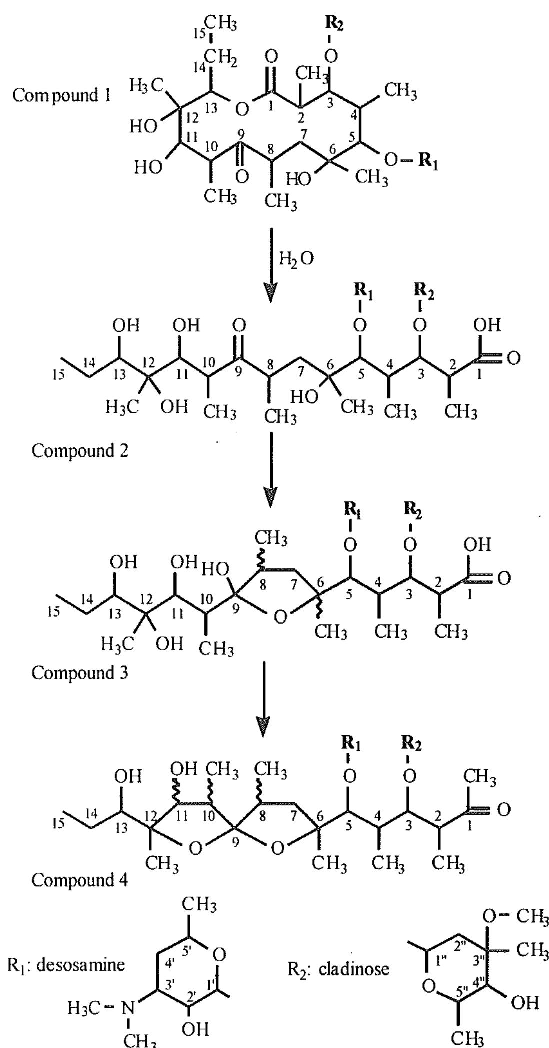

Fig. (15). Reaction catalyzed by erythromycin esterase A and B. Enzymatic hydrolysis of the lactone ring of erythromycin is followed by the formation of an hemiketal by internal condensation and dehydration. Compound 4 is the major end product of detoxification of erythromycin by resting cells at pH 7.0 [57].

resistant to EM (MIC >2000 μg/ml). The *mph*(K) gene on this plasmid is 918 bp long and encodes a protein of 306 amino acids; it is very similar to *mph*(A) (98% identity in DNA and 97% identity in amino acid sequence). The *mph*(B) gene, encoding macrolide 2'-phosphotransferase II (MPH (2')II; 302 amino acids) in *E. coli* BM2506, consists of 906 bp. This enzyme showed potent inactivating activity against 14- and 16-membered macrolides, different from MPH(2')I, which inactivated only 14-membered macrolides [59, 65]. The observation of low similarity between *mph*(A) and *mph*(B) (40% identity in DNA and 37% identity in amino acid sequence) seems to be related to the very different G+C contents of *mph*(A) (65.6%) and *mph*(B) (38.3%). This dissimilarity between the two genes is consistent with the fact that the *mph*(B) gene is only expressed in the Gram-positive bacterium, *S. aureus* [67]. Much work has been done on the *mph*(B) gene [66, 68-72, 84]. The *mph*(C) (formerly *mphBM*) gene, encoding a phosphotransferase, was obtained from *S. aureus* [60] and subsequently from *Stenotrophomonas maltophilia* [74]. The *mph*(C) gene of *S. aureus* is located on plasmid pMS97 in the sequence 5'-*msr*(A)-*mph*(C)-*erm*(Y)-3' and an ATG initiation codon is present 342 bp downstream of the *msr*(A) gene, encoding an ABC-family efflux transporter, while a TAG termination codon is present 414 bp upstream of the *erm*(Y) gene, the product of which dimethylates 23S rRNA [60, 73]. The *mph*(C) gene consists of 897 bp and encodes a deduced protein of 299 amino acids. Furthermore, DNA sequence analysis of *mph*(C) revealed a G+C content of 37.1%, and 62% identity with *mph*(B), despite the 45% identity with *mph*(A). Constructed plasmids pND502, pND5012, pND5013, and pND50A, including the *mph*(C) gene (Fig. **17**), were introduced into the susceptible strain *S. aureus* RN4220, which was then assayed for the inactivation of EM by measurement of the residual activity towards *Micrococcus luteus* ATCC9341. As shown in Fig. (**18**), *S. aureus* RN4220 (pND502) containing only the *mph*(C) gene formed an inhibition zone, although other constructs formed no inhibition zone, suggesting that the expression of *mph*(C) gene is required for the activation of *msr*(A) gene and/or its promoter. Inactivation activity of the *mph*(C) gene was compared with that of the *ere*(A) gene encoding EM-esterase by radioautography and bioautography using [^{14}C]EM and *E. coli* BM694/pAT63 harboring the *ere*(A) gene or *S. aureus* 8325(pMS97) harboring the *mph*(C) gene. Both strains exhibited complete resistance (Fig. **19**). However, the radioautograms showed different peak patterns, indicating that the inactivation mechanism of the *mph*(C) gene product is different from that of the *ere*(A) gene product. The *mph*(C) genes from *Stenotrophomonas maltophilia* D457 [74] and from *S. aureus* harboring plasmid pSR1 showed high similarity to that of pMS97 (more than 98% identity in amino acids) (Fig. **20**). The *mph*(C) gene of *S. maltophilia* D457 was located in a cluster of genes including *mph*(C) and cadmium efflux determinant (*cadA*), together with the gene *cadC* coding for its transcriptional regulator. Nucleotide sequences of plasmid pMS97 (accession number AB179623) resembled those of plasmid pI258, a typical plasmid conferring resistance to macrolides and ß-lactam antibiotics (Fig. **21**). Three plasmids, pI258 containing *erm*(B) (dimethylation of 23S rRNA), pEP2104 containing *msr*(A) (ABC efflux transporter) , and pMS97 containing *msr*(A)-*mph*(C)-*erm*(Y), had the same nucleotide sequences over 20 kb, except for a *Sal*I-*Pst*I fragment including genes conferring macrolide resistance. These observations suggest that genes such as *msr*(A), *mph*(C), and *erm*(Y) have disseminated among bacterial species by providing a growth advantage in the environment.

The range of MLS antibiotics inactivated by the *mph*(C)-encoded phosphotransferase was compared with that inactivated by *ere*(A)-encoded EM esterase (Table 7). Both inactivated all 14-membered macrolides other than telithromycin, which is one of the new ketolide antibiotics. The activity of this ketolide was lost upon phosphorylation, but not upon esterase cleavage of the macrolactone ring (Fig. **22**). Inactivation activity of azithromycin mediated by

```
Ere(A)      1 ------------------------------------------------------------   1
Ere(A2)     1 MTWRTTRTLLQPQKLEFNEFEILNPVVEGARIVGIGEGAHFVAEFSLARASLIRYFVERH  60

Ere(A)      2 -MRL-VWKCGAIQASRLSEWLNSTAGAHELERFSDTLTFSVYGSVLIWLKSYLRESGRKL  58
Ere(A2)    61 DFNAIGLECGAIQASRLSEWLNSTAGAHELERFSDTLTFSSYGSVLIWVKSYLRESGRKL 120
                      ******************************** ******* ***********

Ere(A)     59 QLVGIALPNTLNPRDDLAQLAEIIQLIDHLMKPHVDMLTHLLASIDGQSAVISSAKWGEL 118
Ere(A2)   121 QLVGIDLPNTLNPRDDLAQLAEIIQVIDHLMKPHVDALTQLLTSIDGQSAVISSAKWGEL 180
              ***** ******************* ********** ** ** *****************

Ere(A)    119 ETARQEKAISGVTRLKLRLASLAPVLKKHVNSDLFRKASDRIESIEYTLETLRIMKTFFD 178
Ere(A2)   181 ETAQQEKAISGVTRLKLRLASLAPVLKNHVNSDFFRKASDRIESIEYTLETLRVMKAFFD 240
              *** *********************** ***** ******************** ** ***

Ere(A)    179 GTSLEGDTSVRDSYMAGVVDGMVRANPDVKIILLAHNNHLQKTPVSFSGELTAVPMGQHL 238
Ere(A2)   241 GTSLEGDTSVRDSYMAGVVDGMVRANPDVRIILLAHNNHLQKTPVSFSGELTAVPMGQHL 300
              ***************************** ******************************

Ere(A)    239 AERVNYRAIAFT--HLGPTVPEMHFPSPKSPLGFSVVTTPADAIREDSMEQYVIDACGTE 296
Ere(A2)   301 AEREEGDYRAIAFTHLGLTVPEMHFPSPDSPLGFSVVTTPADAIREDSVEQYVIDACGKE 360
              ***      *    *** ********** ******************* ********* *

Ere(A)    297 NSCLTLTDAPMEAKRMRSQSASVETKLSEAFDAIVCVTSAGKDSLVAL             344
Ere(A2)   361 DSCLTLTDDPMEAKRMRSQSASVETNLSEAFDAIVCVPSAGKDSLVAL             408
               ******* **************** *********** **********
```

Fig. (16). Comparison of the deduced amino acid sequences of erythromycin esterase Ere(A) from *E. coli* (accession number M11277) and Ere(A2) from *Providensia stuartii* (accession number AF099140). Identical residues are indicated by asterisks.

mph(C) was lower that by *ere*(A), and 16-membered macrolide was more strongly inactivated by *mph*(C) than by *ere*(A).

Table 6. Homology (%) of DNA and Amino Acid Sequences for Erythromycin Esterase.

Combination of genes	Homology (%) of	
	DNA	Amino Acid
ere(A) - *ere*(A2)	80	78
ere(A2) - *ere*(B)	49	26
ere(A) - *ere*(B)	47	24

Accession numbers of genes used for homology analysis were the same as in Table 5.

2. Lincosamide Antibiotics

Phosphorylation and nucleotidylation of the hydroxyl group at position 3 of lincosamide have been observed in several species of *Streptomyces* [19, 39]. Among clinical isolates, *Staphylococcus aureus* BM4611 and *Staphylococcus haemolyticus* BM4610 were highly resistant to lincomycin (MIC 64 μg/ml), but susceptible to clindamycin (MIC 0.12 μg/ml). Lincosamide *O*-nucleotidyltransferases coded by two closely related genes, *linA* (lincosamide inactivation nucleotidylation) from *S. haemolyticus* and *linA'* from *S. aureus*, provide resistance to lincosamide [85-88] by inactivation to afford lincomycin 3-(5'-adenylate) and clindamycin 4-(5'-adenylate) (Fig. **23**). Both *linA* and *linA'* encode 3-lincomycin, 4-clindamycin *O*-nucleotidyltransferase, and the two 161-amino acids isozymes differ by only 14 amino acids (Fig. **24**) [88]. A nucleotidyl donor and Mg^{2+} as a cofactor are required for inactivation of lincomycin, as in the case of *Streptomyces coelicolor* [37-40]. Recently, a new resistance gene, *linB*, was identified in *Enterococcus faecium* 1025 [89]. This gene encodes a lincosamide nucleotidyltransferase (267 amino acids) that catalyzes 3-(5'-adenylation) of lincomycin and clindamycin, different from *linA* and *linA'* that catalyze 4-(5'-adenylation) of clindamycin (Fig. **25**). Comparison of nucleotide sequences and deduced amino acid sequences revealed no significant homology with *linA* and *linA'* (Table **8**).

