originated from Gram-positive bacteria such as *Staphylococcus aureus* [60]. Resistance owing to inactivation can be divided into two classes, degradation and modification. The former mechanism includes the hydrolysis of the lactone ring in 14- and 16-membered macrolides and type B streptogramin, and the latter includes phosphorylation of 14- and 16-membered macrolides, nucleotidylation of lincosamides, and acetylation of type A streptogramin.

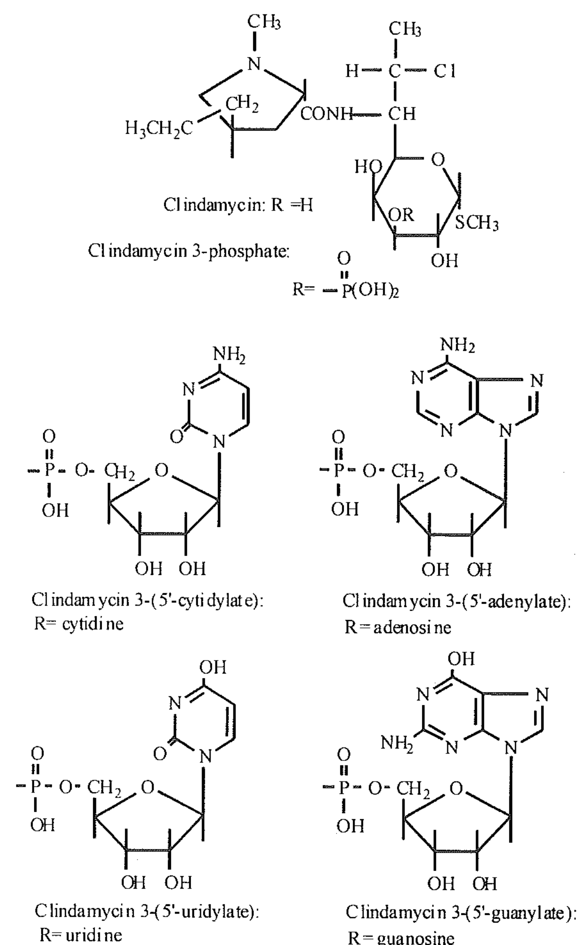

Fig. (11). Structures of clindamycin 3-phosphate and clindamycin 3-ribonucleotides [39].

This brief review outlines the biochemical mechanisms of resistance to MLS antibiotics due to inactivation in human clinical isolates (Table **5**).

1. Macrolide Antibiotics

Bacterial inactivation of macrolides, including 14-, 15-, and 16-membered macrolides, can occur through hydrolysis by erythromycin esterase A [53-55], erythromycin esterase B [57, 61, 62], phosphotransferase A [58, 63, 64], phosphotransferase B [59, 65-72], and phosphotransferase C [60, 73, 74]. Exceptionally, *Nocardia* sp. possesses multiple mechanisms for inactivation of antibiotics, that is, phosphorylation, glycosylation, reduction, and deacylation [75, 76].

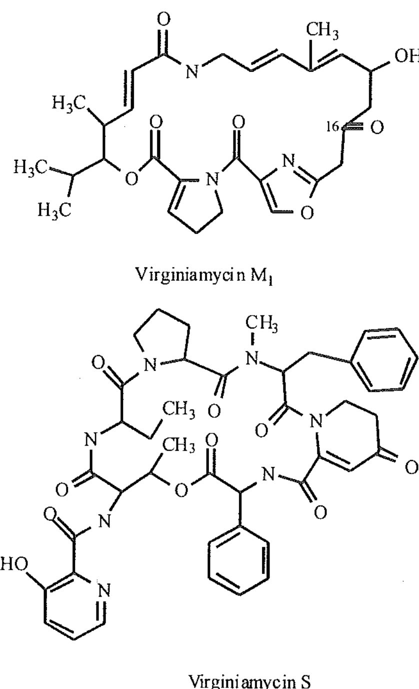

Fig. (12). Structures of virginiamycin M_1 and virginiamycin S [41].

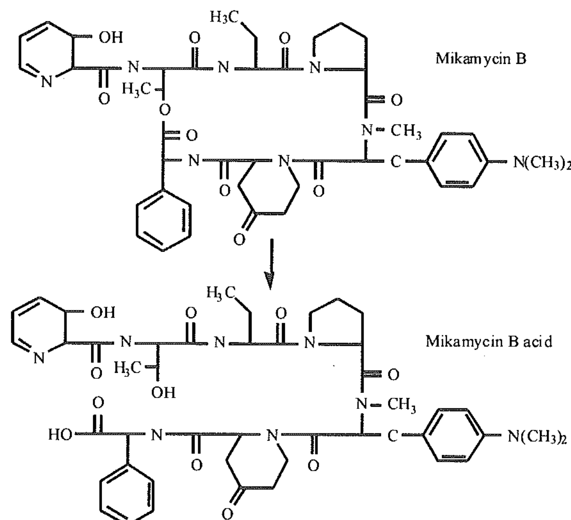

Fig. (13). Reaction of mikamycin B lactonase of *Streptomyces mitakaensis* [43].

1-1. Hydrolysis

Macrolide, lincosamide, and streptogramin (MLS) antibiotics are mainly effective against Gram-positive bacteria. On the other hand, erythromycin (EM) has been used to control the Gram-negative flora of the intestinal tract. Two genes have been identified as being associated with resistance to EM in *E. coli*. One is the *ere*(A) gene, encoding EM esterase A, generated from plasmid pIP1100 in *E. coli* BM2195. The other is the *ere*(B) gene, encoding EM

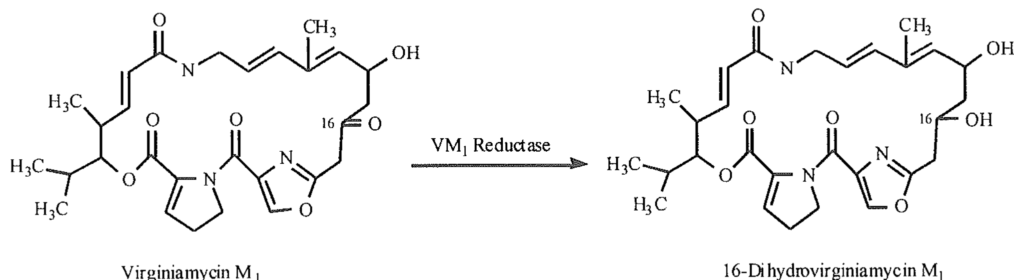

Fig. (14). Structure of 16-dihydrovirginiamycin M_1 [46].

esterase B, generated from plasmid pIP1527 in *E. coli* BM2570. The nucleotide sequence of *ere*(A) on plasmid pI1100, a self-transferable plasmid, revealed that the gene consists of 1032 bp, corresponding to a product with a molecular weight of 37,765, and has a G+C content of 50.5%. Since the G+C content of Gram-positive bacteria is 32-36%, this gene should be indigenous to *E. coli. E. coli* BM2570 was isolated from a clinical specimen in France, like *E. coli* BM2195, and was resistant to high levels of EM (MIC >2000 μg/ml) through two different mechanisms. Plasmid pI1527, a 150-kb self-transferable plasmid, in *E. coli* BM2570 carried two genes, *erxA* and *ereB*. The *erxA* gene, which is closely related to the *ermAM* gene encoding the rRNA-methylating enzyme in *Streptococci*, and encodes a single polypeptide with a molecular weight of 27,000, confers high resistance to MLS antibiotics due to dimethylation of 23S rRNA. The *ere*(B) gene product, an enzyme with a molecular weight of 51,000, inactivated EM and oleandomycin (OL) similarly to the enzyme encoded by *ere*(A) and had a G+C content of 35.9%. The low G+C content of *ere*(B) might be due to a specific codon usage in *E. coli*, suggesting an exogenous origin of *ere*(B). Inactivation products of EM generated by enzymic hydrolysis of the lactone ring due to both the *ere*(A) and *ere*(B) gene products are shown in Fig. (**15**). The two genes, *erxA* and *ere*(B), provide different resistance mechanisms to macrolide antibiotics, i.e., dimethylation of 23S rRNA and inactivation. These genes appear similar to *srmA-gimA* in *Streptomyces ambofaciens* [32], and *lrm-mgt* in *Streptomyces lividans* [12].

In recent years, the *ere*(A2) gene, a variant of *ereA* located in an integron cassette has been found in several bacteria [77-80]. Kim *et al.* [81] characterized EM esterase from *Pseudomonas* sp. and the gene encoding this enzyme

Table 5. Inactivation of MLS Antibiotics by Clinically Isolated Microorganisms.

