25) NEU, H. C. & J. CHOU: release of surface enzymes in Enterobacteriaceae by osmotic shock. J. Bacteriol. 94: 1934~1945, 1967
26) O'HARA, K.; S. HARUTA, T. SAWAI, M. TSUNODA & S.

IYOBE: Novel metallo β-lactamase mediated by *Shigella flexneri* plasmid. FEMS Microb. Lett. 162: 201~206, 1998

27) O'HARA, K.; Y. SHIOMI & M. KONO: Application of nuclear magnetic resonance spectrometry to measure the activity of β-lactamase inhibitor. Jpn. J. Antibiotics 36: 2763~2768, 1983

28) O'HARA, K.: Two different type offosfomycin resistance in clinical isolates of *Klebsiella pneumoniae*. FEMS Microb. Lett. 114: 9~6, 1993

29) SUTCLIFFE, J.; T. GREBE, A. T. KAMRADT & L. WONDRACK: Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. 40: 2562~2566, 1996