3. Streptogramin Antibiotics

3-1. Hydrolysis

Plasmid-mediated pristinamycin IA (PH IA, belonging to the streptogramin type B category) resistance involving

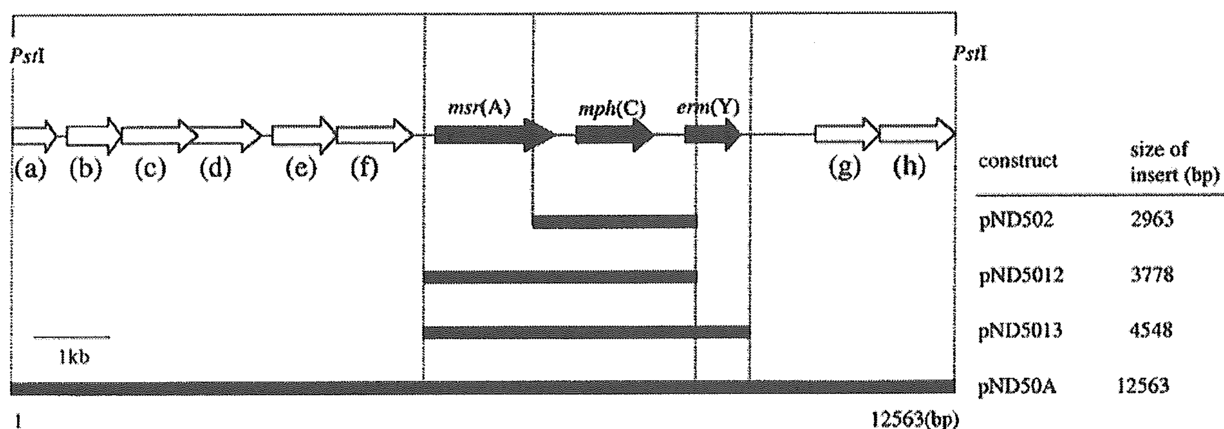

Fig. (17). Schematic representation of constructs containing fragments of genes coding for resistance to macrolides. The constructs pND502, pND5012, pND5013 and pND50A contain *mph*(C), *msr*(A)-*mph*(C), *msr*(A)-*mph*(C)-*erm*(Y), and *msr*(A)-*mph*(C)-*erm*(Y) including the extra region, respectively. The amino acid sequence deduced from the DNA sequence of fragment A (12563 bp) gives eight putative proteins ((a) to (h)) in addition to three macrolide resitance gene products. The constructed fragment is shown by black bars. Arrows show orientation of cloning (5' to 3') and approximate size of the genes of the constructs. Black arrows show the macrolide resistance genes: *msr*(A), *mph*(C), and *erm*(Y). ORFs of (a), (b), (c), (d), (e), (f), (g), and (h) were homologious with reblication-associated protein of *Staphylococcus aureus* plasmid pI9789 with 100%, two-component response regulator of *Clostridium acetoutylicum* with 55%, ABC-type multidrug transport system of *Clostridium acetobutylicum* with 46%, transmembrane protein of *Clostridium acetobutylicum* with 30%, sensory transduction histidine kinase of *Clostridium acetobutylicum* with 36%, hypothetical protein of *Enterococcus faecium* with 61%, transcriptional regulator, AcrR family, of *Clostridium acetobutylicum* with 42%, and ABC transporter ATP-binding protein of *Oceanobacillus iheyensis* with 77% homology, respectively [73].

PH1A hydrolase was first reported in *Staphylococcus aureus* [90]. Thereafter, two genes encoding hydrolases of streptogramin type B antibiotics, *vgb*(A) (formerly *vgb*) from *S. aureus* BM3041 [91] and *vgb*(B) (formerly *vgb*B) from plasmid pI1714 in *Staphylococcus cohnii* subsp. *cohnii* strain were reported [92]. The *vgb*(A) gene consists of 894 bp with a G+C content of 37.5%, and the enzyme (33 kDa) encoded by this gene is similar in molecular weight to an enzyme from *Actinoplanes missouriensis* (35 kDa) inactivating the B component of virginiamycin antibiotics by cleavage of the lactone ring [42]. The *vgb*(B) is linked with *vat*(C), encoding

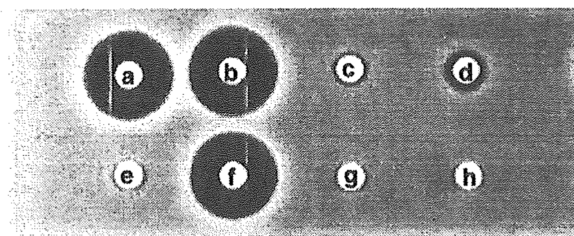

Fig. (18). Inactivation assay of erythromycin (EM) with several *Staphylococcus aureus* strains. EM activity after 24 h incubation with *S. aureus* in the presence of the drug was assayed by the disk plate method using *Micrococcus luteus* ATCC9341 as an indicator microorganism. The residual EM activity was determined in the supernatant (80 µl) of the mixtures incubated with the following bacteria. The supernatants applied to the paper disks were follows: a, 800 ng of EM as a blank; b, *S. aureus* RN4220; c, *E. coli* BM694(pAT63) encoding *ere*(A) gene; d, *S. aureus* 4220(pMS97); e, *S. aureus* 4220(pND50A); f, *S. aureus* 4220(pND502); g, *S aureus* 4220(pND5012); h, *S. aureus* 4220(pND5013).

an acetyltransferase that inactivates streptogramin B on plasmid pIP1714 from *S. cohnii*, and consists of 855 nucleotides encoding a 295-amino acid lactonase. These enzymes, Vgb(A) and Vgv(B), exhibits 67% amino acid identity as shown in Fig. (**26**). A recent study showed that *vgb* from *S. aureus* inactivated streptogramin B antibiotics by elimination, not by hydrolysis of the ester bond [93, 94]. Because the streptogramin B of hexadepsipeptide is linearized through an elimination reaction across the ester bond with generation of an N-terminal dehydrobutyrine group (Fig. **27**), it was suggested that *vgb* is not a hydrolase, but a lyase.

3-2. Acetylation

Acetylation of streptogramin antibiotics is a specific reaction for type A component, whereas hydrolysis reaction is specific for type B *Staphylococcus aureus,* isolated in the virginiamycin production plant from a workman in continuous contact with this antibiotic, inactivated virginiamycin M to the *O*-acetyl derivative [95]. Other strains of *S. aureus* isolated from skin burns inactivated pristinamycin IIA to the *O*-acetylate, as shown in Fig. (**28**) [96].

To date, five different *vat* genes encoding acetyltransferase and eight variations of *vat*(E) from *Enterococcus faecium* have been reported (Table **5**). The genes *vat*(A) [97], *vat*(B) [98], and *vat*(C) [92] (formerly *vat*, *vatB*, and *vatC*, respectively) were isolated from pI680 and pIP1633 on *S. aureus* and from pIP1714 on *Staphylococcus cohnii.* The *vat*(A) gene on plasmid pI680 is linked with *vgb*(A) immediately upstream and with *vga*(A) (formerly *vga*; an ABC–transporter gene) more distantly downstream, while the *vat*(C) gene on plasmid pIP1714 is linked closely downstream of *vgb*(B). The *vat*(D) [99, 100] and *vat*(E)