MLS antibiotics /Resistance Profile/Organisms	Plasmid	Gene[1]	Gene[2]	Size (bp)	Protein (size[3])	GenBank	Antibiotics[4]	Reference
Macrolide								
Hydrolysis								
E. coli BM2195	pIP1100	*ereA*	*ere*(A)	1032bp	Ere(A) (344aa)	M11277	EM	[53-55, 82]
E. coli		*ereA2*	*ere*(A2)					[77]
Pseudomonas sp. GD100		*ereA2*	*ere*(A2)					[81]
E. coli BM2570	pIP1527	*ereB*	*ere*(B)	1257bp	Ere(B) (419aa)	X03988	EM, OL	[57, 61, 82]
Phosphorylation								
E. coli Tf481A		*mphA*	*mph*(A)	903bp	Mph(A) (301aa)	D16251	OL, EM, SPCM MDM, JM, LCM	[58, 63, 64]
E. coli 209K	pSK66	*mphK*	*mph*(A)	918bp	Mph(A) (306aa)	U36578	14-, 16-mac	[83]
E. coli BM2506		*mphB*	*mph*(B)	906bp	Mph(B) (202aa)	D85892	14-, 16-mac	[59, 65-72, 84]
S. aureus	pMS97	*mphBM*	*mph*(C)	897bp	MphBM (299aa)	AB013298	14-, 15-, 16-mac EM	[60, 73]
S. aureus 01A1032	pSR1	*mphBM*	*mph*(C)	897bp		AF167161		
Stenotrophomonas maltophilia D457		*mphBM*	*mph*(C)	897bp		AJ251015		[74]

(Table 5. Contd....)

MLS antibiotics /Resistance Profile/Organisms	Plasmid	Gene[1]	Gene[2]	Size (bp)	Protein (size[3])	GenBank	Antibiotics [5]	Reference
Phosphorylation, Glycosylation, Reduction, Deacylation								
Nocardia sp.							EM, RKM, MDM CHA, TL	[75, 76]
Lincosamide								
Nucleotidylation								
S. haemolyticus BM4610	pIP855	*linA*	*lnu*(A)	483bp	LNT(3)(4) (161aa)	M14039	LCM	[85, 86, 88]
S. aureus BM4611		*linA'*	*lnu*(A)	483bp	LNT(3)(4) (161aa)	J03497	LCM, CLDM	[87, 88]
E. faecium HM1025		*linB*	*lnu*(B)	801bp	LinB (267aa)	AF110130	LCM, CLDM	[89]
Streptogramin								
Hydrolysis								
S. aureus STE						PH IA	PRI-1	[90]
S. aureus BM3041	pIP630	*vgb*	*vgb*(A)	897bp	Vgb(A) (298aa)	M20129	VIR-B	[91, 105]
S. cohnii BM1071	pIP1714	*vgbB*	*vgb*(B)	885bp	Vgb(B) (295aa)	AF015628	STG-B	[92]
Acetylation								
S. aureus 71							VIR-M	[95]
S. *aureus* STE					PAC (IIA)		PRI II-A	[96]
S. aureus	pI680	*vat*	*vat*(A)	657bp	VAT (219aa)	L07778	VER-A	[97]
S. aureus BM3385	pIP1633	*vatB*	*vat*(B)	636bp	VatB (212aa)	L38809	STG-A	[98]
S. cohnii BM10711	pIP1714	*vatC*	*vat*(C)	636bp	VatC (212aa)	AF015628	STG-A	[92]
E. faecium BM4145		*satA*	*vat*(D)	627bp	SatA (209aa)	L12033	STG-A	[99, 105]
E. faecium UW1965		*satG*	*vat*(E)	642bp	SatG (214aa)	AF139725	STG-A	[101, 102]
E. faecium		*vat*(E 1-8)[5]		642bp		AF139735	Q-D	[103]

[1] The gene name is based on the original name.
[2] The gene name follows the nomenclature by Roberts M. C. *et al.* [48]
[3] Number of amino acid (aa) is shown in parentheses.
[4] *E. faecium* generates a series of *vat* genes, *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), and they were deposited as accession numbers AF153312, AY008284, AY043211, AY43209, AY043210, AY043212, and AY043213, respectively.
[5] See Table 1 for abbreviations other than SPCM, spectinomycin; MDM, midecamycin; CHA, charcomycin; STG, streptogramin; Q-D, quinupristin-dalfopristin; mac, macrolide antibiotics.

was similar to the *ere*(A2) gene from *Providencia stuartii* plasmid PLQ1723 [80]. A comparison of EM esterase genes, including *ere*(A), *ere*(A2) and *ere*(B), is shown in Fig. (**16**) and Table **6**. As expected, *ere*(A) and *ere*(A2) showed a high degree of identity in terms of both DNA and amino acid sequences, but *ere*(A) and *ere*(A2) showed relatively low similarity to *ere*(B). EM esterases include *ere*(A) and *ere*(B) appear to be disseminating independently among *Escherichia* sp. *Staphylococcus* sp. and *Pseudomonas* sp. although they are also found together [82].

1-2. Phosphorylation

Inactivation due to phosphorylation of macrolide, lincosamide, and streptogramin (MLS) antibiotics in clinical pathogens was first found by O'Hara *et al.* [63] in *E. coli* with high-level resistance to erythromycin (EM) (MIC >3200 μg/ml), and was similar to the phosphorylation at the 2'-OH group of 14- and 16-membered macrolides in *Streptomyces coelicolor* [19, 20]. Two years later, a similar phosphotransferase gene was discovered in a Gram-positive bacterium, *Staphylococcus aureus*; it was located between two other genes conferring resistance to MLS antibiotics, i.e., *msr*(A), which is related to antibiotic efflux, and *erm*(Y), which is related to methylation of 23S rRNA [73].

To date, three phosphotransferase genes, *mph*(A), *mph*(B), and *mph*(C), have been reported. The *mph*(A) gene, encoding macrolide 2'-OH phosphotransferase I, was found in *E. coli* Tf481. It consists of 903 bp [58] and encodes the inducible enzyme MPH(2')I, which inactivates 14-membered macrolides in the presence of ATP and Mg^{2+} [63, 64] to 2'-OH phosphoryl macrolide antibiotics, as seen in *Streptomyces coelicolor*(Table **1**). *E. coli* 209K harboring the plasmid pSK66 was isolated in Korea [83] and was highly

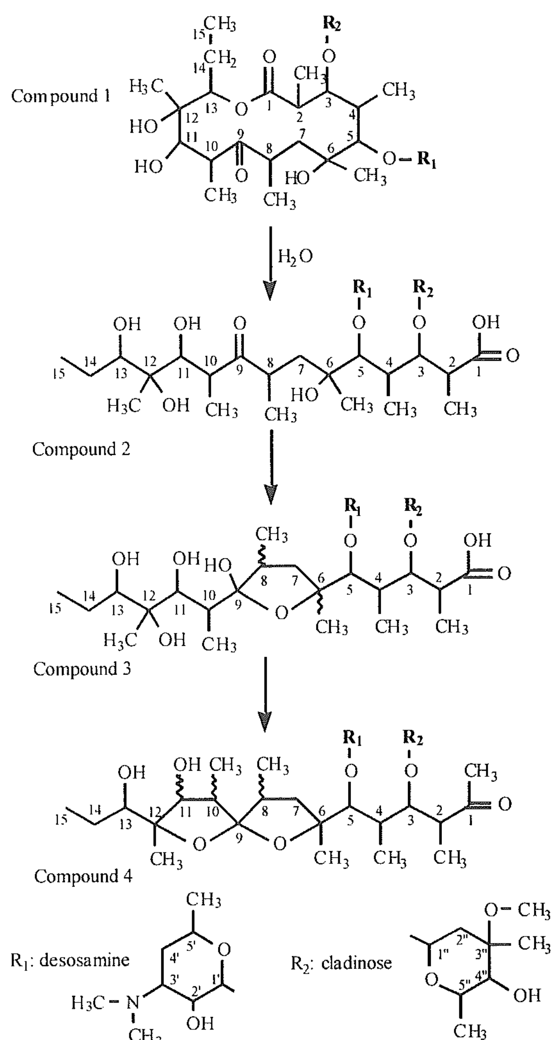

Fig. (15). Reaction catalyzed by erythromycin esterase A and B. Enzymatic hydrolysis of the lactone ring of erythromycin is followed by the formation of an hemiketal by internal condensation and dehydration. Compound 4 is the major end product of detoxification of erythromycin by resting cells at pH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

