表３．インフルエンザ菌が起炎菌であった小児肺炎の抗菌薬治療による比較（ABPCのMICが２μg/ml以上の症例）

MIC(μg/ml)	治療薬による分類	症例数	白血球最高値(μl)	CRP最高値(mg/dl)	解熱までの日数
2．0	A群	14	11,532	4．4	1．8
	B群	7	17,917	1．8	1．6
4．0≦	A群	9	12,945	2．1	2．8
	B群	3	14,333	1．3	1．3

A群：ペニシリン系抗菌薬による治療群

B群：ペニシリン以外の抗菌薬による治療群

表４．ABPC感受性インフルエンザ菌が起炎菌であった小児肺炎の*pbp*-3遺伝子変異数による比較

遺伝子変異数	症例数	年齢（才）	白血球最高値(μl)	CRP最高値(mg/dl)	解熱までの日数
0	6	2．2	15,216	3．5	2．2
1	6	1．3	10,550	2．6	1．5
2	12	1．9	12,276	1．5	1．7

表５．b型インフルエンザ菌による髄膜炎患児における抗PRP抗体（μg/ml）

	年齢	性別	抗PRP抗体値（血清採取日の病日）			
症例1	1歳	M	2.35 (5)	3.08 (11)	5.14 (5M)	1.27(11M)
2	1歳	F	1.43 (0)	2.00 (21)	2.02 (41)	
3	1歳	M	> 9.0 (4)	> 9.0 (19)	> 9.0 (32)	
4	1歳	M	1.03 (1)	2.81(11)	2.88 (20)	
5	7ヶ月	M	1.45 (10)	1.17 (12)		

Ⅲ. 研究成果の刊行に関する一覧表

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雑 誌

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	2) Characterization and molecular analysis of macrolide-resistant *Mycoplasma pneumoniae* clinical isolates obtained in Japan.	Antimicrob. Agents Chemother	48	4624-4630	2004
	3) Use of fluorescent-protein tagging to determine the subcellular localization of *Mycoplasma pneumoniae* proteins encoded by the cytadherence regulatory locus.	J. Bacteriol.	186	6944-6955	2004
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	2) Recombinant cholera toxin B subunit (rCTB) as a mucosal adjuvant enhances inducyion of diphtheria and tetanus antitoxin antibodies in mice by intranasal administration with diphtheria-pertussis-tetanus(DPT) combination vaccine.	Vaccine	22	3061-3068	2004
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	3) Characterization and molecular analysis of macrolide-resistant *Mycoplasma pneumoniae* clinical isolates obtained in Japan.	Antimicrob. Agents Chemother	48	4624-4630	2004
荒川宜親	1) Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan.	J.Clin. Microbiol.	42	5453-5457	2004
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Ⅳ. 研究成果の刊行物・別冊

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Antigenic Divergence Suggested by Correlation between Antigenic Variation and Pulsed-Field Gel Electrophoresis Profiles of *Bordetella pertussis* Isolates in Japan

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Antigenic divergence has been found between *Bordetella pertussis* vaccine strains and circulating strains in several countries. In the present study, we analyzed *B. pertussis* isolates collected in Japan from 1988 to 2001 using pulsed-field gel electrophoresis (PFGE) and sequencing of two virulence-associated proteins. The 107 isolates were classified into three major groups by PFGE analysis; 87 (81%) were type A, 19 (18%) were type B, and 1 (1%) was type C. Sequence analysis of the S1 subunit of pertussis toxin (*ptxS1*) and adhesion pertactin (*prn*) genes revealed the presence of two (*ptxS1A* and *ptxS1B*) and three (*prn1*, *prn2*, and *prn3*) variants, respectively, in the isolates. Among those isolates, 82 (95%) of the 87 type A strains and the type C strain had the same combination of *ptxS1B* and *prn1* alleles (*ptxS1B/prn1*) as the Japanese vaccine strain. On the other hand, 17 (90%) of 19 type B strains had an allele (*ptxS1A/prn2*) distinct from that of the vaccine strain. A correlation was found between the antigenic variation and the PFGE profile in the isolates. In addition, the frequency of the type B strain was 0, 27, 0, 42, and 37% of the isolates in the periods 1988 to 1993, 1994 to 1995, 1996 to 1997, 1998 to 1999, and 2000 to 2001, respectively. In contrast, the number of reported pertussis-like and pertussis cases decreased gradually from 1991 on, suggesting that the antigenic divergence did not affect the efficacy of pertussis vaccination in Japan.

Bordetella pertussis is the primary etiologic agent of the disease pertussis. Whole-cell and acellular pertussis vaccines have been very effective at inducing protection against *B. pertussis* infection (23, 24). In Japan, pertussis vaccination was started in 1950 using whole-cell vaccine. Then, in 1981, acellular pertussis vaccines containing detoxified pertussis toxin (PT) and filamentous hemagglutinin as major antigens were introduced and have successfully controlled the prevalence of pertussis ever since (24). Most people with cases of pertussis (98.7%) reported to the National Epidemiological Surveillance of Infectious Diseases from 1987 to 1996 had no history of pertussis vaccination in Japan (21). However, in recent years, a resurgence of pertussis has been found in several countries despite high vaccination coverage (1, 5, 8). Since Mooi et al. (20) found that the circulating clinical strains had antigens distinct from those of vaccine strains, they proposed that the circulating clinical strains might have escaped the immunity provided by pertussis vaccination. The antigenic divergence between recently circulating strains and vaccine strains has been reported in European and North American countries (3, 6, 9, 16, 19, 22, 29–31) but not in Asian countries.

