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Isolation of Human Monoclonal Antibodies That Neutralize Human Rotavirus

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A human antibody library constructed by utilizing a phage display system was used for the isolation of human antibodies with neutralizing activity specific for human rotavirus. In the library, the Fab form of an antibody fused to truncated cp3 is expressed on the phage surface. Purified virions of strain KU (G1 serotype and P[8] genotype) were used as antigen. Twelve different clones were isolated. Based on their amino acid sequences, they were classified into three groups. Three representative clones—1-2H, 2-3E, and 2-11G—were characterized. Enzyme-linked immunosorbent assay with virus-like particles (VLP-VP2/6 and VLP-VP2/6/7) and recombinant VP4 protein produced from baculovirus recombinants indicated that 1-2H and 2-3E bind to VP4 and that 2-11G binds to VP7. The neutralization epitope recognized by each of the three human antibodies might be human specific, since all of the antigenic mutants resistant to mouse monoclonal neutralizing antibodies previously prepared were neutralized by the human antibodies obtained here. After conversion from the Fab form of an antibody into immunoglobulin G1, the neutralizing activities of these three clones toward various human rotavirus strains were examined. The 1-2H antibody exhibited neutralizing activity toward human rotaviruses with either the P[4] or P[8] genotype. Similarly, the 2-3E antibody showed cross-reactivity against HRVs with the P[6], as well as the P[8] genotype. In contrast, the 2-11G antibody neutralized only human rotaviruses with the G1 serotype. The concentration of antibodies required for 50% neutralization ranged from 0.8 to 20 μg/ml.

Rotavirus is the major cause of severe acute gastroenteritis among infants and young children. Rotavirus infection is life-threatening in developing countries, resulting in 500,000 to 600,000 deaths annually (33). In developed countries, rotavirus infections lead to a high disease burden with considerable medical expense due to the high morbidity. Furthermore, adults, particularly the elderly, are also affected by rotavirus infection (34, 39), and immunocompromised children and adults develop persistent rotavirus diarrhea (12, 42, 43). Thus, vaccination is thought to be the best way to reduce severe rotavirus gastroenteritis worldwide. Tetravalent rhesus rotavirus (RRV) human reassortant vaccine comprising RRV and three RRV-based monoreassortants carrying the VP7 genes from G1, G2, and G4 human rotaviruses (HRVs) was developed (25), and 1.5 million doses of this vaccine had been administered to infants by the end of May 1999 in the United States. However, the vaccine was withdrawn due to the occurrence of gut intussusception, which appeared to be epidemiologically linked to vaccine application (5, 38). Moreover, even if a safe and effective rotavirus vaccine is developed, vaccination would be less effective in immunocompromised patients.

Rotaviruses have two outer capsid proteins, viral protein 4 (VP4) and VP7, encoded on RNA segment 4 and RNA segment 7, 8, or 9, depending on the strain, respectively (19). VP4 and VP7 are known to induce neutralizing antibodies (Abs) in the sera and stools of infected patients, and they are relevant to protection against rotavirus infection (14, 18, 41, 45–47, 51). It is well known that the rotavirus G serotypes and P genotypes defined by VP7 and VP4, respectively, exhibit diversity. A total of 15 G serotypes and 22 P genotypes have been described (11). Although the majority of HRVs prevailing worldwide have G1, G2, G3, or G4 as the G serotype, and P[4] or P[8] as the P genotype, at least 10 G and 10 P types have been reported on HRVs (8). Recently, a number of HRV strains with unusual G or P types and rare combinations of G and P types have been detected worldwide. For example, G9 is increasing rapidly. In contrast, both VP4 and VP7 carry heterotypic (cross-reactive) neutralization epitopes, which are thought to be related to heterotypic protection (29, 30, 45–47, 49). An individual can be repeatedly infected with various strains of HRVs, suggesting that he or she has broadly and strongly effective Abs to HRVs. Although the validity of passive immunization remains unclear (17), oral administration of cross-reactive human immunoglobulins could be one of the measures for both prophylaxis and therapy for HRV diseases.

The natural repertoire formed in the human body should be composed of two different types of Abs. One type, which forms a naive repertoire, should show a wide range of antigen (Ag) specificity, the Ab binding avidity of each Ab being low in general. The other type, which is raised against specific Ags by immunization, should show a narrow range of specificity, the Ab binding avidity of each Ab being strong. In the present study, we used the Ab library called AIMS4 constructed from the B lymphocyte-rich tissues of a few dozen patients. Since this library is human-derived and rotavirus infection is considered to be very common worldwide, we expected that the Ab

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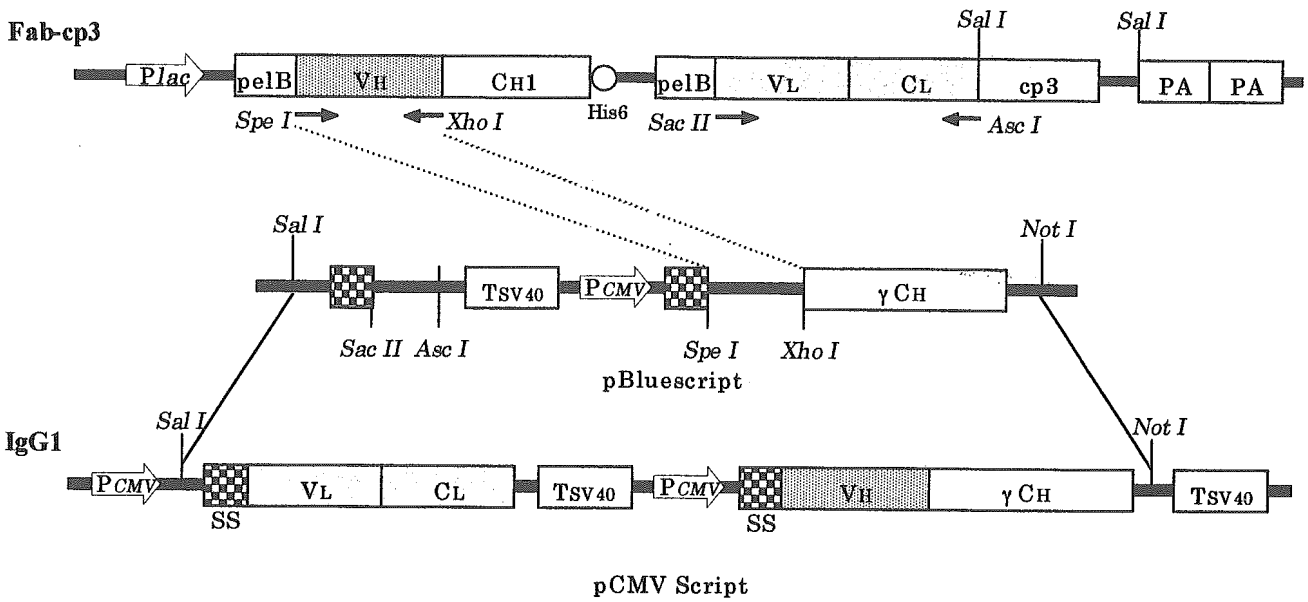

FIG. 1. Scheme for the conversion of a phage Ab (Fab-cp3) to an human IgG1. The V_H and the V_L C_L regions were amplified by PCR with the proper primers listed in Table 1, followed by subcloning into the proper restriction enzyme sites to construct an IgG1 cassette. The IgG1 cassette was then cloned into expression vector pCMVScript. *Plac*, *lac* promoter; *pel*B, *pel*B leader sequence; C_H 1, the first H-chain constant domain of human IgG1; His6, His tag-encoding part; cp3, truncated cp3; PA, Fc-binding domain of protein A; Tsv40, simian virus 40 terminator; P_{CMV}, cytomegalovirus promoter; γC_H, human γC_H domain; SS, signal sequence.

repertoire formed in AIMS4 should reflect a variety of rotavirus-specific Abs acquired through natural exposure. It would be interesting to directly explore the Ab repertoire of humans. In particular, comparison of the neutralization epitopes recognized by humans and mice would be useful for understanding the immune response against rotavirus infection in humans.

We describe here the successful isolation of anti-VP4 cross-reactive Abs and an anti-VP7 G1-specific Ab with neutralizing activities toward rotaviruses.

MATERIALS AND METHODS

Viruses. The following HRV strains and reassortants were used for the present study: KU (G1P[8]), Wa (G1P[8]), M37 (G1P[6]), K8 (G1P[9]), S2 (G2P[4]), 1076 (G2P[6]), YO (G3P[8]), McN13 (G3P[6]), AU-1 (G3P[9]), Hosokawa (G4P[8]), 69M (G8P[10]), WI61 (G9P[8]), L26 (G12P[4]), and two bovine strain UK-based single gene-reassortants, UK/Wa (G1P[5]) and UK/DS1 (G2P[5]), carrying the VP7 gene from HRV strains Wa and DS1, respectively (32). Eleven antigenic KU mutants resistant to each of 11 neutralizing mouse monoclonal Abs (MAbs) were also used in the present study: six (V-YO-1E6, V-ST-1F2, V-YO-1S3, V-YO-2C2, V-KU-4D7, and V-KU-6B11) and five (V-KU-3C7, V-YO-4C2, V-KU-5H1, V-KU-6A11, and V-KU4) mutants have been prepared by cultivating strain KU in the presence of anti-VP4 cross-reactive neutralizing MAbs and anti-VP7 G1-specific neutralizing MAbs, respectively (45, 47). Virus propagation and purification were carried out as described previously (49). Unless otherwise stated, the culture fluids of MA104 cells infected with rotaviruses were used for the assays.

Preparation of virus-like particles (VLPs) and recombinant VP4. Construction of the artificial VLP of HRV KU origin is described elsewhere (K. Taniguchi et al., unpublished data). Briefly, the reverse transcription-PCR products of the VP2, VP4, VP6, and VP7 genes of human strain KU were cloned into a TA cloning vector, pCRII (Invitrogen, San Diego, Calif.), to generate pKU-VP2, pKU-VP4, pKU-VP6, and pKU-VP7. After digestion with restriction enzymes, the fragments were ligated into transfer vector pVL1392 to yield pVL1392/KU-VP2, -VP4, -VP6, and -VP7. Sf9 cells were coinfected with linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen) and either pVL1392/KU-VP2, -VP4, -VP6, or -VP7 by the Lipofectin-mediated method. The baculovirus recombinants thus obtained were used for preparation of recombinant VP4, VLP-VP2/6, and VLP-VP2/6/7 in Tn*5* cells.

Ab library. Abs were isolated from the Abs library called AIMS4, which was constructed in Y. Kurosawa's laboratory. In brief, B lymphocyte-rich fractions of human tissues such as tonsils, umbilical cord blood, peripheral blood, and bone marrow were used as gene sources of Abs (35; Y. Akahori et al., unpublished data). Using a phage-display system, the Fab form of an Ab fused to a truncated cp3 (Fab-cp3) was expressed on the phage surface. The library is composed of 10^{11} independently established clones, and it has been shown that >70% of the phages express Abs.