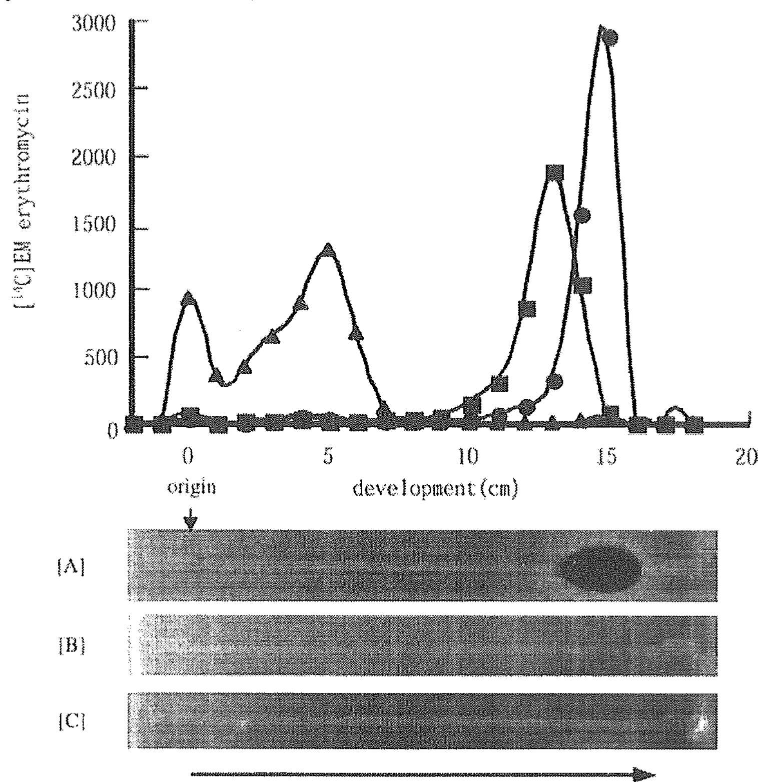

Fig. (19). Radioautogram and bioautogram of [^{14}C]erythromycin (EM). [^{14}C] EM was incubated for 63 h at 37°C with *E. coli* BM694(pAT63) (closed square and [B]) or *Staphylococcus aureus* 8325(pMS97) (closed triangle and [C]). The arrow indicates the direction of development with a solvent system of chloroform-methyanol-34.2% aqueous ammonia (9:1:0.1) [73].

```
pMS97    1 MTRHNEIIKCAEKYQLHIQPQTISLNESGLDFQVAFGKDKHGVEWVLRLPRRPDVYKRTK   60
pSR1     1 MTRHNEIIKCAEKYQLHIQPQTISLNESGLDFQVAFGKDKHGVEWVLRLPRRPDVYKRTK   60
D457     1 MTRHNEIIKCAEKYQLHIQPQTISLNESGLDFQVAFGKDKHGVEWVLRLPRRPDVYKRTK   60
           ************************************************************

pMS97   61 PEKQTVDFLQKNVSFEVPKWKVHERDLIAYPKLTGKPAATIDPEIQNYVWEIEHKPLPEN  120
pSR1    61 PEKQTVDFLQKNVSFEIPKWKVHAKDLIAYPKLTGKPAATIDPEIQNYVWEIEHKPLPEN  120
D457    61 PEKQTVDFLQKNVSFEIPKWKVHAKDLIAYPKLTGKPAATIDPEIQNYVWEIEHKPLPEN  120
           **************** ******  ***********************************
                           .      ..

pMS97  121 FINTLAETLVDLHNIPEENINVQHINIKTIQEIKNDFQRRMNKVKETYGVSDELWNRWKQ  180
pSR1   121 FINTLAETLVDLHNIPEENINVQHINIKTIQEIKNDFQRRMNKVKETYGVSDELWNRWKQ  180
D457   121 FINTLAETLVDLHNIPEENINVQHINIKTIQEIKNDFQRRMNKVKETYGVSDELWNRWKQ  180
           ************************************************************

pMS97  181 WLENDELWPRHATMIHGDLHPGHIMVDNQANVTGLIDWTEATHSDPSMDFMGHHRVFDDE  240
pSR1   181 WLENDELWPRHATMIHGDLHPGHIMVDNQANVTGLIDWTEATHSDPSMDFIGHHRVFDDE  240
D457   181 WLENDELWPRHATMIHGDLHPGHIMVDNQANVTGLIDWTEATHSEPSMDFIGHHRVFDDE  240
           ******************************************** ***** *********
                                                       .     .

pMS97  241 GLEQLITAYGKAGGEIWPRMKEHIIELNAVFPMFIAEFAMESGESAYETMALKELGMKE   299
pSR1   241 GLEQLITAYGKAGGEIWPRMKEHIIELNAVFPMFIAEFAMESGESAYETMALKELGMKE   299
D457   241 GLEQLITAYGKAGGEIWPRMKEHIIELNAVFPMFIAEFAMESGESAYETMALKELGMKE   299
           ***********************************************************
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Fig. (20). Comparision of the deduced amino acid sequences of Mph(C) conferring erythromycin resistance by plasmid pMS97 (accession number AB013298) and pSR1 (accession number AF167161) from *Staphylococcus aureus* and by *Stenotrophomonas maltophillia* D457 (accession number AJ251015).

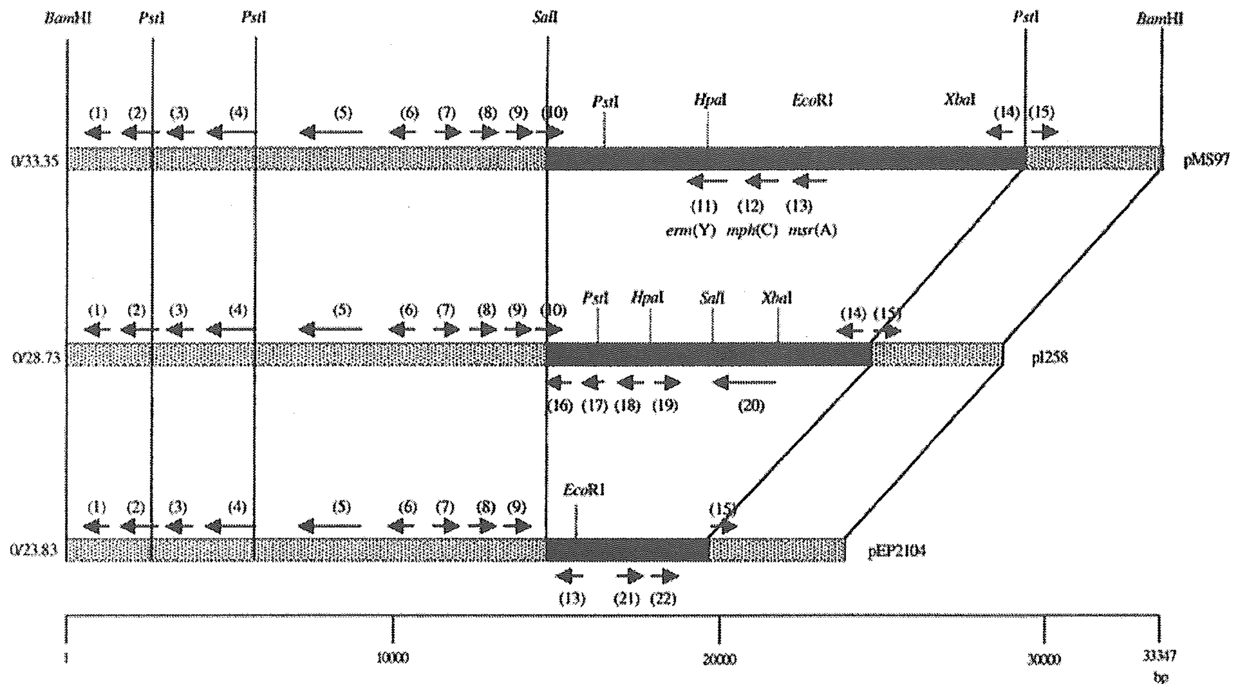

Fig. (21). Genetic maps of macrolide resistance plasmids, pMS97 (accession number AB179623), pI258 [112] and pEP2104 (accession numberAB016613) from *Staphylococcus aureus*. Genes are indicated by the following numbers: (1), *merB*; (2), *merA*; (3), *merT*; (4), *merR*; (5), *cadA*; (6), *blaZ*; (7) *blaRI*; (8), *blaI*; (9), *binR*; (10), *bin3*, (11), *erm*(Y); (12), *mph*(C); (13), *msr*(A), replication associated protein; (15), replication initiation protein; (16), *arsC*; (17), *arsB*: (18), *arsR*; (19), *erm*(B); (0), transposase; (21), *stp*(A); (22) *smp*(A).

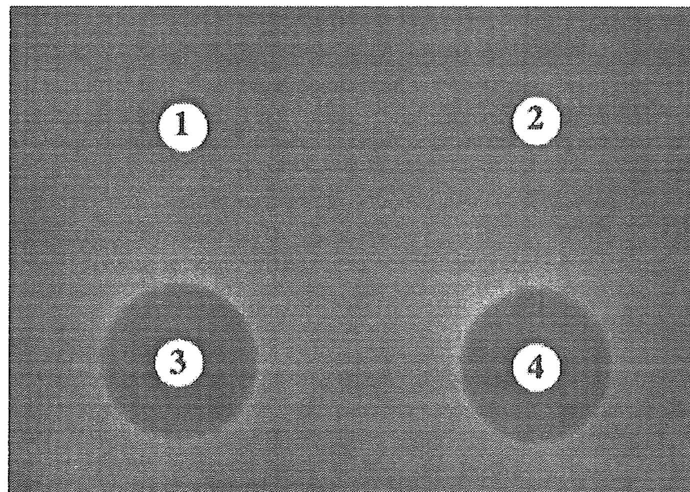

Fig. (22). Inactivation assay for telithromycin. Disks contained incubation supernatant of bacteria and telithromycin as follows: *Staphylococcus aureus* 8325(pMS97) encoding *mph*(C) (disk 1), *S. aureus* 8325(pMS97) induced by EM (disk 2), *E. coli* BM694/pAT63 encoding *ere*(A) (disk 3), and 800 ng of telithromycin as a blank (disk 4).

[101, 102] genes (former *satA* and *satG*, respectively) were isolated from *E. faecium*. The *vat*(D) is homologous with a family of chloramphenicol acetyltransferases present in *Agrobacterium tumefaciens*, *E. coli*, *Pseudomonas aerginosa*, and *S. aureus*. Another Enterococcal gene, *vat*(E) were isolated from quinupristin-dalfopristin-resistant *E. faecium* UW1965 at a sewage treatment plant in Germany [101]. The DNA and amino acid sequences of five *vat* genes were compared (Table **9**, Fig. **29**), and the amino acid sequences of eight variants of the *vat*(E) gene product of *E. faecium* isolated from retail poultry samples [103, 104], *vat*(E-1) to *vat*(E-8), were also compared (Fig. **30**). The prevalence of streptogramin resistance gene in *Enterococci* found in retail poultry [105] might be due to the use of virginiamycin in

Table 7. Comparison of the Inactivation Spectra of Macrolide, Lincosamide, and Streptogramin B Antibiotics for *S. aureus* 8325(pMS97) Containing *mph*(C) and *E. coli* BM694(pAT63) Containing *ere* (A) Gene [73].

Antibiotics	Group	Inactivation (%)*	
		S. aureus 8325(pMS97)	*E. coli* BM694(pAT63)
Erythromycin	14-membered	100	100
Oleandomycin	14-membered	100	100
Roxithromycin	14-membered	100	100
Clarithromycin	14-membered	100	100
Telithromycin	14-membered	100	4
Azithromycin	15-membered	54	100
Rokitamycin	16-membered	6	13
Mycinamicin	16-membered	53	6
Spiramycin	16-membered	8	17
Tylosin	16-membered	12	4
Rosamicin	16-membered	100	0
YM133	16-membered	63	8
Clindamycin	lincosamide	6	3
Lincomycin	lincosamide	5	4
Mikamycin-B	streptograminB	0	0

* Extent of inactivation (%) is expressed as the ratio of decrease in amount of drug activity (i.e. difference between initial drug activity and residual drug activity after incubation) to the initial amount of drug before incubation with bacteria.

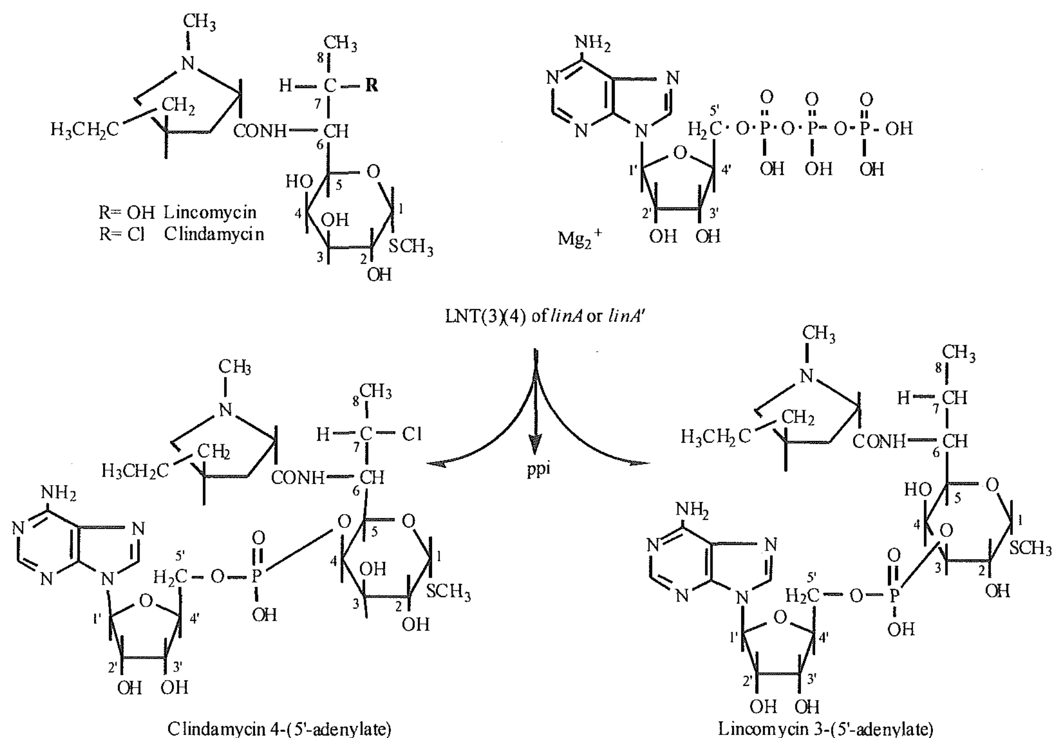

Fig. (23). Adenylation of lincosamides by *Staphylococcus haemolyticus* BM4610 and *Staphylococcus aureus* BM4611. LNT (3)(4); 3-lincomycin, 4-clindamycin nucleotidyl transferase [88].

```
LNT(3)(4)/linA      1 MKNNNVTEKELFYILDLFEHMKVTYWLDGGWGVDVLTGKQQREHRDIDIDFDAQHTQKVI  60
LNT(3)(4)/linA'     1 MKIDNVTEKDLFYILDLFEKMEVTHWLDGGWGVDVLTGKQQREHRDIDIDFDAQHTQKVI  60
                      **  ***** ********* * ** ***********************************

LNT(3)(4)/linA     61 QKLEDIGYKIEVHWMPSRMELKHEEYGYLDIHPINLNDDGSITQANPEGGNYVFQNDWFS 120
LNT(3)(4)/linA'    61 KKLEDIGYKIEVDWMPSRMELKHKEYGYLDIHPINLNDDGSITQANPEGGNYIFQNEWFS 120
                       *********** ********** **************************** *** ***

LNT(3)(4)/linA    121 ETNYKDRKIPCISKEAQLLFHSGYDLTETDHFDIKNLKSIT                   161
LNT(3)(4)/linA'   121 ETNYKGRKIPCISKEAQLLFHSGYELTEKDHFDIKNLKSIT                   161
                      ***** ****************** *** ************
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Fig. (24). Comparision of amino acid sequences of LNT(3)(4), 3-lincomycin, 4-clindamycin O-nucleotidyl transferase encoded by *linA* (accession number M14039) or *linA'* (accession number J03497).