In the United States and France, genetic diversity of circulating *B. pertussis* isolates has been observed using pulsed-field gel electrophoresis (PFGE) (10, 31). In addition, the genetic divergence between circulating strains and vaccine strains can also be detected by DNA typing analyses, by IS*1002*-based DNA fingerprinting, and by sequencing the structural genes encoding *B. pertussis* virulence factors. The antigenic variants have been observed in the genes encoding the S1 subunit of PT (*ptxS1*) and pertactin (*prn*), which are important virulence factors of *B. pertussis*. Recently, polymorphism was also found in the genes encoding the S3 subunit of PT (*ptxS3*) and tracheal colonization factor (*tcf*) in circulating clinical strains (28). However, it is not clear whether the antigenic variations and DNA types in *B. pertussis* are associated with each other. For example, van Loo et al. (29, 30) found congruence between clustering based on IS*1002*-based DNA fingerprint types of Dutch clinical strains and the *ptxS1* allele but not the *prn* allele. In contrast, in those strains found recently in France, most of the circulating strains showed a correlation between the PFGE profile and *prn* allele but not *ptxS1* (31). Moreover, there was no high correlation between the PFGE types and the combinations of *ptxS1* and *prn* alleles in the strains found in Canada or the United States (3, 22).

In a previous study, most Japanese *B. pertussis* isolates (83%) collected in the period 1975 to 1996 had the same *ptxS1* and *prn* alleles as the Japanese vaccine strain, whereas the two recently circulating isolates exhibiting a different PFGE profile had *ptxS1* and *prn* alleles different from those of the vaccine strain (7). Since a limited number of isolates (n = 12) were used in the study, it was not clear whether the antigenic divergence in *ptxS1* and *prn* alleles has progressed in circulating strains in Japan. In the present study, we collected 107 Japanese clinical isolates, including recently circulating strains, and the antigenic divergence in those strains was investigated using

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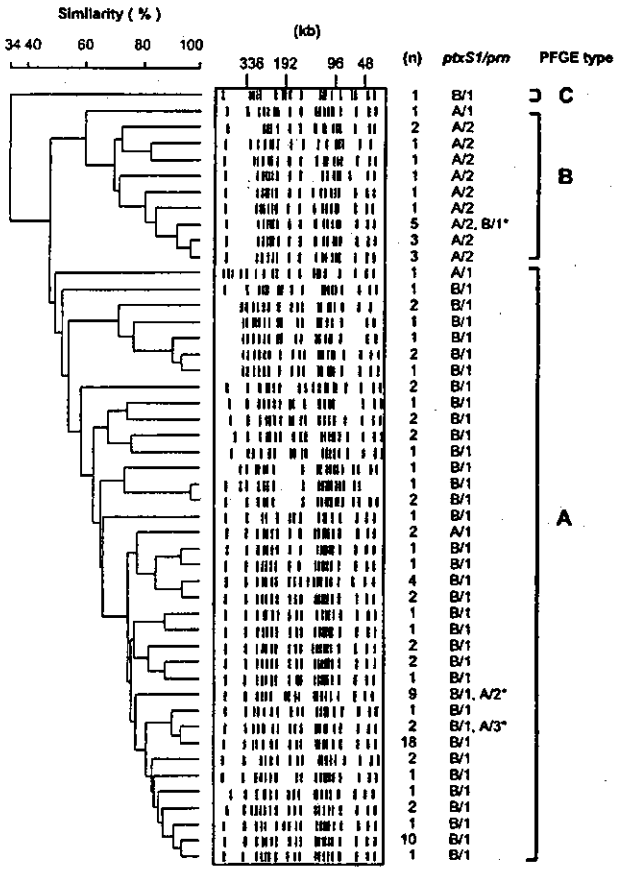

FIG. 1. Dendrogram of PFGE profiles of 107 Japanese *B. pertussis* isolates from 1988 to 2001. The dendrogram was calculated by UPGMA. The regions including the *ptxS1* and *prn* repeat were sequenced, and the combination of *ptxS1* and *prn* alleles is shown as *ptxS1/prn*, e.g., B/1 indicates *ptxS1B/prn1*. *, one isolate had different *ptxS1/prn* alleles in the PFGE profile.

PFGE analysis and sequencing of *ptxS1* and *prn* alleles. The possible association of the antigenic variations with the PFGE profiles in the isolates was also investigated.

MATERIALS AND METHODS

Isolates. One hundred seven *B. pertussis* clinical isolates, collected from 1988 to 2001 in Japan, were obtained from 22 medical institutes, hospital laboratories, private clinical laboratories, and prefectural public health institutes. The periods of isolation and numbers of strains isolated were as follows: 1988 to 1993, 35; 1994 to 1995, 15; 1996 to 1997, 18; 1998 to 1999, 12; 2000 to 2001, 27. All of the isolates were confirmed as *B. pertussis* in our laboratory using PCR identification (14). For comparison, the Japanese acellular vaccine strain *B. pertussis* Tohama was used as a reference strain. The strains were cultured on Bordet-Gengou agar (Difco) supplemented with 1% glycerol and 15% defibrinated horse blood and incubated at 36°C for 2 to 3 days.