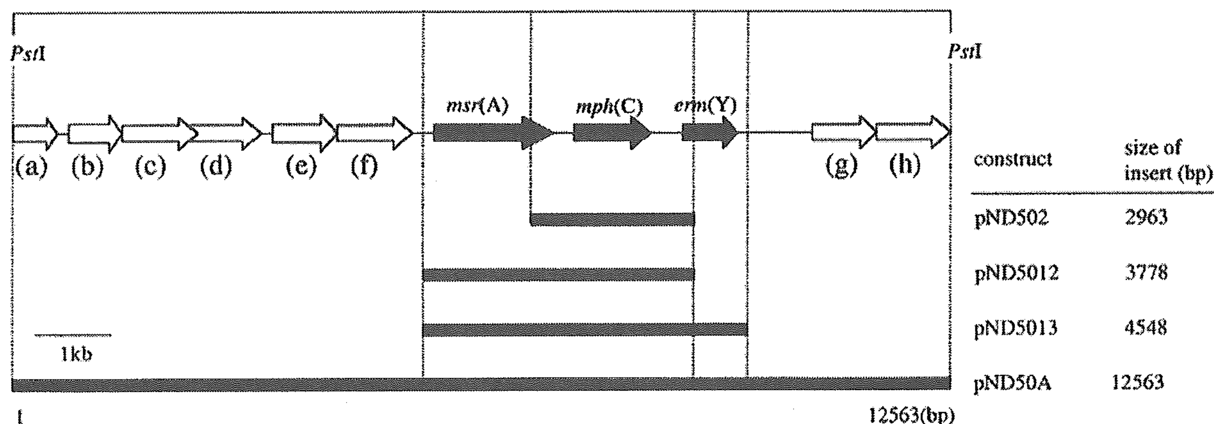

Fig. (17). Schematic representation of constructs containing fragments of genes coding for resistance to macrolides. The constructs pND502, pND5012, pND5013 and pND50A contain *mph*(C), *msr*(A)-*mph*(C), *msr*(A)-*mph*(C)-*erm*(Y), and *msr*(A)-*mph*(C)-*erm*(Y) including the extra region, respectively. 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 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.

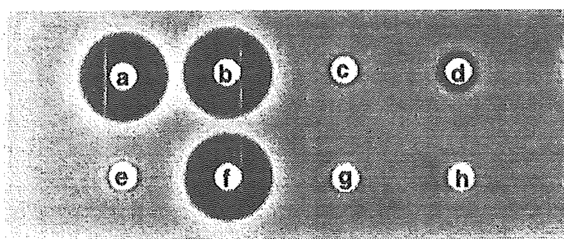

Fig. (18). Inactivation assay of erythromycin (EM) with several *Staphylococcus aureus* strains. EM activity after 24 h incubation with *S. aureus* in the presence of the drug was assayed by the disk plate method using *Micrococcus luteus* ATCC9341 as an indicator microorganism. The residual EM activity 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).

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)

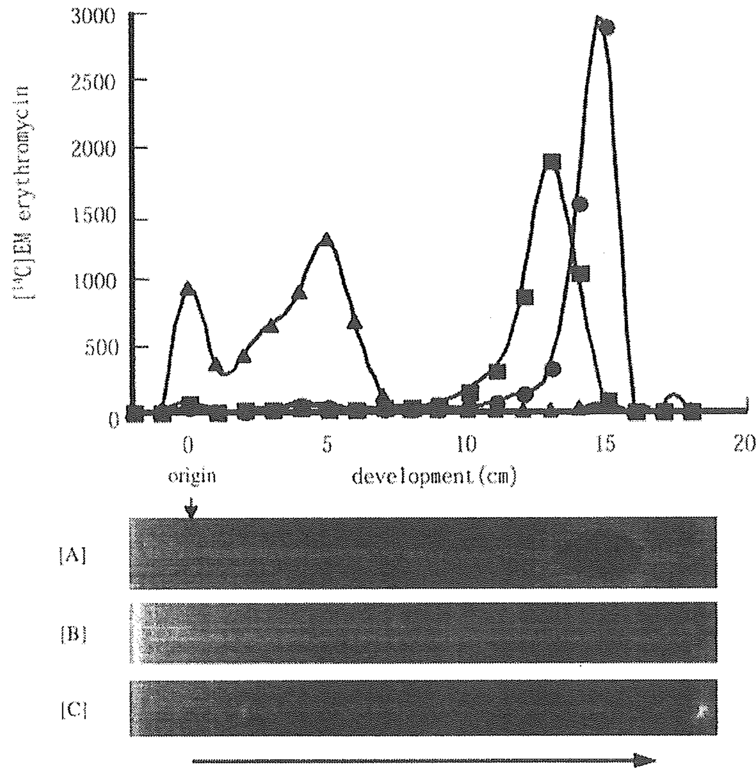

Fig. (19). Radioautogram and bioautogram of [^{14}C]erythromycin (EM). [^{14}C] EM was incubated for 63 h at 37°C with *E. coli* BM694(pAT63) (closed square and [B]) or *Staphylococcus aureus* 8325(pMS97) (closed triangle and [C]). The arrow indicates the direction of development with a solvent system 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
            ***********************************************************
```

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).

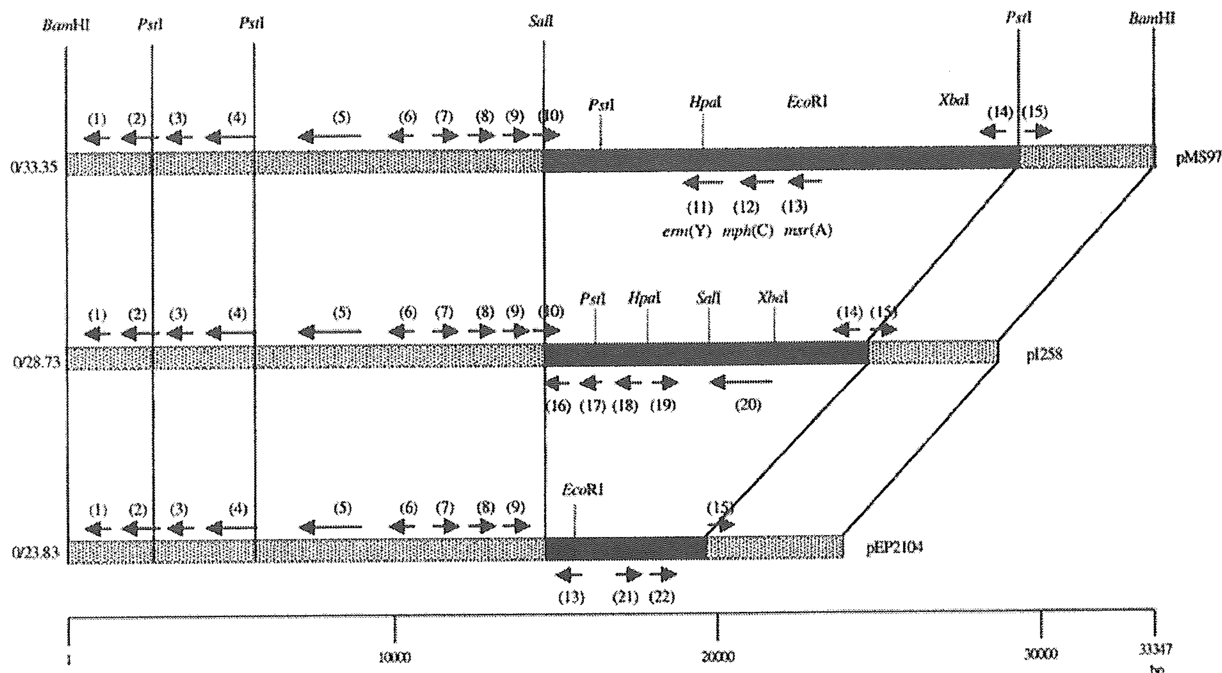

Fig. (21). Genetic maps of macrolide resistance plasmids, pMS97 (accession number AB179623), pI258 [112] and pEP2104 (accession numberAB016613) from *Staphylococcus aureus*. Genes are indicated by the following numbers: (1), *merB*; (2), *merA*; (3), *merT*; (4), *merR*; (5), *cadA*; (6), *blaZ*; (7) *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).

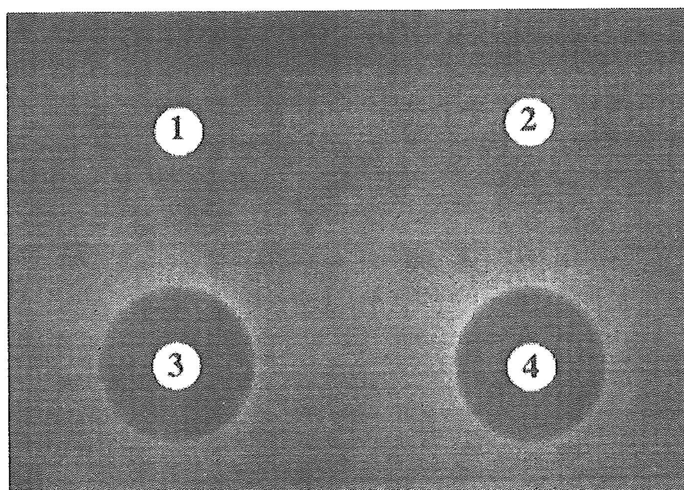

Fig. (22). Inactivation assay for telithromycin. Disks contained incubation supernatant of bacteria and telithromycin as follows: *Staphylococcus aureus* 8325(pMS97) encoding *mph*(C) (disk 1), *S. aureus* 8325(pMS97) induced by EM (disk 2), *E. coli* BM694/pAT63 encoding *ere*(A) (disk 3), and 800 ng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

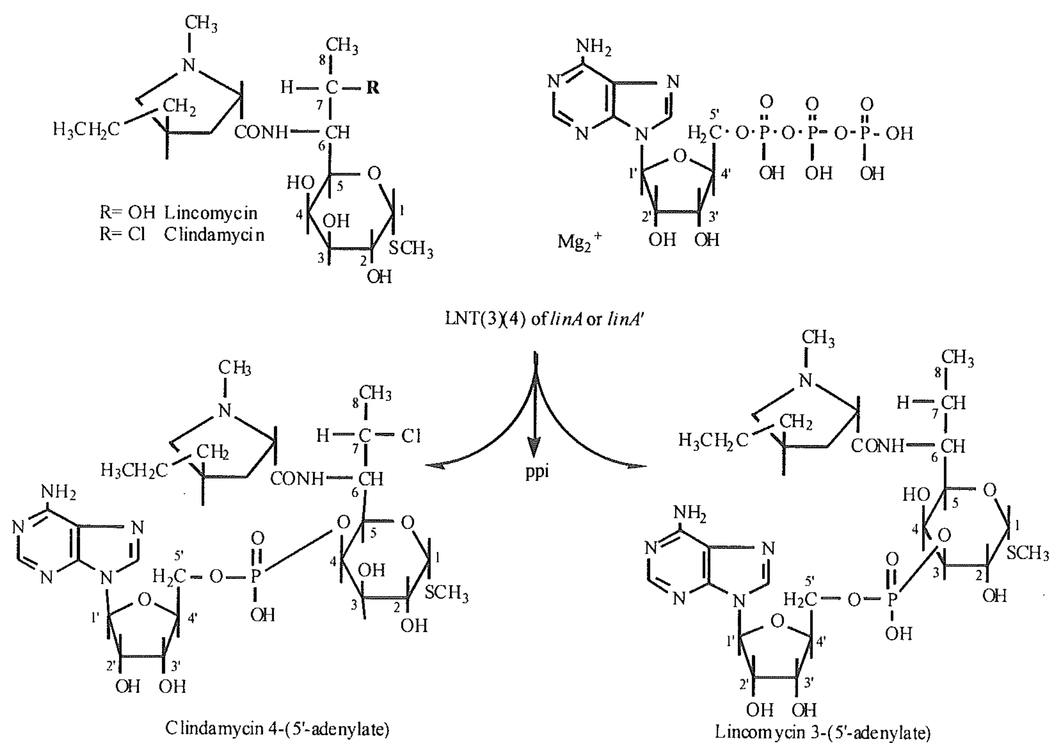

Fig. (23). Adenylation of lincosamides by *Staphylococcus haemolyticus* BM4610 and *Staphylococcus aureus* BM4611. LNT (3)(4); 3-lincomycin, 4-clindamycin nucleotidyl transferase [88].

