2002 and 2003 and collected relevant epidemiological and clinical data. We observed the presence of new *tst*-positive MRSA clones responsible for both hospital- and community-acquired infections.

MATERIALS AND METHODS

Bacterial isolates. Among the 1,550 unconstrained strains sent to the French National Reference Center for Staphylococci during 2002 and 2003, 103 isolates from 42 towns were *tst* positive. As controls we used nine *tst*-positive MRSA isolates from Australia (one isolate, provided by Graeme Nimmo), Switzerland (three isolates), and Japan (five isolates causing neonatal toxic shock-like exanthematous diseases, TWCC3812, TWCC390861, TWCC4082, TWCC4382, and TWCC4410) (16). We also used an isolate representative of the Pediatric clone and an isolate representative of the New York/Japan clone.

Data collection. For each *S. aureus* strain we collected relevant clinical information (age, sex, type, and site of infection) by using a standard form provided by the French National Reference Center for Staphylococci. TSS, staphylococcal scarlet fever, and neonatal toxic shock syndrome-like NTED were diagnosed by using published criteria (11, 21, 30, 32). For this study, MRSA infection was considered to be community acquired if the specimen was obtained outside the hospital setting or less than 2 days after hospital admission of a patient with no direct or indirect exposure to the healthcare system in the previous year (2).

DNA extraction. Strains were grown on brain heart infusion agar or in brain heart infusion broth at 37°C overnight. Genomic DNA was extracted with a standard procedure, and its concentration was estimated spectrophotometrically (18). Amplification of *gyrA* was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. All PCR products were analyzed by electrophoresis on ethidium bromide-stained 1% agarose gels (Sigma, France).

Identification of *agr* alleles. The *agr* group (*agr1* to *-4*) was determined by PCR as previously described (15).

Detection of the *mecA* gene and SCC*mec* typing. The *mecA* gene coding for methicillin resistance was detected by PCR as described by Murakami et al. (20). The staphylococcal chromosomal cassette *mec* (SCC*mec* I to IV) was detected by using the method of Oliveira et al. (23). The following reference strains, kindly provided by Herminia de Lencastre and Alexander Tomasz, were used as controls: COL (SCC*mec* I), BK2464 (SCC*mec* II), HU106 (SCC*mec* III), and BK2529 (SCC*mec* IV).

Detection of toxin and adhesin genes. Sequences specific for staphylococcal enterotoxin genes (*sea-e* and *seg-o*), the toxic shock syndrome toxin gene (*tst*), exfoliative toxin genes (*eta* and *etb*), PVL genes (*lukS*-PV-*lukF*-PV), the LukE-lukD leukocidin genes (*lukE-lukD*), the class F *lukM* leukocidin gene (*lukM*), and hemolysin genes (gamma [*hlg*], gamma variant [*hlgv*], and beta [*hlb*]) and for nine MSCRAMM genes (microbial surface components recognizing adhesive matrix molecules), bone sialoprotein binding protein (*bsp*), clumping factors A and B (*clfA* and -B), collagen binding protein (*cna*), elastin binding protein (*ebpS*), laminin binding protein (*eno*), fibronectin binding proteins A and B (*fnbA* and *-B*), and extracellular fibrinogen binding protein (*efb*), were detected by PCR as described elsewhere (15, 23, 26, 27, 35, 37).

Antimicrobial susceptibility testing. Susceptibility tests were performed with the ATB System (bioMérieux, France).

Capsular typing. Capsular serotyping was performed for all MRSA strains and for randomly selected MSSA strains. The strains were grown for 24 h at 37°C on Columbia agar plates containing 2% $MgCl_2$ and 0.5% $CaCl_2$. Several colonies of each strain were suspended in 0.9% saline and tested by slide agglutination with rabbit polyclonal antibodies specific for capsular polysaccharide types 5 and 8 (8, 9).

Fingerprinting by PFGE. SmaI macrorestriction patterns were obtained by using a contour-clamped homogeneous electric field DR-II apparatus (Bio-Rad), as described elsewhere (19). Strain NCTC 8325 was used as a pulsed-field gel electrophoresis (PFGE) control. Resolved macrorestriction patterns were compared as recommended by Tenover et al. (33). Isolates were assigned to a single clonal group if they differed by less than six bands. PFGE patterns with more than six band differences (<75% similarity) were considered to correspond to different types.

The *mecA* gene was tested for in one of the PFGE bands, as follows: the fragment was cut out from the agarose gel, DNA was extracted by using the MinElute gel extraction kit protocol (QIAGEN), and PCR with the *mecA* primers and multiplex PCR for SCC*mec* typing were performed on the extract as described above.

***spa* typing.** *spa* typing was performed on MRSA isolates and on *agr2* MSSA isolates, as previously described (14). The x region of the *spa* gene was amplified by PCR. *spa* types were determined with Ridom Staph Type software (Ridom GmbH, Germany), which automatically detects *spa* repeats and assigns a *spa* type.

MLST. MLST was performed on strains representative of each clonal group, as described elsewhere (6, 36). The allelic profile of each strain was obtained by sequencing internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) and entering them on the MLST home page (http://saureus.mlst.net), where seven numbers depicting the allelic profile were assigned which defined an ST (6). To determine genetic relationships, MLST data were examined with BURST software (*b*ased *u*pon *r*elated *s*equence *t*ypes; details are available from http://www.mlst.net/BURST/burst.htm). The algorithm places STs that share five out of seven MLST alleles in a common clonal complex (7).