PFGE analysis. PFGE was performed according to standardized recommendations for typing of *B. pertussis* (18), with minor modifications. Chromosomal DNA was digested with the restriction enzyme XbaI, and the digested fragments were separated using a CHEF DR II apparatus (Bio-Rad). Electrophoresis was performed at 6 V/cm at 16°C with the following ramped switch times: block 1, 4 to 8 s for 12 h; block 2, 8 to 50 s for 10 h. The PFGE patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using Diversity Database version 1.1 software (PDI, Inc.).

DNA sequencing. DNA sequencing of relevant regions of the pertactin (*prn*) and S1 subunit of pertussis toxin (*ptxS1*) genes was performed on PCR fragments as described previously (18). Chromosomal DNA of *B. pertussis* was isolated using a QIAGEN genomic tip (20G) and genomic DNA buffer set. Sequence reactions were carried out with a BigDye terminator version 3.1 cycle-sequencing kit (Applied Biosystems), and the products were sequenced on a PRISM 3100 genetic analyzer (Applied Biosystems). Regions 1 of *prn* and *ptxS1* were sequenced for all 107 clinical isolates. On the other hand, region 2 of *prn* was sequenced for 40 of the 107 isolates, since polymorphism has been reported infrequently in the region (19, 20). The carbohydrate recognition domain (fragment A) of the *fhaB* gene was also sequenced between positions 3421 and 3837 (17). The sense primer fraAF (5′-CGACATCATCATGGATGCGA-3′) and the antisense primer fraAR (5′-TCTGGAAGGTGCCCCTGTTC-3′) were used for the sequencing.

Purification of PT variants and analysis of biological activity. Two PT variants, PT-194M and PT-194I, encoded by *ptxS1B* and *ptxS1A*, respectively, were purified from each culture supernatant using an Affi-gel blue column and a fetuin-Sepharose column as described previously (25). The protein concentrations of the purified PT variants were measured by Lowry assay with bovine serum albumin as a standard. The purity of PT-194M and PT-194I was >95%, as estimated by sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis, followed by Coomassie blue R-250 staining. The biological activities of the purified PT variants were determined by the Chinese hamster ovary (CHO) cell-clustering test (13).

RESULTS

PFGE typing and polymorphism in PT-S1 and pertactin. Among 107 *B. pertussis* isolates collected in Japan during the period 1988 to 2001, 48 PFGE profiles were identified (Fig. 1). The 48 PFGE profiles were classified into three major groups using UPGMA; type A comprised 37 PFGE profiles, type B comprised 10 profiles, and type C had one profile. The type A strain was a major group of isolates, since 87 (81%) of the 107 isolates were type A. On the other hand, types B and C were minor groups: 19 isolates (18%) were type B and 1 isolate (1%) was type C. Surprisingly, the type C strain had the same profile as the Japanese vaccine strain, *B. pertussis* Tohama.

The *ptxS1* and *prn* genes of all 107 isolates were sequenced. Two PT-S1 subunit alleles (*ptxS1A* and *ptxS1B*) and three pertactin alleles (*prn1*, *prn2* and *prn3*) were identified among the isolates (Fig. 2). Four *ptxS1* and eight *prn* variants have been

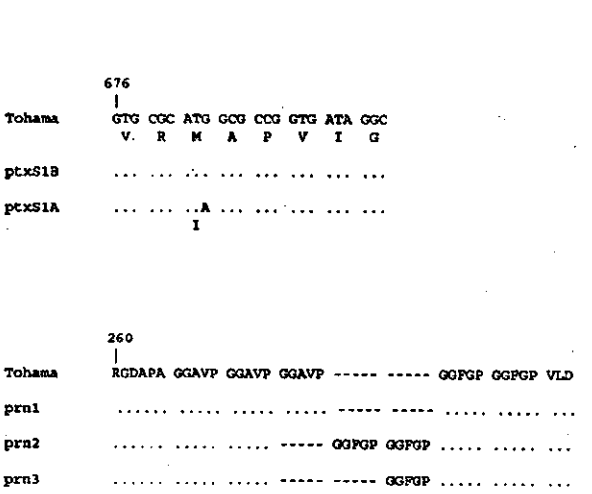

FIG. 2. Variants of pertussis toxin S1 subunit and pertactin observed in Japanese *B. pertussis* isolates. (A) Primary structure of pertussis toxin S1 subunit gene showing regions of polymorphism. (B) Deduced amino acid sequence of region 1 of pertactin. The dots indicate sequence identity with the Japanese vaccine strain, *B. pertussis* Tohama. The dashed lines indicate gaps. The vaccine strain has a combination of *ptxS1B/prn1* alleles.