Screening of the library. Selection of phages exhibiting rotavirus (strain KU)-binding activity was performed by a panning method that was essentially the same as that described previously (20, 27). The immunotubes (Nunc-Immunomodules Polysorp) were coated with 200 μg of a purified KU virion/ml in phosphate-buffered saline containing 100 μg of Ca^{2+} and Mg^{2+}/ml [PBS(+)] overnight at 4°C. After a blocking step with 2% skim milk, a solution of phages (10^{14} CFU) was added to each tube, followed by incubation at room temperature for 2 h. The unbound clones were washed out four times with PBS(+). Bound phages were eluted with 0.1 M triethylamine (pH 12.3), and the eluent was then immediately neutralized with 1 M Tris-HCl (pH 6.8). *E. coli* DH12S cells cultured in 2xYT medium were infected with the eluted phages, precipitated by centrifugation, and then resuspended in 2xYT containing 1% glucose and 100 μg of ampicillin/ml, followed by superinfection with helper phages and further cultivation under kanamycin-selective conditions (70 μg/ml) in order to replicate phage clones harboring KU-reactive Abs. The phage clones obtained through this process were used for the next round of panning. The input titers of the phages and the number of washings with PBS(+) were 1.06×10^{13} and 8 for the second panning and 3.46×10^{13} and 16 for the third panning, respectively. After the third round of panning, DH12S cells infected with the selected phages were spread on LB plates containing 1% glucose and 100 μg of ampicillin/ml and incubated at 30°C overnight.

Preparation of various forms of Abs. The individual clones of *E. coli* infected with phages were grown in 2xYT medium containing 0.1% glucose and 100 μg of ampicillin/ml. After the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), the Fab-cp3 molecules were initially accumulated in the periplasm of *E. coli* and then gradually secreted and/or released into the culture medium (crude Fab-cp3). On average, 1 μg of Fab-cp3 molecules/ml is present in the culture fluid. The Fab-cp3 molecules can be purified with anti-cp3 MAb-conjugated Sepharose beads. After isolation of phage particles, the gene encoding an Fab-cp3 molecule can be easily converted into another gene encoding an Fab-PP (P denotes a single Fc-binding domain of protein A) form of Ab by digestion with SalI followed by self-ligation (Fig. 1) (22). The Fab-PP molecules can be purified

TABLE 1. Oligonucleotide primers used for the conversion of phage antibodies (Fab-cp3) to human IgG1

Primer	Orientation[a]	Sequence
1-2HVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCCAGGTGCAGCTGGTGCAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCAGGGTTC-3′
1-2HVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGACAGTCTGTGTTGACGCAGCCG-3′
	R	5′-TCGACTGGCGCGCCCTATGAACATTCTGTAGGGGCCACTGTCTTC-3′
2-3EVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCGAGGTGCAGCTGGTGGAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCATTGTCC-3′
2-3EVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGAGAAACGACACTCACGCAGTCT-3′
	R	5′-TCGACTGGCGCGCCCTAACACTCTCCCCTGTTGAAGCTCTTTGTG-3′
2-11GVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCGAGGTGCAGCTGGTGGAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCGTGGTCC-3′
2-11GVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGAGAAACGACACTCACGCAGTCT-3′
	R	5′-TCGACTGGCGCGCCCTAACACTCTCCCCTGTTGAAGCTCTTTGTG-3′

[a] F, forward; R, reverse. SpeI, XhoI, SacII, and AscI sites are underlined.

with an immunoglobulin G (IgG)-conjugated column. For conversion of an Fab to a human IgG1, the variable heavy-chain region (V_H) and the variable and constant light-chain region (V_L C_L) of an Fab fragment were amplified by PCR (15 cycles of amplification at 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min) with primers designed for each clone (Table 1). After digestion with SpeI and XhoI for the V_H region and SacII and AscI for the V_L C_L region, the PCR products were subcloned into an IgG1 construction vector. The constructed IgG1 cassette was further cloned into the pCMV-Script expression vector (Fig. 1) (Akahori et al., unpublished). Expression of IgG1 molecules was performed by transfection of the IgG1 expression vectors into CHO-K1 cells by using GenePORTER (Gene Therapy Systems), which allows production and/or secretion of a whole human IgG1 type of Ab in culture fluid.

Enzyme-linked immunosorbent assay (ELISA). To determine the reactivity to or titer of the Abs for strain KU, immunoplates (96-well; Nalgen Nunc International) were coated with highly purified virions (500 ng/well) suspended in PBS(+) for 1 day at 4°C. After a blocking treatment with 5% bovine serum albumin in PBS(+), the plates were washed with PBS(+). For the reactivity study, the plates were then incubated with 50 ng of purified Fab-cp3 in PBS(+) at 4°C overnight. For the titration study, the plates were then incubated with serial dilutions of purified IgG1-formed Abs ranging from 0.01 to 5,000 ng/well in PBS(+) at 4°C overnight. In both cases, after the plates had been washed with PBS(+), a 1:2,500 dilution of peroxidase-conjugated goat anti-human IgG (H+L chain; MBL) was added to each well. One optical density at 492 nm (OD_{492}) unit was defined as the Ab concentration at which the OD_{492} reading was 1.0. To identify virus proteins recognized by the isolated Abs, the immunoplates were coated with 500 ng of purified Fab-cp3 at 4°C overnight, followed by blocking with 5% BSA in PBS(+). Then, unpurified VLPs or recombinant VP4 in the culture supernatant were added. After incubation at 4°C overnight and additional washing with PBS(+), 50 μl of 1:4,500 diluted rabbit anti-HRV antiserum (a mixture of 1:1,500 diluted rabbit anti-KU, anti-AK13, and anti-YO antiserum) was added to each well, followed by incubation at 4°C for 1 day. After the plates had been washed with PBS(+), a 1:2,500 dilution of peroxidase-conjugated goat anti-rabbit IgG (H+L chain; MBL) was added to each well. In all cases, the reactivity of the Abs to Ags was assessed after addition of the substrate.

Assay for virus-neutralizing activity. Screening of crude Fab-cp3 Abs for preliminary selection as to neutralizing activity and determination of the titers of the purified Abs against HRVs or antigenic mutants were determined by the fluorescent focus reduction (FF) method. A total of 25 μl of crude Fab-cp3 or purified Abs in PBS(+) at various concentrations (0.4 to 20 μg/ml) was mixed with an equal volume of virus suspension containing 3.6×10^4 to 14.4×10^4 focus-forming units in Eagle minimum essential medium, followed by reaction at 37°C for 1 h. Aliquots (50 μl) of the mixtures of Abs and viruses were inoculated onto MA104 cells in 96-well culture plates and, after an additional 1 h of incubation at 37°C, 100 μl of fresh Eagle minimum essential medium was added, followed by 16 to 18 h cultivation at 37°C. Fixation in the cold (−80°C), and reaction with the first and second Abs were performed as described previously (49). The neutralization assays were performed in duplicate and at least twice.

Sequence analysis. The nucleotide sequences of V_L and V_H regions were determined with an ABI Prism 320 genetic analyzer by using a BigDye terminator cycle sequencing kit (Applied Biosystems). The T7 primer (5′-TGTAATAC GACTCACTATAG-3′) and the huCH1J primer (5′-ATTAATAAGAGCTAT CCCGG-3′) were used for V_H and V_L sequencing, respectively.

Nucleotide sequence accession numbers. The nucleotide sequence data for the HRV neutralizing Abs reported in the present study have been submitted to the DDBJ database and were assigned the following accession numbers: AB114449 (for 1-2H H chain), AB114450 (2-1D H chain), AB114451 (2-2D H chain), AB114452 (2-3E H chain), AB114453 (2-4F H chain), AB114455 (2-7G H chain), AB114456 (2-9B H chain), AB114457 (2-9D H chain), AB114458 (2-11G H chain), AB114459 (2-12B H chain), AB114460 (3-1G H chain), AB114461 (4-3-C H chain), AB114461 (1-2H L chain), AB114462 (2-1D L chain), AB114463 (2-2D L chain), AB114464 (2-3E L chain), AB114465 (2-4F L chain), AB114466 (2-7G L chain), AB114467 (2-9B L chain), AB114468 (2-9D L chain), AB114469 (2-11G L chain), AB114470 (2-12B L chain), AB114471 (3-1G L chain), and AB114472 (4-3C L chain).

RESULTS

Isolation of Fab forms of Abs with neutralizing activities toward HRVs. After three rounds of panning, the recovered phages were used to infect *E. coli*, which was spread on plates containing ampicillin without infection with helper phages. We picked up 321 colonies and cultured them in 96-well plates. The supernatants, crude Fab-cp3, were directly subjected to analysis of neutralizing activities by means of the FF assay. Among the 321 clones analyzed, 24 appeared to exhibit neutralizing activities toward rotavirus strain KU.

Amino acid sequences of Fab H and L chains. In order to confirm the successful selection of phages with Fab-cp3 specific to strain KU and also to classify the 24 clones, we determined the sequences of variable regions of both their H and L chains. Some redundants were included in the 24 clones (Fig. 2), indicating the specific and successful selection and enrichment of KU-reactive phage Abs. The amino acid sequences of the H chains could be divided into 7 clones and the L chains could be divided into 16 clones (Fig. 2). As a result, 16 of the 24 clones were found to be independent.

Neutralizing activity of the purified Fab fragments. Since the use of crude Fab-cp3 in the FF assay gave ambiguous

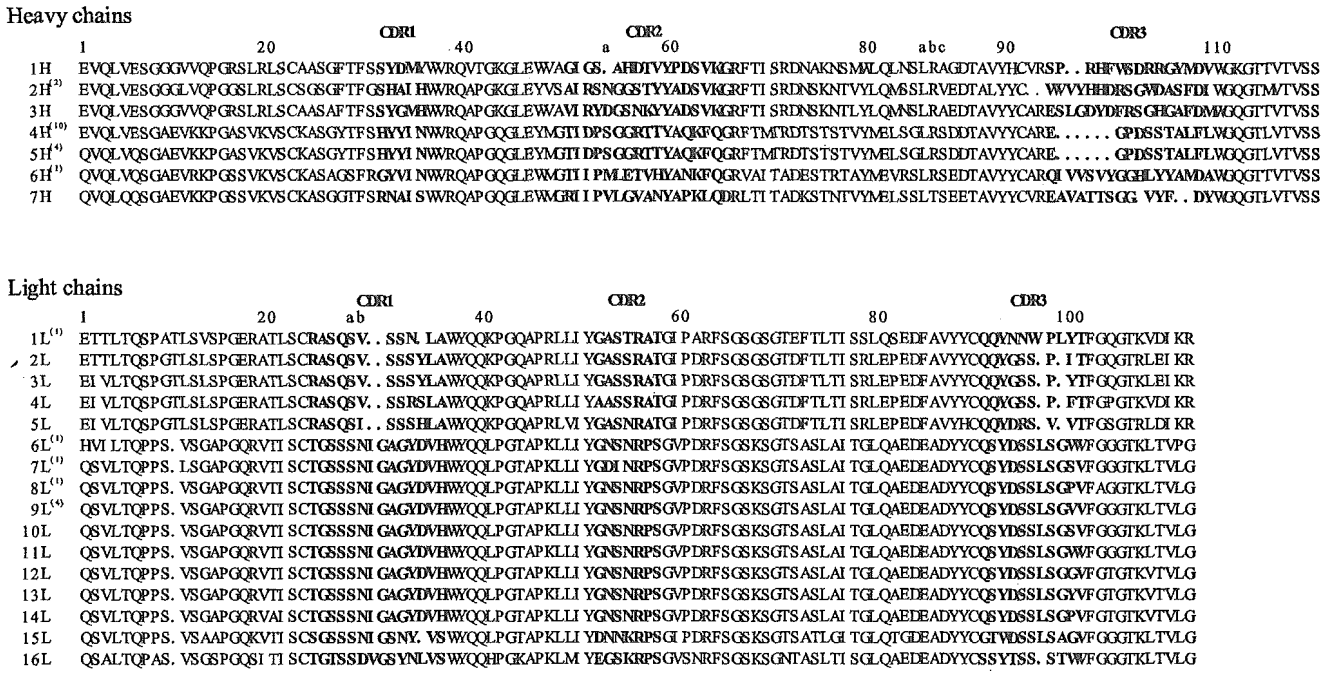

FIG. 2. Amino acid sequences of variable regions of the H and L chains of Abs that appeared to exhibit neutralizing activities toward strain KU. The sequences of CDRs are shown in boldface. Numbers in parentheses indicate number of redundant clones found among the 24 Abs analyzed, with 16 Abs being independent. The combinations of H and L chains in the independent Abs are indicated in Table 2. The numbering of amino acid positions is according to the method of Kabat et al. (23).