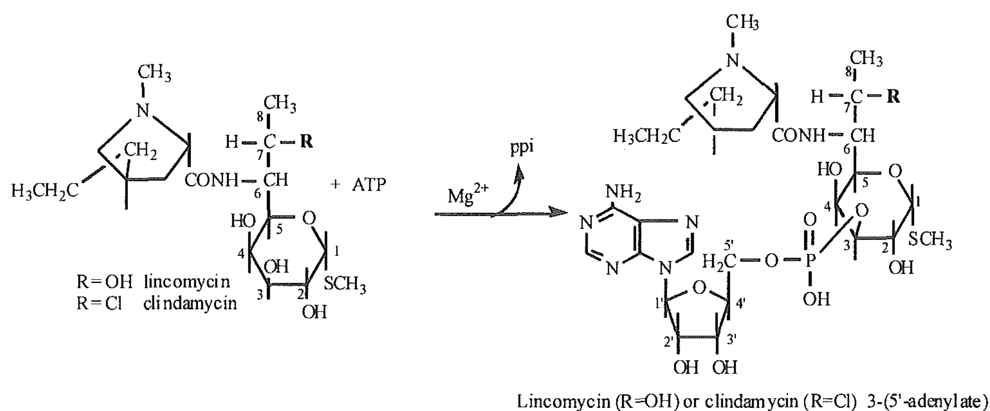

Fig. (25). Adenylation of lincomycin and clindamycin by *linB* gene product of *Enterococcus faecium* HM1025 [89].

Table 8. Homology (%) of DNA and Amino Acid Sequences for the Lincosamide Nucleotidyl Transferase.

Combination of genes	Homology (%) of	
	DNA	Amino Acid
linA - *linA'*	93	91
linA' - *linB*	43	19
linA - *linB*	42	17

Accession numbers of genes used for homology analysis were the same as in Table 5.

many countries for many years as a feed additive to enhance growth of food animals. Consequently, large numbers of virginiamycin-resistant *E. faecium* have been isolated from the feces of food animals, and these were also resistant to quinupristin-dalfopristin, indicating the presence of cross-resistance between virginiamycin and quinupristin-dalfopristin. Many instances of resistance to MLS antibiotics originating from animals have been reported, as shown in *Lactobacillus* [106-108], *Staphylococci* [109], and *Clostridium perfringens* [110].

As MLS antibiotics are widely used as human and veterinary medicines [111], human and veterinary bacteria may acquire resistance genes against MLS antibiotics. In considering the resistance mechanisms of human pathogens, it is always important to ask "Where did the resistance gene come from?" Clinical isolates resistant to MLS antibiotics possess similar resistance mechanisms to those of antibiotic-producing bacteria, except for the hydrolytic inactivation of 14-, 15- and 16-membered macrolides and acetylation of streptogramin A. Resistance genes of antibiotic-producing bacteria might have been transferred into human bacteria through any of the pathways shown in Fig. (**31**). Pathway [I] shows resistance gene transfer to human bacteria mediated by soil bacteria, animal bacteria, and so on [106, 107, 109, 111]. Pathway [II] shows gene transfer involving human bacteria found in hospitals where antibiotics are used as chemotherapeutic agents [92]. Pathway [III] shows transfer

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Vgb(A)     1 MEFKLQELNLTNQDTGPYGITVSDKGKVWITQHKANMISCINLDGKITEYPLPTPDAKVM   60
Vgb(B)     1 MNFYLEEFNLSIPDSGPYGITSSEDGKVWFTQHKANKISSLDQSGRIKEFEVPTPDAKVM   60
             * * * * **   * ****** *  **** ****** **     * * *   ********

Vgb(A)    61 CLTISSDGEVWFTENAANKIGRITKKGIIKEYTLPNPDSAPYGITEGPNGDIWFTEMNGN  120
Vgb(B)    61 CLIVSSLGDIWFTENGANKIGKLSKKGGFTEYPLPQPDSGPYGITEGLNGDIWFTQLNGD  120
             **  ** *  ***** *****   ***   ** ** *** ******* *******  **

Vgb(A)   121 RIGRITDDGKIREYELPNKGSYPSFITLGSDNALWFTENQNNAIGRITESGDITEFKIPT  180
Vgb(B)   121 RIGKLTADGTIYEYDLPNKGSYPAFITLGSDNALWFTENQNNSIGRITNTGKLEEYPLPT  180
             ***  * ** * ** ******** ****************** *****  *   *   **

Vgb(A)   181 PASGPVGITKGNDDALWFVEIIGNKIGRITPLGEITEFKIPTPNARPHAITAGAGIDLWF  240
Vgb(B)   181 NAAAPVGITSGNDGALWFVEIMGNKIGRITTTGEISEYDIPTPNARPHAITAGKNSEIWF  240
              *  ***** *** ******* ********  *** *  **************     **

Vgb(A)   241 TEWGANKIGRLTSNNIIEEYPIQIKSAEPHGICFDGETIWFA-MECDKIGKLT-LIKDNM  298
Vgb(B)   241 TEWGANQIGRITNDKTIQEYQLQTENAEPHGITFGKDGSVWFALKCKIG-KLNLNE----  295
             ****** *** *    * **  *   ****** *           *    **

Vgb(A)   299 E
Vgb(B)   296 -
```

Fig. (26). Comparision of amino acid sequences of Vgb(A) [91] and Vgb(B) [92], which hydrolyze mikamycin B antibiotics.

Pristinamycin IA

Hydrolysis generates
N-terminal threonine

Elimination generates
N-terminal dehydrobutyrine

Fig. (27). Structures of possible products of lactone cleavage of pristinamycin IA [93].

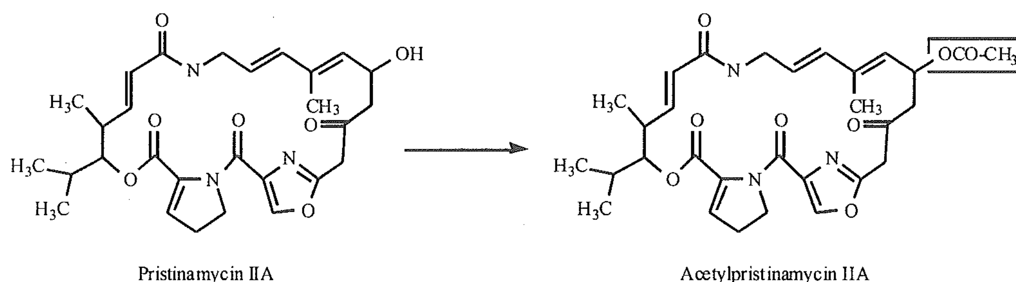

Fig. (28). Acetylation of pristinamycin IIA [96].

```
Vat(A)     1 MNLNNDHGP--DPENILPIKGNRNLQFIKPTITNENILVGEYSY-YDSKRGESFEDQVLY   57
Vat(B)     1 ------MKYGPDPNSIYPHEEIKSVCFIKNTITNPNIIVGDYTYYSDVNGAEKFEEHVTH   54
Vat(C)     1 MKWQNQQGP--NPEEIYPIEGNKHVQFIKPSITKPNILVGEYSY-YDSKDGESFESQVLY   57
Vat(D)     1 ------MGP--NPMKMYPIEGNKSVQFIKPILEKLENVEVGEYSYYDSKNGETFDKQILY   52
Vat(E)     1 ------MTIP-DANAIYPNSAIKEVVFIKNVIKSPNIEIGDYTYYDDPVNPTDFEKHVTH   53
                              *        ***                 *    *
                   ...  ..  .. ..... ..   . .. ... .. . ... .. . . ....

Vat(A)    58 HYEVIGDKLIIGRFCSIGPGTTFIMNGANHRMDGSTYPF-HLFRMGWEKYMPSLKDLPLK  116
Vat(B)    55 HYEFRGDKLVIGKFCAIAEGIEFIMNGANHRMNSITTYPFNIMGNGWEKATPSLEDLPFK  114
Vat(C)    58 HYELIGDKLILGKFCSIGPGTTFIMNGANHRMDGSTFPF-NLFGNGWEKHTPTLEDLPYK  116
Vat(D)    53 HYPILNDKLKIGKFCSIGPGVTIIMNGANHRMDGSTYPF-NLFGNGWEKHMPKLDQLPIK  111
Vat(E)    54 HYEFLGDKLIIGKFCSIASGIEFIMNGANHVMKGISTYPFNILGGDWQQYTPELTDLPLK  113
             **    ***  * ** *  *   ******* *              *    * *  ** *
               .  .   .. .  . ..  ..       . .... .. ...... ..      .

Vat(A)   117 GDIEIGNDVWIGRDVTIMPGVKIGDGAIIAAEAVVTKNVAPYSIVGGNPLKFIRKRFSDG  176
Vat(B)   115 GDTVVGNDVWIGQNVTVMPGIQIGDGAIVAANSVVTKDVPPYRIIGGNPSRIIKKRFEDE  174
Vat(C)   117 GNTEIGNDVWIGRDVTIMPGVKIGNGAIIAAKSVVTKNVDPYSVVGGNPSRLIKIRFSKE  176
Vat(D)   112 GDTIIGNDVWIGKDVVIMPGVKIGDGAIVAANSVVVKDIAPYMLAGGNPANEIKQRFDQD  171
Vat(E)   114 GDTVVGNDVWFGQNVTVLPGVKIGDGAIIGANSVVTKDVAPYTIVGGNPIQLIGPRFEPE  173
             *    ***** *  *   **  ** ***  *  ** *   **   ****   *  **
              .. .     .  . ...  ..  .   .. ..  . ...   ..        .     .

Vat(A)   177 VIEEWLALQWWNLDMKIINENLPFIING----------------------DIEMLKRKR  213
Vat(B)   175 LIDYLLQIKWWDWSAQKIFSNLET---------------LCSSDLEKIKSIRD-------  212
Vat(C)   177 KIAALLKVRWWDLEIETINENIDCILNG----------------------DIKKVKRS-  212
Vat(D)   172 TINQLLDIKWWNWPIDIINENIDKILDN----------------------SIIREVIWK  208
Vat(E)   174 VIQALENLAWWNKDIEWITANVPKLMQTTPTLELINSLMEK------------------  214
              *        **     *  *
                 ..      . .   ..    .                          .

Vat(A)   214 KLLDDT                                                       219
Vat(B)   213 ------                                                       218
Vat(C)   213 ------                                                       218
Vat(D)   209 K-----                                                       214
Vat(E)   215 ------                                                       220
```

Fig. (29). Alignment of amino acid sequences of acetyltransferases Vat(A) [97], Vat(B) [98], Vat(C) [92], Vat(D) [99], and Vat(E) [101] from *Staphylococci* and *Enterococci*, conferring resistance to streptogramin A antibiotics. Identical residues are indicated by asterisks. Highly conserved regions are blodfaced.

```
Vat(E-1)  1 MTIPDANAIYPNSAIKEVVFIKNVIKSPNIEIGDYTYYDDPVNPTDFEKHVTHHYEFLGD  60
Vat(E-2)  1 ..........H.................................................  60
Vat(E-3)  1 ............P...............................................  60
Vat(E-4)  1 ..............MI............................................  60
Vat(E-5)  1 ............L...............................................  60
Vat(E-6)  1 ............L...............................................  60
Vat(E-7)  1 ...............I............................................  60
Vat(E-8)  1 ........V...LILI.LD.........................................  60
            ******** * *    *  *****************************************
                    . .  ...  ..
```

(Fig. (30). Contd....)