TABLE 1. Correlation between PFGE type and *ptxS1/prn* alleles in *B. pertussis* isolates collected from 1988 to 2001 in Japan

PFGE type	No. of isolates	No. (%) of *ptxS1/prn* alleles			
		Old *ptxS1B/prn1*	Transitional *ptxS1A/prn1*	New *ptxS1A/prn2*	New *ptxS1A/prn3*
A	87	82 (95)	3 (3)	1 (1)	1 (1)
B	19	1 (5)	1 (5)	17 (90)	
C	1	1			
Total	107	84 (78)	4 (4)	18 (17)	1 (1)

described (6); however, only two *ptxS1* and three *prn* variants were found in the Japanese isolates. The Japanese vaccine strain had the *ptxS1B* and *prn1* alleles described previously (2, 7). The *fhaB* genes from the 40 selected isolates (type A, 20 isolates; type B, 19 isolates; type C, 1 isolate) were sequenced between bases 3421 and 3837. No polymorphism was observed in the sequence.

Correlation between PFGE type and combination of *ptxS1* and *prn* alleles. As shown in Fig. 1, there was a correlation between the PFGE profile and the combination of *ptxS1* and *prn* alleles in the Japanese isolates, although the smallest genetic distance (10%) was observed between the type A and type B groups. Table 1 summarizes the correlation between the PFGE type and the combination of *ptxS1* and *prn* alleles. For convenience, *ptxS1* and *prn* alleles have been placed in one of three groups: *ptxS1B/prn1*, old; *ptxS1A/prn1*, transitional; and *ptxS1A/prn2* and *ptxS1A/prn3*, new (3, 22). Of the 87 type A strains, 82 (95%) isolates had old *ptxS1B/prn1* alleles and 3 and 2 isolates had transitional and new alleles, respectively. The Japanese vaccine strain also had the old *ptxS1B/prn1*. In contrast, 17 (90%) of 19 type B strains had new *ptxS1A/prn2* alleles, and each isolate had old and transitional alleles.

We also analyzed the PFGE data using the neighbor-joining clustering method instead of UPGMA. All of the type B strains were classified in the same group, and the same result was obtained (data not shown).

Trend in type B strain in Japan. As shown in Fig. 3, type B strains were collected in various areas of Japan, as were type A strains. Four type B strains were first collected in the Chugoku and Hokkaido districts in 1994 and 1995. Five were collected consecutively in the Kinki and Kanto districts in 1998 and 1999, and 10 were collected in the Tohoku, Kanto, and Kinki districts in 2000 and 2001 (data not shown). Thus, type B strains were widely distributed throughout Japan during these times.

Figure 4B shows the temporal trend of the frequency of the type B strain according to the year of collection. Surprisingly, the percentage of the type B strain changed between 1994 and 1995 and between 2000 and 2001 (0% from 1988 to 1993, 27% from 1994 to 1995, 0% from 1996 to 1997, 42% from 1998 to 1999, and 37% from 2000 to 2001), although the numbers of reported pertussis-like and pertussis cases had decreased gradually from 1991 (Fig. 4A). The temporal trend of the type B strain harboring new *ptxS1A/prn2* was the same as the trend of the type B strain. The frequency of the type B strain harboring *ptxS1A/prn2* was 20, 0, 42, and 33% of the isolates in the periods 1994 to 1995, 1996 to 1997, 1998 to 1999, and 2000 to 2001, respectively.

Biological activities of PT variants. The biological activities of vaccine-type PT (PT-194M) and nonvaccine-type PT (PT-194I) were assessed. Two PT variants, PT-194M and PT-194I, encoded by the old *ptxS1B* and the new *ptxS1A*, respectively, were purified from each culture supernatant, and the biological activities of the purified PT variants were determined by the CHO cell-clustering test. The minimum concentrations required for the clustering of PT-194M and PT-194I were assessed to be 68 (95% confidence interval, 52 to 89) and 44 pg/ml (95% confidence interval, 25 to 76 pg/ml), respectively. No significant difference was observed between the biological activities.

DISCUSSION

In a previous study, most Japanese *B. pertussis* isolates collected from 1975 to 1996 had the same *ptxS1B* and *prn1* alleles as the Japanese vaccine strain (7). However, since a limited number of isolates were used in the study, it was not clear whether the antigenic divergence between recently circulating strains and the vaccine strain has progressed. In the present study, 107 Japanese *B. pertussis* isolates, including recently circulating strains, were collected, and the antigenic divergence in those isolates was investigated. We found that the type B