results due to contaminants in the *E. coli* culture supernatant, the Fab-cp3 coding phagemid DNAs of the 16 independent clones (2-7G, 3-1G, 2-9D, 2-4F, 2-9B, 1-2H, 4-3C, 2-12B, 2-2D, 2-3E, 2-1D, 2-11G, 1-8A, 2-2G, 2-5G, and 1-4D) were reconstructed to produce Fab-PP fragments (Fig. 1) to facilitate purification. After purification by means of affinity selection, their neutralizing activities toward strain KU were assessed by FF assay. Except for 1-4D, 1-8A, 2-2G, and 2-5G, we could detect the neutralizing activity against strain KU (Table 2). Although the Ag-binding site of each Ab is formed by amino acid residues in the six complementarity-determining regions (CDRs) of the H and L chains, the contribution of CDR3 of the H chain to Ag specificity is greatest among them in a usual case, especially that to protein Ags (21). Judging from this, the two H chains, 4H and 5H, are essentially the same (Fig. 2), and the 12 clones exhibiting the neutralizing activities toward KU could be classified into three groups (Table 2). We finally selected 1-2H, 2-3E, and 2-11G as representative clones of the three group, and further investigations were carried out on these three.

Virus proteins recognized by Abs. The reactivity of the three representative Fab fragments was examined by means of ELISA, their specific reactivity with strain KU being shown (Fig. 3A). Although an isolated VP4 molecule could expose its neutralizing epitope on the surface, VP7 exposed its epitope only when the molecule was embedded in inner proteins VP2 and VP6 (49). Therefore, we prepared two kinds of VLP, VLP-V2/6 and VLP-2/6/7, and recombinant VP4. As shown in Fig. 3B, the targeted virus protein of 1-2H and 2-3E was found to be VP4, and that of 2-11G was found to be VP7. A Western blot analysis to confirm the ELISA results was unsuccessful (data not shown).

Neutralizing activities toward various HRV strains. After conversion of the Fab form into an IgG1 Ab, we analyzed the neutralizing activities of the three Abs against 13 HRVs by means of the FF assay (Table 3). The 1-2H Ab neutralized 7 strains—S2, L26, KU, Wa, YO, Hosokawa, and WI61—all of which exhibited either P[4] or P[8] type specificity on VP4. The 2-3E Ab showed neutralizing activities toward 8 strains—M37,

TABLE 2. Neutralization activity of Fab-PP forms of Abs to strain KU

Clone (*n*)[a]	H chain	L chain	Antibody concn (μg/ml)[b]
2-7G (1)	4H	6L	1.6
3-1G (1)		7L	8.0
2-9D (4)		9L	1.6
2-4F		10L	1.6
2-9B		12L	1.6
1-2H	5H	13L	1.6
4-3C (1)		8L	1.6
2-12B		11L	1.6
2-2D		14L	1.6
2-3E (1)	2H	1L	1.6
2-1D		15L	1.6
2-11G	1H	2L	1.6
1-8A	3H	5L	>40.0
2-2G	6H	4L	>40.0
2-5G		3L	>40.0
1-4D	7H	16L	>40.0

[a] *n* = number of redundant clones found among the 24 clones analyzed.
[b] Concentration of antibodies required for a 50% reduction of the fluorescent focus in the FF assay.

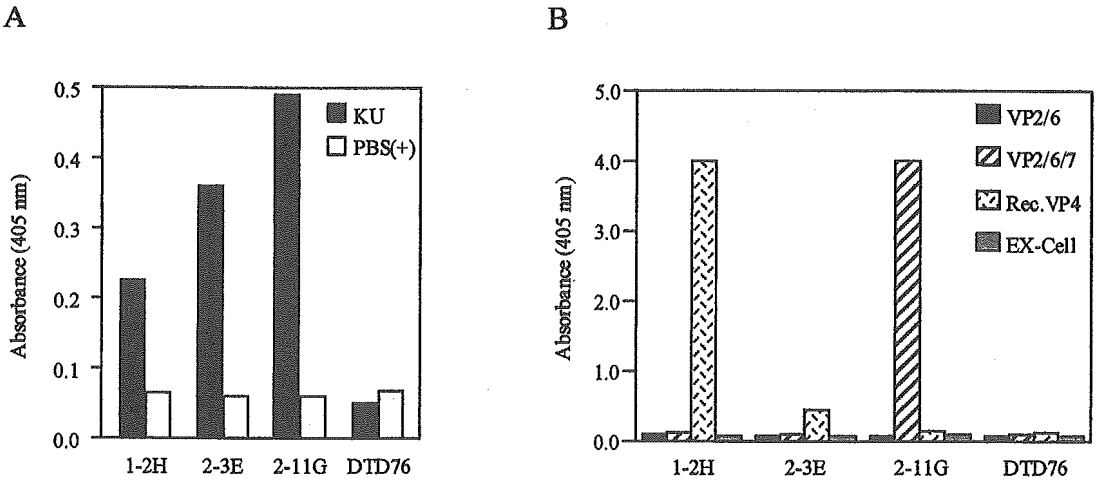

FIG. 3. ELISA of the three Abs (purified Fab-cp3 forms). (A) Reactivities of the three Abs to strain KU. Purified virions suspended in PBS(+) were used as Ags. (B) Virus proteins directed by the three Abs. The culture fluids of Tn5 cells containing VLP-VP2/6, VLP-VP2/6/7, or recombinant VP4 were used as Ags. EX-Cell was the culture medium of Tn5. DTD76 is anti-diphtheria toxin Ab isolated from AIMS4, which was used as a negative control. VP2/6, VLPs constructed with VP2 and VP6; VP2/6/7. VLPs constructed with VP2, VP6, and VP7; Rec.VP4, recombinant VP4.

1076, McN13, KU, Wa, YO, Hosokawa, and WI61—all of which are classified as either a P[6] or P[8] virus strain. The 2-11G Ab reacted with four strains—M37, KU, Wa, and K8—all of which are G1 strains. The conclusion that the 2-11G Ab reacts with VP7 with the G1 serotype was further supported by using two VP7 single gene reassortants, UK/Wa and UK/DS1. The former has VP7 of G1 and VP4 of P[5] specificity, and the latter has VP7 of G2 and VP4 of P[5] specificity. The 2-11G Ab neutralized only the former strain. Of the 13 HRVs examined, 11 were neutralized by any of the three Abs. The concentration of Abs required for 50% neutralization ranged from 0.8 to 20 μg/ml, which corresponds to 5.3 to 133 nM.

Neutralizing activities toward antigenic mutants. We also examined the neutralizing activities of the three representative Abs isolated in the present study with the mouse MAb-resistant mutants prepared in our previous studies (45–47). All of the three Abs of the Fab-cp3 form turned out to neutralize all of the mutants examined (Table 4). The concentration of the each Ab required for 50% reduction of the fluorescent focus in the FF assay was <1.6 μg/ml.

Titers of the Abs to strain KU. We determined the titers of three Abs (IgG1 form) to strain KU by ELISA (Fig. 4). All three Abs showed dose-dependent binding. There was no correlation between the absorbance density and the neutralizing activity of each Ab. The values for one OD_{492} unit for the 1-2H, 2-3E, and 2-11G Abs were 0.70, 0.75, and 1.8 μg/ml, respectively.

DISCUSSION

Ab libraries constructed with a means of a phage-display system are convenient for the rapid isolation of Abs specific for various Ags (2, 27, 31, 44, 52). Recently, several recombinant human Fab fragments exhibiting neutralizing activity toward viruses such as human immunodeficiency virus types 1 and 2, Ebola virus, measles virus, Puumala virus, and respiratory syncytial virus were prepared by means of a phage display system (1, 3, 6, 7, 28, 37, 50). Since the amount of surface proteins with neutralization epitopes on viruses is small and the immunogenicity of inner proteins is quite high, it is generally much more difficult to obtain MAbs with neutralizing capacity than non-neutralizing MAbs. This requires some modifications of the panning and/or screening processes. For example, blockade of a common, nonneutralizing epitope with a representative Fab has been used in panning assays to isolate respiratory syncytial virus-neutralizing human MAbs (50). In the present study, we used highly purified virion for panning and directly screened numerous clones by means of rapid microneutralization FF tests, which have been found to be very efficient for screening neutralizing MAbs with common mouse hybridoma technology (49).

We used HRV strain KU as the Ag for panning, since strain KU exhibits representative G1 and P[8] specificity, which is the

TABLE 3. Neutralization of HRVs and reassortants by purified human IgG1s

Strain	P genotype	G serotype	Antibody concn (μg/ml)[a]		
			1-2H	2-3E	2-11G
S2	P[4]	G2	4–20	>20	>20
L26	P[4]	G12	0.8	>20	>20
M37	P[6]	G1	>20	4	0.8
1076	P[6]	G2	>20	4	>20
McN13	P[6]	G3	>20	4	>20
KU	P[8]	G1	8	1.6	0.4
Wa	P[8]	G1	4	4	4–20
YO	P[8]	G3	8	1.6	>8
Hosokawa	P[8]	G4	4	0.8	>20
WI61	P[8]	G9	4–20	0.8–4	>20
AU-1	P[9]	G3	>20	>20	>20
K8	P[9]	G1	>20	>20	4
69M	P[10]	G8	>20	>20	>20
UK/Wa	P[5]	G1	>20	>20	4
UK/DS-1	P[5]	G2	>20	>20	>20

[a] Antibody concentration that reduced the fluorescent focus count by >50% in the neutralization test (FF assay).