```
Vat(E-1)   61 KLIIGKFCSIASGIEFIMNGANHVMKGISTYPFNILGGDWQQYTPELTDLPLKGDTVVGN  120
Vat(E-2)   61 .........L..................................................  120
Vat(E-3)   61 .........................................K..................  120
Vat(E-4)   61 ............................................................  120
Vat(E-5)   61 ............................................................  120
Vat(E-6)   61 ............................................................  120
Vat(E-7)   61 ............................................................  120
Vat(E-8)   61 ............................................................  120
              ********* ******************************* ******************

Vat(E-1)  121 DVWFGQNVTVLPGVKIGDGAIIGANSVVTKDVAPYTIVGGNPIQLIGPRFEPEVIQALEN  180
Vat(E-2)  121 ............................................................  180
Vat(E-3)  121 ............................................................  180
Vat(E-4)  121 ............................................................  180
Vat(E-5)  121 ............................................................  180
Vat(E-6)  121 ................................................S...........  180
Vat(E-7)  121 ............................................................  180
Vat(E-8)  121 ............................................................  180
              ************************************************ ***********

Vat(E-1)  181 LAWWNKDIEWITANVPKLMQTTPTLELINSLMEK                            214
Vat(E-2)  181 ..................................                            214
Vat(E-3)  181 .......V....V...........V.........                            214
Vat(E-4)  181 ..................................                            214
Vat(E-5)  181 ..................................                            214
Vat(E-6)  181 ..................................                            214
Vat(E-7)  181 ..................................                            214
Vat(E-8)  181 ..................................                            214
              ******* **** *********** *********
```

Fig. (30). Amino acid sequences variations of Vat(E) alleles encoded by streptogramin A acetyltransferase gene found in Enterococcus faecium from retail samples of poultry. GenBank accession numbers of *vat*(E-1), *vat* (E-2), *vat* (E-3), *vat*(E-4), *vat*(E-5), *vat*(E-6), *vat*(E-7), and *vat*(E-8) are AF242872, AF153312, AY008284, AY043211, AY0430209, AY043210, AY043212, and AY043213, respectively.

Table 9. Homology (%) of DNA and Amino Acid Sequences for Virginiamycin Acetyltransferase.

Combination of genes	Homology (%) of	
	DNA	Amino Acid
vat(A) - *vat*(B)	63	52
vat(A) - *vat*(C)	70	69
vat(A) - *vat*(D)	64	59
vat(A) - *vat*(E)	59	49
vat(B) - *vat*(C)	63	57
vat(B) - *vat*(D)	63	51
vat(B) - *vat*(E)	62	61
vat(C) - *vat*(D)	64	64
vat(C) - *vat*(E)	60	50
vat(D) - *vat*(E)	59	49

Accession numbers of genes used for homology analysis were the same as in Table 5.

of a resistance gene into human bacteria directly from antibiotic-producing bacteria, as found in a workman who had continuous exposure to the antibiotic at the virginiamycin production plant; however, this route may occur only rarely [95].

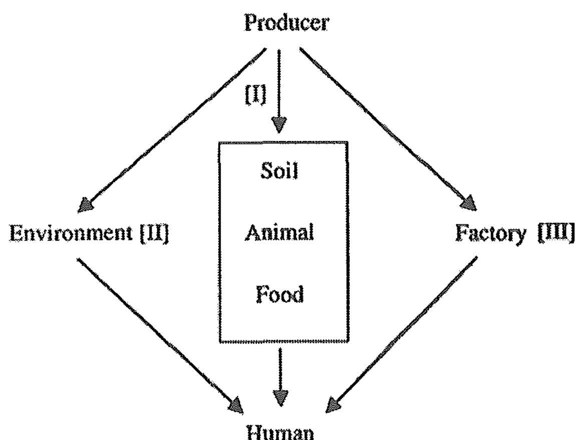

Fig. (31). Proposed pathway [I] 106, 107, 109, 111], pathway [II] [92], and pathway [III] [95] of transfer of resistance genes from producers to humans.

ABBREVIATIONS

ABC transporter	=	ATP-binding cassette transporter
DNA	=	Deoxyribonucleic acid
EM	=	Erythromycin
ere	=	Erythromycin resistance esterase
G+C content	=	GC content
HJM	=	3'''-hydroxyjosamycin
HMDM I	=	3'''-hydroxylmaridomycin
JM	=	Josamycin
kDa	=	Kilo Dalton
MDM I	=	Hydroxyl maridomycin I
MDM III-M	=	4''-depropionylmaridomycin III
MDM III	=	Maridomycin III
MLS	=	Macrolide, lincosamide and streptogramin
mph	=	Macrolide phosphotransferase
OL	=	Oleandomycin
ORF	=	Open reading frame
PH IA	=	Pristinamycin IA
PMDM III	=	9-propionylmaridomycin III
PMDM III-M	=	4''-depropionyl-9-propionylmaridomycin III
rRNA	=	Ribosomal ribonucleic acid
sp.	=	Species
UDP	=	Uridine 5'-diphosphate

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2004, p. 4624–4630 Vol. 48, No. 12
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.12.4624–4630.2004
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Characterization and Molecular Analysis of Macrolide-Resistant *Mycoplasma pneumoniae* Clinical Isolates Obtained in Japan

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Received 28 April 2004/Returned for modification 11 July 2004/Accepted 1 August 2004

In recent years, *Mycoplasma pneumoniae* strains that are clinically resistant to macrolide antibiotics have occasionally been encountered in Japan. Of 76 strains of *M. pneumoniae* isolated in three different areas in Japan during 2000 to 2003, 13 strains were erythromycin (ERY) resistant. Of these 13 strains, 12 were highly ERY resistant (MIC, ≥256 μg/ml) and 1 was weakly resistant (MIC, 8 μg/ml). Nucleotide sequencing of domains II and V of 23S rRNA and ribosomal proteins L4 and L22, which are associated with ERY resistance, showed that 10 strains had an A-to-G transition at position 2063 (corresponding to 2058 in *Escherichia coli* numbering), 1 strain showed A-to-C transversion at position 2063, 1 strain showed an A-to-G transition at position 2064, and the weakly ERY-resistant strain showed C-to-G transversion at position 2617 (corresponding to 2611 in *E. coli* numbering) of domain V. Domain II and ribosomal proteins L4 and L22 were not involved in the ERY resistance of these clinical *M. pneumoniae* strains. In addition, by using our established restriction fragment length polymorphism technique to detect point mutations of PCR products for domain V of the 23S rRNA gene of *M. pneumoniae*, we found that 23 (24%) of 94 PCR-positive oral samples taken from children with respiratory infections showed A2063G mutation. These results suggest that ERY-resistant *M. pneumoniae* infection is not unusual in Japan.

Mycoplasma pneumoniae is a pathogen causing human respiratory infections such as atypical pneumonia, mainly in children and younger adults. In the chemotherapy of *M. pneumoniae* infection in children, erythromycin (ERY) and clarithromycin (CLR) among 14-membered macrolides and the 15-membered macrolide azithromycin (AZM) are usually considered the first-choice agents in Japan. Although there was no report on the isolation of ERY-resistant *M. pneumoniae* before 2000 in Japan, we found that ca. 20% of *M. pneumoniae* strains isolated from patients from 2000 to 2003 were ERY resistant. These results are consistent with pediatricians' impression that antibiotics such as ERY, CLR, and clindamycin (CLI) are not effective for some patients with *M. pneumoniae* infection.

It is well known that the macrolide-lincosamide-streptogramin B (MLS) antibiotics inhibit protein synthesis by binding to domain II and/or domain V of 23S rRNA (3, 26). Lucier et al. (10) and Okazaki et al. (17) found that an A-to-G transition or A-to-C transversion at position 2063 (corresponding to 2058 in *Escherichia coli* numbering) or 2064 of the 23S rRNA gene resulted in high resistance to macrolide antibiotics. No point mutation was found in domain II of 23S rRNA of the ERY-resistant *M. pneumoniae* strains used in the present study.

We report here the prevalence of macrolide-resistant *M. pneumoniae* infection in Japan. By using 13 ERY-resistant *M. pneumoniae* strains, we investigated the mechanisms of resistance to MLS antibiotics. Furthermore, we established restriction fragment length polymorphism (RFLP) techniques to detect point mutations in domain V of 23S rRNA of *M. pneumoniae* by using throat swabs or sputum samples.

MATERIALS AND METHODS

Mycoplasmas. Three types of *M. pneumoniae* strains were used in the present study, i.e., ERY-resistant strains isolated from children infected with *M. pneumoniae* in Japan from 2000 to 2003, ERY-resistant strains induced with ERY in vitro, and three reference strains: M129, Mac, and FH. The ERY-resistant clinical isolates are listed in Table 1, with details regarding patient age, year of isolation, symptoms, and the administration of antibiotics. Most of the isolates

TABLE 1. Macrolide-resistant *M. pneumoniae* strains isolated from patients, along with patient information

Strain no.	Patient		Antimicrobial agent(s)[a]	
	Age (yr)	Symptoms and/ or disease	First choice/effect	Second choice/ effect
350	9	Pneumonia	CLI/−	CLR/+
374	3	Pneumonia	Unknown	Unknown
375	4.5	Pneumonia	Unknown	Unknown
376	12	Pneumonia	CLR/−	AZM/+
377	7	Fever and cough	AZM/+	
378	2	Fever and cough	Cefditoren pivoxil/−	AZM/+
379	9	Pneumonia	CLR/−	AZM/−
380	11	Pneumonia	CLR/−	Minocycline/+
381	11	Pneumonia	AZM/+	
382	7	Pneumonia	RKM/−	AZM/−
383	5	Bronchitis	Cefaclor/−	ERY/+
384	7	Pneumonia	Cefdinir, Fosfomycin/−	ERY/+
385	NI[b]	Pneumonia, pleurisy	CLR/+	

[a] −, No effect from antimicrobial agent; +, improvement of symptoms.
[b] NI, no information.

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TABLE 2. Primers used for PCR amplification and sequencing of domains II and V of 23S rRNA and ribosomal proteins of L4 and L22 in *M. pneumoniae*

PCR and primer designation	Sequence (5′ to 3′)	Position[a]	Amplicon size (bp)