```
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
                     ***** ****************** *** ************
```

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).

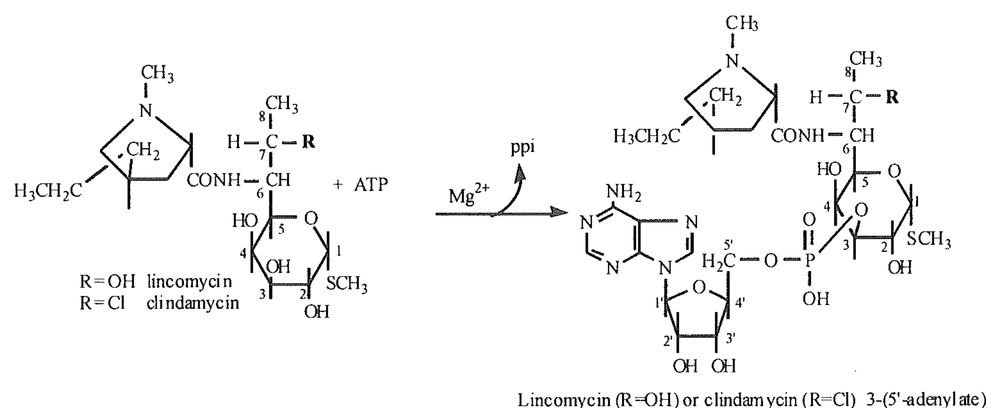

Fig. (25). Adenylation of lincomycin and clindamycin by *linB* gene product of *Enterococcus faecium* HM1025 [89].

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

```
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.

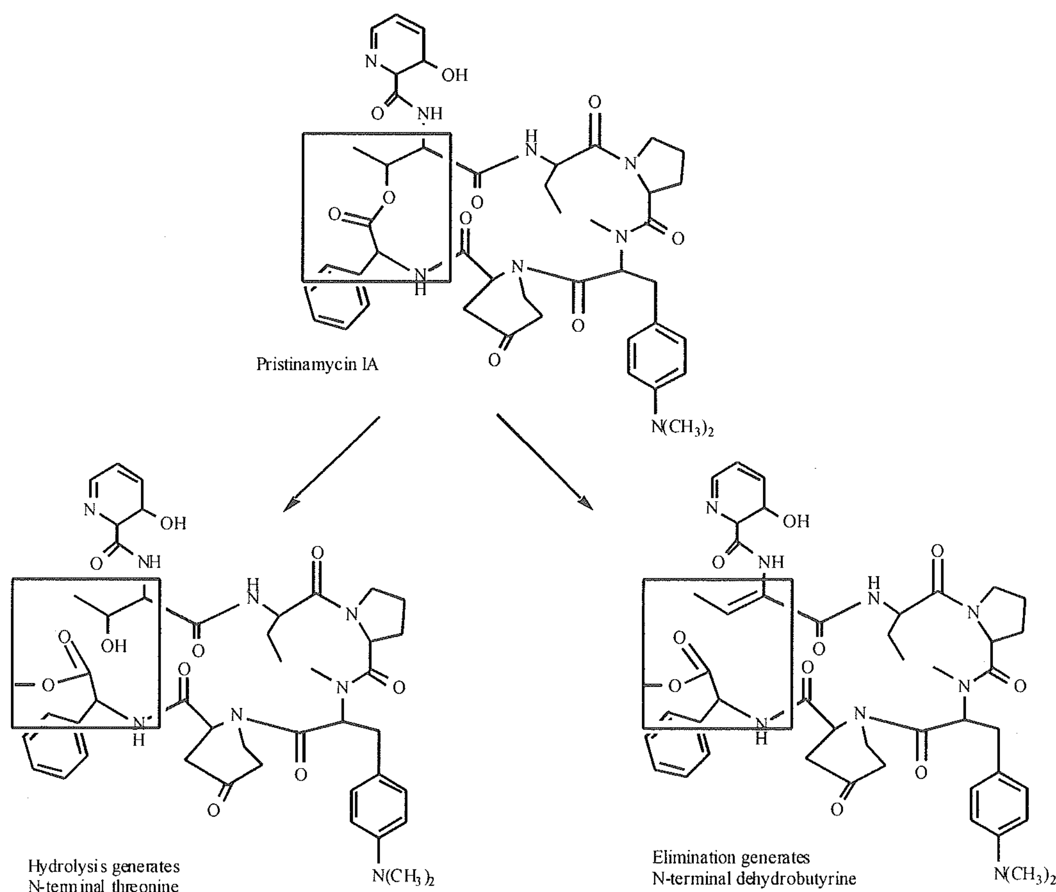

Fig. (27). Structures of possible products of lactone cleavage of pristinamycin IA [93].