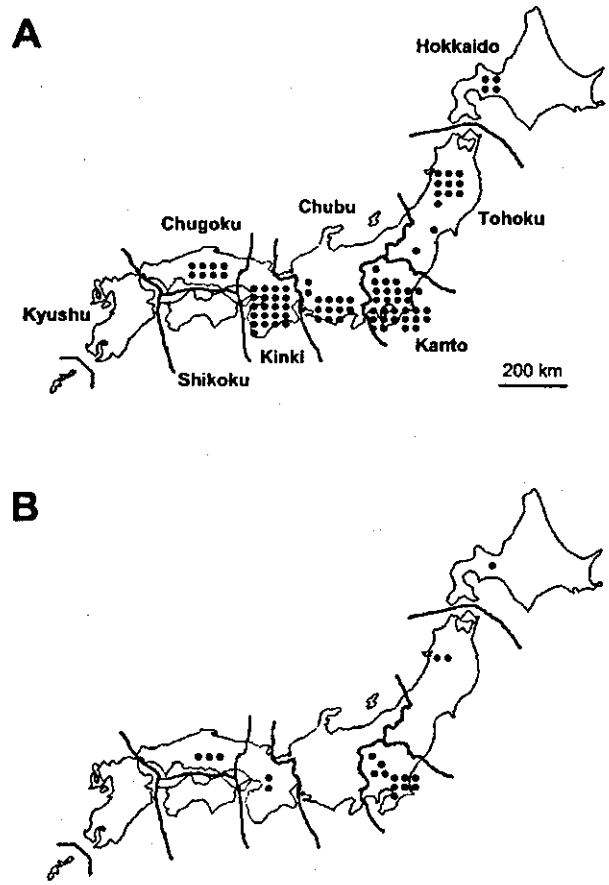

FIG. 3. Geographic distribution of type A (A) and type B (B) strains collected from 1988 to 2001 in Japan. The numbers of symbols indicate the numbers of isolates.

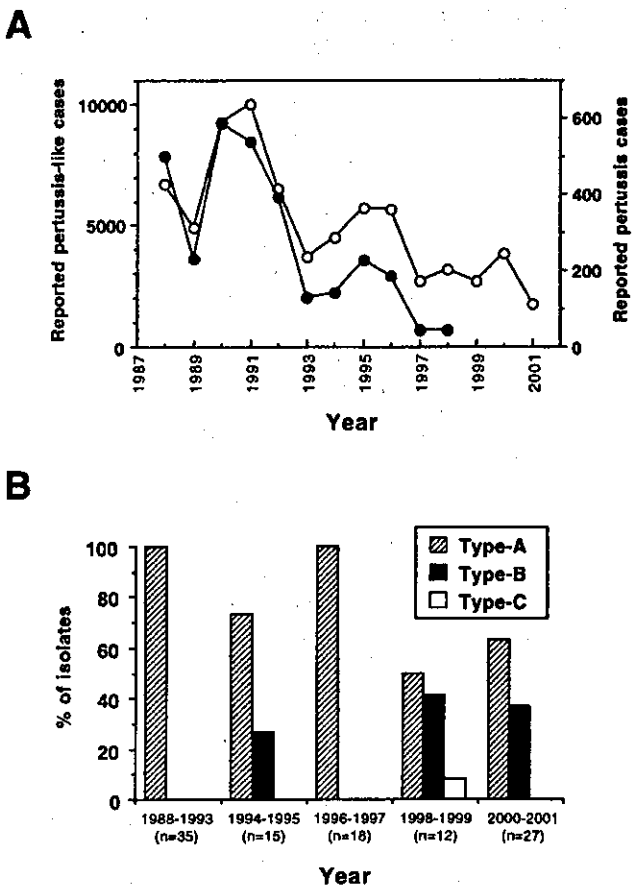

FIG. 4. Temporal trend in isolation of type B strains and reported pertussis cases in Japan. (A) Pertussis-like cases (○) and pertussis cases (●) reported by sentinel clinics and hospitals in Japan from 1988 to 2001. The data were obtained from Infectious Disease Surveillance data of the Ministry of Health, Labor and Welfare of Japan. The reporting of pertussis cases was discontinued in 1999. (B) Changes in the frequencies of type A, type B, and type C strains.

strain harboring nonvaccine *ptxS1A* and *prn2* alleles appeared during the period 1994 to 1995. Thus, the antigenic divergence has progressed since the mid-1990s in Japan, similar to other countries, such as European and North American countries.

Despite high vaccination coverage, the resurgence of pertussis has been reported in several countries (1, 5, 8). In The Netherlands, the incidence of pertussis increased dramatically in 1996 and 1997 (5), and the antigenic divergence in the protective antigens encoded by *ptxS1* and *prn* was observed in the circulating strains (20). In regard to the resurgence, Mooi et al. (20) proposed that circulating strains distinct from the vaccine strain might have escaped the immunity provided by vaccination. In contrast, in the United Kingdom, an antigenic divergence in the *prn* allele was observed in recently circulating strains (6), despite the relatively few pertussis cases there (27). These observations suggested that the presence of nonvaccine *prn2* has not been associated with a resurgence of pertussis. In the present study, the type B strain harboring *ptxS1A/prn2* distinct from those of the vaccine strain appeared in the mid-1990s, although the reported pertussis and pertussis-like cases had decreased since 1991 (Fig. 4). Most type B strains (90%) had not only *prn2* but also nonvaccine *ptxS1A*. Therefore, our finding suggested that (i) the presence of nonvaccine *ptxS1A*, as with *prn2*, has not been associated with a resurgence of pertussis and (ii) the type B strain might not have escaped the immunity provided by vaccination in Japan.

