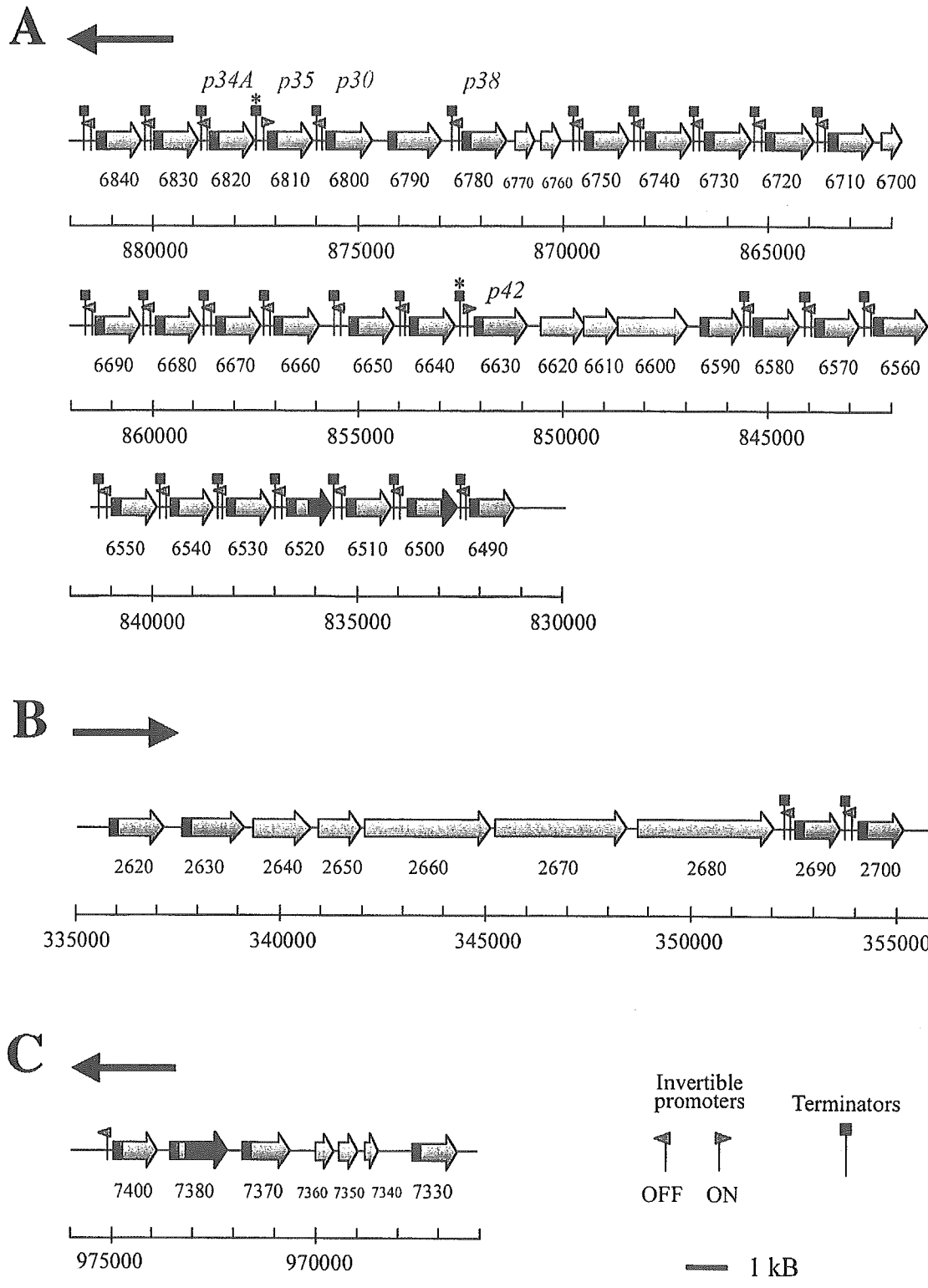

FIG. 8. Genomic organization of *mpl* genes in *M. penetrans* HF-2. Three *mpl* gene clusters are illustrated. The gray and red boxes in the arrows represent *mpl* genes. The red regions indicate signal sequence regions of *mpl* genes. The yellow arrows represent non-*mpl* genes. The numbers under the genes are MYPE serial numbers that are used to describe *M. penetrans* ORFs in the whole-genome sequencing analysis. The approximate nucleotide positions of *mpl* genes in the genome are also shown with the scale. The genes MYPE6500, -6520, and -7380 have frameshift mutations and are disrupted by internal stop codons. In these genes, the regions after the stop codons are shown as dark green. The small green arrowheads indicate invertible promoter-like sequences and their directions. The small purple squares represent 16-bp inverted-repeat terminator-like sequences. Two terminator-like sequences (upstream of the *p35* and *p42* genes that are marked with asterisks) are inactivated because of promoter inversions (see the text). The genes for P35, P42, and previously reported lipoproteins (P30, P38, and P34A) are labeled. The blue arrows above each cluster indicate relative directions in the genome.

sequences were fairly well conserved but the sequences of the 12- to 14-bp inverted repeats were not conserved between intergenic sequences (data not shown). In most cases, the 133- to 138-bp DNA regions contained a highly conserved promoter-like sequence that was similar to those of the *p35* and *p42* promoters. Although the −35-like consensus regions of these promoter-like sequences were not conserved, the −10-like consensus and transcriptional-start-site regions were almost identical to those of the *p35* and *p42* promoters (data not shown). As the lack of a −35-like consensus sequence in mycoplasmal promoters has been reported in *Mycoplasma pneumoniae* (44), these promoter-like sequences may be active. These results suggested that most of the *mpl* genes possessed independent invertible promoter-like sequences in their upstream regions (Fig. 8). (See also the genome sequence data for *M. penetrans* HF-2 and their annotation. This will appear in databases.)

In summary, we have identified the invertible promoter-like sequences for 31 *mpl* genes (including *p35* and *p42*) but not those for 7 other *mpl* genes (MYPE 6790, -6590, -2620, -2630, -7380, -7370, and -7330) (Fig. 8). Interestingly, of these 31 invertible promoter-like sequences, only two (for the *p35* and *p42* genes) were in the ON orientation. The other promoter-like sequences were in the OFF orientation (Fig. 8). These patterns of the promoter-like sequences of *mpl* genes are consistent with the expression patterns of P35 family lipoproteins of *M. penetrans* HF-2 (i.e., P35 and P42 are major lipoproteins expressed in strain HF-2) (Fig. 2).

We have also identified 16-bp inverted-repeat sequences upstream of all invertible promoter-like sequences, with the exception of MYPE7400 (Fig. 8). These 16-bp inverted-repeat sequences were homologous to those found upstream of the *p42* gene of GTU (Fig. 4 and 5).

DISCUSSION

In this report, we have confirmed that the LAMP profiles of *M. penetrans* are different in strains GTU and HF-2. The 46-kDa protein (P42 lipoprotein) is present only in strain HF-2, and the 34-kDa protein is found only in strain GTU. Röske et al. also recently reported the difference between the LAMP profiles of GTU and HF-2 (33). They reported that the P35 lipoprotein is found in both strains, as observed in our study. However, the 34-kDa protein found in our GTU is not present in their GTU. In addition, a 40-kDa protein is present in their HF-2 strain, but this is smaller than the P42 of our HF-2. Although we cannot fully explain the reason for these differences, the presence and absence of a 34-kDa protein in GTU may be due to antigenic variation between two laboratory strains caused by phase-variable expression of *mpl* genes during the culture passages. On the other hand, in HF-2, the electrophoresis patterns of LAMPs are essentially identical in both studies. Judging from the fact that the calculated molecular mass of P42 is 41.8 kDa, it is possible that the 40-kDa protein in Röske's HF-2 is the P42 protein in our HF-2. The size discrepancy may be caused by different gel conditions and size marker systems.

We have shown that the P42 protein in our HF-2 strain is the product of the putative *mpl* gene pepIMP13 and found that promoter inversion was the cause of the antigenic variation of this protein between GTU and HF-2. Although the reversible inversion event of this promoter region has not yet been demonstrated directly, the data presented in this study strongly suggest that the promoter-containing 135-bp DNA region is an invertible element that functions as a genetic switch. DNA inversion is a mechanism commonly used by bacteria to regulate gene expression and generate antigenic variation (8, 15). There are many examples of DNA inversion systems, including the Hin system for flagellar gene expression of *Salmonella enterica* serovar Typhimurium and the type 1 fimbrial gene (*fim*) expression by *Escherichia coli* (1, 52). DNA inversion systems have also been reported in some mycoplasma species (22, 34, 36). The characteristic feature of the promoter inversion of the *p42* gene is the length of the invertible DNA region. To our knowledge, the 135-bp region is the shortest invertible element discovered so far, although an invertible promoter region consisting of 192 bp of DNA was recently identified in *Bacteroides fragilis* (17). One hundred thirty-five base pairs seems quite short for an invertible element that functions as a frequent genetic switch, because an inversion reaction requires the looping of DNA and close contact between two recombination sites. Looping of the 135-bp DNA results in tight DNA curvature and might not occur frequently. One solution to this problem is a participation of the bacterial histone-like protein HU that mediates tight DNA curvature (16, 41). It has been reported that DNA ring closure by self-ligation does not occur in 126-bp linear DNA, but it occurs in the presence of the HU protein (16). It is possible that the HU-like protein stimulates 135-bp DNA inversion of the *p42* promoter region. The gene for the HU-like protein was found in the *M. penetrans* genome (MYPE2490). Another possible factor in inversion stimulation is DNA bending. Since the *p42* promoter region is highly AT rich (Fig. 4), DNA bending is suggested in this region (40). The DNA bending may directly enhance the formation of DNA loops or binding of some regulatory proteins that support DNA looping (27, 39). In the inversion of the *p42* promoter, the main enzymes that directly mediate the recombination reaction are not yet known. However, we could identify two candidate ORFs, MYPE2900 and -8180, for invertase genes in the *M. penetrans* HF-2 genome. These ORFs have homology to the integrase-recombinase family of genes that has been identified in the *Ureaplasma urealyticum*, *M. pulmonis*, and *Bacillus subtilis* genomes (4, 11, 18).