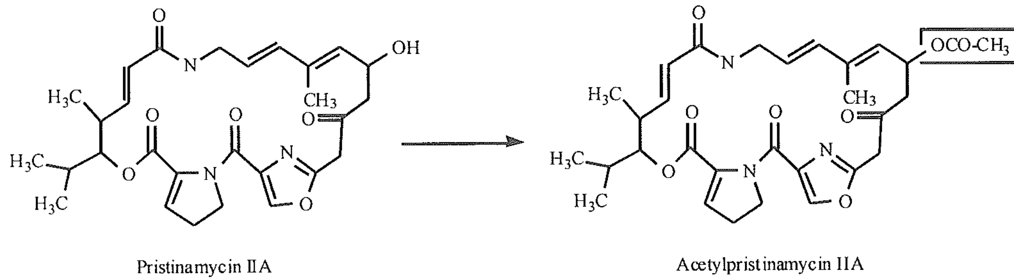

Fig. (28). Acetylation of pristinamycin IIA [96].

```
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].

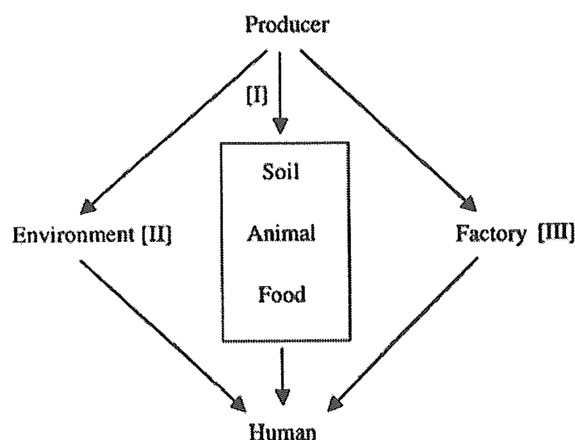

Fig. (31). Proposed pathway [I] 106, 107, 109, 111], pathway [II] [92], and pathway [III] [95] of transfer of resistance genes from producers to humans.

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

REFERENCES

[1] Skinner, R.H. *J. Gen. Microbiol.*, **1982**, *128*, 2411.
[2] Zalacain, M.; Cundliffe, E. *J. Bacteriol.*, **1989**, *171*, 4254.
[3] Zalacain, M.; Cundliffe, E. *Gene*, **1991**, *97*, 137.
[4] Zalacain, M.; Cundliffe, E. *Eur. J. Biochem.*, **1990**, *189*, 67.
[5] Rosteck, P.R. Jr.; Reynolds, P.A.; Hersberger, C.L. *Gene*, **1991**, *102*, 27.
[6] Schoner, B.; Geistlich, M.; Rosteck, P. Jr.; Rao, R.N.; Seno, E.; Reynolds, P.; Cox, K.; Burgett, S.; Hershberger, C. *Gene*, **1992**, *115*, 93.
[7] Rodriguez, A.M.; Olano, C.; Vilches, C.; Mendez, C.; Salas, J.A. *Mol. Microbiol.*, **1993**, *8*, 571.
[8] Olano, C; Rodriguez, A.M.; Mendez, C.; Salas, J.A. *Mol. Microbiol.*, **1995**, *16*, 333.
[9] Vilches, C.; Hernandez, C.; Mendez, C.; Salas, J.A. *J. Bacteriol.*, **1992**, *174*, 161.
[10] Quiros, L.M.; Salas, J.A. *J. Biol. Chem.*, **1995**, *270*, 18234.
[11] Quiros, L.M.; Hernandez, C.; Salas, J.A. *Eur. J. Biochem.*, **1994**, *222*, 129.
[12] Jenkins, G.; Cundliffe, E. *Gene.*, **1991**, *108*, 55.
[13] Sasaki, J.; Mizoue, K.; Morimoto, S.; Omura, S. *J. Antibiot.*, **1996**, *49*,1110.
[14] Feldman, L.I.; Dill, I.K.; Holmlund, C.E.; Whaley, H.A; Paterson, E.L.; Bohonos, N. *Antimicrob. Agents Chemother.*, **1963**, *161*, 54.
[15] Flickinger, M.C.; Perlman, D. *J. Antibiot.*, **1975**, *28*, 307.
[16] Nakahama, K.; Izawa, M.; Muroi, M.; Kishi, T.; Uchida, M.; Igarashi, S. *J. Antibiot.*, **1974**, *27*, 425.
[17] Nakahama, K.; Kishi, T.; Igarasi, S. *J. Antibiot.*, **1974**, *27*, 433.
[18] Nakahama, K.; Kishi, T.; Igarasi, S. *J. Antibiot.*, **1974**, *27*, 487.
[19] Wiley, P.F.; Baczynskyj, L.; Dolak, L.A.; Cialdella, J.I.; Marshall, V.P. *J. Antibiot.*, **1987**, *40*, 195.
[20] Marshall, V.P.; Cialdella, J.I.; Baczynskyj, L.; Liggett, W.F.; Johnson, R.A. *J. Antibiot.*, **1989**, *42*, 132.
[21] Kuo, M.S.; Chirby, D.G.; Argoudelis, A.D.; Cialdella, J.I.; Coats, J.H.; Marshall, V.P. *Antimicrob. Agents Chemother.*, **1989**, *33*, 2089.
[22] Cundliffe, E. *Gene*, **1992**, *115*, 75.
[23] Cundliffe, E. *Antimicrob. Agents Chemother.*, **1992**, *36*, 348.
[24] Hernandez, C.; Olano, C.; Mendez, C.; Salas, J.A. *Gene*, **1993**, *134*, 139.
[25] Salas, J.A.: Hernandez, C.; Mendez, C.; Olano, C.; Quiros L.M.; Rodriguez, A.M.; Vilches, C. *Microbiologia*. **1994**, *10*, 37.
[26] Aparicio, G.; Buche, A.; Mendez, C.; Salas, J.A. *FEMS Microbiol. Lett.* **1996**, *141*, 157.
[27] Olano, C.; Rodriguez, A.M.; Mendez, C.; Salas, J.A. *Mol. Microbiol.* **1995**, *16*, 333.
[28] Walsh, C. *Antibiotics-actions, origins, resistance*; ASM press: Washington, D. C., **2003**; pp. 89-234.
[29] Quiros, L.M.; Aguirrezabalaga, I.; Olano, C.; Mendez, C.; Salas, J.A. *Mol. Microbiol.*, **1998**, *28*, 1177.
[30] Quiros, L.M.; Carbajo, R.J.; Salas, J.A. *FEBS Lett.*, **2000**, *476*, 186.
[31] Schulman, M.; Doherty, P.; Arison, B. *Antimicrob. Agents Chemother.*, **1993**, *37*, 1737.
[32] Gourmelen, A.; Blondelet-Rouault, M.H.; Pernodet, J.L. *Antimicrob. Agents Chemother.*, **1998**, *42*, 2612.
[33] Fierro, J.F.; Hardisson, C.; Salas, J.A. *J. Gen. Microbiol.*, **1987**, *133*, 1931.
[34] Argoudelis, A.D.; Coats, J.H.; Mason, D.J.; Sebek, O.K. *J. Antibiotics*, **1969**, *22*, 309.
[35] Argoudelis, A.D.; Mason, D.J. *J. Antibiotics*, **1969**, *22*, 289.
[36] Argoudelis, A.D.; Coats, J.H. *J. Antibiot.*, **1969**, *22*, 341.
[37] Coats, J.H.; Argoudelis, A.D. *J. Bacteriol.*, **1971**, *108*, 459.
[38] Argoudelis, A.D.; Coats, J.H. *J. Am. Chem. Soc.*, **1971**, *93*, 534.
[39] Argoudelis, A.D.; Coats, J.H.; Mizsak, S.A. *J. Antibiot.*, **1977**, *30*, 474.
[40] Marshall, V.P.; Patt, T.E.; Argoudelis, A.D. *J. Ind. Microbiol.*, **1986**, *1*, 17.
[41] Lee, C.K.; Minami, M.; Sakuda, S.; Nihira, T.; Yamada, Y. *Antimicrob. Agents Chemother.*, **1996**, *40*, 595.

[42] Hou, C.T.; Perlman, D.; Schallock, M.R. *J. Antibiot.*, **1970**, *23*, 35.
[43] Kim, C.H.; Otake, N.; Yonehara, H. *J. Antibiot.*, **1974**, *27*, 903.
[44] Kim, C.H.; Endo, T.; Yonehara, H. *J. Antibiot.*, **1988**, *41*, 73.
[45] Fernando Fierro, J.; Vilches, C.; Hardisson, C.; Salas, J.A. *FEMS Microbiol. Lett.*, **1989**, *58*, 243.
[46] Suzuki, N.; Lee, C.K.; Nihira, T.; Yamada, Y. *Antimicrob. Agents Chemother.*, **1998**, *42*, 2985.
[47] Nakajima Y. *J. Infect. Chemother.* **1999**, *5*, 61.
[48] Roberts, M.C.; Sutcliffe, J.; Courvalin, P.; Jensen, L.B.; Rood, J.; Seppala, H. *Antimicrob. Agents Chemother.*, **1999**, *43*, 2823.
[49] Leclercq, R. *Clin. Infect. Dis.*, **2002**, *34*, 482.
[50] Weisblum, B. *Drug Resistance Updates*, **1998**, *1*, 29.
[51] Leclercq, R.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1991**, *35*, 1273.
[52] Arthur, M.; Brisson-Noel, A.; Courvalin, P. *J. Antimicrob. Chemother.*, **1987**, *20*, 783.
[53] Barthelemy, P.; Autissier, D.; Gerbaud, G.; Courvalin, P. *J. Antibiot.*, **1984**, *37*, 1692.
[54] Ounissi, H.; Courvalin, P. *Gene*, **1985**, *35*, 271.
[55] Andremont, A.; Gerbaud, G.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1986**, *29*, 515.
[56] Biskri, L.; Mazel, D. *Antimicrob. Agents Chemother.*, **2003**, *47*, 3326.
[57] Arthur, M.; Autissier, D.; Courvalin, P. *Nucleic Acids Res.*, **1986**, *14*, 4987.
[58] Noguchi, N.; Emura, A.; Matsuyama, H.; O'Hara, K.; Sasatsu, M.; Kono, M. *Antimicrob. Agents Chemother.*, **1995**, *39*, 2359.
[59] Noguchi, N.; Katayama, J.; O'Hara, K. *FEMS Microbiol. Lett.*, **1996**, *144*, 197.
[60] Matsuoka, M.; Endou, K.; Kobayashi, H.; Inoue, M.; Nakajima, Y. *FEMS Microbiol. Lett.*, **1998**, *167*, 221.
[61] Arthur, M.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1986**, *30*, 694.
[62] Wondrack, L.; Massa, M.; Yang, B.V.; Sutcliffe, J. *Antimicrob. Agents Chemother.*, **1996**, *40*, 992.
[63] O'Hara, K.; Kanda, T.; Kono, M. *J. Antibiot.*, **1988**, *41*, 823.
[64] O'Hara, K.; Kanda, T.; Ohmiya, K.; Ebisu, T.; Kono, M. *Antimicrob. Agents Chemother.*, **1989**, *33*, 1354.
[65] Kono, M.; O'Hara, K.; Ebisu, T. *FEMS Microbiol. Lett.*, **1992**, *97*, 89.
[66] O'Hara, K.; Yamamoto, K. *Antimicrob. Agents Chemother.*, **1996**, *40*, 1036.
[67] Noguchi, N.; Tamura, Y.; Katayama, J.; Narui K. *FEMS Microbiol. Lett.*, **1998**, *159*, 337.
[68] Katayama, J.; Okada, H.; O'Hara, K.; Noguchi, N. *Biol. Pharm. Bull.*, **1998**, *21*, 326.
[69] Katayama, J.; Noguchi, N. *Biol. Pharm. Bull.*, **1999**, *22*, 227.
[70] Taniguchi, K.; Nakamura, A.; Tsurubuchi, K.; Ishii, A.; O'Hara, K.; Sawai, T. *Antimicrob. Agents Chemother.*, **1999**, *43*, 2063.