In a previous study, Weber et al. (31) found no correlation between the PFGE type and the *ptxS1* allele in French *B. pertussis* isolates but revealed a correlation with the *prn* allele. In the Canadian and U.S. isolates, there was no high correlation between the PFGE type and the combination of *ptxS1* and *prn* alleles (3, 22). However, among the U.S. isolates, all isolates harboring *ptxS1A/prn2* clustered in a relatedness group at the phylogenetic tree, whereas isolates harboring *ptxS1B/prn1* and *ptxS1A/prn1* were scattered throughout the tree (3). In contrast, among Japanese isolates, most (90%) type B strains had a combination of *ptxS1A* and *prn2* (*ptxS1A/prn2*) and most (95%) type A strains and the type C strain had the same *ptxS1B/prn1* as the Japanese vaccine strain (Table 1). Thus, there was a correlation between the PFGE types and *ptxS1/prn* alleles in the Japanese isolates. Strains harboring *ptxS1B* and *prn1* had been collected prior to the 1970s in European countries and the United States, whereas strains harboring *ptxS1A*, *prn2*, and *prn3* had been collected in those countries since the early 1980s (3, 19, 20). Therefore, *ptxS1B* and *prn1* alleles have been called old alleles, while *ptxS1A*, *prn2*, and *prn3* are called new alleles (3, 22). Moreover, strains harboring a combination of new and old alleles (*ptxS1A/prn1*) have been called transitional strains. Among strains collected in the United States from 1935 to 1999, 34 (22%) of 152 isolates were transitional strains (3). Similarly, among strains collected in Canada from 1985 to 1994, 17% of the isolates were transitional strains (22). These observations suggested that the *B. pertussis* strain had evolved from an old strain into a new strain by selective pressure from vaccination. However, only four transitional strains (4%) were found among the 107 Japanese isolates in this study. This finding strongly suggested that there was no genetic relationship between old strains (type A and type C) and the new strain (type B), i.e., the type B strain did not derive from the type A or type C strain genetically. One possible explanation for the appearance of the type B strain is that it was imported to Japan from other countries.

Among recently circulating strains in The Netherlands, polymorphism was observed only in *prn*, *ptxS1*, *ptxS3*, and *tcfA* by sequencing 15 genes coding for surface proteins (28). Polymorphism in *prn* is essentially limited to region 1, which has an important role in immunity (12, 15). However, Boursaux-Eude et al. (2) showed that acellular vaccine was highly effective against *B. pertussis* strains harboring nonvaccine *ptxS1* and *prn* alleles by using a mouse intranasal challenge model. On the other hand, Hausman and Burns (11) suggested that significant amino acid changes could occur in PT sequence without affecting antibody neutralization. Thus, polymorphism in pertussis toxin may have no influence on the efficacy of antibody neutralization. In the present study, we also investigated the virulence of two PT variants, PT-194 M, encoded by vaccine *ptxS1B*, and PT194I, encoded by nonvaccine *ptxS1A*, but no significant difference between their biological activities was detected. These findings suggested that the virulence of PT-194I had not been associated with the wide spread of the type B strain harboring *ptxS1A/prn2*. The type B strain may have a more important virulent allele(s) than the *ptxS1A* and *prn2*

alleles associated with the wide spread. For analyzing differences in gene expression between bacterial strains, the proteomic approach is a powerful tool (4, 26). Thus, the comparative proteomic analysis of *B. pertussis* strains (type A and type B) was thought to be worth trying, and an attempt is now under way.

In conclusion, in the Japanese *B. pertussis* strains, the antigenic divergence between recently circulating strains and the vaccine strain has been observed since the mid-1990s, although reported pertussis-like and pertussis cases have decreased in number. In addition, the strains showed a correlation between the PFGE profile and the combination of *ptxS1/prn* alleles. Our findings strongly suggested that the antigenic divergence had no influence on the efficacy of pertussis vaccination in Japan. However, the reason for the appearance of the type B strain harboring nonvaccine *ptxS1A/prn2* has remained unclear. Continuous surveillance and further analyses are needed to determine the virulence of the type B strain.

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Characterization and Molecular Analysis of Macrolide-Resistant *Mycoplasma pneumoniae* Clinical Isolates Obtained in Japan

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

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

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

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

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MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