The analysis of the genomic organization of the P35 family lipoprotein genes revealed that there were 38 *mpl* genes in the *M. penetrans* HF-2 genome. These genes were clustered at three positions in the genome (Fig. 8). The surprising finding was that most of the *mpl* genes possess the promoter-like sequences that seem to be invertible (Fig. 8). These promoter-like sequences may be active because of their high sequence similarity to the functional *p35* and *p42* promoters and may express downstream genes when they are turned on by inversion. The presence of independent invertible promoters may allow every *mpl* gene to be switched ON↔OFF simply. This conclusion, based on the analysis of *mpl* gene structure, accounts well for the phenotypic observations of lipoprotein variation that show frequent, independent phase variation of the *mpl* gene products (24, 33).

The lipoprotein gene families that mediate surface antigenic variations have been reported in several mycoplasma species.

These are the *vlp*, *vsp*, *vsa*, *pMGA*, *vlh*, and *vpma* families of *M. hyorhinis*, *Mycoplasma bovis*, *M. pulmonis*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Mycoplasma agalactiae*, respectively (4, 6, 12, 13, 23, 26). These gene families employ different molecular mechanisms to change the expression patterns of lipoprotein genes. The *vlp* and *pMGA* families employ nucleotide insertion and deletion mechanism in their promoter regions (7, 12). The *vlh* family changes gene expression by gene conversion (26). The *vsp* and the *vsa* families utilize DNA inversions for modulation of lipoprotein gene expression (22, 34, 35). Although the *vsp* and *vsa* families developed the sophisticated genetic-switch systems with DNA inversions, these systems are not like the *mpl* gene system of *M. penetrans*. In the *vsp* and *vsa* systems, the multiple lipoprotein genes are also clustered in the genome, but these genes are not oriented in the same direction and there is only one active promoter in the cluster. Only a selected lipoprotein gene that is properly connected to an active promoter can be expressed in this locus. DNA inversions exchange the lipoprotein genes that connect to active promoters and generate antigenic variations (22, 34). In contrast to the *vsp* and *vsa* systems, the *mpl* gene system of *M. penetrans* is a system that employs multiple invertible promoters. This system is a novel mechanism for the generation of antigenic variation of mycoplasmal lipoproteins.

The use of multiple promoters seems to be an effective way of generating antigenic variation. The multiple promoters can express lipoprotein genes in a large number of combinations, generating additional antigenic diversity. However, in this system, some problems are predicted to exist. One problem is the inversion frequency of promoters. Although 31 invertible promoters were predicted in *mpl* gene clusters, only two promoters (*p35* and *p42*) are in the ON orientation in the HF-2 genome (Fig. 8). If all of the promoters invert randomly and at the same probability forward and backward, the number of ON promoters may be nearly half of the total promoters. So it is likely that there are some biases in promoter inversion reactions, such as selective inversion of some promoters or directional bias in ON↔ OFF reactions. Therefore, the frequency of each promoter inversion must be assessed by further study. The other problems come from the structure of the *mpl* gene clusters. In the *mpl* gene clusters, all genes are oriented in the same direction and every *mpl* gene is about 1 kb in length (Fig. 8). When one of the promoters turns on in these clusters, it is likely that the transcription from the ON promoter may reach downstream genes, reading through the intergenic sequences, and produce polycistronic mRNA containing several *mpl* gene sequences. This may cause the expression of some downstream genes even if the promoter of downstream genes is in the OFF orientation. If this situation really occurred, it would not make sense for *mpl* genes to have independent invertible promoters. Moreover, if the transcription was initiated from promoters in the OFF orientation to upstream genes, it might produce a large number of useless mRNAs that would not be translated because the *mpl* genes are not bi-directional. The production of excess untranslated mRNA (antisense *mpl* RNAs) is not advantageous for optimum use of cell metabolic energy. However, the *mpl* gene system seems to possess the mechanisms to overcome these serious problems. We could identify conserved 16-bp inverted-repeat sequences in the regions just upstream of most invertible promoters (Fig. 8) that were consistent with the pattern of terminator sequences (Fig. 5), although their activity has not been demonstrated experimentally. In addition, it was found that the formation of these 16-bp inverted repeats depended upon the promoter inversions (Fig. 5). If these 16-bp inverted-repeat sequences really function as terminators, the *mpl* gene system will work effectively. An explanation of the function of the 16-bp inverted-repeat sequences, using the *p42* promoter region as an example, is shown in Fig. 9. When the promoter is in the OFF orientation (in strain GTU), the active form of the terminator sequence (the 16-bp inverted repeat) exists just upstream of the promoter. This active terminator blocks transcription both from the *p42* gene promoter and from upstream if there are promoters in the ON orientation. In this configuration, the *p42* gene is kept OFF, regardless of the ON↔ OFF orientation of the upstream promoters. Alternatively, when the promoter turns on by inversion (in strain HF-2), the terminator structure is divided into two parts and inactivated. In this configuration, the transcription of the *p42* gene can initiate from the *p42* promoter. Also, if there was transcription from the upstream ON promoter, this might read through the inactive terminators and transcribe the *p42* gene, resulting in efficient expression of the *p42* gene. Although our model requires experimental proof, the coupling of the promoter inversion and the terminator activation is a compelling mechanism for the regulation of gene expression.

Our results show that the *p35* promoter also seems to be invertible, because it has inverted-repeat sequence for inversion. However, Neyrolles et al. (24) previously analyzed the *p35* gene regions from $P35^+$ and $P35^-$ phenotype strains and reported that the P35 phase variation was not associated with DNA sequence change, although the phase variation occurred practically at the transcriptional level. This observation does not seem to agree with our promoter inversion model. However, in Neyrolles' study, the *p35* promoter region was cloned by PCR and sequenced. Because the upstream primer sequence for this PCR was located within the invertible DNA region (24), the *p35* promoter region of $P35^-$ strains might have been amplified from $P35^+$ revertants arising in the $P35^-$ strain population, and thus, no inversion could have been detected by sequencing. The frequency of phase variation of P35 family lipoproteins was reported to be about 10^{-2} to 10^{-4} per cell per generation (24, 33). This frequency is sufficiently high for DNA amplification by PCR from revertants.

In addition to ON↔ OFF phase variation, variable surface lipoproteins of mycoplasma species frequently exhibit size variation (2, 48). These size variations are mainly created by the increase or decrease of repetitive sequences within the lipoprotein genes (23, 49, 50, 51). Unlike other variable surface lipoprotein genes of mycoplasmas, the *mpl* genes do not contain certain repeated regions in their sequences. There is no evidence of size variation in *mpl* gene products. However, we found that the coding lengths of one of the *mpl* genes, *p30* (MYPE6800), in GTU and HF-2 are different because of a frameshift mutation. In GTU, the P30 protein consists of 318 aa (9), while it is 376 aa in HF-2 (data not shown). Although the promoter of the *p30* gene is in the OFF orientation in both strains (Fig. 8A), P30 proteins with different sizes must be expressed when the promoters of these genes are turned on. In addition, we identified frameshift mutations in three other *mpl* genes of the HF-2 genome (Fig. 8). Thus, the frequency of

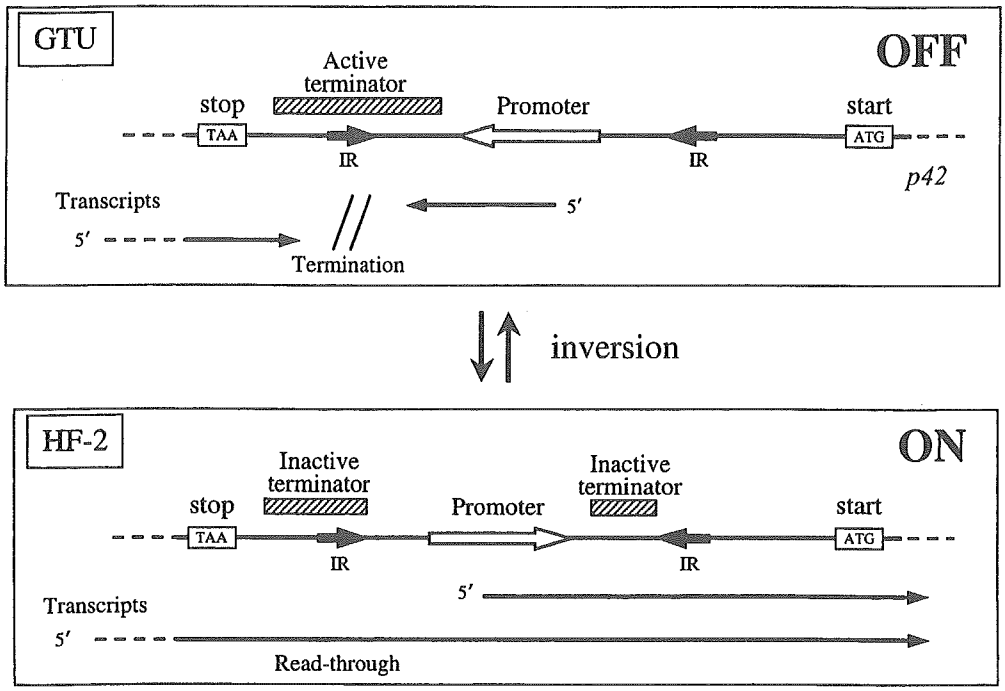

FIG. 9. Model for genetic switch of *mpl* genes by promoter inversion. The upstream intergenic regions of the *p42* genes are illustrated. The open arrows represent promoter sequences and their directions. The short solid arrows represent 12-bp inverted repeats (IR). The hatched boxes represent terminator-like sequences (16-bp inverted repeats). When the promoter is in the OFF orientation (GTU), an active terminator is formed. The inversion between 12-bp inverted-repeat sequences changes the promoter direction from OFF to ON and splits the terminator sequence. RNA transcripts from the promoter and from upstream are represented by thin solid arrows.

frameshift mutation seems to be high in the *mpl* gene locus. It is possible that frequent frameshift mutation generates size variations in *mpl* gene products.