Domain II of 23S rRNA			
MN23SDIIF	AGTACCGTGAGGGAAAGGTG	491–510	816
MN23SDIIR	TCCCAAGCGTTACTCATGCC	1287–1306	
Domain V of 23S rRNA			
MN23SDVF	GCAGTGAAGAACGAGGGG	1758–1775	927
MN23SDVR	GTCCTCGCTTCGGTCCTCTCG	2664–2684	
Ribosomal protein L4			
MNL4F	AAAAGCAGCACCAGTTGTAG	1231–1250	722
MNL4R	GGTTAGAACTGGTTTTAGCA	1933–1952	
Ribosomal protein L22			
MNL22F	GTACATAACGGCAAGACCTT	3640–3659	627
MNL22R	GCAAGCCGTTGGAGTTTACT	4247–4266	
Nested PCR for 23S rRNA of 2063, 2064 region			
MN23SF1937	ACTATAACGGTCCTAAGGTA	1918–1937	210
MN23SR2128	ACCTATTCTCTACATGATAA	2108–2177	
Nested PCR for 23S rRNA of 2617 region			
MN23SF2577	TACGTGAGTTGGGTTCAAA	2577–2595	108
MN23SR2664	GTCCTCGCTTCGGTCCTCTCG	2664–2684	

[a] The positions of domain II and V of 23S rRNA are based on accession no. X68422 of the *M. pneumoniae* gene, and those of ribosomal proteins L4 and L22 are based on accession no. AE000061 of the *M. pneumoniae* M129 section 19 of 63 of the complete genome.

were obtained during the patient's first visit to the hospital, except in a few cases in which the isolates were obtained within a week after an initial treatment failure. Modified Hayflick medium (6) were used for the isolation of *M. pneumoniae* from patients. The broth medium was composed of 7.5 parts PPLO broth (Difco), 1.5 parts heat-inactivated horse serum, and 1 part aqueous extract (25%) of baker's yeast, penicillin G (1,000 U/ml), thallium acetate (0.025%), glucose (0.5%), and phenol red (0.002%). The composition of agar medium was the same as that of the broth medium except that glucose and phenol red were omitted and 1.2% agar was added. A throat swab was immersed several times in 0.5 ml of PPLO broth; then, 0.2 ml of the suspension was transferred to the diphasic (agar/broth) medium, and 0.1 ml of the suspension was transferred onto the agar medium. The agar medium was incubated under 5% CO_2 in air with moisture, and the diphasic medium was incubated aerobically at 37°C for 5 to 14 days. When a color change was observed in the diphasic medium, 0.1 ml of the broth was subcultured onto the agar medium. When typical colonies were observed on the agar medium, a single colony was inoculated into the broth medium. After cloning of the colonies, *M. pneumoniae* was identified serologically or by using PCR.

MIC determination. MICs of MLS antibiotics were determined by a broth microdilution method based on the method of the National Committee for Clinical Laboratory Standards. Serial twofold dilutions of MLS antibiotics prepared in PPLO broth containing 10^4 to 10^5 CFU/ml of *M. pneumoniae* were put in 96-well microplates (17). The microplates were sealed with adhesive sheets and incubated at 37°C. The MIC was determined as the lowest concentration of antimicrobial agent at which the color of the control medium was changed. A number of antibiotics were tested. ERY, oleandomycin (OL), josamycin (JM), spiramycin (SPM), midekamycin (MDM), leucomycin (LM), and lincomycin (LCM) were purchased from Wako Pure Chemical Industries, Ltd., Japan; roxithromycin (RXM) and quinupristin-dalfopristin were provided by Aventis Pharm Japan, Ltd.; CLR was provided by Abbott Co., Ltd. (Japan); rokitamycin (RKM) was provided by Asahi Kasei Co. Japan; CLI was provided by Upjohn Co. (Japan); and AZM was provided by Pfizer Japan, Inc.

PCR amplification and sequencing of domains II and V of the 23S rRNA gene and L4 and L22 ribosomal protein genes. The ERY-resistant *M. pneumoniae* strains were screened on the basis of MIC of ERY. A 0.5-ml aliquot of growth culture of *M. pneumoniae* was centrifuged at 17,500 × *g* for 20 min at 4°C. After

TABLE 3. MICs of MLS antibiotics for *M. pneumoniae* isolated from patients and reference strains

Strain no.	23S rRNA mutation[a]	MIC (μg/ml)												
		ERY	OL	RXM	CLR	AZM	JM	MDM	LM	RKM	SPM	LCM	CLI	Q-D[b]
350	A2063G	>256	>256	>256	256	32	8	16	4	0.5	8	>256	>256	1
374	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	>256	256	0.5
375	A2063G	>256	>256	>256	>256	32	16	16	8	0.5	16	>256	256	0.5
376	A2063C	>256	>256	>256	>256	16	64	64	64	4	256	64	32	1
377	C2617G	8	64	8	1	0.031	0.25	0.25	0.25	0.0625	1	16	2	0.25
378	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	256	256	1
379	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	256	256	0.5
380	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	0.5
381	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	0.5
382	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	1
383	A2064G	256	>256	128	32	16	256	>256	>256	32	>256	64	32	0.25
384	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	>256	256	1
385	A2063G	>256	>256	>256	>256	64	16	16	16	1	16	>256	256	1
FH		0.0625	0.25	0.0625	0.0156	0.00098	0.0156	0.25	0.0625	0.0625	0.25	16	4	0.0625
M129		0.0156	0.125	0.0156	0.0156	0.00195	0.125	0.0625	0.0625	0.0625	0.125	8	4	0.25
Mac		0.0156	0.25	0.0156	0.0156	0.00098	0.0625	0.0625	0.0625	0.0625	0.0625	4	4	0.25

[a] According to *M. pneumoniae* numbering.
[b] Q-D, quinupristin-dalfopristin.

TABLE 4. Nucleotide substitution by point mutation of genes of ribosomal protein and 23S rRNA for macrolide-resistant *M. pneumoniae* strains and *M. pneumoniae* FH and Mac compared to *M. pneumoniae* M129[a]

Strain no.	Substitution(s) in ribosomal protein						Mutation in 23S rRNA		Type of P1 gene
	Position of L4		Position of L22						
	162	430	62	279	341	508	Domain II	Domain V	
M129	C	A	C	T	C	T	–	–	I
350	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
374	–	–	–	–	–	T→C	–	A2063G	I
375	–	–	–	–	–	T→C	–	A2063G	I
376	C→A	A→G	–	T→C	–	T→C	–	A2063C	II
377	C→A	A→G	–	T→C	–	T→C	–	C2617G	II
378	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
379	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
380	–	–	–	–	–	T→C	–	A2063G	I
381	–	–	–	–	–	T→C	–	A2063G	I
382	–	–	–	–	–	T→C	–	A2063G	I
383	–	–	–	–	–	T→C	–	A2064G	I
384	–	–	–	–	–	T→C	–	A2063G	I
385	–	–	–	–	–	T→C	–	A2063G	I
1020-EMR3	–	–	–	–	–	T→C	–	C2617G	I
1020	–	–	–	–	–	T→C	–	A2064G	I
1253	–	–	C→A	–	C→T	T→C	–	A2064G	I
1552	–	–	–	–	–	T→C	–	A2064C/C2617A	I
1653	–	–	–	–	–	T→C	–	A2064G	I
FH	C→A	A→G	–	T→C	–	T→C	–	–	II
Mac	C→A	A→G	–	T→C	–	T→C	–	–	II

[a] –, No mutation compared to the sequence of *M. pneumoniae* M129.

removal of the supernatant, the sediment was suspended in 20 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer containing 1.0% (vol/vol) Triton X-100 and boiled for 5 min. Specific primers were designed for the detection of the point mutations of domain II of 23S rRNA and of L4 (*rplD*) and L22 (*rplV*) ribosomal proteins (Table 2). Primers for domain V of 23S rRNA were as reported by Lucier et al. (10). To identify the mutation in domain II containing nucleotide A752 interacting with the macrolide 3-cladinose moiety, 23SDIIF-23SDIIR primer pairs were used. For domain V (peptidyltransferase region), MH23SDVF-MH23SDVR primer pairs were used. Amplification of ribosomal protein L4 and L22 fragments was performed with the MNL4F-MNL4R and MNL22F-MNL22R primer pairs, respectively. The composition of the PCR mixture was as follows: 2 μl of template, 30 pmol of forward and reverse primers, and 25 μl of premix *Taq* (TaKaRa Ex *Taq* Version; Takara Bio, Inc.) and water in a final reaction volume of 50 μl. PCR conditions were 2 min at 94°C first, followed by 45 s at 94°C for denaturation, 1 min at 55°C for annealing, and 80 s at 72°C for elongation for 30 cycles, and followed finally by 5 min at 72°C. The

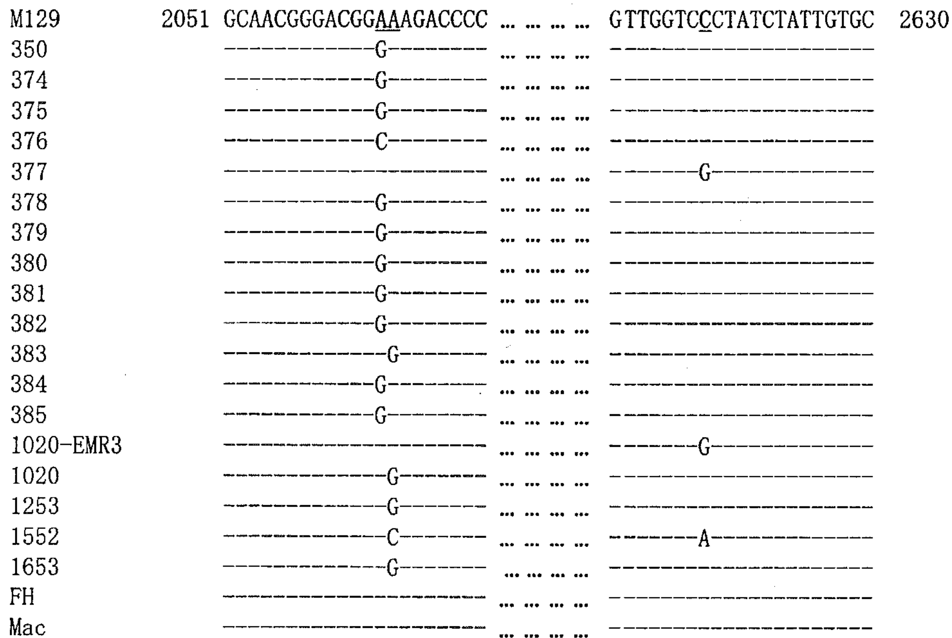

FIG. 1. Multiple alignment of 23S rRNA gene of ERY-resistant *M. pneumoniae* strains and *M. pneumoniae* M129, FH, and Mac. Partial sequences of the peptidyltransferase (domain V) from positions 2051 to 2081 and 2601 to 2630 are presented. The nucleotides are numbered on the basis of *M. pneumoniae*. The nucleotide sequence of *M. pneumoniae* M129 was according to GenBank accession no. X68422. Identical nucleotides are indicated by dashes. The positions of 2063, 2064, and 2617 are underlined.

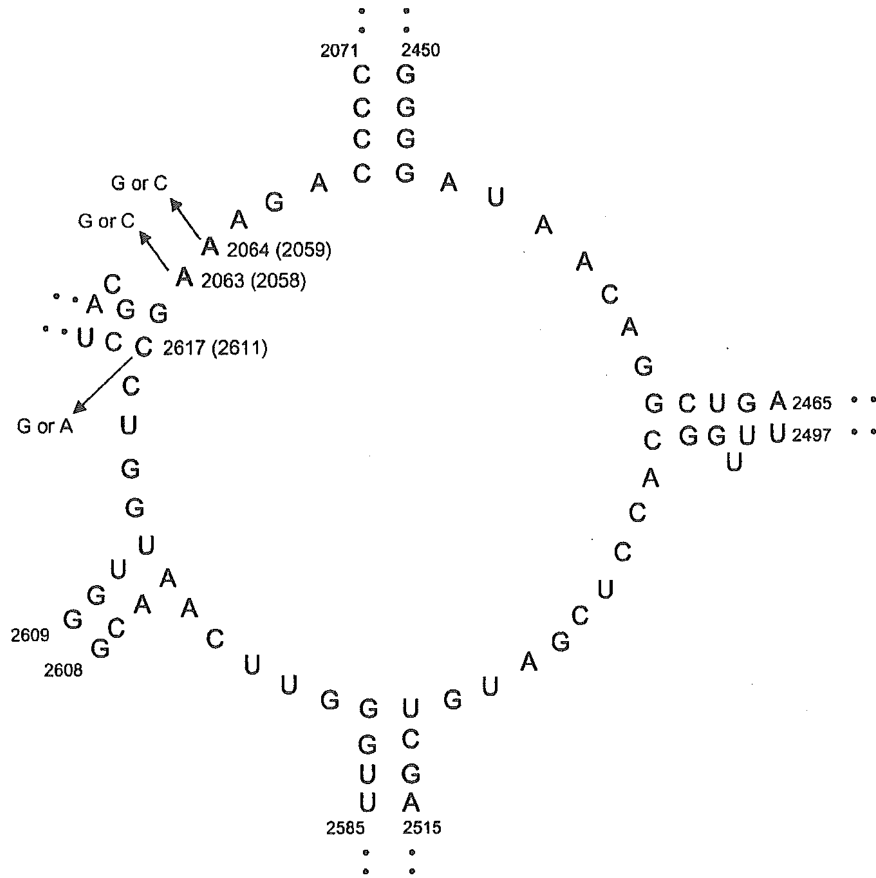

FIG. 2. Secondary structure of the peptidyltransferase loop in domain V of *M. pneumoniae* 23S rRNA. Positions of the newly found mutations (A2063C and C2617G), as well as previously reported in vitro mutations (A2063G, A2064G, and A2064C), in clinical isolates are indicated by using the numbering for *M. pneumoniae* 23S rRNA (accession no. X68422). The numbers in parentheses indicate *E. coli* numbering.

products were purified with a MiniElute PCR purification kit (Qiagen, Hilden, Germany), labeled with a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems), and applied to an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The primers used for sequencing were the same as those used for PCR (Table 2). DNA sequences of PCR products were compared to the sequence of *M. pneumoniae* M129 (accession no. X68422) by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