TABLE 4. Neutralization activity of purified antibodies (cp3 form) to antigenic variants

Strain	Mutant protein	No. of virus-infected cells with:[a]											
		1-2H at:			2-3E at:			2-11G at:			DTD76 at:		
		1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml
V-1E6	VP4	63	69	60	4	2	1	14	2	14	296	378	358
V-1F2	VP4	64	76	43	45	108	21	10	5	1	350	462	368
V-1S3	VP4	31	32	19	127	91	18	26	9	4	312	302	304
V-2C2	VP4	87	46	56	9	3	4	16	0	2	296	342	344
V-4D7	VP4	68	13	12	6	1	0	35	3	4	396	222	354
V-6B11	VP4	21	17	11	8	63	4	3	1	0	212	324	240
V-3C7	VP7	121	123	81	14	17	11	19	3	2	482	532	554
V-4C2	VP7	96	87	120	20	9	5	23	2	3	596	660	656
V-5H1	VP7	146	156	90	13	4	4	46	1	1	372	395	355
V-6A11	VP7	57	84	79	57	23	27	24	8	6	425	554	564
V-KU4	VP7	47	22	14	3	4	0	6	0	3	206	186	206
KU	–	26	15	13	3	3	3	52	3	5	282	300	305

[a] That is, the numbers of virus-infected cells detected by the FF method (see Materials and Methods). Virus-positive cells in a one-ninth area of one well of a 96-well tissue culture plate were counted. Results are presented as the means for two independent experiments performed in duplicate.

most prevalent HRV serotype worldwide (24), and since the neutralization epitopes on VP4 and VP7 of the strain have well been characterized by using mouse MAbs (36, 45, 47, 49). The three human Abs characterized (1-2H, 2-3E, and 2-11G) are specific to P[8], P[4]; P[8], P[6]; and G1 HRVs, respectively. In particular, the former two are broadly reactive with a wide spectrum of HRVs. Since a total of 15 G serotypes have been defined for rotavirus and at least 10 G serotypes have been isolated from humans, it is desirable to prepare such broadly reactive heterotypic Abs for therapeutic purposes. The reactivity of the three Abs covered most HRV strains, and they indeed neutralized 11 of the 13 HRVs examined. In previous studies, an Ab response to cross-reactive neutralization epitopes (YO-2C2 epitopes) was observed much more frequently in schoolchildren and adults than in infants (13, 48). Since the library was constructed from the tissues of adults, who would have been repeatedly infected with HRVs with distinct serotypes and would have immunological memory for cross-reactive neutralization epitopes, cross-reactive Abs may have been readily selected in the present study. In other words, a cross-reactive immune response should be common in the immune system in humans, particularly in adults, infected with rotaviruses.

A number of murine MAbs have been prepared for rotaviruses by means of conventional hybridoma technology. Although many of them were directed to the inner protein VP6,

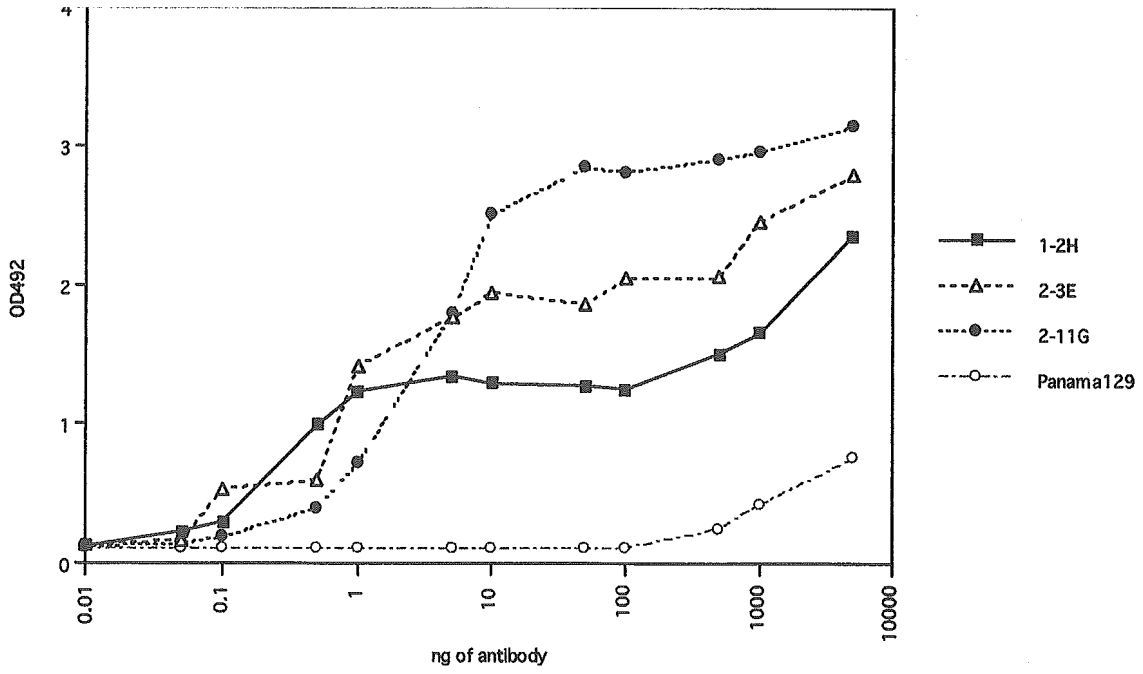

FIG. 4. Abs titration curves on ELISA. The reactivities between purified IgG1-formed Abs and purified virions of strain KU were assessed. The assay was performed in duplicate, and the mean data are plotted. Panama129 is the IgG1-formed anti-influenza virus Ab isolated from the AIMS4 library and converted to the IgG1 form as described in Materials and Methods.

some were directed to VP7 and VP4. However, the number of neutralizing MAbs with hetetrotypic specificity was limited. In particular, ones directed to HRVs were few; i.e., there was only one to VP7 and seven to VP4 (26, 36, 45–47, 49). They have been useful for the analysis of heterotypic neutralization epitopes on VP7 and VP4. By analyzing the mutants resistant to each of the neutralizing mouse MAbs directed to VP4 or VP7 of HRVs, critical amino acids in the neutralization epitopes have been identified (29, 45, 46). It has been shown that the cross-reactive neutralization epitopes on VP4 are the 305th, 385th, 392nd, 428th, 433rd, and 439th amino acid residues (26, 46, 47) and that the G1-specific epitopes on VP7 are the 94th and 96th residues (45). Furthermore, by means of neutralization tests on various combinations of MAb-resistant mutants and MAbs, operational maps of the neutralization epitopes have been constructed (26, 36). We examined the reactivities of the three human Abs isolated in the present study with the MAb-resistant mutants prepared in our previous studies (45–47): mutants resistant to anti-VP4 MAbs (YO-2C2, KU-6B11, YO-1S3, ST-1F2, KU-4D7, and YO-1E6) and mutants resistant to anti-VP7 MAbs (KU-2, KU-4, KU-3C7, KU5H1, KU-6A11, and YO-4C2). These three Abs turned out to neutralize all of the mutants examined (Table 4). This finding strongly suggests that the human Abs isolated in the present study recognize neutralization epitopes distinct from those recognized by mouse MAbs obtained to date. These results could have been predicted, since the specificity showed by the human Abs, such as 1-2H Ab to P[4] and P[8] and 2-3E Ab to P[6] and P[8], had not been shown by mouse MAbs isolated to date. These results imply that the cross-reactive neutralization epitopes recognized by humans, in particular adults, infected with rotaviruses and by mice immunized with rotaviruses are quite distinct. We are now attempting to prepare mutants resistant to each human Ab for analysis of the neutralization epitopes recognized by them.

The mechanism of protective immunity against rotavirus infection has not been well elucidated. Both humoral and cellular immunity are likely to be involved in the protection from rotavirus infection (11, 24, 40). The mucosal Ab response has been believed to be effective for such protection. Furthermore, passive immunity has also been found to be effective (40). Maternal transfer of anti-rotavirus immunoglobulins protects babies from rotavirus infection. Oral administration of bovine immunoglobulins, mouse MAbs, and human immunoglobulins has been found to be effective for protecting suckling mice from rotavirus infection (9, 30, 41). In addition, therapeutic reports on the passive immunity of children with rotavirus diarrhea have also been published. Guarino et al. reported that the oral administration of human serum immunoglobulins to children with rotavirus-induced diarrhea resulted in a faster recovery from the disease (15), even though the children were immunocompromised due to human immunodeficiency virus infection (16). In contrast, there have also been reports showing no clinical effect of oral administration of bovine immunoglobulins for prophylaxis or therapy for HRV infection (4, 10). We are now examining a mouse model to determine whether the human MAbs prepared in the present study are effective and practically relevant to immunotherapy and/or prophylaxis for diseases caused by rotavirus.

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Inactivation of Macrolides by Producers and Pathogens

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Abstract: Inactivation, one of the mechanisms of resistance to macrolide, lincosamide and streptogramin (MLS) antibiotics, appears to be fairly rare in clinical isolates in comparison with target site modification or efflux. However, inactivation is one of the major mechanisms through which macrolide-producing organisms avoid self-damage during antibiotic biosynthesis.

The inactivation mechanisms for MLS antibiotics in pathogens are mainly hydrolysis, phosphorylation, glycosylation, reduction, deacylation, nucleotidylation, and acetylation. The *ere* (erythromycin resistance esterase) and *mph* (macrolide phosphotransferase) genes were originally found in *Escherichia coli*. Subsequently, Wondrack *et al.* (Wondrack, L.; Massa, M.; Yang, B.V.; Sutcliffe, J. *Antimicrob. Agents Chemother.*, **1996**, *40*, 992) reported *ere*-like activity in *Staphylococcus aureus*. In addition, a variant of erythromycin esterase was found in *Pseudomonas* sp. from aquaculture sediment by Kim *et al.* (Kim, Y.H.; Cha, C.J.; Cerniglia, C.E. *FEMS Microbiol. Lett.*, **2002**, *210*, 239). Although the *mph* genes, including *mph*(K), were first characterized in *E. coli*, a recent study revealed that *S. aureus* and *Stenotrophomonas maltophilia* have *mph*(C). The *mph*(C) has a low G+C content, like *mph*(B), and has high homology with *mph*(B), but not with *mph*(A) or *mph*(K). Consequently, the *mph*(C) and *ere*(B) genes seem to have originated from Gram-positive bacteria and been transferred between Gram-positive and Gram-negative bacteria.

In this chapter, the genes and the mechanisms involved in the inactivation of MLS antibiotics by antibiotic-producing bacteria are reviewed.

Key Words: Macrolide antibiotics, macrolide resistance, inactivation, erythromycin esterase, phosphotransferase, glycosyltransferase, acetylation, hydrolysis.

(A) PRODUCERS

Most macrolides are produced by *Streptomycete* species, and inevitably the biosynthesis of a potentially lethal antibiotic in these microorganisms requires self-defence mechanisms to avoid suicide. To date, three distinct self-defence mechanisms have been reported in macrolide-producing organisms. The first mechanism is modification of the ribosome (the antibiotic target site) by monomethylation or dimethylation of a single adenine residue in the 23S rRNA gene; this results in resistance to erythromycin [1], tylosin [2, 3] and carbomycin [4]. The second mechanism is active efflux. Some macrolide producers have ABC (ATP-binding cassette) transporters that pump out macrolides through ATP-dependent pathways, thereby providing resistance to these macrolides [5-8]. The third is the existence of antibiotic-modifying enzymes. *Streptomyces antibioticus*, an oleandomycin (OL) producer, possesses a glycosyltransferase that inactivates OL by glycosylation of a hydroxyl group of the sugar desosamine attached to the aglycone [9, 10]. It also possesses a glycosidase that converts inactive glycosylated OL into the active antibiotic [9, 11]. *Streptomyces lividans* is a non-macrolide producer [12], but can inactivate macrolides by glycosylation [13].

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The resistance mechanisms of MLS antibiotic-producing microorganisms are summarized in Table **1**.

1. Macrolide Antibiotics

The study of bacterial ability to modify or degrade macrolide antibiotics started in 1964. The disappearance of erythromycin (EM) in the culture media of steroid-transforming strains of *Streptomyces* or *Nocardia* and EM-A inactivation by *Pseudomonas* in soil were reported by Feldman *et al.* [14] and Flickinger *et al.* [15], respectively. Nakahama *et al.* [16-18] then reported the deacylation and the hydroxylation of maridomycin, spiramycin, and josamycin (16-membered macrolides).