In this study, we have investigated the molecular mechanism of antigenic profile changes of *M. penetrans* P35 family lipoproteins and reported the presence of novel systems for the regulation of lipoprotein gene expression. Although the actual biological functions of P35 family surface lipoproteins remain unclear, they are abundant, major surface antigens recognized by the human immune system during infection (14, 25, 42). The generation of extensive variations of these surface antigens by multiple promoter inversions may contribute to the survival of *M. penetrans* in the host. The *M. penetrans mpl* gene system may be a useful model for expanding our understanding of bacterial host adaptation and mechanisms for the evasion of immune responses.

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Evaluation of a new serotyping kit for *Streptococcus pneumoniae*

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A new serotyping test kit (*Streptococcus pneumoniae* antisera 'Seiken' set; Denka kit) was evaluated for 285 strains of *Streptococcus pneumoniae* in comparison with the standard capsular reaction (Quellung test). This new kit is based on the slide-agglutination method and is composed of eight pool sera, 40 group or type sera and 41 specific type sera. All serotyping results by using the Denka kit were completely identical to those obtained by using the conventional Quellung test. For types and groups, sensitivity and specificity were 100 and 100 %, respectively. For specific types, sensitivity and specificity were 100 and 100 %, respectively. The Denka kit is relatively rapid (mean test time, 5 min, versus 15 min by Quellung test), cheap (0·5 US$ per test, versus 1·4 US$ per Quellung test), easy to perform and does not require special equipment. The Denka kit may be useful for fieldworkers in developing countries involved in epidemiological surveys and vaccine development.

INTRODUCTION

Streptococcus pneumoniae is a major community-acquired pathogen that causes serious pneumonia, otitis media, septicaemia and meningitis worldwide (Roberts, 1979). In adults, types 1–8 are responsible for about 75 % of pneumococcal pneumonia cases and for more than half of pneumococcal bacteraemia cases, whereas in children, types 6, 14, 19 and 23 are frequently responsible (Brooks *et al.*, 1998). Infections caused by *S. pneumoniae* can be life-threatening, particularly to children aged < 2 years; the highest risk is to children aged 0–11 months and elderly people (Cant, 2002).

Pneumococcal vaccines are made from purified capsular polysaccharides. More than 90 capsule types and serotypes are known in *S. pneumoniae* (Hausdorff *et al.*, 2002). The polysaccharide vaccine for *S. pneumoniae* (Pneumovaccus^{R}) is composed of 23 different capsule antigens. This vaccine is very useful for prevention of pneumococcal infection in adults. However, in children aged < 2 years, the acquisition rate of immunity to *S. pneumoniae* is insufficient because of low immunogenic activity of the bacterium and premature immunity of the subjects (Hausdorff *et al.*, 2000). Several new conjugated pneumococcal vaccines have been developed, but the antigens selected for these vaccines are limited to only 7, 9 or 11 different serotypes, mainly of penicillin-resistant *S. pneumoniae* (Joloba *et al.*, 2001). Serotyping of *S. pneumoniae* is very important for epidemiological analysis and pneumococcal vaccination.

The capsular reaction (Quellung test) is the gold standard used to determine serotypes of *S. pneumoniae* (Sorensen, 1993). It is very reliable and reproducible, but is also labour-intensive and not easily affordable. Denka Seiken has developed a new method (*Streptococcus pneumoniae* antisera 'Seiken' set; Denka kit) based on slide-agglutination, which is cheap, simple, rapid and reliable. We evaluated this kit for epidemiological surveys of *S. pneumoniae*.

Abbreviations: CIEP, counterimmunoelectrophoresis; COA, coagglutination; LA, latex agglutination.

METHODS

Bacterial strains and culture methods. In total, 285 strains of *S. pneumoniae* were used (130 from Nepal and 155 from Japan). These isolates were stored at −85 °C in 10 % skimmed milk until use. Each strain was maintained on Columbia agar with 5 % sheep blood at 35 °C for 16 h in 5 % CO_2. *S. pneumoniae* was identified by conventional methods, including optochin susceptibility and bile solubility tests (Doern *et al.*, 1999). These identification results were confirmed by PCR amplification of *lytA*, the pneumococcal-specific autolysin gene (Nagai *et al.*, 2001).

Quellung test. The Quellung test was performed as described previously by using anti-pneumococcal capsule serum from the Statens Serum Institute, Denmark (Lund & Henrichsen, 1978; Sorensen, 1993).

Slide-agglutination method for serotyping. The Denka kit is supplied by Denka Seiken. It is intended for determination of *S. pneumoniae* serotypes.

Sera were prepared by hyperimmunizing healthy rabbits with standard strains of known serotypes of pneumococci, which were obtained from the American Type Culture Collection (ATCC). After bleeding, antisera were separated, heated at 56 °C for 30 min, absorbed to remove cross-agglutinins and sterilized by antibacterial filtration.

The Denka kit is composed of eight pool sera, 40 group or type sera and 41 specific type factor sera. The first kit contains eight vials of polyvalent (pool) antisera and 40 vials of monovalent group or type antisera. The Denka kit covers all vaccine types and several major non-vaccine types of pneumococci (Table 1). Pool sera recognize several antigens, which are serogroups or types within each pool. For example, pool 1 recognizes pneumococcal antigens types 1, 2, 3, 4 and 5, whilst pool 2 recognizes 6, 8, 9 and 10 type or group sera. Similarly, pools 3–8 recognize type or group sera as shown (Table 1). Factor sera work on the same principle, but are more specific; they recognize antigens within a particular serotype or group. The second kit contains 41 specific type factor sera (Table 2).

The Denka kit is used as follows. A single colony is obtained after culturing bacteria. A glass slide is divided into several parts for testing the isolate to be typed. A drop (25 µl) of each antiserum to be tested is placed onto the respective section of the prepared slide. The same is done for a control with normal saline solution. By using a toothpick or sterile microbacteriological loop, a small but visible amount of bacteria is picked and mixed directly with the drop of antiserum, spreading over the partitioned area. The glass slide is then tilted back and forth to look for agglutination (Fig. 1). Agglutination occurs only when bacteria are mixed with specific sera, which react with bacterial antigens to cause an antigen–antibody reaction if the serotype of the isolate is contained within the antiserum being used. Agglutination that occurs within 1 min can be observed visually and is considered to be a positive reaction (Fig. 2). For the control, bacteria are mixed with physiological saline solution to check if agglutination occurs spontaneously. Reactions are performed at room temperature.

In this study, we first used pool sera to determine the pool to which pneumococci belonged by using the procedure described above. Thereafter, bacteria were tested with each type or group serum within the pool; for instance, if the bacteria agglutinated with pool 1 serum, we next picked bacterial colonies and checked them against type or group sera 1, 2, 3, 4 and 5 (Table 1). Finally, the specific type was checked similarly by using specific type factor sera (Table 2). Some types or groups do not have specific types, e.g. 1, 2, 3, 4, 5, 8, 13, 14, 20, 21, 25, 27, 29, 31, 34, 36, 37, 38, 39, 40, 42, 43, 44, 45, 46 and 48. For these types, the procedure of checking for agglutination stopped at the level of serotype or group. For the rest, we determined the specific type by using specific factor sera that recognized certain antigens or antigenic groups with reactions as shown (Table 2). For example, if a bacterial colony formed agglutination with pool 2 serum and then with type 6 serum, if on further reaction it formed agglutination with specific factor serum 6b and not with 6c (6b+ 6c–), then the specific type is 6A; the pattern 6b– 6c+ would indicate specific type 6B. Such reactions were carried out for all bacteria tested and patterns of agglutination were interpreted with the aid of the list provided with the kit. Typical slide-agglutination positive and negative controls are shown in Fig. 2.

We also checked the Denka kit for reaction against 18 type strains of streptococci other than pneumococci: *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus constellatus*, *Streptococcus cristatus*, *Streptococcus dysgalactiae*, *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus sobrinus* and *Streptococcus vestibularis*.

RESULTS AND DISCUSSION

All serotyping results for types or groups by the Denka kit were completely identical to those obtained from serotyping by using the conventional Quellung test (Table 3). The predominant serotypes were 19, 23, 3, 6, 15, 18 and 11. There was excellent correlation of the results. Sensitivity and specificity were 100 and 100 %, respectively.

The results of serotyping for specific types by the Denka kit were identical to those obtained by the Quellung test. Some serogroups, e.g. 3, 4 and 14, do not have specific types. Specific types were determined for 191 pneumococcal strains and there was excellent correlation of the results. The Denka kit cannot distinguish between specific types 9A and 9V or 24B and 24F. In this study, one pneumococcal strain was identified as 9V by the Quellung test and as 9A or 9V by the Denka kit. Two strains that were identified as 24B by the Quellung test were identified by the Denka kit as either 24B or

Table 1. Composition of the Denka kit: pool sera and type or group sera contained

Pool serum	Type or group sera									
	Vaccine-related					Non-vaccine-related				
1	1	2	3	4	5					
2	6	8	9	10						
3	11	12	14	15		16				
4	17	18	22			21				
5	20	33				29	31	34	35	47
6	23					25	28	41	46	
7						27	32	36	38	39
8	7	19				24	40			
						37*				

*37 is not included in any of the eight pool sera.