RFLP analysis of point mutation in domain V of 23S rRNA. To detect the point mutations A2063G, A2063C, A2064G, and A2617G in domain V of 23S rRNA, BbsI, BceAI, BsaI, and BsmFI (New England BioLabs) were used. Second PCR products from domain V for tested *M. pneumoniae* strains were used for digestion with the four restriction enzymes. After the first PCR product (927 bp) was obtained with the MH23SDVF-MH23SDVR primer pair, a second PCR product (210 bp) was obtained with the MN23SF1937-MN23SR2128 primer pair to detect the point mutation at 2063 or 2064 in domain V of 23S rRNA. For the detection of point mutation at 2617 in domain V, the primer set of MN23SF2577 and MN23SF2664 was used, and a 108-bp PCR product was obtained. A portion of the second PCR product was digested with BbsI (5 U for 1 μl of PCR product) for the A2063G mutation, BceAI (1 U for 1 μl of PCR product) was used for the A2063C mutation, BsaI (10 U for 1 μl of PCR product) was used for the A2064G mutation, and BsmFI (2 U for 1 μl of PCR product) was used for the C2617G mutation. Digested products were electrophoresed on a 10 to 15% gradient polyacrylamide gel (Nikkyo Technos Co., Ltd.) or on a 4% Nusieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, Maine).

RESULTS

Antimicrobial susceptibility. In all, 13 (17%) of the 76 clinical isolates obtained in Japan during the period from 2000 to 2003 showed various degrees of elevation of MICs against macrolides, including the ERY MIC. The in vitro activities of the MLS antibiotics against ERY-resistant clinical isolates and reference strains of *M. pneumoniae* are summarized in Table 3. *M. pneumoniae* reference strains, including M129, showed low ERY, OL, RXM, CLR, AZM, JM, MDM, LM, RKM, and SPM (0.0156 to 0.25 μg/ml) MICs. Of the ERY-resistant strains, strain 377 (C2617G) showed low resistance to macrolide antibiotics except for OL. The 15-membered macrolide AZM and most of the 16-membered macrolides were more effective than the 14-membered macrolides for strain 377. Although ERY-resistant clinical strains, except for strain 377, tended to show resistance to all of the macrolides, some of them showed different responses to RKM. That is, for strains with an A-to-G mutation at position 2063 the RKM MICs were not so high (<1 μg/ml). LCM and CLI, lincosamide antibiotics, and streptogramin antibiotics showed no marked activity toward the reference strains or some of the clinical isolates.

Sequencing analysis of ribosomal protein and 23S rRNA genes. PCR amplification and sequence analysis of ribosomal proteins and 23S rRNA were performed for all *M. pneumoniae* strains used in the present study. The results are summarized in Table 4. In domain II of the 23S rRNA containing position 752, there was no difference in sequence from that of *M.*

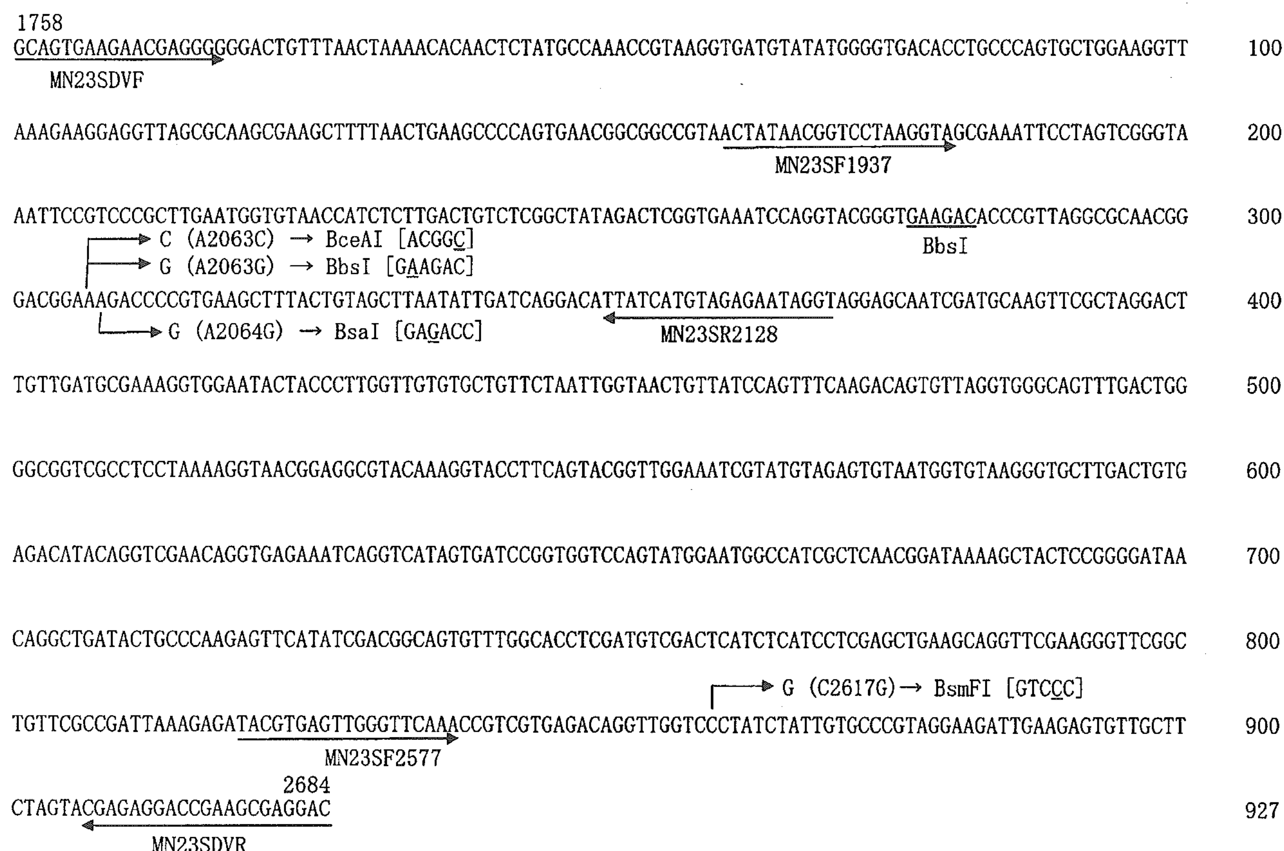

FIG. 3. Nucleotide sequence of the 927-bp amplicon from positions 1758 to 2684 of the 23S rRNA gene from *M. pneumoniae* M129. A long arrow indicates a primer sequence with direction. A short arrow indicates a site of mutation with a substituted base, i.e., A2063G, A2063C, A2064G, or C2617A. A newly constructed restriction site and the responsible base change with underline is shown in parentheses with the corresponding restriction enzyme.

pneumoniae M129. Figure 1 shows the results of the nucleotide sequence analysis of domain V, called the peptidyltransferase region, in the 23S rRNA of the *M. pneumoniae* strains. Five ERY-resistant strains (1020-EMR3, 1020, 1253, 1552, and 1653) were induced with ERY in vitro, as previously reported (17). Figure 2 shows the position of a point mutation on the peptidyltransferase loop in domain V of *M. pneumoniae* 23S rRNA. Of 13 ERY-resistant clinical isolates, 10 (77%) showed A2063G transition, and the remaining 3 showed one A2064G transition, one A2063C transversion, and one A2617G transversion. Of the ERY-resistant strains obtained in vitro, strain 1020-EMR3 had C2617G and strain 1552 had two point mutations: A2064C and C2617A. Compared to the sequence of the M129 strain, different nucleotides were found in some strains (350, 376, 377, 378, 379, FH, and Mac) at positions 162 and 430 of L4 and 279 of L22 ribosomal protein genes. These differences are related to two different types of *M. pneumoniae* strains (19). Mutation T508C of the L22 ribosomal protein gene was observed in all strains used in the present study except for M129. Thus, these nucleotide differences are not involved in the ERY resistance of *M. pneumoniae*. Although C62A and C341T mutations were found in strain 1253, it is uncertain whether these mutations are involved in ERY resistance because of the A2064G mutation, which imparts high ERY resistance.

RFLP analysis of ERY-resistant *M. pneumoniae* strains. To detect a point mutation at position 2063 or 2064 of the 23S rRNA gene, a second PCR product (210 bp) was digested from the first PCR product (927 bp) with suitable restriction enzymes. Digestion with BsaI generated two fragments of 124 and 86 bp for ERY-susceptible strain M129, whereas three fragments of 124, 57, and 29 bp were obtained in the case of the A2063G mutation (lanes 2 and 3 in Fig. 4A). Two fragments of 158 and 52 bp were generated with BceAI in the case of the A2063G mutation (lane 5 in Fig. 4A), and two fragments were generated with BsaI in the case of the A2064G mutation (lane 7 in Fig. 4A). Strain M129 has no cut site for the second PCR product with BceAI and BsaI (lanes 4 and 6 in Fig. 4A). To detect a point mutation at position 2617, the PCR primer pair MN23SF2577 and MN23SDVR was used, generating a 108-bp product (Fig. 3). Although there was no restriction enzyme to digest C2617A or C2617G mutation, the M129 strain had a restriction site with BsmFI and generated two fragments of 81 and 27 bp (Fig. 4B).

DISCUSSION

In general, macrolides such as ERY, CLR, and AZM are used as the first-choice therapeutic agent for treating *M. pneumoniae* infections in children, as well as in adults. We isolated

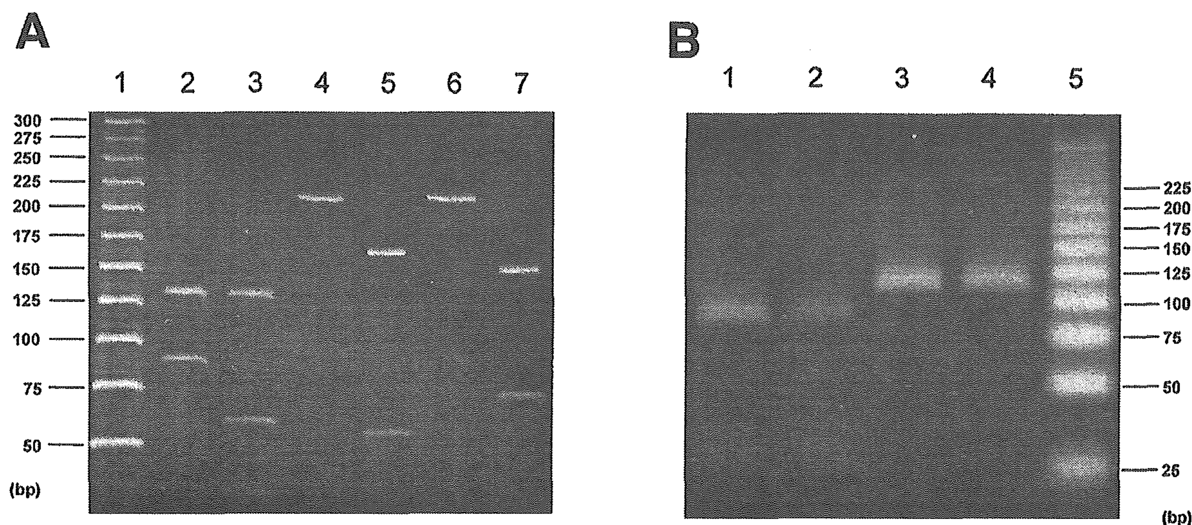

FIG. 4. Restriction analysis of 210-bp (A) and 108-bp (B) amplicons from the peptidyltransferase region (domain V) in 23S rRNA of *M. pneumoniae*. (A) Restriction profile for detection of the A2063G, A2063C, and A2064G mutations. Lanes: 1, DNA size marker (25-bp DNA step ladder; Promega); 2, 4, and 6, *M. pneumoniae* M129 (susceptible strain) treated with BbsI (lane 2, 124-, and 86-bp products) and BceAI and BsaI (lanes 4 and 6, respectively; uncut 210-bp product); 3, strain 375 (A2063G) treated with BbsI (124-, 57-, and 52-bp products); 5, strain 376 (A2063C) treated with BceAI (158- and 52-bp products); 7, strain 1020 (A2064G) treated with BsaI (141- and 69-bp products). (B) Restriction profile for detection of C2617 mutation with BsmFI digestion. Although *M. pneumoniae* M129 and strain 375 (A2063G) produced two fragments of 81 and 27 bp (lanes 1 and 2), the 108-bp fragment remained uncut in strains 377 and 1020-EMR3 (C2617G) as a result of loss of the restriction site for BsmFI (lanes 3 and 4). Lane 5, DNA size marker (25-bp DNA step ladder; Promega).

76 *M. pneumoniae* strains from three geographically distant regions in Japan (Hokkaido in the northern island, Kanagawa in the central region, and Kochi in south) and found that 13 strains (17%) were ERY resistant. Although resistance to ERY was observed many years ago in a few *M. pneumoniae* strains (16, 20), when we investigated the ERY MICs for 296 *M. pneumoniae* strains isolated in Japan from 1983 to 1998, no ERY-resistant strain was found among them (data not shown). Thus, we concluded that ERY-resistant *M. pneumoniae* had appeared in 2000 and spread rapidly in Japan. We applied our established RFLP analysis to ca. 1,000 sputum samples taken from patients with respiratory infections from 2000 to 2002 and found that 23 (24%) of 94 PCR-positive samples for *M. pneumoniae* DNA had the ERY resistance-inducing point mutation A2063G (unpublished data). Whether or not the prevalence of ERY-resistant *M. pneumoniae* and the predominance of A2063G among the isolates are peculiar to Japan needs to be clarified by future studies outside Japan.

The mechanisms of resistance to MLS antibiotics in various microorganisms have been reviewed and include modification of the target site, active efflux, or inactivation (13, 24–26). The MLS antibiotics inhibit protein synthesis by binding to domains II and V of 23S rRNA (3, 26). In particular, it has been clearly shown that ribosomal mutations in domains II and V of 23S rRNA and mutations in ribosomal protein L4 (*rplD*) and L22 (*rplV*) are related to resistance to MLS antibiotics (2, 4). In L4 and L22 ribosomal proteins, no mutation that clearly contributed to resistance to macrolide antibiotics was found, although one strain (strain 1253) exhibited mutations of the L22 protein, such as C62A and C341T, in vitro. We found several point mutations in domain V of 23S rRNA in ERY-resistant *M. pneumoniae* but none in domain II of 23S rRNA. Among them, the point mutations at position 2063 or 2064 in domain V have been reported in several pathogens such as *E. coli*, *H. pylori*, *Mycobacterium* spp., and *S. pneumoniae* (24) and generated strong resistance to macrolide antibiotics. Transversions of C to G and C to A at position 2617 of domain V were observed in a clinical isolate (strain 377) and ERY-induced strains (1020-EMR3 and 1552), respectively. On the other hand, it has been reported that C-to-U transition at position 2611 (corresponding to 2617 in *M. pneumoniae* numbering) in clinical pathogens such as *Neisseria gonorrhoeae* (15), *Streptococcus pyogenes* (11), *Mycoplasma hominis* (18), *Chlamydia trachomatis* (12), and *E. coli* (23) was associated with macrolide resistance. *M. pneumoniae* strain 1552, derived by incubation with ERY in vitro, showed A2064C transversion and C2617A transversion. The mutation at position 2617 produced less resistance to macrolide antibiotics than did the mutation at position 2063 or 2064 of domain V. Based on our results, it is considered that transition is the predominant type of mutation in *M. pneumoniae*. This may be due to the structural difference between purine and pyrimidine. These results support the observation in *E. coli* that the apparent dissociation constant (K_d) for ERY of C2611U (corresponding to 2617 in *M. pneumoniae*) [$K_d = (4.4 \pm 0.9) \times 10^{-7}$] is ca. 480 times higher than that of the A2058G (2063 in *M. pneumoniae*) *E. coli* strain [$K_d = (1.9 \pm 0.3) \times 10^{-4}$] (3). As mentioned above, macrolide resistance of *M. pneumoniae* has been explained thus far in terms of mutation of 23S rRNA. However, *M. hominis* was associated with an absence of intracellular accumulation and ribosomal binding of macrolide antibiotics (18). These results suggest that several different mechanisms of macrolide resistance exist in *Mycoplasma* species.