[71] Taniguchi, K.; Nakamura, A.; Tsurubuchi, K.; Ishii, A.; O'Hara, K.; Sawai, T. *Microbios.*, **1999**, *97*, 137.
[72] Noguchi, N.; Katayama, J.; Sasatsu, M. *FEMS Microbiol. Lett.*, **2000**, *192*, 175.
[73] Matsuoka, M.; Inoue, M.; Endo, Y.; Nakajima, Y. *FEMS Microbiol. Lett.*, **2003**, *220*, 287.
[74] Alonso, A.; Sanchez, P.; Martinez, J.L. *Antimicrob. Agents Chemother.*, **2000**, *44*, 1778.
[75] Yazawa, K.; Mikami, Y.; Sakamoto, T.; Ueno, Y.; Morisaki, N.; Iwasaki, S.; Furihata, K. *Antimicrob. Agents Chemother.*, **1994**, *38*, 2197.
[76] Morisaki, N.; Hashimoto, Y. *J. Antibiot.*, **2001**, *54*, 157.
[77] Chang, C.Y.; Chang, L.L.; Chang, Y.H.; Lee, T.M.; Chang, S.F. *J. Med. Microbiol.*, **2000**, *49*, 1097.
[78] Peters, E.D.; Leverstein-van Hall, M.A.; Box, A.T.; Verhoef, J.; Fluit, A.C. *Antimicrob. Agents Chemother.*, **2001**, *45*, 2961.
[79] Thungapathra, M.; Amita; Sinha, K.K.; Chaudhuri, S.R.; Garg, P.; Ramamurthy, T.; Nair, G.B.; Ghosh, A. *Antimicrob. Agents Chemother.*, **2002**, *46*, 2948.
[80] Plante, I.; Centron, D.; Roy, P.H. *J. Antimicrob. Chemother.*, **2003**, *51*, 787.
[81] Kim, Y.H.; Cha, C.J.; Cerniglia, C.E. *FEMS Microbiol. Lett.*, **2002**, *210*, 239.
[82] Arthur, M.; Andremont, A.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1987**, *31*, 404.
[83] Kim, S.K.; Baek, M.C.; Choi, S.S.; Kim, B.K.; Choi, E.C. *Mol. Cells*, **1996**, *6*, 153.
[84] Nakamura, A.; Nakazawa, K.; Miyakozawa, I.; Mizukoshi, S.; Tsurubuchi, K.; Nakagawa, M.; O'Hara, K.; Sawai, T. *J. Antibiot.*, **2000**, *53*, 516.
[85] Leclercq, R.; Carlier, C.; Duval, J.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1985**, *28*, 421.
[86] Brisson-Noel, A.; Courvalin, P. *Gene.* **1986**, *43*, 247.
[87] Leclercq, R.; Brisson-Noel, A.; Duval, J.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1987**, *31*, 1887.
[88] Brisson-Noel, A.; Delrieu, P.; Samain, D.; Courvalin, P. *J. Biol. Chem.*, **1988**, *263*, 15880.
[89] Bozdogan, B.; Berrezouga, L.; Kuo, M.S.; Yurek, D.A.; Farley, K.A.; Stockman, B.J.; Leclercq, R. *Antimicrob. Agents Chemother.*, **1999**, *43*, 925.
[90] Le Goffic, F.; Capmau, M.L; Abbe, J.; Cerceau, C.; Dublanchet, A.; Duval, J. *Ann. Microbiol.* **1977**, *128B*, 471.
[91] Allignet, J.; Loncle, V.; Mazodier, P.; El Solh, N. *Plasmid.*, **1988**, *20*, 271.
[92] Allignet, J.; Liassine, N.; El Solh, N. *Antimicrob. Agents Chemother.*, **1998**, *42*, 1794.
[93] Mukhtar, T.A.; Koteva, K.P.; Hughes, D.W.; Wright, G.D. *Biochemistry*, **2001**, *40*, 8877.
[94] Bateman, K.P.; Thibault, P.; Yang, K.; White, R.L.; Vining, L.C. *J. Mass Spectrom.*, **1997**, *32*, 1057.
[95] De Meester, C.; Rondelet, J. *J. Antibiot.*, **1976**, *29*, 1297.
[96] Le Goffic, F.; Capmau, M.L.; Bonnet, D.; Cerceau, C.; Soussy, C.; Dublanchet, A.; Duval, J. *J. Antibiot.*, **1977**, *30*, 665.
[97] Allignet, J.; Loncle, V.; Simenel, C.; Delepierre, M.; El Solh, N. *Gene*, **1993**, *130*, 91.
[98] Allignet, J.; El Solh, N. *Antimicrob. Agents Chemother.*, **1995**, *39*, 2027.
[99] Rende-Fournier, R.; Leclercq, R.; Galimand, M.; Duval, J.; Courvalin, P. *Antimicrob. Agents Chemother.*, **1993**, *37*, 2119.
[100] Hammerum, A.M.; Jensen, L.B.; Aarestrup, F.M. *FEMS Microbiol. Lett.* **1998**, *168*, 145.
[101] Werner, G.; Witte, W. *Antimicrob. Agents Chemother.*, **1999**, *43*, 1813.
[102] Simjee, S.; White, D.G.; Meng, J.; Wagner, D.D.; Qaiyumi, S.; Zhao, S.; Hayes, J.R.; McDermott, P.F. *J. Antimicrob. Chemother.*, **2002**, *50*, 877.
[103] Soltani, M.; Beighton, D.; Philpott-Howard, J.; Woodford, N. *Antimicrob. Agents Chemother.*, **2001**, *45*, 645.
[104] Simjee, S.; McDermott, P.F.; Wagner, D.D.; White, D.G. *Antimicrob. Agents Chemother.* **2001**, *45*, 2931.
[105] Jensen, L.B.; Hammerum, A.M.; Aerestrup, F.M.; van den Bogaard, A.E.; Stobberingh, E.E. *Antimicrob. Agents Chemother.*, **1998**, *42*, 3330.
[106] Dutta, G.N.; Devriese, L.A. *Ann. Microbiol.*, **1981**, *132A*, 51.
[107] Dutta, G.N.; Devriese, L.A. *J. Appl. Bacteriol.*, **1981**, *51*, 283.
[108] Devriese, L.A.; Gutta, G.N. *J. Vet. Pharmacol. Therap.*, **1984**, *7*, 49.
[109] Devriese, L.A. *Ann. Microbiol.*, **1980**, *131B*, 261.
[110] Dutta, G.N.; Devriese, L.A. *J. Appl. Bacteriol.*, **1981**, *51*, 283.
[111] Onan, L.J.; LaPara, T.M. *FEMS Microbiol. Lett.* **2003**, *220*, 15.
[112] Murphy, E.; Novick, R.P. *J. Bacteriol.* **1980**. *141*. 316.

Analysis of major antigens of *Mycoplasma penetrans* by using proteomics: development of a new ELISA system for diagnosis.

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Keywords : *Mycoplasma penetrans,* proteomics, antigen

Abstract

Major antigens of *M. penetrans* were identified by proteomics analysis. Since the antigenicity of lipid-associated membrane proteins (LAMPs) that have been used as antigens for serological diagnosis is predicted to be changeable based on *p35* paralog gene family for lipoproteins, major antigens except lipoproteins were identified. Major proteins reacted with anti-*M. penetrans* sera were components of pyruvate dehydrogenase (PDH). An ELISA system using a recombinant protein for partial *pdh-c*, that encodes PDH-E2, clearly distinguished sera of *M. penetrans*-LAMPs positive individuals from that of healthy donors by its OD value. With no significant cross reactivity of the recombinant PDH-E2 antigen to hyperimmunized rabbit sera for several human-related mycoplasma species, this recombinant protein could be a useful tool for serological diagnosis of *M. penetrans* infection.

Introduction

M. penetrans has been isolated from both genital and respiratory tracts in humans (1-4). A systemic illness with respiratory distress requiring intensive care caused by *M. penetrans* infection in previously healthy young woman has also reported. In that case, rapid diagnosis for *M. penetrans* infection made a suitable selection of antibiotics, and the patient was cured (5). In diagnosis of *M. penetrans* infection, lipid-associated membrane proteins (LAMPs) are used as antigens for serological test. In the *M. penetrans* genome we analyzed, there is a paralog gene family, the *p35* gene family consisting from 38 genes, encoding antigenic lipoproteins that are major components of the LAMPs (6). Of the 38, only two genes are expressed as lipoproteins at one time in some condition (7), and the expression of the *p35* paralog genes is thought to be random. The antigenicity of the 38 genes is probably different because of low homology of amino acid sequence from 34 to 70%. From this genomic construction, it is predicted that *M. penetrans* has a sophisticated antigenic variation system for lipoproteins, and antigenicity of the LAMPs is not constant. In this study, in order to establish a new serological diagnostic tool instead of LAMPs, we analyzed major antigens of *M. penetrans*.