Table 2. Composition of the Denka kit: specific type factor sera and pattern of reaction to factor sera

Specific type	Reaction to factor serum			
Type 6	6b	6c		
6A	+	–		
6B	–	+		
Type 7	7b	7c	7e	7f
7A	+	+	–	–
7B	–	–	+	–
7C	–	–	–	+
7F	+	–	–	–
Type 9	9d	9e	9f	
9A	+	–	–	
9V	+	–	–	
9N	–	+	–	
9L	–	–	+	
Type 10	10b	10c		
10A	–	+		
10F	+	–		
Type 11	11c	11f	11g	
11A	+	–	–	
11B	–	+	+	
11C	+	+	–	
11F	–	–	+	
Type 15	15b	15e	15f	15g
15A	–	–	–	+
15B	+	+	–	–
15C	–	+	–	–
15F	+	–	+	–
Type 18	18c	18d	18e	18f
18A	–	+	–	–
18B	–	–	+	–
18C	+	–	+	–
18F	+	–	–	+
Type 19	19b	19d	19e	19f
19A	–	+	–	–
19B	–	–	+	–
19C	–	–	–	+
19F	+	+	–	–
Type 22	22b	22c		
22A	–	+		
22F	+	–		
Type 23	23b	23c	23d	
23A	–	+	–	
23B	+	–	+	
23F	+	–	–	
Type 24	24b	24c		
24A	–	+		
24B	+	–		
24F	+	–		
Type 32	32b			
32A	+			
32F	–			
Type 33	20b	33b	33e	33f
33A	+	–	–	–
33B	–	–	+	+
33C	–	–	+	–
33F	–	+	–	–
Type 35	20b	29b	35b	42a
35A	+	–	–	–
35B	–	+	–	–
35C	+	–	–	+
35F	–	–	+	–

24F (Table 4). In this study, a Quellung result of 9V or 24B was determined to be identical to the Denka result of 9V/9A or 24B/24F, respectively.

There was no agglutination with the Denka kit against all 18 type strains of streptococci other than pneumococci. All tested negative with all polyvalent pool sera 1–8; hence, there was no need for further serotyping.

Since the 1940s, the *Streptococcus* Unit at Statens Serum Institute, Denmark, has manufactured diagnostic pneumococcal antisera, intended for identification and typing of pneumococci by the capsular reaction test. This method has proved to be reliable and has been used extensively by medical and research centres as well as vaccine industries worldwide. However, the procedure of serotyping is labour-intensive, time-consuming, requires experience and technical skill for interpretation of results and the reagents are not easily affordable, especially by developing countries. Our results show that the Denka kit has excellent correlation with the Quellung test and has several advantages: it is more rapid (mean test time, 5 min, vs 15 min by Quellung test) and easy to perform, requires no special equipment and is much cheaper (0·5 US$ per test, vs 1·4 US$ per Quellung test).

In the past, other serotyping techniques have been described, including the counterimmunoelectrophoresis (CIEP) (Ayyagari *et al.*, 1984), latex agglutination (LA) and coagglutination (COA) methods (Kronvall, 1973; Kaldor *et al.*, 1988; Lalitha *et al.*, 1996). CIEP has the drawback that the polysaccharide capsules of a small number of important serotypes fail to form precipitin lines (Henrichsen *et al.*, 1980). The COA method for serotyping pneumococci was first developed by Kronvall (1973), who reported that it exhibited weak cross-reactions with one or more serotypes in addition to the Quellung serotype. The LA and COA methods rely on elaborate, time-consuming, self-prepared reagents (Kaldor *et al.*, 1988). More recently, a multiplex PCR assay for determination of *S. pneumoniae* serotypes and serogroups has been described (Lawrence *et al.*, 2003). This method can only determine five serotypes, 1, 3, 14, 19F and 23F, and serogroups 6, 19 and 23. It is not yet available for commercial use and would not be easily affordable by developing countries as it is expensive. In contrast, the Denka kit is commercially prepared, cheap and ready for use. The Denka kit offers hope to the majority of developing countries to be able to perform serotyping and obtain the appropriate conjugate vaccine, which will help to prevent high morbidity and mortality from pneumococcal infections in paediatrics (Wafula & Onyango, 1986; Graham, 1990; Garenne *et al.*, 1992; Onyango & Wafula, 1995; Murray & Lopez, 1996). It will also be very useful when conducting large epidemiological surveys. The only limitation is that the Denka kit cannot distinguish between specific types 9A and 9V or 24B and 24F. This kit also does not include some minor non-vaccine types, such as 13, 42, 43, 44, 45 and 48, and some specific types, e.g. 12A, 12B, 12F, 11D, 10B, 10C and 33D. However, this point is not a major problem because the types or groups to which these specific types belong are covered in

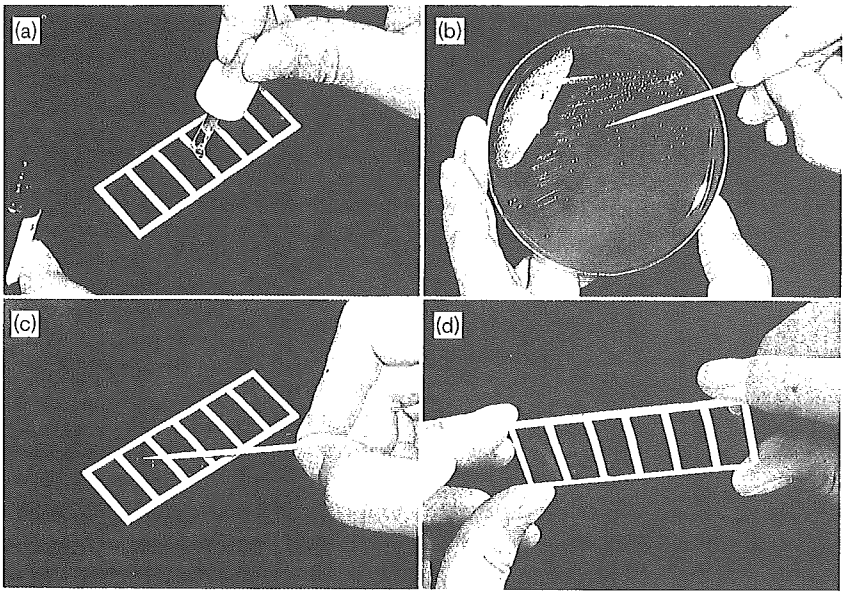

Fig. 1. Procedure of slide-agglutination by using the Denka kit. (a) Drop antiserum onto glass slide; (b) pick colonies of bacteria; (c) mix colonies with antiserum; (d) tilt glass slide back and forth and observe for agglutination.

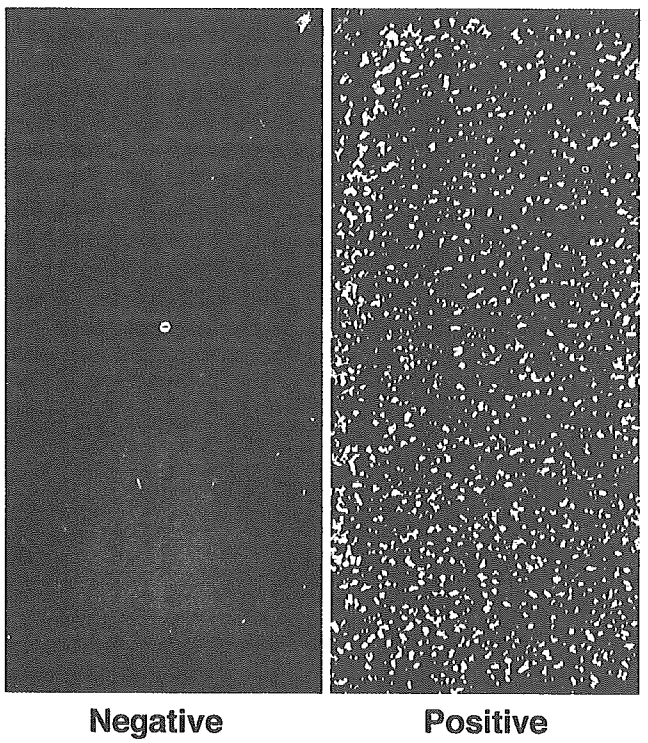

Fig. 2. Results of slide-agglutination by the Denka kit. Left, negative reaction; right, positive reaction.

the kit (Table 1). Serogroups that are not included in the new serotyping kit are minor non-vaccine types that are not important for serotyping.