Table 1 summarizes information about the patients from whom ERY-resistant *M. pneumoniae* strains were isolated. Although these patients were actually infected with ERY-resis-

tant *M. pneumoniae*, macrolides were apparently effective after their first administration in six (ERY in cases 383 and 384, CLR in case 350, and AZM in cases 377, 378, and 381) of the ten patients for whom the clinical course was known. One possible explanation may be the anti-inflammatory effects of macrolides, which inhibit the production of cytokines such as proinflammatory tumor necrosis factor alpha, interleukin-1β (IL-1β), IL-6, IL-8, and so on rather than the antimicrobial effect (1, 7, 8, 21). Much more information is available about the immunopathological mechanisms of *M. pneumoniae* pneumonia, particularly with regard to a wide variety of cytokines. Among them, Th1-type cytokines (22) and IL-8 (14) might play significant roles in the pathomechanism. In this context, recent investigations have revealed that macrolides modulate the actions of these cytokines (5, 9). It is therefore a reasonable proposition that macrolides, particularly 14- and 15-membered macrolides, exert their clinical efficacy in the treatment of *M. pneumoniae* pneumonia through immunomodulation. Our results obtained for patients with ERY-resistant *M. pneumoniae* infection strongly suggest that the beneficial effects of macrolides in the treatment of *M. pneumoniae* pneumonia are not solely due to direct antimicrobial activity and support the idea that immunomodulatory effects of macrolides play an important role in recovery from the illness.

In conclusion, we found 13 strains of macrolide-resistant *M. pneumoniae* among 76 clinical isolates obtained during the period from 2000 to 2003, despite the fact that no resistant strain was found among 296 isolates from 1983 to 1998. The predominant mutation was A2063G in domain V of 23S rRNA (10 of 13 resistant strains), and mutations involving either A2063 or A2064 resulted in high MICs to macrolide antibiotics. On the other hand, mutations involving C2617 in domain V of 23S rRNA generated less resistance to ERY than mutations involving A2063 or A2064. Our results indicate that macrolide-resistant *M. pneumoniae* is spreading in Japan, and it will be necessary to reconsider the effectiveness of macrolides in the treatment of patients with *M. pneumoniae* pneumonia.

ACKNOWLEDGMENTS

We thank Satoshi Yamada of Tonden Children's Clinic, Sapporo, Japan, and Shohei Harada of Ikeda Municipal Hospital for isolating the *M. pneumoniae* clinical strains.

This study was partly supported by a Grant for Studies on Emergency and Re-emergency Infectious Diseases (H15-Shinko-24) from the MHLW.

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JOURNAL OF BACTERIOLOGY, Oct. 2004, p. 6944–6955 Vol. 186, No. 20
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.20.6944–6955.2004
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Use of Fluorescent-Protein Tagging To Determine the Subcellular Localization of *Mycoplasma pneumoniae* Proteins Encoded by the Cytadherence Regulatory Locus

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Received 13 May 2004/Accepted 15 July 2004

Mycoplasma pneumoniae **lacks a cell wall but has internal cytoskeleton-like structures that are assumed to support the attachment organelle and asymmetric cell shape of this bacterium. To explore the fine details of the attachment organelle and the cytoskeleton-like structures, a fluorescent-protein tagging technique was applied to visualize the protein components of these structures. The focus was on the four proteins—P65, HMW2, P41, and P24—that are encoded in the *crl* operon (for "cytadherence regulatory locus"), which is known to be essential for the adherence of *M. pneumoniae* to host cells. When the P65 and HMW2 proteins were fused to enhanced yellow fluorescent protein (EYFP), a variant of green fluorescent protein, the fused proteins became localized at the attachment organelle, enabling visualization of the organelles of living cells by fluorescence microscopy. The leading end of gliding *M. pneumoniae* cells, expressing the EYFP-P65 fusion, was observed as a focus of fluorescence. On the other hand, when the P41 and P24 proteins were labeled with EYFP, the fluorescence signals of these proteins were observed at the proximal end of the attachment organelle. Coexpression of the P65 protein labeled with enhanced cyan fluorescent protein clearly showed that the sites of localization of P41 and P24 did not overlap that of P65. The localization of P41 and P24 suggested that they are also cytoskeletal proteins that function in the formation of unknown structures at the proximal end of the attachment organelle. The fluorescent-protein fusion technique may serve as a powerful tool for identifying components of cytoskeleton-like structures and the attachment organelle. It can also be used to analyze their assembly.**

Mycoplasma pneumoniae, one of the smallest self-replicating bacteria known, is a causative agent of bronchitis and primary atypical pneumonia in humans (43, 44). *M. pneumoniae* lacks a cell wall and hence has a pleomorphic cell shape. However, a majority of *M. pneumoniae* cells in cultures are filamentous and have a differentiated terminal structure at one pole. This terminal structure, the attachment organelle, is a tapered membrane protrusion responsible for the adherence of *M. pneumoniae* to host respiratory epithelium (cytadherence) (23, 24). The attachment organelle renders *M. pneumoniae* cells asymmetric and functions as a leading end for gliding motility. This organelle also may have a role in initiating cell division in *M. pneumoniae*, because the bifurcation of the attachment organelle seems to occur prior to the binary fission of *M. pneumoniae* (2, 6, 7, 25, 26, 34, 36, 48).

The attachment organelle and polar filamentous cell shape of *M. pneumoniae* are thought to be stabilized by intracellular cytoskeleton-like structures, which have been observed in electron micrographs of *M. pneumoniae* (5, 25, 33). The most remarkable architectural feature of the cytoskeleton-like structures is the electron-dense core, a rod-like structure that exists longitudinally at the center of the attachment organelle (33). This rod-like structure, measuring about 300 nm long and 80 nm thick, has a knob at the distal end (terminal button) (33, 45). A network of fibrous structures is also observed in the cytoplasm of *M. pneumoniae* (33). These cytoskeleton-like structures are major components of the Triton X-100-insoluble fraction of *M. pneumoniae* cells (Triton shell) and are thought to have a scaffold-like function upon which other cell components construct *M. pneumoniae* cells (45, 51).

A recent report indicated that the Triton X-100-insoluble fraction contains about 100 proteins, including most of the known proteins required for cytadherence (P1, B, C, HMW1, HMW2, and HMW3) (45). These cytadherence-related proteins are believed to be the main components of the attachment organelle and are encoded in three operons, designated *p1*, *hmw*, and *crl*, in the genome (24, 25). Protein P1 (encoded in the *p1* operon) is a major adhesin molecule responsible for cytadherence and is densely clustered at the surface of the attachment organelle (9, 18, 26, 48). Proteins B, C, HMW1, HMW2, and HMW3, called cytadherence accessory proteins, are not adhesin molecules but are required for the formation of functional attachment organelles (2, 3, 25). Proteins B and C, also named P90 and P40 (2, 26), are products of open reading frame 6, which exists just downstream of the *p1* gene in the *p1* operon (19). Proteins B and C associate with protein P1 at the attachment organelle and may support the proper structural configuration of P1 (29, 30). HMW1, HMW2, and HMW3 are large proteins necessary for the localization of P1

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