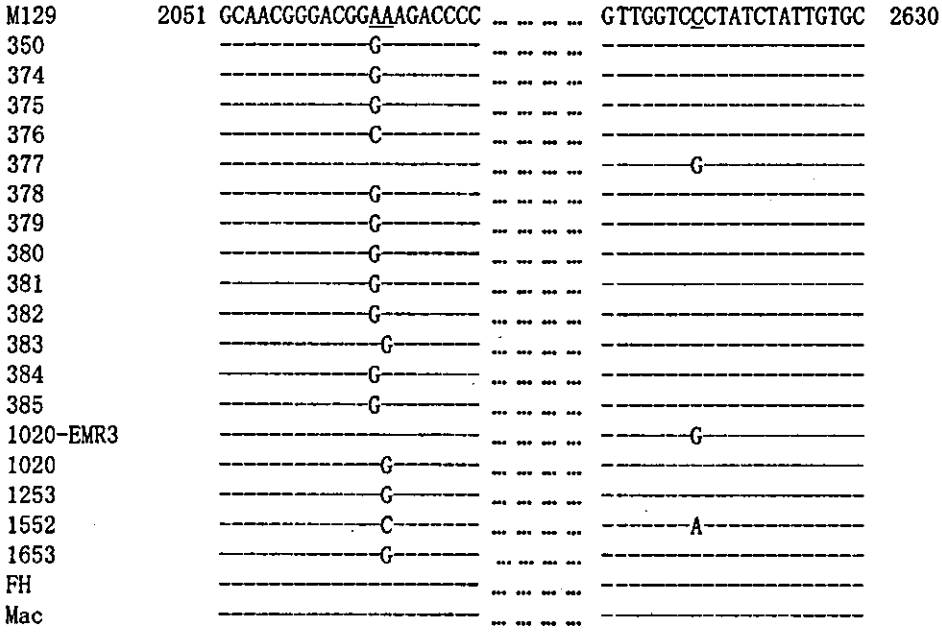

FIG. 1. Multiple alignment of 23S rRNA gene of ERY-resistant *M. pneumoniae* strains and *M. pneumoniae* M129, FH, and Mac. Partial sequences of the peptidyltransferase (domain V) from positions 2051 to 2081 and 2601 to 2630 are presented. The nucleotides are numbered on the basis of *M. pneumoniae*.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

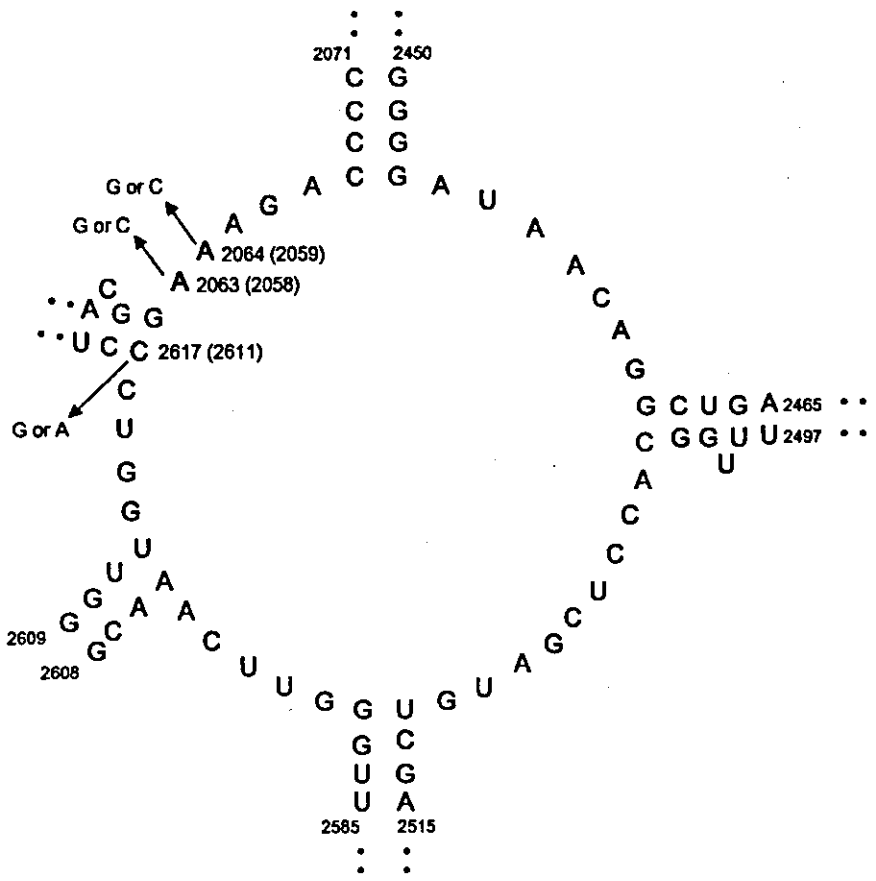

FIG. 2. Secondary structure of the peptidyltransferase loop in domain V of *M. pneumoniae* 23S rRNA. Positions of the newly found mutations (A2063C and C2617G), as well as previously reported in vitro mutations (A2063G, A2064G, and A2064C), in clinical isolates are indicated by using the numbering for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