Our results showed excellent correlation with the conventional method, with sensitivity and specificity of 100 % by

Table 3. Determination of type or group of *S. pneumoniae* strains

Data are no. (%) of isolates serotyped by the Denka kit or Quellung test

Type or group	Denka kit	Quellung test
3	31 (10·9)	31 (10·9)
4	2 (0·7)	2 (0·7)
6	27 (9·5)	27 (9·5)
7	4 (1·4)	4 (1·4)
8	6 (2·1)	6 (2·1)
9	4 (1·4)	4 (1·4)
10	1 (0·4)	1 (0·4)
11	13 (4·6)	13 (4·6)
12	4 (1·4)	4 (1·4)
14	12 (4·2)	12 (4·2)
15	26 (9·1)	26 (9·1)
17	2 (0·7)	2 (0·7)
18	15 (5·3)	15 (5·3)
19	49 (17·2)	49 (17·2)
20	3 (1·1)	3 (1·1)
21	7 (2·5)	7 (2·5)
23	39 (13·7)	39 (13·7)
24	2 (0·7)	2 (0·7)
25	2 (0·7)	2 (0·7)
27	2 (0·7)	2 (0·7)
28	1 (0·4)	1 (0·4)
31	3 (1·1)	3 (1·1)
33	1 (0·4)	1 (0·4)
34	12 (4·2)	12 (4·2)
35	7 (2·5)	7 (2·5)
39	2 (0·7)	2 (0·7)
40	1 (0·4)	1 (0·4)
41	5 (1·8)	5 (1·8)
46	1 (0·4)	1 (0·4)
Total	285 (100)	285 (100)

Table 4. Determination of specific types of *S. pneumoniae* strains

Data are no. (%) of isolates serotyped by the Denka kit or Quellung test

Specific type	Denka kit	Quellung test
6A	14 (7·4)	14 (7·4)
6B	14 (7·4)	14 (7·4)
7A	1 (0·5)	1 (0·5)
7C	3 (1·6)	3 (1·6)
9A/9V	1 (0·5)	
9V		1 (0·5)
9L	1 (0·5)	1 (0·5)
9N	2 (1·1)	2 (1·1)
10A	1 (0·5)	1 (0·5)
11A	11 (5·8)	11 (5·8)
11B	2 (1·1)	2 (1·1)
11C	2 (1·1)	2 (1·1)
15A	8 (4·2)	8 (4·2)
15B	3 (1·6)	3 (1·6)
15C	9 (4·7)	9 (4·7)
15F	5 (2·6)	5 (2·6)
18A	1 (0·5)	1 (0·5)
18B	2 (1·1)	2 (1·1)
18C	1 (0·5)	1 (0·5)
18F	11 (5·8)	11 (5·8)
19A	4 (2·1)	4 (2·1)
19B	3 (1·6)	3 (1·6)
19F	41 (21·6)	41 (21·6)
23A	6 (3·1)	6 (3·1)
23B	1 (0·5)	1 (0·5)
23F	32 (16·8)	32 (16·8)
24B		2 (1·1)
24B/24F	2 (1·1)	
33F	1 (0·5)	1 (0·5)
35B	1 (0·5)	1 (0·5)
35C	4 (2·1)	4 (2·1)
35F	2 (1·1)	2 (1·1)
Total	191 (100)	191 (100)

using the Denka kit. In conclusion, the Denka kit is easy to use, simple, cheap and reliable. This kit may be very useful, especially for fieldworkers in developing countries who are involved in large epidemiological studies or vaccine development.

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Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* Strains Causing Neonatal Toxic Shock Syndrome-Like Exanthematous Disease in Neonatal and Perinatal Wards

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Neonatal toxic shock syndrome-like exanthematous disease (NTED) is a new neonatal disease caused by toxic shock syndrome toxin 1 (TSST-1). We conducted a prospective surveillance study and characterized the methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from patients with NTED and compared them with the strains from patients with other MRSA infections and asymptomatic carriers. The study was performed in the neonatal intensive care unit and a general neonatal and maternal ward in the Tokyo Women's Medical University Hospital (TWMUH) from September to December 1998. Among 103 patients eligible for the study, MRSA was detected in 62 (60.2%) newborns; of these 62 newborns, 8 (12.9%) developed NTED, 1 (1.6%) had another MRSA infection, and 53 (85.5%) were asymptomatic MRSA carriers. Sixty-nine MRSA strains were obtained from the 62 newborns. DNA fingerprinting by pulsed-field gel electrophoresis showed two clusters: clone A with 8 subtypes and clone B. Sixty-seven of the 69 MRSA strains (97.1%) belonged to clone A, and type A1 was the most predominant (42 of 69 strains; 60.9%) in every neonatal and perinatal ward. All but one of the clone A strains had the TSST-1 and staphylococcal enterotoxin C genes. We also analyzed eight MRSA strains from eight NTED patients in five hospitals in Japan other than TWMUH. All the MRSA strains from NTED patients also belonged to clone A. These results suggest that a single clone that predominated in the neonatal wards of six hospitals might have caused NTED. However, the occurrence of NTED might not be dependent on the presence of an NTED-specific strain.

Toxic shock syndrome (TSS) is an acute life-threatening illness (39) caused by TSS toxin 1 (TSST-1), produced by *Staphylococcus aureus* (10, 40–42). Large outbreaks of TSS were seen in the United States from 1980 to 1985 (10). Cytokines produced by T cells activated by TSST-1, now classified as superantigenic toxins (10, 27, 40, 41), have been implicated in the pathogenesis of this illness (10, 40–42). The massive expansion in the number of TSST-1-reactive vβ2-positive T cells observed in patients with TSS supports this view (6, 10, 40). Since 1992, a number of neonates in Japan have been reported to have developed systemic exanthema, thrombocytopenia, and fever in the first week of life (36; N. Takahashi and H. Nishida, Arch. Dis. Child. 77:F79, 1997). Subsequently, microbiological examinations showed that most of the neonates with this illness were colonized by methicillin-resistant *S. aureus* (MRSA) strains that produced TSST-1 (36), suggesting that the pathogenesis of this illness is the same as that for TSS. Actually, the neonates exhibited a marked polyclonal expansion of vβ2-positive T cells in the acute phase of this illness (35, 36). As this neonatal disease did not match the clinical criteria for TSS, the disease was named neonatal toxic shock syndrome-like exanthematous disease (NTED) (36).

MRSA remains a major problem in nosocomial infections. The incidence of MRSA infection has been increasing among inpatients in many hospitals worldwide, especially neonatal, perinatal, and pediatric wards, as well as among outpatients in Japan (1, 20, 37). On the other hand, only 10 to 20% of MRSA carriers develop symptoms of NTED (35). Several recent reports have suggested that the development of NTED is dependent on host factors such as serum anti-TSST-1 levels (35). However, few reports have compared the epidemiological data for MRSA strains isolated from NTED patients with those for isolates from non-NTED patients. The question as to whether a specific clone(s) of MRSA is involved as the etiologic agent of NTED has been raised. Molecular typing techniques have introduced new possibilities for evaluation of the epidemiology of MRSA strains (1, 8, 11, 28, 32, 34).

In the present study, we characterized the MRSA strains isolated from NTED patients and compared them with MRSA strains isolated from patients with other MRSA infections and neonatal asymptomatic MRSA carriers.

MATERIALS AND METHODS

Prospective surveillance. A prospective study was performed in two areas of a neonatal intensive care unit (NICU; 25 beds with 300 admissions per year) and a general maternal and perinatal ward (general ward; 37 beds with 700 admissions per year) in the Tokyo Women's Medical University Hospital (TWMUH; 1,500 beds) between 13 September and 28 December 1998. The NICU is divided into two areas: an intensive care area (with nine incubators) and an intermediate care area (with 15 beds). Each incubator in the intensive care area occupies 7.0

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m^2. A total of 103 patients were analyzed. Informed consent was obtained from all the parents. NTED was diagnosed on the basis of the clinical criteria established by Takahashi et al. (36), which consisted of erythema plus at least one of the following three conditions: thrombocytopenia with platelet counts below 150 $\times$ 10^9/liter, a C-reactive protein concentration above 10 mg/liter, and a rectal body temperature above 38°C. Nasal, oral mucosal, umbilical, and rectal swab specimens or fecal specimens were obtained from the neonates in the NICU on postnatal day 3 and once a week later. The same samples were collected from the neonates in the general maternal and perinatal ward on postnatal day 3. Other specimens requested by the patients' doctors were also used in the analyses.

Bacterial strains. Each sample was plated onto a Trypticase soy agar plate with 5% defibrinated sheep blood (blood agar; Nissui Pharmaceutical Co, Ltd., Tokyo, Japan), and the plates were incubated at 35°C aerobically for 24 h. OPA *Staphylococcus* agar (Nippon Beckton-Dickinson Co., Ltd., Tokyo, Japan), which contained oxacillin, polymyxin B, aztreonam, amphotericin B, and egg yolk in brain heart infusion agar base, was also used for the selective isolation of MRSA strains if staphylococcal colonies were not found on the blood agar plates. *S. aureus* was identified by standard microbiological methods, including Gram staining, the catalase test, the latex slide agglutination test for clumping factor and protein A (PS test; Eiken Chemistry Co. Ltd., Tokyo, Japan), and the tube coagulase test (22). If similar *S. aureus* strains were obtained from different sites, the order of priority was blood, other infection sites, umbilicus, sputum, nostril, and rectal swab or feces, because the umbilicus is the primary site of neonatal colonization and infection (18). If different colonies of *S. aureus* were recovered from one patient, further antimicrobial susceptibility testing and molecular analysis were performed. Then, if phenotypic or genotypic differences were confirmed, both strains were counted and were included in the analysis as multiple isolates from the same patient. We also analyzed eight MRSA strains from eight patients with NTED in the NICUs of five other hospitals. Seibo Hospital (SH) is a TWMUHH-affiliated community hospital (192 beds) located 6.5 km from TWMUH. The other four hospitals are not affiliated with TWMUH. The status, number of beds, and distance from TWMUH are as follows: International Medical Center of Japan (IMCJ), a teaching hospital with 925 beds 1 km from TWMUH; Kawaguchi Municipal Medical Center (KMMC), a teaching hospital with 540 beds 12.5 km from TWMUH; Tokyo Metropolitan Kiyose Pediatric Hospital (TMKPH), a teaching hospital with 303 beds 20 km from TWMUH; and Nagoya Second Red Cross Hospital (NSRCH), a teaching hospital with 835 beds 380 km from TWMUH. Coagulase typing (44) was carried out with the MRSA strains isolated from NTED patients by using a coagulase typing kit (Denka Seiken, Niigata, Japan). The strains were stocked in 10% skim milk at −85°C until use.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed with the Pos Combo Panel Type 2J, and the results were read on a WalkAway System (Dade Behring Inc., West Sacramento, Calif.). The antibiotics tested were gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), ofloxacin (OFX), vancomycin (VAN), and trimethoprim-sulfamethoxazole (SXT). The isolates were interpreted as sensitive or resistant according to the MICs, as described previously (12). Resistance to several drugs was determined by plating on Trypticase soy agar containing spectinomycin (SPT; 500 μg/ml) and tetracycline (TET; 40 μg/ml). After a 24-h incubation, growth of more than two colonies was determined as resistance (17).