1-1. Phosphorylation

Phosphorylation of antibiotics by microorganisms is a well-known inactivation mechanism *in vivo*. Phosphorylating enzymes are widely distributed among *Streptomyces* spp. and are also found in other genera. Crude phosphorylating enzyme of *Streptomyces coelicolor* in the presence of ATP and Mg^{2+} catalyzes the conversion of oleandomycin, tylosin, and spiramycin to inactive 2'-*O*-phosphates. Under the same conditions, erythromycin was converted to anhydroerythromycin 2'-*O*-phosphate [19, 20]. Fig. (**1**) shows the structures of the substrate antibiotics and phosphorylation products.

1-2. Glycosylation

Microbial glycosylation of erythromycin A (EM-A) was observed in *Streptomyces vendargensis* [21] and the inacti-

Table 1. Inactivation of MLS-antibiotics by Antibiotic Producers and Other Organisms.

MLS antibiotics Resistance profile	Organism	Inactivated antibiotics	Gene	GenBank number	Reference
Macrolide					
Phosphorylation	*Streptomyces coelicolor* Muller	EM-A, OL, TL, LMA_3, SPM		M74717	[19, 20]
Glycosylation	*Streptomyces lividans* TK21	EM, TL, RSM, AZM, TL	*mgt*	AF055579	[12, 22, 23]
	Streptomyces antibioticus ATCC11891	OL, RSM, MET, LAN	*oleI*	Z22577	[9-11, 29, 30]
	Streptomyces vendargensis UC5315	EM		AJ223970	[21]
	Streptomyces antibioticus	OL	*oleD*		[24]
	Saccharopolyspora erythraea ATCC11635	AVE			[31]
	Streptomyces ambofaciens ATCC23877	RSM, OL, CHA, TL	*gimA*		[32]
Deacylation	*Bacillus megaterium* 91277	MAR, SPM			[16]
	Streptomyces olivaceus 219	MAR, JM			[17, 18]
	Streptomyces ptistinaespiralis IFO13074	MAR			[18]
N. C.	*Pseudomonas* 56	EM-A			[15]
Lincosamide					
Phosphorylation	*Streptomyces rochei*	LCM			[36]
Phosphorylation/ Ribonucleotidylation	*Streptomyces coelicolor* Muller	CLDM, LCM, PIR			[37-40]
Streptogramin					
Hydrolysis	*Actinoplanes missouriensis*	DHS-S			[42]
	Streptomyces mitakaensis	MKM-B			[43, 44]
	Streptomyces diastaticus NRRL2650	VER-A and B, PR-I and II,			[45]
	Streptomyces loidensis ATCC11415	OST-A and B, VM-1 and M2			
	Streptomyces olivaceus ATCC12019				
Reduction	*Streptomyces virginiae*	VIR-M1			[41, 46]

Abbreviations: EM, erythromycin; OL, oleandomycin; TL, tylosin; LMA_3, leucomycin A_3; SPM, spiramycin; RSM, rosamicin; AZM, azithromycin; MET, methymycin; LAN, lankamycin; AVE, avermectin, CHA, chalcomycin; MAR, maridomycin; JM, rosamycin; LCM, lincomycin; CLDM, clindamycin; PIR, pirlimycin; DHS, dihydrostaphylomycin; MKM, mikamycin; VER, vernamycin; PR, pristinamycin; OST, osteogrycin; V, vernamycin; VIR, virginiamycin. N. C., not clear.

vated product was identified as 2'-(*O*-[β-D-glucopyranosyl] EM-A (Fig. **2**). This product lacked antibiotic activity when tested against several Gram-positive pathogens, as well as *S. vendargensis* (Table **2**).

In 1991, Cundliffe [22, 23] reported an inducible gene, *mgt*, in *Streptomyces lividans*, which inactivates macrolides by UDP-glucose-dependent glycosylation at the 2'-OH of the sugar moieties attached to C-5 of 14- and 15-membered lactones and to C-3 of 12-membered lactones. This enzyme (Mgt) shows a preference for monosaccharide derivatives over disaccharide derivatives. The substrates of Mgt are 12-, 14-, and 15-membered macrolides, or 16-membered lactones (as in methymycin, erythromycin, azithromycin, or tylosin), although spiramycin and carbomycin were apparently not modified. This organism expresses another gene, *lrm* that is linked with *mgt*, encoding a 23S rRNA methyltransferase that confers high resistance to lincomycin, together with lower resistance to macrolides. The deduced *lrm* product is a 26-kDa protein with considerable similarity to other 23S rRNA methyltransferases, such as the *carB*, *tlrA* and *ermE* gene products. The *mgt* gene consists of 1257 bp and encodes a 42-kDa protein. The *lrm* and *mgt* genes occur in tandem in the chromosome, and their expression may be transcriptionally and translationally coupled, since their coding sequences overlap.

A 3.3-kb DNA fragment from the oleandomycin (OL) producer, *Streptomyces antibioticus*, was found to consist of the 3' end of a gene (ORF1) and two complete ORFs (ORF2 and OleD) [24, 25]. The deduced product of the sequenced region of ORF1 contained transmembrane domains characteristic of transport proteins. The ORF2 product contained an N-terminal leader peptide region characteristic of a secretory protein, and a lipid attachment site motif characteristic of membrane lipoproteins synthesized with a precursor signal peptide. The *oleD* gene product showed clear similarity with several UDP-glucuronosyl and UDP-glycosyl transferases of various origins and was especially similar to the *S. lividans mgt* gene product, which is thought to encode a glycosyltransferase capable of inactivating macrolides. The *orf1*, *orf2*, and *oleD* gene products may participate in the intracellular glycosylation of OL and the secretion of glycosylated OL during antibiotic production.

In the study on *oleD*, it was reported that cell extracts of *S. antibioticus* could inactivate OL in the presence of UDP-glucose [9-11]. This enzyme also inactivated other macrolides (rosamicin, methymycin and lincomycin) containing a free 2'-OH group in a monosaccharide linked to the lactone ring (except for erythromycin) (Fig. **3**), but not those containing a disaccharide (tylosin, spiramycin, carbomycin, josamycin, and neomycin), and seems to function in the biosynthetic

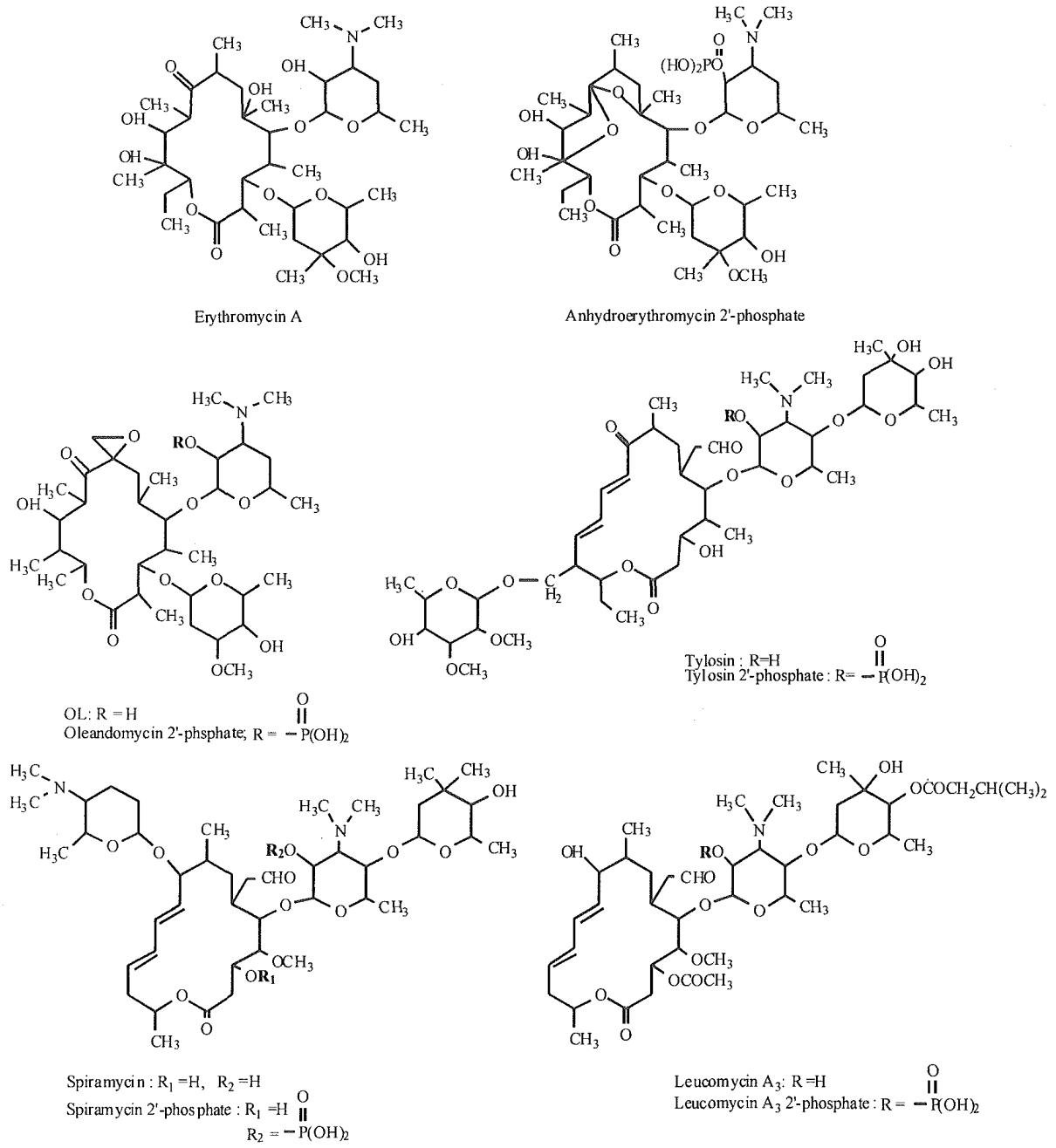


Fig. (1). Structures of substrate and product of erythromycin A, oleandomycin, tylosin, spiramycin I, and leucomycin A_3.

pathway to OL. The culture supernatant of *S. antibioticus* contained another enzyme activity capable of reactivating the glycosylated OL and regenerating the biological activity with the release of a glucose molecule. These two enzyme activities could be an integral part of the OL biosynthetic pathway. OL binds first to the enzyme, followed by UDP-glucose. The ternary complex is thus formed prior to transfer of glucose. UDP is released, followed by the glycosylated OL, and this is the final intracellular product (Fig. 4). The OleB transporter would be responsible for transmembrane secretion [25-28] of this inactive molecule, which would then be extracellularly reactivated by the product of the *oleR* gene. Sequencing analysis of a 5.2-kb region from the OL gene cluster located between the OL polyketide synthesis gene and sugar biosynthetic gene in *S. antibioticus* revealed the presence of three open reading frames (designated *oleI*,

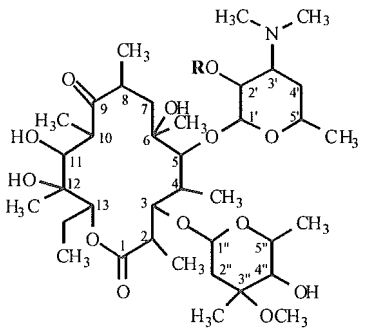


Fig. (2). Structures of erythromycin A (R =H) and 2'-(*O*-[ß-D-glucopyranosyl] erythromycin A (R= glucose).

oleN2 and *oleR*) [29]. The *oleI* gene product resembles other glycosyl-transferases involved in macrolide inactivation, including the *oleD* gene product, a previously described glycosyltransferase from *Streptococcus antibioticus*. The *oleN2* gene product showed similarity with various aminotransferases involved in the biosynthesis of 6-deoxyhexose, and the *oleR* gene product was similar to glucosidases of several origins. The sugar moieties are transferred to a different aglycon by glycosyltransferases such as OleI [30]. The sugar donor was UDP-α-glucose, though the glycosylated product showed a ß-glycoside linkage. The changes in the sugar composition of these molecules may influence the important biological actions of glucosylated OL on ribosomes [29].