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

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

RESULTS

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

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

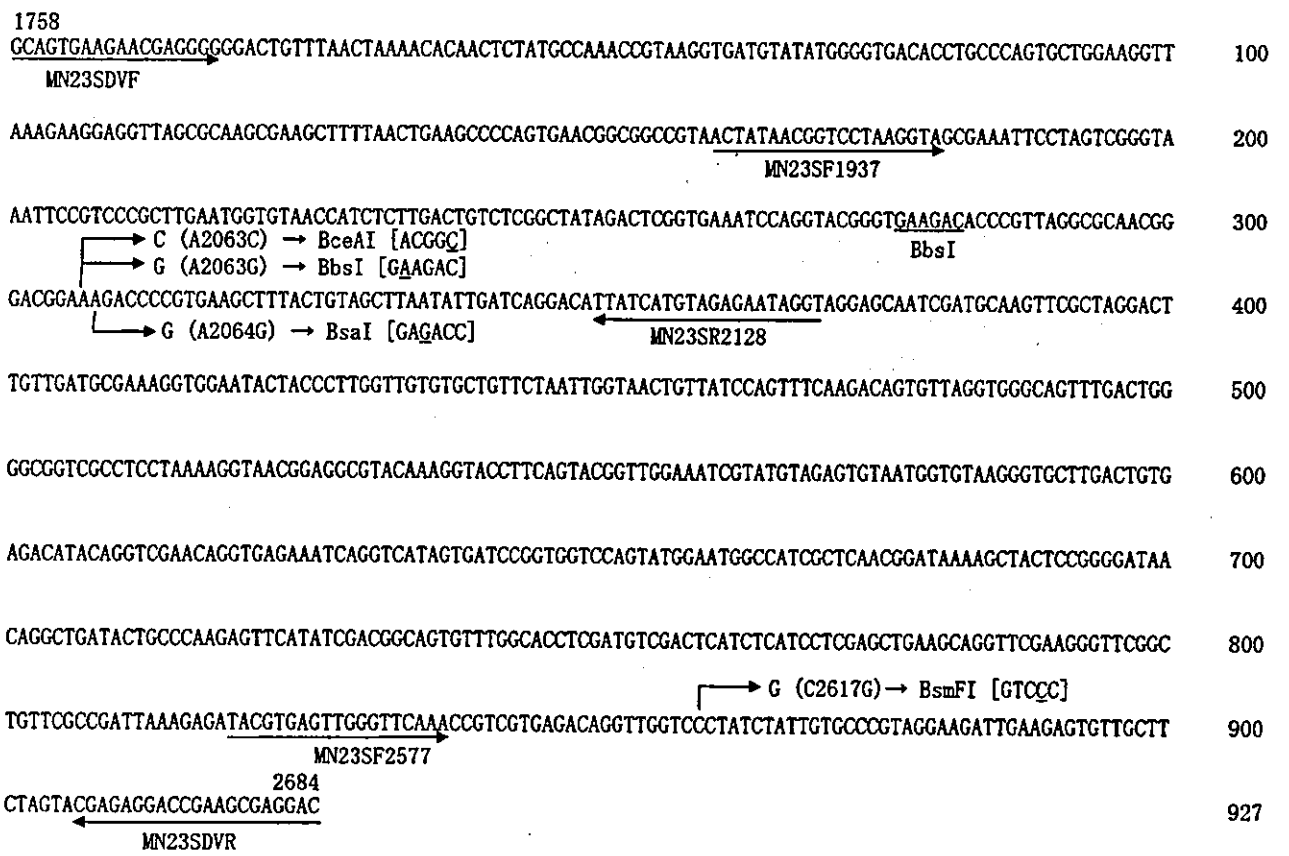

FIG. 3. Nucleotide sequence of the 927-bp amplicon from positions 1758 to 2684 of the 23S rRNA gene from *M. pneumoniae* M129. A long arrow indicates a primer sequence with direction. A short arrow indicates a site of mutation with a substituted base, i.e., A2063G, A2063C, A2064G, or C2617A. A newly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

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

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

DISCUSSION

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

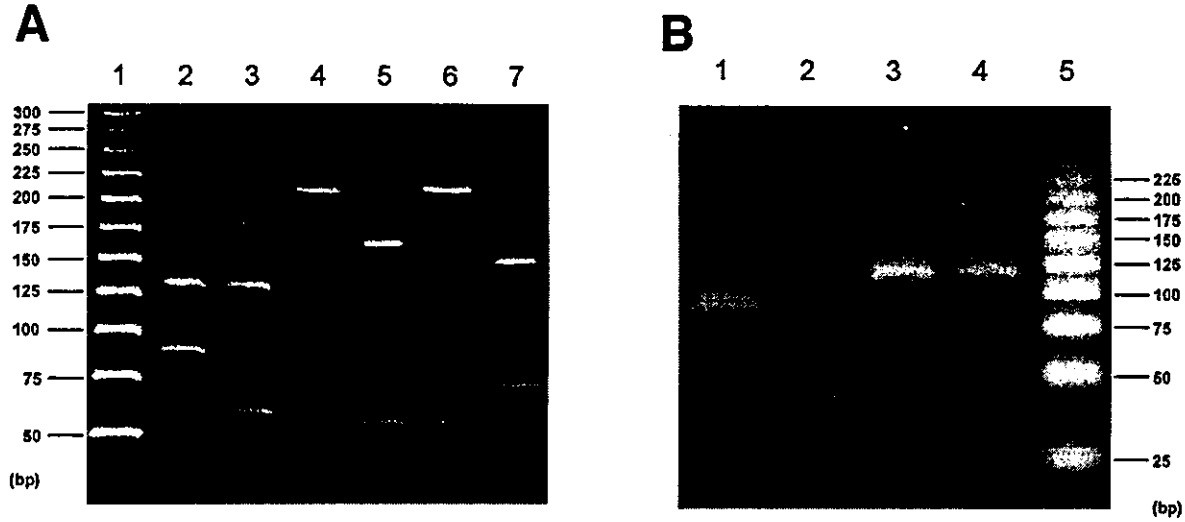

FIG. 4. Restriction analysis of 210-bp (A) and 108-bp (B) amplicons from the peptidyltransferase region (domain V) in 23S rRNA of *M. pneumoniae*. (A) Restriction profile for detection of the A2063G, A2063C, and A2064G mutations. Lanes: 1, DNA size marker (25-bp DNA step ladder; Promega); 2, 4, and 6,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

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

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

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

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

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

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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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1568-0053/04 $45.00+.00

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