PFGE analysis. Preparation of chromosomal DNAs and pulsed-field gel electrophoresis (PFGE) analysis were carried out as described previously (8), with minor modifications, by using InCert agarose and SeaKem Gold agarose (FMC BioProducts, Rockland, Maine) instead of SeaPlaque and SeaKem LE agarose (FMC BioProducts), respectively. The PFGE run time was 20 h. The PFGE profiles were analyzed by visual inspection of the patterns according to the criteria of Tenover et al. (38). Isolates showing fragment differences of six or less were considered to be the same clone with different subtypes (38).

DNA extraction for amplification. A single colony was suspended to a McFarland 1.0 standard in 100 μl of TE buffer (20 mM Tris chloride, 2 mM EDTA [pH 7.5]) with 10 U of achromopeptidase (Wako Chemical, Co. Ltd., Osaka, Japan), and the suspension was incubated at 55°C for 10 min. After centrifugation at 18,500 $\times$ *g* for 5 min, the supernatants were used as crude DNA extracts for PCR.

Detection of enterotoxins, exfoliative toxins, and TSST-1 by multiplex PCR. The genes for staphylococcal enterotoxin A (SEA [*sea*]), SEB (*seb*), SEC (*sec*), SED (*sed*), SEE (*see*), exfoliative toxin A (ETA [*eta*]), ETB (*etb*), and TSST-1 (*tst*) were detected by using the multiplex PCR system with the oligonucleotide primers described by Becker et al. (3), with minor modifications. The PCR conditions were 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

TABLE 1. Results of prospective surveillance for MRSA in neonates

Results of screening cultures	No. of patients			
	Ward			Total
	General	NICU		
		Intermediate care	Intensive care	
MRSA isolated from:				
NTED patients	0	2	6	8
Patients with other infections	0	0	1	1
Carrier	23	15	15	53
Total	23	17	22	62
MRSA not detected[a]	19	19	3	41
Total	42	36	25	103

[a] No patients with NTED were in this category.

Determination of SCC*mec* type. The staphylococcal cassette chromosome *mec* (SCC*mec*) types of the MRSA strains isolated from NTED patients were determined by PCR of the *mec* and *ccr* gene complexes, as described previously (31).

Statistical analysis. Differences between groups were analyzed by the Mann-Whitney U test (StatView 5.0; Abacus Concepts Inc., Berkeley, Calif.). *P* values less than 0.05 were considered significant.

RESULTS

Prospective surveillance. The results of the prospective surveillance for *S. aureus* in neonates are summarized in Table 1. Among 103 patients eligible for the study, MRSA was detected in 62 (60.2%) newborns. No MRSA strain was isolated from the other 41 newborns. Among the 62 MRSA-positive newborns, 8 (12.9%) developed NTED, 1 (1.6%) had another MRSA infection, and 53 (85.5%) were asymptomatic MRSA carriers without any signs of infection. Six patients with NTED were in the intensive care area and two were in the intermediate care area. NTED did not occur in any of the newborns in the general ward. MRSA was isolated from all the NTED cases. The other case of MRSA infection was bacteremia and umbilicitis, which occurred in a patient in the intensive care area. The incidence of MRSA isolation in the intensive care area (22 of 25; 88.0%) was significantly higher than those in the intermediate care area (17 of 36; 47.2%; $P < 0.002$) and the general ward (23 of 42; 54.8%; $P < 0.02$). Methicillin-sensitive *S. aureus* (MSSA) was isolated from only six patients (one with umbilicitis in the intermediate care area and three and two asymptomatic carriers in the intermediate care area and the general ward, respectively), and only three patients were infected or colonized with MSSA strains without infection or colonization with MRSA strains (data not shown). Ten patients were simultaneously infected or colonized with multiple isolates of *S. aureus*. Two different MRSA strains were isolated from seven patients, and both MSSA and MRSA strains were isolated from three patients. Therefore, we obtained 69 MRSA strains from 62 patients and analyzed those strains in this study.

Antimicrobial susceptibility profiles. The 69 MRSA strains from TWMUH and 8 MRSA strains from five other hospitals showed five different antibiogram types with a panel of eight antibiotics (GEN, ERY, CLI, OFX, VAN, SXT, SPT, and TET). Antibiogram type I was resistance to all drugs except VAN and SXT, and isolates with this antibiogram type were

TABLE 2. Clonal distribution of MRSA strains from patients of different statuses

PFGE type and subtype	Toxin type	Antibiogram type	No. of strains from:			
			NTED patients	Patients with other infections	Carriers	Total
A						
A1	*tst*, *sec*	I	3	0	20	23
A1	*tst*, *sec*	II	2	0	15	17
A1	*tst*, *sec*	III	0	0	2	2
A2	*tst*, *sec*	I	1	0	2	3
A2	*tst*, *sec*	II	0	0	1	1
A3	*tst*, *sec*	I	5	1	7	13
A3	*tst*, *sec*	II	1	0	1	2
A4	*tst*, *sec*	II	1	0	1	2
A5	*tst*, *sec*	I	0	0	1	1
A6	*tst*, *sec*	II	0	0	1	1
A8	*tst*, *sec*	II	0	0	1	1
A9	Negative	II	0	0	1	1
Total			12	1	54	67
B	Negative	V	0	0	2	2
Total			12	1	56	69

found in both areas in the NICU and the general ward in TWMUH (data not shown). Types I and II (type II was similar to type I, except for sensitivity to GEN) were also predominant (in all hospitals, 44 of 77 [57.1%] and 27 of 77 [35.1%] isolates, respectively; in TWMUH, 40 of 69 [57.9%] and 25 of 69 [36.2%] isolates, respectively; and in the other hospitals, 4 of 8 [50%] and 2 of 8 [25%] isolates, respectively). Only minor populations were of antibiogram type III (resistance to ERY, CLI, OFX, and SPT), type IV (resistance to ERY, OFX, and SPT), and type V (resistance to ERY and SPT): three, one, and two strains, respectively. Of all the antimicrobial agents tested, the ones to which resistance was most frequently observed were ERY (100%), SPT (100%), OFX (75 of 77; 97.4%), CLI (74 of 77; 96.1%), and TET (71 of 77; 92.2%). The percentage of isolates resistant to GEN was lower (44 of 77; 57.1%). None of the isolates were resistant to SXT or VAN. There was one unique MSSA strain (TWCC4372), which was resistant to GEN, ERY, CLI, OFX, SPT, and TET but sensitive to oxacillin (data not shown).

Molecular analysis of *S. aureus* strains. Table 2 shows the genotypic and phenotypic characteristics of 69 MRSA strains evaluated in the prospective study conducted at TWMUH. The PFGE patterns of MRSA strains revealed a single major clone, clone A (67 of 69 isolates; 97.1%), with eight subtypes (subtypes A1 to A6, A8, and A9), and a minor clone, clone B (2 of 89 isolates; 2.9%), according to the criteria of Tenover et al. (38) (Fig. 1). By using the antibiogram, clone A (types I, II, and III) could easily be differentiated from clone B (type V). The most common subtype found was A1 (42 of 69 isolates; 60.9%), followed by A3 (15 of 69 isolates; 21.7%). The remaining PFGE subtypes were found at low frequencies: A2, 4 of 69 isolates (6.0%); A4, 2 of 69 isolates (2.9%); and A5, A6, A8, and A9, 1 isolate each. Clone A strains were detected in all 62 patients colonized or infected with MRSA. Among all clone A strains, all except one A9 strain had *tst* and *sec*. None of the MRSA strains possessed *sea*, *seb*, *sed*, *see*, *eta*, or *etb*. No enterotoxin, *tst*, or exfoliative toxin gene was detected in the type B MRSA strains. Twelve MRSA strains were obtained from eight NTED patients. These strains belonged to subtype A1 ($n = 5$), A2 ($n = 1$), or A3 ($n = 6$). No specific MRSA clone(s) was associated exclusively with NTED. We found no correlation between the PFGE subtype and an MRSA carrier state,

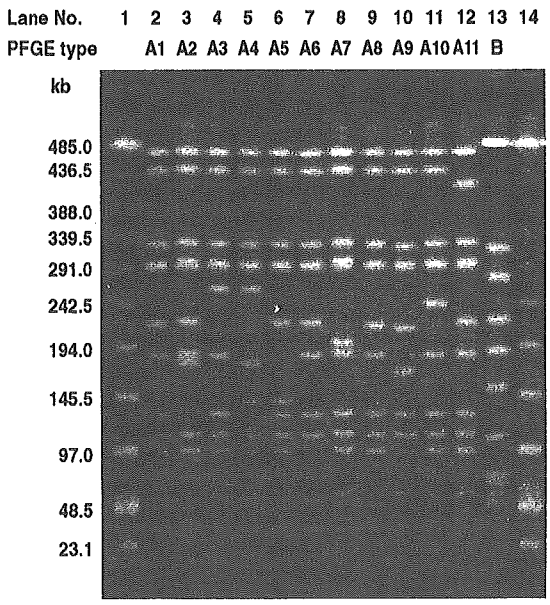

FIG. 1. PFGE patterns of MRSA and isogenic MSSA strains isolated from newborns. Lanes 1 and 14, low-molecular-weight bacteriophage λ DNA ladder markers; lanes 2 to 13, results obtained with the different strains. Each lane shows a different PFGE type, with the patient's clinical status indicated in parentheses below. Lane 2, TWCC4382 (NTED); lane 3, TWCC3794 (asymptomatic carrier); lane 4, TWCC3908-1 (NTED); lane 5, TWCC3957 (asymptomatic carrier); lane 6, TWCC4142 (asymptomatic carrier); lane 7, TWCC4173 (asymptomatic carrier); lane 8, TWCC4372 (MSSA infection other than NTED); lane 9, TWCC3904 (asymptomatic carrier); lane 10, TWCC4077-1 (asymptomatic carrier); lane 11, TWCC4082 (NTED, from SH); lane 12, TWCC4410-1 (NTED, from IMCJ); and lane 13, TWCC3861 (asymptomatic carrier).