Another glycosyltransferase, specific for macrolide monosaccharide, generates a disaccharide linked to the aglycon [31]. Avermectin and ivermectin, a family of oleandrose-containing disaccharide derivatives of 16-membered macrolides produced by *Streptomyces avermitilis*, are glycosylated at C-4" and C-4' by both growing and resting cells of *Saccharopolyspora erythraea* (Fig. **5**). The specificity of the *S. erythraea* enzyme of the glycosyl donor is different from that of the *S. lividans* enzyme. Glycosylated products were formed *in vitro* with UDP-glucose, UDP-galactose, UDP-mannose, and UDP-glucouronic acid, but not with UDP-xylose, UDP-N-acetylglucosamine, UDP-*N*-acetylgalactosamine, or UDP-galactouronic acid. Only the product with UDP-glucose was formed in sufficient quantity to permit its identification. The enzyme in *Streptomyces lividans* utilized only UDP-glucose and UDP-galactose (Table **3**). The *S. lividans* glycosyltransferase, which confers resistance, was induced by substrate macrolides and not by non-substrate macrolides, though that of *S. erythraea* appeared to be constitutive.

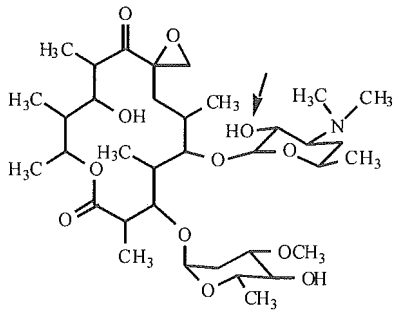


Fig. (3). Chemical structure of oleandomycin. The arrow indicates the hydroxyl group considered to be a potential site for glycosylation.

Streptomyces ambofaciens produces spiramycin, a 16-membered macrolide, and has the *gimA* gene (conferring resistance to spiramycin) downstream of the *srmA* gene, which encodes an rRNA monomethylase [32]. The *gimA* gene product shows a high degree of similarity to the *mgt* gene product in *S. lividans*. In a cloning experiment using a susceptible host mutant of *S. lividans* lacking macrolide-inactivating glycosyltransferase activity, the cloned *gimA* gene was expressed in the presence of UDP-glucose. That is, cell extracts from mutated *S. lividans* could inactivate various macrolides by glycosylation. Spiramycin was not inactivated, but forocidine, a spiramycin precursor, was modified. In *S. ambofaciens*, *gimA* could confer a low level of resistance to some macrolides even under conditions where spiramycin was produced. The putative GTG start codon of the *gimA* gene overlapped with the *srmA* stop codon (GTGA). The ORF of the *gimA* gene consisted of 1254 bp and encoded a 45-kDa protein. The deduced protein showed high homology with the deduced proteins derived from *mgt* of *S. lividans* (82%) [12] and *oleD* of *S. antibioticus* (72%) [24]. Table **4** shows the degree of identity of the DNA and amino acid sequences for four

Table 2. MIC Values of Erythromycin A (EM-A) and 2'-(*O*-[ß-D-glucopyranosyl])EM- A for *Staphylococcus and Streptococcus* Strains [21].

Organism	MIC (μg/ml)	
	EM-A	2'-(*O*-[ß-D-glucopyranosyl]EM-A
Staphylococcus aureus 9218 and 9271	0.5	>32
Streptococcus faecalis 9217	2	>32
Streptococcus pneumoniae 41	0.06	32
Streptococcus pyogenes 152	0.03	16
Streptococcus vendargensis 5315	50	1000

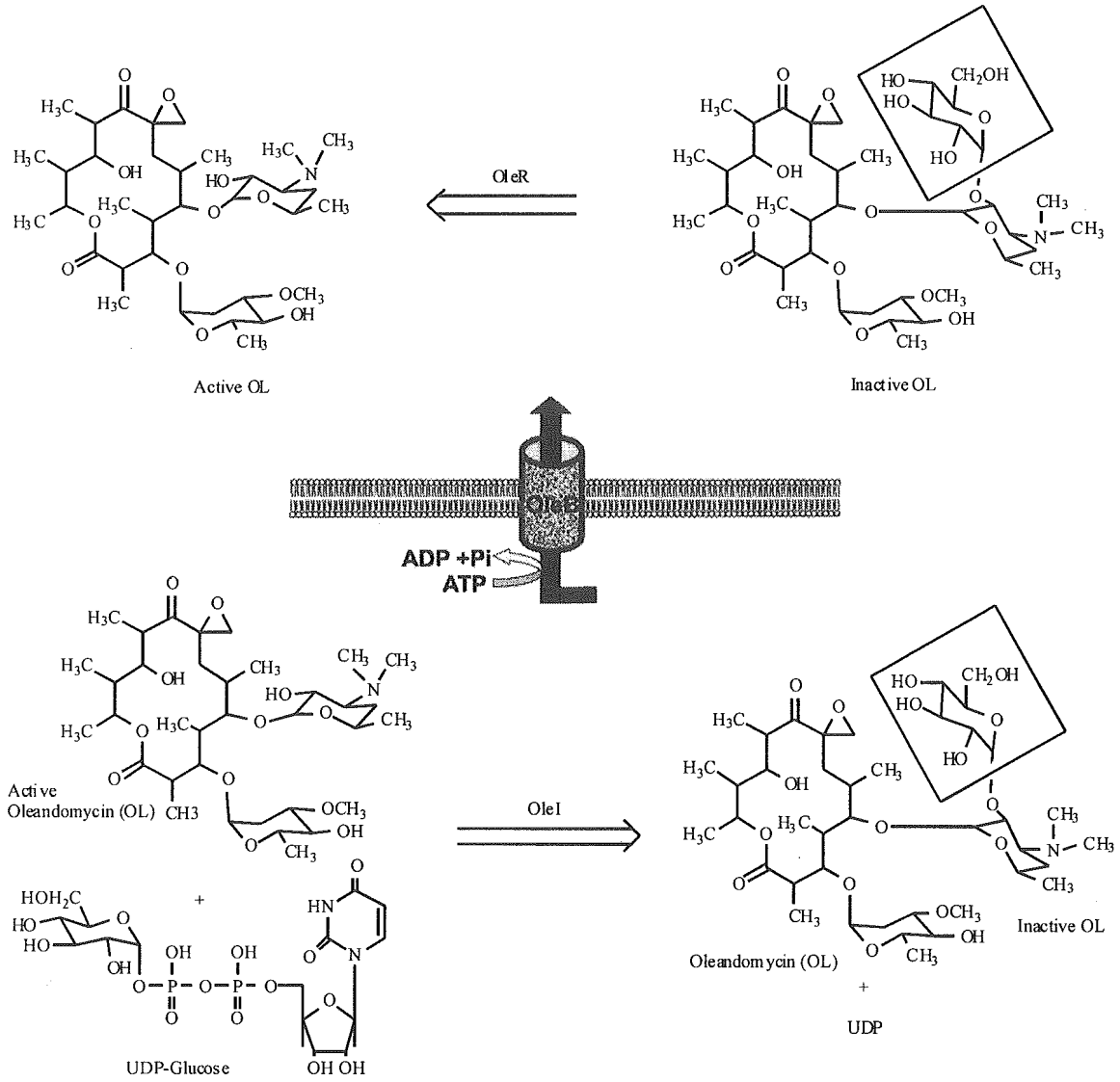


Fig. (4). Strategy for self-protection by oleandomycin producers. Intracellular glucosylation of oleandomycin to an inactive precursor is catalyzed by OleI, then the precursor is pumped out by OleB, and reactivated extracellularly by the glycosidase OleR [28, 29].

glycosyltransferase genes, *mgt, oleI, oleD,* and *gimA*, from different *Streptomyces* spp. In *Streptomyces ambofaciens*, *gimA* was found just downstream of *srmA*, which encodes an rRNA methyltransferase that confers macrolide resistance by target modification [32]. As shown in Fig. (**6**), the glycosyltransferase genes *gimA* and *mgt* are linked with methyltransferase genes, *srmA* in *S. ambofaciens* and *lrm* in *S. lividans*. For *S. antibioticus,* no methyltransferase gene was found upstream of *oleD* or *oleI*. This is in agreement with the observation that ribosomes from *S. antibioticus* are sensitive to OL, even during macrolide production [33]. Upstream of *oleD* are two ORFs, ORF1 and ORF2, which have high degrees of similarity to those of upstream of *srmA*. Thus, some deletion or insertion events may have occurred at the corresponding loci in *S. ambofaciens* or in *S. antibioticus*. The region located upstream of *lrm* in *S. lividans* is completely different from those in the other two strains. This observation suggests that glycosyltransferases like that in *S. vendargensis* are widespread among *Streptomyces* spp [13].

The alignment of the deduced amino acid sequences of three macrolide glycosyltransferases, Mgt from *S. lividans* [12], GimA from *S. ambofaciens* [32] and OleD from *S. antibioticus* [24], is shown in Fig. (7). These proteins contain well-conserved amino acid sequences. Analysis of the amino acid sequences of several glycosyltransferases involved in the biosynthesis of polyketides also showed very well conserved regions, including one of two histidine residues [30]. The conserved histidine seems to play an important role in the catalytic activity of the enzyme and in substrate binding