Materials and methods

M. penetrans HF-2 strain isolated from the patient with severe respiratory distress was used. *M. penetrans* HF-2 strain was cultivated in PPLO broth (Becton Dickinson Microbiology Systems) supplemented with 10% (v/v) heat-inactivated horse serum (GIBCO/BRL), 0.5% glucose, 0.002% of phenol red and 100 U/ml of penicillin G (Meiji Seika Kaisya, Japan). Harvested mycoplasma cells by centrifugation (8000 x g for 20 minutes) were washed with phosphate buffered saline (PBS) three times and suspended in Destreak Rehydration Solution ™ (Amersham Biosciences) after demineralization. Mycoplasma proteins developed by both SDS-PAGE and two-dimensional electrophoresis were visualized by staining with either cumassie brilliant blue (CBB) or silver for proteomics analysis (Invitrogen). Protein spots digested as peptides by trypsin were separated on a MAGIC 2002 system. The elutant was analyzed by an ion trap mass spectrometer, LCQ deca XP (Thermo Electron Bioresources). To identify peptides from MS/MS scans, data files were generated by program Bioworks, version 3.0 using the SEQUEST algorithm (Thermo Electron corp), and searched against the complete *M. penetrans* amino acid database derived from its genome database. Mycoplasma cell proteins developed by electrophoresis were also transferred to nitrocellulose membrane, then the membrane was reacted with anti-*M. penetrans* mouse sera by the method of the Western blotting.

In order to producing a recombinant protein for *pdh-c,* a

partial region of the gene amplified by PCR, including non conserved region in the genes of *M. penetrans, M. pneumoniae* and *Ureaplasma parvum,* was cloned into pDONR™. Then, the vector was changed to pDEST17™ expression vector which has His-tag at its N-terminal (Gateway system, Invitrogen). After transformation, induced product with alabinose in *Eschericia coli* was purified by His-tag column under the existence of urea.

Antibody against both *M. penetrans* whole cells and the recombinant protein were obtained from BALB/c mice after immunization. Hyperimmunized rabbit sera for several mycoplasma species were previously prepared in our laboratory. Sera from *M. penetrans* LAMPs-positive individuals were kindly provided from Dr. A. Blanchard, INRA, France. Human sera were performed to enzyme-linked immunosorvent assay (ELISA) with 1:100 dilution.

Results and discussion

1) Analysis of major antigens of *M. penetrans* except lipoproteins by proteomics

In SDS-PAGE and immuno blotting analysis of *M. penetrans* whole cell lysates, adding to P35 family lipoproteins, about 53 kDa protein strongly reacted with anti-*M. penetrans* sera. Among 60 major protein spots stained by CBB in two-dimensional electrophoresis and identified by LC-MS/MS, several protein spots were identified as antigens reacted with anti -*M. penetrans* sera in Western blotting analyzed of the transferred membrane from gel in two-dimensional electrophoresis. These major antigens were components for pyruvate dehydrogenase (PDH) E 1-alpha, E1 beta, E2 and E3, and especially PDH-E2 was the largest spot reacted. The identification of the major antigenic spot as PDH-E2 component was also analyzed by Edman-degradation for its amino acid sequences at the N-terminal.

2) Confirmation of the results from LC-MS/MS by producing a recombinant protein for partial *pdh-c* encoding PDH-E2 component

Then, we made a reconbinant protein for *pdh-c* that is encoding the PDH-E2. Mouse antisera for the recombinant pdh-c clearly reacted with about 53kDa protein of *M. penetrans* whole cell lysates (Fig. 1, lane 2-4). The reacted protein band was most likely the same molecular weight with that reacted with anti-M. penetrans sera (Fig. 1, lane 1). This data suggested that one of the major antigens detected by SDS-PAGE and Western blotting, 53kDa protein, might be PDH-E2 component.

3) Utility of recombinant *pdh-c* encoding PDH-E2 for serological diagnosis

The recombinant protein for *pdh-c* encoding PDH-E2 did not react with hyperimmunized rabbit sera for some human-

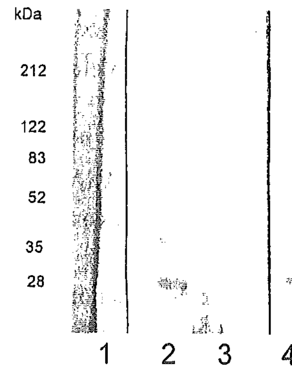

Fig. 1 Immuno blotting image of *M. penetrans* whole cell lysates reacted with sera for both anti-*M. penetrans* and anti-rPDH-E2. *M. penetrans* whole cell lysates reacted with anti-*M. penetrans* whole cells (lane 1) and with anti-recombinant PDH-E2 protein from three different mice (lane 2-4).

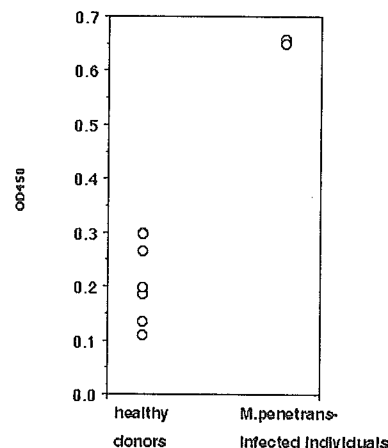

Fig. 2 The results of an ELISA system using rPDH-E2 as antigen. Sera were diluted at 1:100

related Mycoplasma species in 1:100-dilution by Western blotting. In addition, antibody against the recombinant protein did not react with whole cell lysates of *Ureaplasma urealyticum* serobar 3 by Western blotting. In ELISA system using the recombinant protein as antigen, OD value of all three sera from *M. penetrans* LAMPs-positive individuals showed two times higher than that of sera from healthy donors (Fig. 2). These data suggested the utility of recombinant *pdh-c* encoding PDH-E2 as a serological diagnostic tool.

In literatures, a surface-exposing membrane protein of *M. gallisepticum,* P52, that is PDH-E2 has been reported as a remarkable antigen and its antigenicity is species-specific to *M. gallisepticum* (8). A membrane-bound antigen P64 K of Neisseria meningitides is also PDH-E2/3, it has both lipoyl domains of the dihydrolipoyl acyltransferase (E2) and dihydrolipoyl dehydrogenase (E3) for PDH (9). This unusual

multidomain form was previously found in *M. capricolum*, PDH-E3 of *M. capricolum* has an aminoterminal extension corresponding to a lipoyl domain (E2) (10). Even though PDH-E2 of *M. penetrans* has no multidomain form, the PDH complex of *M. penetrans* is probably membrane associated since E3 component is predicted as an integral membrane protein, and E2 has an E3-binding domain.

Referencess

1. Lo, S. C., Hayes, M. M., Wang, R. Y., Pierce, P. F., Kotani, H. and Shih, J. W. (1991) Newly discovered mycoplasma isolated from patients infected with HIV. *Lancet,* **338,** 1415-8.
2. Lo, S. C., Hayes, M. M., Tully, J. G., Wang, R. Y., Kotani, H., Pierce, P. F., Rose, D. L. and Shih, J. W. (1992) *Mycoplasma penetrans* sp. nov., from the urogenital tract of patients with AIDS. *Int. J. Syst. Bacteriol.,* **42,** 357-64.
3. Grau, O., Slizewicz, B., Tuppin, P., Launay, V., Bourgeois, E., Sagot, N., Moynier, M., Lafeuillade, A., Bachelez, H., Clauvel, J. P., Blanchard, A., Bahraoui, E. and Montagnier, L. (1995) Association of *Mycoplasma penetrans* with HIV infection. J. *Infect.,* **172,** 672-681.
4. Grau, O., Tuppin, P., Slizewicz, B., Launay, V., Goujard, C., Bahraoui, E., Delfraissy, J. F. and Montagnier, L. (1998) A longitudinal study of seroreactivity against *Mycoplasma penetrans* in HIV-infected homosexual men: association with disease progression. *AIDS Res. Hum. Retroviruses,* **14,** 661-667.