TABLE 3. Typing of MRSA strains isolated from NTED patients

Patient no.	Hospital	TWCC strain	Site	PFGE type		Toxin type	Coagulase type	SCC*mec* type	Antibiogram
				Clone	Subtype				
1	TWMUH	3812	Umbilicus	A	A2	*tst*, *sec*	II	II	I
2	TWMUH	3908-1	Nose	A	A3	*tst*, *sec*	II	II	I
2	TWMUH	3908-2	Nose	A	A3	*tst*, *sec*	II	II	II
3	TWMUH	3800	Umbilicus	A	A3	*tst*, *sec*	II	II	I
4	TWMUH	4087-1	Umbilicus	A	A3	*tst*, *sec*	II	II	I
5	TWMUH	4350	Nose	A	A1	*tst*, *sec*	II	II	I
5	TWMUH	4382	Umbilicus	A	A1	*tst*, *sec*	II	II	II
6	TWMUH	4373	Oropharynx	A	A1	*tst*, *sec*	II	II	II
7	TWMUH	4032-2	Umbilicus	A	A1	*tst*, *sec*	II	II	I
7	TWMUH	4032-1	Umbilicus	A	A3	*tst*, *sec*	II	II	I
8	TWMUH	4055-2	Umbilicus	A	A1	*tst*, *sec*	II	II	I
8	TWMUH	4055-1	Umbilicus	A	A3	*tst*, *sec*	II	II	I
9	SH	3631	Umbilicus	A	A1	*tst*, *sec*	II	II	I
10	SH	3633	Nose	A	A1	*tst*, *sec*	II	II	I
11	SH	4082	Umbilicus	A	A10	*tst*, *sec*	II	II	I
12	SH	4139	Umbilicus	A	A10	*tst*, *sec*	II	II	I
13	NSRCH	4148	Umbilicus	A	A1	*tst*, *sec*	II	II	IV
14	IMCJ	4410-1	Blood	A	A11	*tst*, *sec*	II	II	II
15	TMKPH	4442	Umbilicus	A	A11	*tst*, *sec*	II	II	II
16	KMMC	4451-1	Umbilicus	A	A1	*tst*, *sec*	II	II	III

other MRSA infection, or NTED. Five different PFGE types (types A7, C, D, E, and F) were obtained for the MSSA strains (except for the data for subtype A7 in Fig. 1, data not shown). Except for the PFGE type A7 strain, the MSSA strains of the five PFGE types did not have any enterotoxin, exfoliative toxin, or TSST-1 genes; type A7 had *sec* and *tst*. The PFGE pattern of the multidrug-resistant MSSA strain (strain TWCC4372, which was resistant to GEN, ERY, CLI, OFX, SPT, and TET) was designated A7 and was quite similar to those of the other A subtypes. Strains of subtypes A1, A2, and A3 (n = 59) were gentamicin resistant and susceptible (subtype A1, 23 resistant and 17 susceptible strains; subtype A2, 3 resistant strains and 1 susceptible strain; subtype A3, 13 resistant and 2 susceptible strains). Two major subtypes (subtypes A1 and A3) were widely distributed in the general ward (15 and 4 of 23 strains, respectively), in the intermediate care area (13 and 4 of 19 strains, respectively), and in the intensive care area 12 and 8 of 25 strains, respectively).

The genotypic and phenotypic characteristics of the 20 MRSA strains isolated from NTED patients at TWMUH and the five other hospitals are listed in Table 3. Four patients in TWMUH harbored multiple MRSA strains with different genotypes or phenotypes. All the strains were identified to be the same clone (clone A), to belong to coagulase type II and SCC*mec* type II, and to have *tst* and *sec*. Two new PFGE subtypes were detected (subtype A10 from SH and subtype A11 from IMCJ and TMKPH) (Fig. 1). Most of the strains showed multidrug resistance (antibiogram type I or II, 18 of 20 isolates [90%]). Fourteen of 20 strains (70%) were isolated from the umbilicus. Subtype A1 (9 of 20 isolates [45%]) was the most predominant PFGE subtype, followed by A3 (6 of 20 isolates [30%]). The remaining PFGE types were found at low frequencies: A10 (2 of 20 isolates [10%]), A11 (2 of 20 isolates [10%]), and A2 (1 isolate). The same PFGE subtype (subtype A1) was found in TWMUH, SH, NSCRH, and KMMC. NSCRH, KMMC, and TWMUH do not share medical staff. Strain TWCC4410-1 from IMCJ and strain TWCC4442 from TMKPH could not be differentiated by PFGE type, toxin type, or antibiogram.

DISCUSSION

NTED was first described in 1995 as a novel exanthematous disease caused by the staphylococcal superantigen TSST-1 (36). TSST-1-producing MRSA strains were isolated from most cases of NTED (36). In our study all the MRSA strains isolated from eight NTED patients at TWMUH had the TSST-1 and SEC genes. Moreover, the TSST-1 and SEC genes were also detected in the eight strains isolated from eight NTED patients at the five other hospitals. These results confirm those presented in a previous report (36) and support the evidence implicating TSST-1 as an etiologic factor for NTED.

Since 1992, the number of hospitals in Japan in which this disease has been encountered has increased, from 25.7% (19 of 74) in 1995 to 70.8% (63 of 89) in 1998 (36). The rate of methicillin resistance among the *S. aureus* strains in many hospitals in Japan was greater than 70% (1, 20, 37). Most of the strains produced TSST-1 and SEC and belonged to coagulase type II and *mecA*-Tn*554* polymorph I-A (1, 20, 34, 37). In the 1990s, this new major clone replaced the former clone that was dominant and widespread during the 1980s, with less resistance to β-lactams and without TSST-1 production (1, 15, 37). These data suggest that the time of emergence and the increase in the incidence of NTED coincide with the emergence and spread of a new MRSA strain. In our hospital, methicillin resistance rates among *S. aureus* strains were 60 to 65% from 1996 to 1998, on the basis of one strain per one inpatient (20). In this study, MRSA was more prominent in the neonatal wards than in other wards, despite the implementation of several control measures. The reason is not fully known. However, overcrowding, understaffing, and increased workloads are well-known risk factors for the nosocomial transmission of MRSA (9, 19, 43). In our neonatal intensive care area, each incubator occupies 7.0 m^2, which is slightly below the American Academy of

Pediatrics standard of ≧7.4 m^2/incubator in intensive care units (14).

The vast majority of MRSA strains belonged to a single clone that spread in three neonatal and perinatal wards. This clone was isolated from all patients with newly developed cases of NTED. However, in all three wards, many patients infected or colonized with this clone did not develop NTED. We could not identify any NTED-specific PFGE type that was not isolated from patients with other staphylococcal infections or asymptomatic carriers. The production of TSST-1 depends on environmental conditions such as the oxygen or carbon dioxide concentration (33, 46, 47) and is regulated by various regulatory access systems (23, 47). The possibility that some strains with the same PFGE subtype produce different amounts of TSST-1 remains to be examined. However, these results rather support the notion that the development of NTED is highly dependent on the patient's immunological background (35, 36). Neonates are generally protected against various infectious agents by specific immunoglobulin G (IgG) antibodies transferred transplacentally from their mothers (35). Exposure to TSST-1-producing *S. aureus* is commonplace in the general population, yet TSS is rare (5). The reason for this is that host susceptibility plays a key role in determining the occurrence of TSS (5). Anti-TSST-1 antibodies play a protective role against the development of TSS in adults (10, 45). The development of NTED was also dependent on the serum anti-TSST-1 IgG antibody titer (35). Whether MRSA carriers developed NTED was partly dependent on the presence of IgG-type anti-TSST-1 antibodies from their mothers (35).

Interestingly, the PFGE patterns of the MRSA strains from NTED patients in the other hospitals closely resembled those of the strains found in TWMUH (Table 3). This means that a single MRSA clone might be widespread in the neonatal wards of many hospitals, in association with an increase in the incidence of NTED. Saito et al. (34) also reported that a single PFGE type of MRSA producing TSST-1 and SEC was widely distributed in the NICUs of three different hospitals in Japan. Musser et al. (29) also reported on the isolation of a single clone from the majority of patients affected by TSS with a urogenital focus.

Clone A has also frequently been isolated in the United States (1, 32). However, there were only two reports of probable NTED suggested by laboratory data and clinical findings from countries other than Japan (7, 13). Both reports were postpartum cases among mother-infant pairs. Both the mothers and infants in each pair had thrombocytopenia, fever, and macular rash without desquamation, and the infections were self-limited. These characteristics are compatible with NTED. The *S. aureus* strain isolated from one patient did not produce TSST-1 but produced SEC, another superantigen of *S. aureus* (7). NTED cases might be underrecognized in other countries. Some investigators have reported that the prevalence of serum anti-TSST-1 antibodies was very high in adults (45). Therefore, newborns who have anti-TSST-1 antibodies transferred from the mothers may be protected from NTED. Moreover, *tst* and *sec* of MRSA N315 and Mu50, which were coagulase type II and SCC*mec* type II (31), were found to be located in the same pathogenic island (24). RN4282 had another different pathogenic island that encoded *tst* without *sec* (24, 30). The potential for TSST-1 production in Japanese clone A MRSA strains might be different from that for U.S. clone A strains. Further investigations of toxin production or the genetic background of *tst*-containing pathogenic islands should be conducted with clone A MRSA strains from different countries or continents.