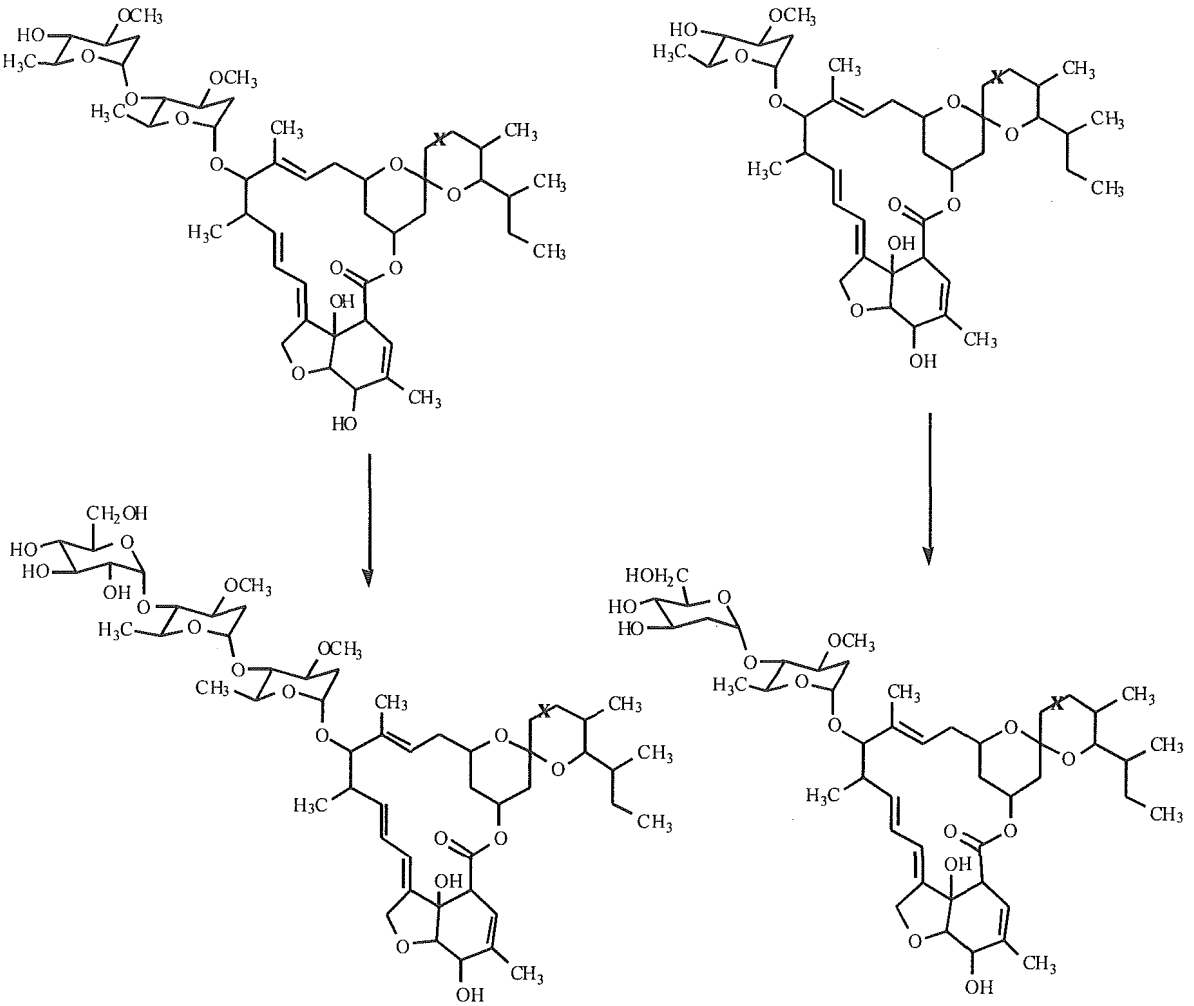


Fig. (5). Glycosylations of evermectin disaccharide and monosaccharide by *Saccharopolyspora*. X, CH_2-CH_2 in invermectin derivatives and CH=CH in avermectin derivatives.

or transition site stabilization in some oligosaccharide-dependent glycosyltransferases.

Table 3. Glycosyl Donors for Glycosyltransferases of *Streptomyces* spp [31].

Glycosyltransferase of	
Streptomyces lividans	*Streptomyces erythraea*
UDP-glucose UDP-galactose	UDP-glucose UDP-galactose UDP-mannose UDP-glucouronic acid

1-3. Deacylation

Deacylation of 16-membered macrolide antibiotics was reported by Nakahama *et al.* [16, 18]. Maridomycin, as well as spiramycin, was deacylated by esterase from *Bacillus megaterium*, *Streptomyces pristinaespiralis* and *Streptomyces olivaceus*. Figure (**8**) shows the deacylation of maridomycin III (MDM III), 9-propionylmaridomycin III (PMDM III) and

Table 4. Homology (%) of DNA and Amino Acid Sequences of Glycosyltransferases from *Streptomyces* spp.

Combination of genes	Homology (%) of	
	DNA	Amino Acid
mgt - gimA	86	82
mgt - oleD	75	72
mgt - oleI	60	40
gimA- oleD	75	72
gimA- oleI	62	41
oleD- oleI	58	38

Accession numbers of genes used for homology analysis were the same as in Table 1.

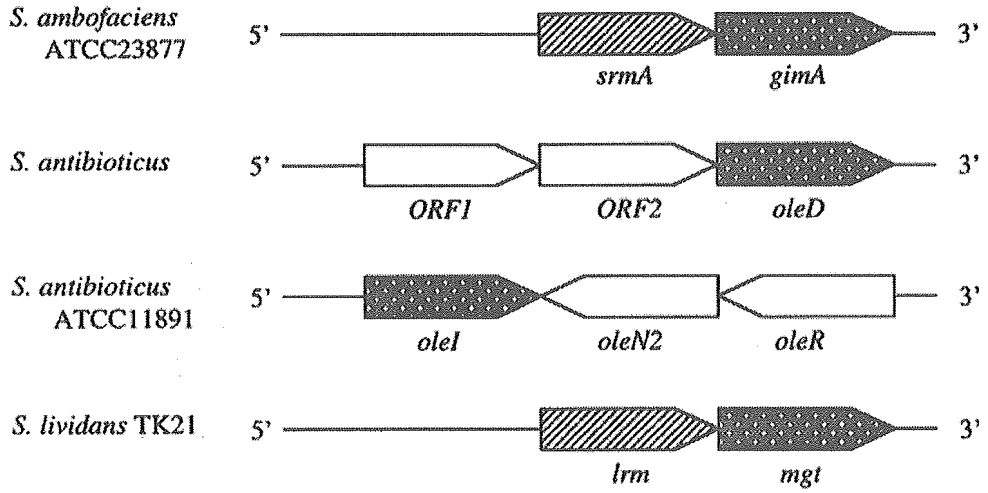


Fig. (6). Comparison of the genetic environments around macrolide resistance genes in various *Streptomyces* spp. *gimA* [32], *oleD* [24], *oleI* [29], and *mgt* [12] encode glycosyltranferase and *srmA* and *lrm* encode methyltransferase.

4"-depropionyl-9-propionylmaridomycin III (PMDM III-M) to 4"-depropionylmaridomycin III (MDM III-M) by an esterase of *Streptomyces* spp. In *B. megaterium*, MDM III and PMDM III are hydrolyzed to MDM III-M and PMDM III-M [16]. *S. olivaceus* also participates in the hydroxylation of maridomycin I (MDM I) to 3"'-hydroxylmaridomycin (HMDM I), as shown in Fig. (**9**) [17]. Josamycin (JM) was also hydroxylated to 3"'-hydroxyjosamycin (HJM) by the same strain, and hydroxylated derivatives of HMD I and HJM exhibited weaker antimicrobial activities [17].

2. Lincosamide Antibiotics

Inactivation studies on lincosamide antibiotics, including lincomycin and clindamycin, have been performed by Argoudelis *et al.* They examined the transformation of lincomycin to lincomycin sulfoxide and 1'-demethylthio-1-hydroxylincomycin by *Streptomyces lincolnensis* [34, 35] and the phosphorylation of lincomycin to lincomycin-3-phosphate by *Streptomyces rochei* [36] (Fig. **10**). These compounds showed loss of the antimicrobial activity during fermentation or growth in a synthetic medium containing *S. lincolnensis* or *S. rochei*, indicating that several *Streptomyces* spp. possess an intrinsic ability to inactivate lincomycin.

Clindamycin is a clinically useful derivative of lincomycin. *Streptomyces coelicolor* completely inactivated clindamycin in less than 48 hr when the antibiotic was added to 24-hr cultures of the organism grown in a complex medium [37]. Clindamycin could be regenerated by treatment of the inactivated fermentation broth with either crude alkaline phosphatase or snake venom phosphodiesterase. This enzymatic behavior suggests that *S. coelicolor* converted clindamycin to compound(s) containing a phosphodiester bond(s). Argoudelis *et al.* have proposed that clindamycin is phosphorylated to clindamycin-3-phosphate [38] and ribonucleotidylated to clindamycin 3-ribonucleotide by *S. coelicolor* [37-39] (Fig. **11**). Enzymic nucleotidylation of lincosamide antibiotics [40] required Mg^{2+} and nucleoside-5'-triphosphates when examined with crude enzyme preparation from *S. coelicolor* in medium maintained at around pH 6.

3. Streptogramin Antibiotics

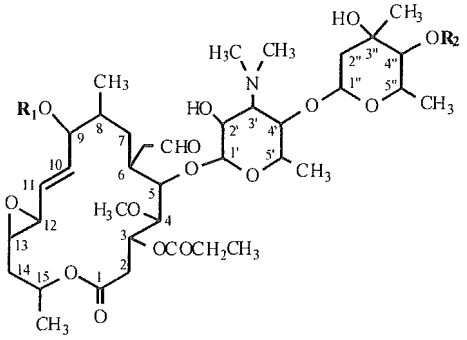
Streptogramin family antibiotics consist of two groups of components, type A and type B, exemplified by virginiamycin M and virginiamycin S, respectively [41] (Fig. **12**). To date, very few investigations of inactivation in this family have been reported. Hou *et al.* obtained an antibiotic lactonase with a molecular weight of 35,000 from *Actinoplanes missouriensis*, and this enzyme hydrolyzed the lactone bond of dihydrostaphylomycin S (a type B streptogramin antibiotic) [42]. Mikamycin lactonase from *Streptomyces mitakaensis*, a producer of mikamycin A and B, was purified and characterized, and the structure of the reaction product, mikamycin-B acid, was identified [43, 44] (Fig. **13**). This enzyme has a molecular weight of 29,000 and catalyzes the hydrolytic degradation of mikamycin B. The reason for the difference of molecular weight is of interest, since both enzymes appear to hydrolyze the lactone linkage of type B streptogramin acid.

The inactivation of streptogramins in *Streptomyces* spp. other than *S. mitakaensis* was examined by Fernando Fierro *et al.* [45]. *Streptomyces diastaticus*, *Streptomyces loidensis*, and *Streptomyces olivaceus* were quite sensitive to most of the macrolides and lincosamides examined, but resistant to both A (or M) and B (or S) streptogramin components. This phenotypical trait was also observed in inactivation assay using cell-free extracts of the producers and type A and B streptogramin antibiotics. Though the mechanism of this inactivation is not clear, it could be lactone hydrolysis, as mentioned above.

In contrast to the lactone ring hydrolysis by mikamycin B-lactonase, inactivation of virginiamycin M1 from *Streptomyces virginiae* involved reduction of the C-16 carbonyl group [41, 46] (Fig. **14**).