5. Yáñez, A., Cedillo, L., Neyrolles, O., Alonso, E., Prévost, M.-C., Rojas, J., Watson, H. L., Blanchard, A. and Cassell, G. H. (1999) *Mycoplasma penetrans* bacteremia and primary antiphospholipid syndrome. *Emerg. Infect. Dis.,* **5,** 164-167.
6. Sasaki, Y., Ishikawa, J., Yamashita, A., Oshima, K., Kenri, T., Furuya, K., Yoshino, C., Horino, A., Shiba, T., Sasaki, T. and Hattori, M. (2002) The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. *Nucleic Acids Res.,* **30,** 5293-5300.
7. Horino, A., Sasaki, Y., Sasaki, T. and Kenri, T. (2003) Multiple promoter inversions generate surface antigenic variation in *Mycoplasma penetrans. J. Bacteriol.,* **185,** 231-242.
8. Jan, G., Hénaff, M. L., Fontenelle, C. and Wróblewski, H. (2001) Biochemical and antigenic characterisation of *Mycoplasma gallisepticum* membrane proteins P52 and P67 (pMGA). *Arch Microbiol,* **177,** 81-90.
9. Tozawa, K., Broadhurst, R. W., Raine, A. R. C., Fuller, C., Alvarez, A., Guillen, G., Padron, G. and Perham, R. N. (2001) Solution structure of the lipoyl domain of the chimeric dihydrolipoyl dehydrogenase P64K from *Neisseria meningitidis. Eur. J. Biochem.,* **268,** 4908-4917.
10. Zhu, P.-P. and Peterkofsky, A. (1996) Sequence and organization of genes encoding enzymes involved in pyruvate metabolism in *Mycoplasma capricolum. Protein Science,* **5,** 1719-1736.

MipR catalyses the promoter inversions in P35 family lipoprotein genes of *M. penetrans*

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Keywords : *Mycoplasma penetrans*, site-specific recombinase, promoter inversion

Abstract

Mycoplasma penetrans frequently changes its surface antigenicity by changing expression patterns of the P35 family lipoproteins. The genes of P35 family lipoproteins are called as *mpl* genes and present as a large gene cluster in the genome. Most of *mpl* genes (31 out of 38 genes) possess a promoter-like sequence independently and inversions of theses sequences are thought to be responsible for phase variable expression. In this study, we have developed a PCR-based method to detect the promoter inversions and demonstrated that at least 9 promoters were reversibly inverted in the genome. In addition, we identified the gene coding for recombinase that catalyze these inversions. We designated this recombinase as MipR (Recombinase for Multiple Invertible promoters).

Introduction

P35 family lipoproteins are major surface antigens of *M. penetrans* and undergo independent phase variations. We previously reported that *M. penetrans* HF-2 genome contains 38 genes of P35 family lipoprotein (*mpl* genes) and 30 of them are present as a large gene cluster in the genome (1). Most of the *mpl* genes (30 genes) have invertible promoter-like sequences that are thought to be responsible for phase variations of P35 family lipoproteins. However, direct evidence for the inversion events of these promoters had not been obtained except for one promoter (*p42* promoter). In this study, to detect the promoter inversion events of the *mpl* genes, we designed a PCR-based assay system. In addition, we also searched the genes that encode DNA recombinase responsible for the promoter inversions of *mpl* genes

Materials and Methods

M. penetrans HF-2 and GTU strains were used to analyze promoter inversion. The PCR-based assay systems to detect the promoter inversions were designed by using the primers that are specific to the three regions of each *mpl* genes (1.2 kb upstream and 2 kb downstream regions of the promoter and the promoter sequence itself). The direction of each *mpl* promoter sequences of HF-2 and GTU strains was assessed by the PCR with combinations of these primers. To analyze DNA recombinase activities of MYPE2900 and MYPE8180, *p42* promoter sequence was introduced into *M. pneumoniae* M129 and *E. coli* DH5α with the Tn*4001*mod vector and pENTR/D-TOPO plasmids, respectively. The MYPE2900 and MYPE8180 were introduced into *M. pneumoniae* also by Tn*4001*mod vector. pSC 101 plasmid was used for this purpose in *E. coli*. The *M. pneumoniae tuf* and *E. coli lac* promoters were used to express MYPE2900 and MYPE8180 in respective organisms. The inversion of *p42* promoter was assessed by PCR-based assay system.

Result and Discussion

We analyzed 9 promoters out of 31 predicted promoters of *mpl* genes by PCR-based inversion assay. PCR amplification results showed that all of these promoter sequences were actually inverted in *M. penetrans* cells. This result suggested that the inversions of all of 31 *mpl* promoter may occur in *M. penetrans* cells because the structural motif of these promoters were very similar each other. To further investigate this inversion system, we cloned *p42* promoter region and introduced this sequence into *E. coli* and *M. pneumoniae*. The PCR assay clearly showed that *p42* promoter sequence was not inverted in *E. coli* and *M. pneumoniae*. We then cloned two ORFs (MYPE2900 and MYPE 8180) of *M. penetrans*. These ORFs show homology to the genes for the site-specific recombinase XerC/D of lambda integrase family. In some other Mycoplasma species, it has been reported that these kind of recombinase protein catalyze DNA

inversions that are involved in the control of lipoprotein expression, (2, 3, 4, 5). MYPE2900 or MYPE8180 were introduced and expressed in *M. pneumoniae* and *E. coli* carrying the *p42* promoter sequence. In the both of *M. pneumoniae* and *E. coli* cells, the inversion of *p42* promoter sequence occurred in the presence of MYPE2900 but not in the presence of MYPE 8180. These results indicated that MYPE2900 is the recombinase involved in the promoter inversions of P35 family lipoprotein genes. We designated MYPE2900 as a MipR (Recombinase for Multiple Invertible Promoters).

References

1) Horino A., Sasaki Y., Sasaki T. and Kenri T., Multiple promoter inversion generate surface antigenic variation in *Mycoplasma penetrans,* J. Bacteriol., 2003, vol.185, 1, 231-242

2) Ron, Y., Flitman-Tene, R., Dybvig, K. and Yogev, D, Identification and characterization of a site-specific tyrosine recombinase within the variable loci of *Mycoplasma bovis*, *Mycoplasma pulmonis* and *Mycoplasma agalactiae,* Gene, 2002, vol.292, 1-2, 205-211

3) Glew, M. D., Marenda, M., Rosengarten, R. and Citti, C., Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus, J. Bacteriol., 2002, vol.184, 21, 5987-5998

4) Nussbaum, S., Lysnyansky, I., Sachse, K., Levisohn, S. and Yogev, D., Extended repertoire of genes encoding variable surface lipoproteins in *Mycoplasma bovis* strains, Infect. Immun., 2002, vol.70, 4, 2220-2225

5) Sitaraman, R., Denison, A. M. and Dybvig, K., A unique, bifunctional site-specific DNA recombinase from *Mycoplasma pulmonis,* Mol. Microbiol., 2002, Vol.46, 4, 1033-1040