There were some discrepancies between the antibiograms and the PFGE patterns, especially in terms of resistance to GEN. Resistance to GEN in *S. aureus* is mainly due to a bifunctional aminoglycoside-modifying enzyme, aminoglycoside-6′-*N*-acetyltransferase–2″-*O*-phosphoryltransferase [AAC(6′)-APH(2″)] (2, 4); and the gene for this enzyme, *aac(6′)-aph(2″)*, is present in 84.5% of MRSA strains in Japan (16). Lelièvre et al. (26) reported that some GEN-sensitive and GEN-resistant MRSA strains had closely related PFGE profiles and that the GEN-sensitive strains could have emerged from resistant ones by excision or deletion of the *aac(6′)-aph(2″)* gene. Although we did not check our strains for the presence of aminoglycoside-modifying enzymes, the different GEN susceptibility patterns among strains with the same PFGE pattern may be attributed to the spontaneous loss or acquisition of this plasmid. Moreover, one MSSA strain (strain TWCC4372) was closely related to clone A (subtype A7) with *tst* and *sec* and resistance to GEN, ERY, OFX, SPT, and TET. It is well known that MRSA strains spontaneously lose methicillin resistance in vitro and in vivo (11, 17, 21, 25).

NTED regresses spontaneously without any active anti-staphylococcal treatment, and the prognosis is good (36; Takahashi and Nishida, Arch. Dis. Child. 77:F79, 1997). We also did not experience any fatal cases in the period covered by this study. However, the high incidence of NTED and the asymptomatic carrier state reflect the wide dissemination of a single MRSA clone in our hospital. Strict infection control measures and new strategies (9, 43) are required to control and eliminate NTED.

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蛍光タンパク質を用いた *M. pneumoniae* の細胞構造の観察

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【要　旨】

M. pneumoniae の接着器官や細胞骨格の構造を理解するために、蛍光タンパク質を利用したタンパク質の視覚化と細胞構造の観察を検討した。*M. pneumoniae* の接着器官に局在するP65タンパク質の遺伝子に、蛍光タンパク質EYFP（enhanced yellow fluorescent protein）やECFP（enhanced cyan fluorescent protein）の遺伝子をつないで発現させると、発現したタンパク質は接着器官に局在し、接着器官を蛍光顕微鏡下で光らせて観察することができた。HMW1、HMW2、HMW3タンパク質にEYFPを連結しても同様な観察を行うことが可能だった。さらに *crl* オペロンに遺伝子が存在するP41とP28タンパク質に蛍光タンパク質をつないで観察すると、これらのタンパク質は接着器官の基幹部に局在していることが観察された。P41とP28も細胞骨格を構成するタンパク質であると考えられた。蛍光タンパク質を使用したタンパク質局在の観察は、今後 *M. pneumoniae* の接着器官や細胞骨格の構造を調べる有効な手段になると考えられる。

【序　文】

M. pneumoniae は細胞壁を持たず、形態は多形性である。しかし、培養中に最も多く観察されるのは、細長いフィラメント状の細胞であり、その一方の端には接着器官（Tip構造）が存在している(1)。このような *M. pneumoniae* の非対称な細胞形態は、細胞内に存在する細胞骨格構造によってささえられていると考えられている(2)。電子顕微鏡では、接着器官の中心に電子密度の高い棒状の構造（rod）が観察され、細胞骨格の一部だと考えられている。また、rodの後方の細胞内には繊維状の構造が広がっているのも観察されている(1)。*M. pneumoniae* の細胞骨格成分の候補はTriton X-100（TX-100）に不溶なタンパク質の一部や、細胞接着性に関与するタンパク質として同定されてきている。しかし、これらの構成タンパク質がどのように組み合わさって細胞骨格構造を形成しているのかは全くわかっていない。本研究では *M. pneumoniae* の細胞骨格の詳しい構造を理解するのを目的として、蛍光タンパク質を使用して *M. pneumoniae* のタンパク質を視覚化する検討を行った。

【材料と方法】

蛍光タンパク質EYFPとECFPの遺伝子はClontech社のプラスミドpEYFPとpECFPから得た。*M. pneumoniae* M129株の遺伝子はPCRで増幅しEYFPまたはECFPの遺伝子に連結した。これらの蛍光タンパク質遺伝子との融合遺伝子はTn*4001*ベクターを使用して *M. pneumoniae* M129株に導入した。遺伝子が導入された株は、それぞれの蛍光タンパク質用のフィルターを取り付けた蛍光顕微鏡で観察した。

【結果と考察】

蛍光タンパク質を連結して視覚化を試みる最初の *M. pneumoniae* のタンパク質として、P65を選んだ。P65はその遺伝子のすぐ上流にプロモーターがあり、これを利用すれば組換えタンパク質として発現させやすい。P65はTX-100不溶画分に見いだされたタンパク質で、細胞接着関連タンパク質HMW1やHMW3と類似したアミノ酸組成領域を有している。また、接着器官に局在することもわかっており(3)、細胞接着性に関与する細胞骨格の成分の一つだと考えられている。P65の遺伝子とEYFPの遺伝子を連結し、Tn*4001*ベクターで *M. pneumoniae* に導入した。この菌を蛍光顕微鏡で観察すると、接着器官の部位にEYFPの蛍光が観察された。P65のN末端とC末端側のどちらにEYFPをつないでもほぼ同様な蛍光が観察されたが、N末端側につないだ方がやや明るかった。P65遺伝子のプロモーターを、より活性の強い *tuf* 遺伝子のプロモーターに変えて実験を行うと、さらに明るく接着器官部位を光らせて観察することができた。ウェスタンブロットによる分析では、蛍光の強さに対応してEYFP-P65融合タンパク質の発現量の増加がみられた。この菌を37℃に保ったまま顕微鏡で観察すると、蛍光を発する接着器官の方向へ *M. pneumoniae* が滑走運動するのを観察することもできた。EYFPを使用してP65タンパク質を視覚化することができたので、この方

法をHMW1、HMW2、HMW3タンパク質にも試した。その結果、HMW1、HMW2、HMW3のN末端側EYFPを連結させたタンパク質を発現させても接着器官部位に蛍光が観察された。他の方法で明らかにされているように、これらのタンパク質の接着器官への局在をEYFPで視覚化できた。

蛍光タンパク質の使用は*M. pneumoniae*においてもタンパク質の局在を視覚化する有効な手段になりうることがわかった。そこで次に、細胞内での局在が未知なタンパク質についてこの方法を応用した。P65の遺伝子の下流にはHMW2の遺伝子があり、さらにその下流にはP41とP24タンパク質の遺伝子がある。このP65からP24までの4つの遺伝子はオペロンを形成しており、*crl* (cytadherence regulatory locus)オペロンと呼ばれている(2)。このうち、P41とP24タンパク質が*M. pneumoniae*細胞内で局在するかは不明だった。このP41とP24タンパク質にEYFPを連結したタンパク質を発現させた。その結果EYFPの蛍光は接着器官の基幹部に観察された。P65にECFPをつないだものと同時発現させると、ECFPとEYFPの蛍光は重ならず、明らかにP41とP24はP65よりも接着器官の基幹部側に存在していると考えられた。さらに、低いレベルでの発現ではP41とP24に連結したEYFPの蛍光は接着器官の基幹部のほぼ同じ位置に限定されたが、高いレベルの発現では、P41の方は頻繁に接着器官とは反対側の尾部にも蛍光が観察された。また、P24は高いレベルでは接着基幹部から後方に尾をひくように蛍光が観察された。これらのタンパク質の機能は現時点では不明だが、接着器官の基幹部に局在が見られることから、この部位で何らかの構造を形成するのに働いている可能性が考えられる。最近、電子顕微鏡による観察から接着器官の基幹側に輪のような構造が存在することが報告され、これも*M. pneumoniae*の細胞骨格の一部をなしていると考えられている(4)。P41とP24がこの構造を形成する因子であることは十分に考えられる。(共同研究：宮田真人、瀬戸真太郎　大阪市大)

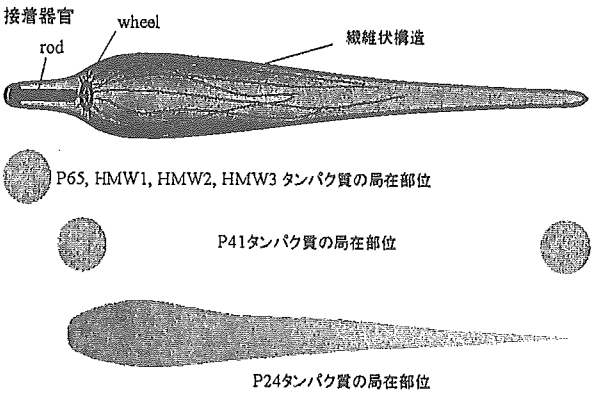

図1．*M. pneumoniae* 細胞におけるタンパク質の局在

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Observation of cell structure of *M. pneumoniae* with fluorescent protein fusion technique

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Key words : attachment organelle, cytoskeleton, green fluorescent protein

Mycoplasma pneumoniae lacks cell wall but has an internal cytoskeleton-like structure that is assumed to support the attachment organelle and the asymmetric cell shape of this bacterium. To understand fine structure of the cytoskeleton and the attachment organelle, fluorescent protein fusion technique was applied to visualize protein components of these structures. The genes for fluorescent proteins EYFP or ECFP were fused to the gene coding for P65 that is known to localize to the attachment organelle and was expressed in *M. pneumoniae* cells. The fusion proteins localized at the attachment organelle and were detectable by fluorescence microscopy enabling visualization of the organelle in living condition. The attachment organelle was also visualized by expressing EYFP fusions with HMW 1, HMW2 and HMW3 proteins. We also constructed EYFP fusions of P41 and P24 proteins that are encoded in *crl* operon of *M. pneumoniae* genome. These fusion proteins were localized at the proximal position of the attachment organelle by fluorescent microscopy, suggesting that P41 and P24 are also cytoskeletal proteins. The fluorescent protein fusion technique may serve as a powerful tool to analyze the structures of cytoskeleton and the attachment organelle by identifying their components.