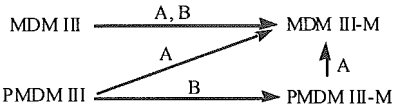
```
Mgt      1 MKRKELHETSRLAYGRRMTTRPAHIAMFSIALHGHVNPSLEVIRELVARGHRVTYAIPRL   60
GimA     1 VRRGDLHETYRLDYAPHMHDPAHIAMFS-IAAHGHVNPSLEVIRELVARGHRVTYAIPPL   59
OleD     1 ---------------VTTQTTPAHIAMFSIAAHGHVNPSLEVIRELVARGHRVTYAIPPV   45

Mgt     61 LADKVAEAGAEPKLWNSTLPGPDADPEAWGSTLLDNVEPFLADAIQSLPQLAQAYEGDEP   120
GimA    60 FAEKVAETGAEPKLWNSTLPGPDADPDAWGTTPLDNVEPFLDDAIQALPQLIAAYEGDEP   119
OleD    46 FADKVAATGPRPVLYHSTLPGPDADPEAWGSTLLDNRRTFLNDAIQALPQLADAYADDIP   105

Mgt     121 DLVLHDIASYTARVLGRRWEVPVISLSPCMVAWEGYEQEVGEPMWEEPRKTERGQAYYAR   180
GimA    120 DLVLHDITSYPARVLAHRWGVPAVSLSPNLVAWEGYEEEVGRPTWEEPLKTERGRAYDAR   179
OleD    106 DLVLHDITSYPARVLARRWGVPAVSLSPNLVAWKGYEEEVAEPMWREPRQTERGRAYYAR   165

Mgt     181 FHAWLEENGITDHPDPFIGRPDRSLVLIPKALQPHADRVDETTYTFVGACQGDRTAEGDW   240
GimA    180 FRGWLKENGITEDPDPFVGRPDRSLVLIPKALQPHADRVDEKTHTFVGACQGDRAAEGDW   239
OleD    166 FEAWLKENGITEHPDTFASHPPRSLVLIPKALQPHADRVDEDVYTFVGACQGDRAEEGGW   225

Mgt     241 ARPEGAEKVVLVSLGSAFTKQPAFYRECVRAFGELPGWHTVLQVGRHVDPAELGDVPDNV   300
GimA    240 RRPEGAEKVVLVSLGSSFTKRPAFYRACVEAFGALPGWHVVLQVGRHVDPAELGDVPENV   299
OleD    226 QRPAGAEKVVLVSLGSAFTKQPAFYRECVRAFGNLPGWHLVLQIGRKVTPAELGELPDNV   285

Mgt     301 EVRTWVPQLAILQQADLFVTHAGAGGSQEGLATATPMIAVPQAADQFGNADMLQGLGVAR   360
GimA    300 EVRSWVPQLAILKQADLFVTHAGAGGSQEGLATATPIVAVPQAVDQFGNADMLQGLGVGR   359
OleD    286 EVHDWVPQLAILRQADLFVTHAGAGGSQEGLATATPMIAVPQAVDQFGNADMLQGLGVAR   345

Mgt     361 TLPTEEATAKALRTAALALVDDPEVAARLKEIQARMAQEAGTRGPADLIEAELAAARG--   418
GimA    360 HLPTEEATAEALRAAGLALVEDPEVARRLKEIQAGMAREGGTRRAADLIEAELAAART--   417
OleD    346 KLATEEATADLLRETALALVDDPEVARRLRRIQAEMAQEGGTRRAADLIEAELPARHERQ   405

Mgt     419 -------------------------                                      443
GimA    418 -------------------------                                      442
OleD    406 EPVGDRPNVGDRPAGVRSDRQRSAL                                      430
```

Fig. (7). Alignment of the deduced amino acid sequences of three macrolide glycosyltransferases, Mgt [12], GimA [32] and OleD [24]. The histidine residue shown by the arrow seems to play an important role in the catalytic activity of the enzyme and in substrate binding or transition site stabilization in some oligosaccharide-dependent glycosyltransferases.



(Fig. (8). Contd....)

	R_1	R_2
Maridomycin II (MDM III)	H	$COCH_2CH_3$
4"-depropionylmaridomycin III (MDM III-M)	H	H
9-propionylmaridomycin III (PMDM III)	$COCH_2CH_3$	$COCH_2CH_3$
4"-depropionyl-9-propionylmaridomycin III (PMDM III-M)	$COCH_2CH_3$	H



[Deacetylation by *Streptomyces pristinaespiralis* IFO13074 (A) and *Streptomyces olivaceus* 219 (B)]

Fig. (8). Deacylation of maridomycin III (MDM III), 9-propionylmaridomycin III (PMDM III) and 9-propionylmaridomycin III (PMDM III-M) by *Streptomyces pristinaespiralis* IFO13074 and *Streptomyces olivaceus* 219 [18].

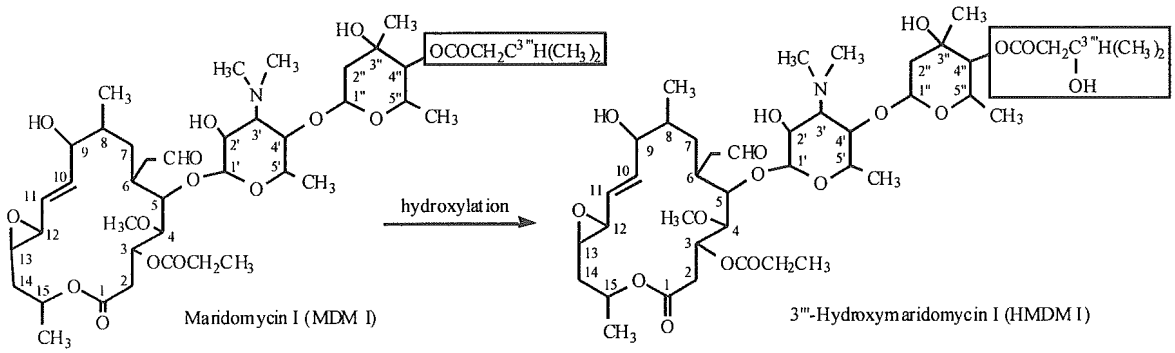


Fig. (9). Hydroxylation of MDM I to HMDM I by *Streptomyces olivaceus* 219 [17].

(B) PATHOGENS

Bacterial inactivation of antibiotics affects only structurally related antibiotics, and is different in this respect from target modification, such as dimethylation of 23S rRNA (the target site of macrolide, lincosamide and streptogramin (MLS) antibiotics). Interestingly, enzymatic inactivation of macrolides appears to be unusual in clinical isolates compared with resistance due to target site modification or efflux [47-52].

Hydrolysis by esterase [53-57] and phosphorylation [58, 59] by phosphotransferase were observed in Gram-negative bacteria such as *E. coli* originally, and in *Providencia stuartii* [56] in recent years. However, the G+C content suggests that the phosphotransferases from Gram-negative bacteria

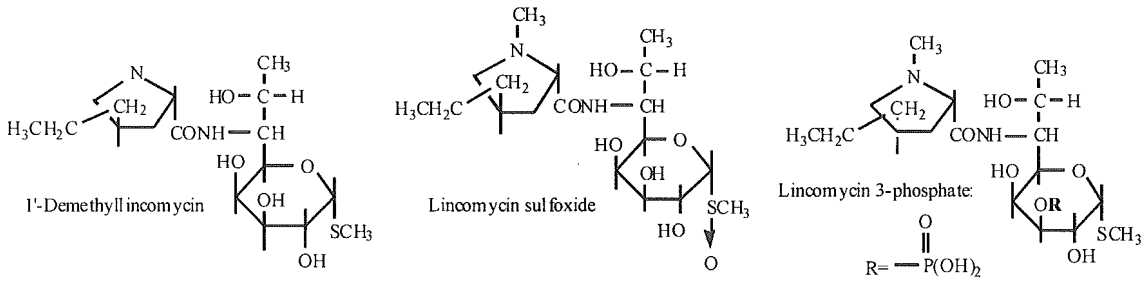


Fig. (10). Structures of 1'-demethylated lincomycin [34], lincomycin sulfoxide [35], and 3-phosphorylated lincomycin [36].

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.

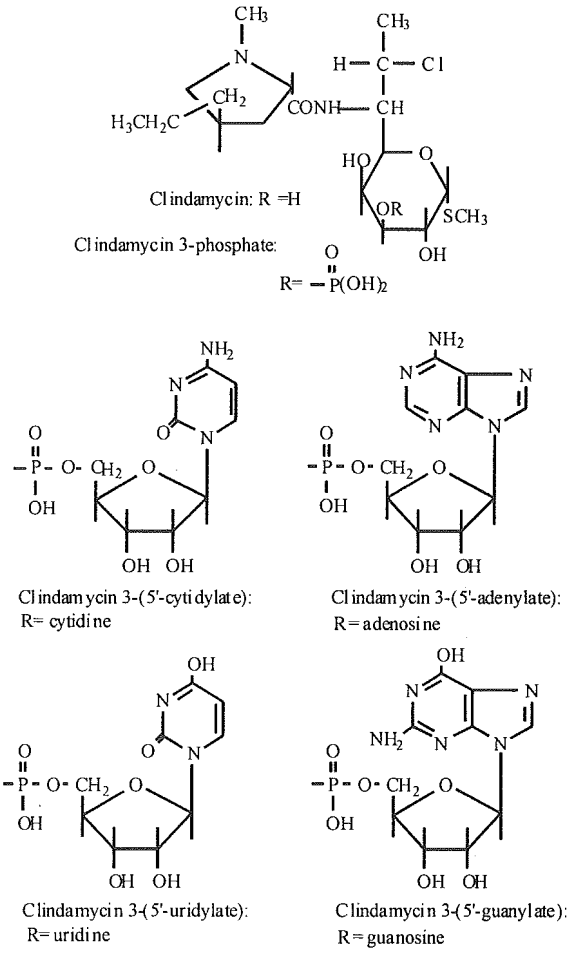


Fig. (11). Structures of clindamycin 3-phosphate and clindamycin 3-ribonucleotides [39].

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].

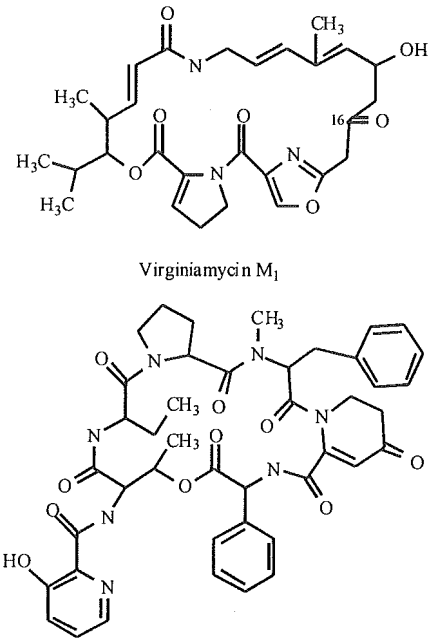


Fig. (12). Structures of virginiamycin M_1 and virginiamycin S [41].

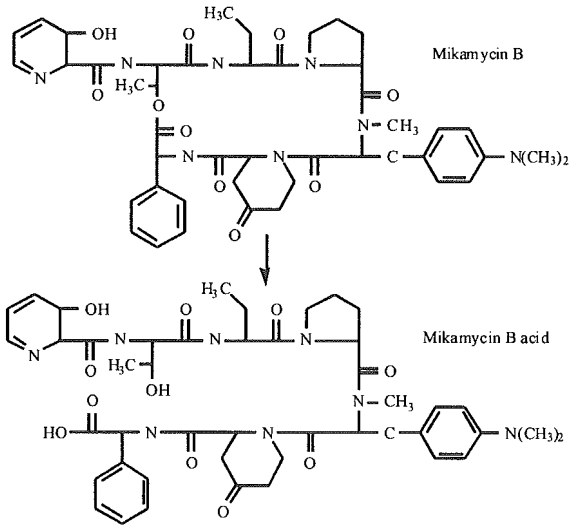
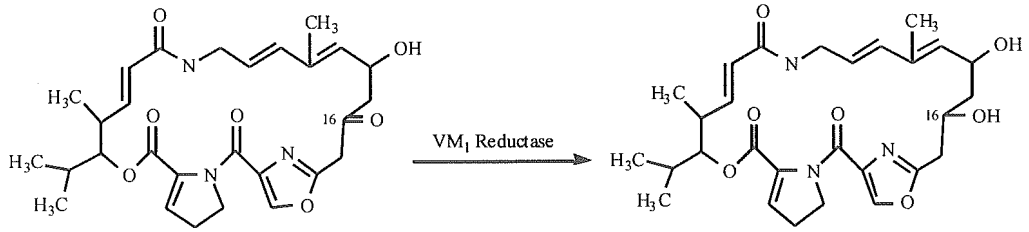


Fig. (13). Reaction of mikamycin B lactonase of *Streptomyces mitakaensis* [43].

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



Virginiamycin M_1 16-Dihydrovirginiamycin M_1

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