TABLE 1. Distribution of the *mecA* gene and *agr* alleles among 103 French *S. aureus* isolates containing the *tst* gene collected between 2002 and 2003

agr allele type	No. (%) of isolates		
	mecA$^+$ (n = 27)	*mecA* deficient (n = 76)	Total
1	0 (0)	1 (1)	1
2	25 (93)	5 (7)	30
3	2 (7)	70 (92)	72
4	0 (0)	0 (0)	0

RESULTS

Distribution of isolates according to methicillin resistance and *agr* group. Among the 103 *tst*-positive *S. aureus* isolates, 27 were methicillin resistant (*mecA*$^+$), and 76 were methicillin susceptible (*mecA* deficient) (Table 1). Twenty-five *tst*-positive MRSA isolates had *agr* allele type 2, and two had *agr* allele type 3. Seventy *tst*-positive MSSA isolates had *agr* allele type 3, five isolates had *agr* allele type 2, and one isolate had *agr* allele type 1.

Clinical characteristics of *tst*-positive MRSA infections. The median age of the 27 patients with *tst*-positive MRSA infections was 3 years (range, <1 month to 84 years), and the sex ratio was 1. Five patients had toxic shock syndrome, two had NTED (31), and one had staphylococcal scarlet fever; nine patients had toxic shock syndrome but did not fulfill all the criteria of a TSST-1-mediated syndrome (i.e., fever and rash without shock) (Table 2). Five skin infections occurred in patients with varicella. Eight patients had deep-seated infections (pneumonia or osteoarthritis), and no clinical information was available for two other patients. Two deaths occurred. The isolates were recovered from skin and soft tissues (14 isolates), blood (7 isolates), the umbilicus (2 isolates from cases of NTED), bronchopulmonary secretions (2 isolates), a prosthesis (1 isolate), and a ligament (1 isolate).

Information on the hospital or community acquisition of the infection was available for 20 MRSA infections and 51 MSSA infections. The origin of MRSA infection was unknown in seven cases. Eight of the 27 patients with *tst*-positive MRSA isolates had no known link to healthcare facilities and no known risk factors for MRSA acquisition; these cases were considered to be community acquired. Twelve cases were hospital acquired.

Microbiological characteristics of *tst*-positive MRSA isolates. The 25 *tst*-positive *agr2* MRSA strains all harbored the *sec*, *sed*, *sel*, *sem*, *seo*, *lukDE*, and *hlgv* toxin genes and the *clfA-B*, *ebpS*, *eno*, and *efb* adhesin genes (Table 3). These

TABLE 2. Clinical data on *tst*-positive MRSA infections

Clinical presentation	Patient and isolate no.[a]	City of isolation	Age (yrs)	Sex[b]	Samples	Site of acquisition	*agr* type	PFGE type
True TSS[c]	1, HT20020212	Lyon	2	M	Skin	Community	2	A1
	2, HT20020255	Bordeaux	42	F	Blood	Nosocomial	2	A1
	3, HT20020256	Bordeaux	0	F	Blood	Nosocomial	2	A1
	4, HT20030603	Lyon	25	F	Blood	Nosocomial	2	A1
	5, HT20030159	Geneva	0	ND[e]	Skin	Unknown	2	A3
Possible TSS[d]	6, HT20030119	Marseille	3	F	Skin	Nosocomial	2	A1
	7, HT20030369	Lyon	0	M	Skin	Unknown	2	D
	8, HT20030416	Lyon	9	F	Skin	Community	2	A7
	9, HT20030618	Lyon	8	F	Skin	Unknown	2	A2
	10, HT20030434	Marseille	0	F	Blood	Unknown	2	A1
	11, HT20030727	Lyon	84	M	Skin	Community	2	A1
	12, HT20030769	Fréjus	2	F	Skin	Unknown	2	A3
	13, HT20030695	Lausanne	2	ND	Skin	Unknown	2	A10
	14, HT20030849	Geneva	2	ND	Skin	Community	2	A9
Superinfection of varicella	15, HT20020188	Lyon	1	M	Skin	Community	2	A1
	16, HT20020277	Paris	1	M	Skin	Community	2	A8
	17, HT20020369	Lyon	7	M	Skin	Community	2	E
	18, HT20030228	Lyon	0	M	Skin	Community	2	A3
	19, HT20030651	Lyon	4	M	Skin	Community	2	A12
NTED	20, HT20020780	Tours	0	ND	Umbilicus	Nosocomial	2	A3
	21, HT20020781	Tours	0	ND	Umbilicus	Nosocomial	2	A3
Staphylococcal scarlet fever	22, HT20030157	Lille	0	M	Skin	Unknown	2	A3
Pneumonia	23, HT20020132	Boulogne	27	F	Skin	Nosocomial	2	A5
	24, HT20020417	Annecy	1	M	Bronchopulmonary secretion	Unknown	2	A12
	25, HT20030216	Lyon	65	M	Blood	Nosocomial	2	A1
	26, HT20030639	Lyon	31	M	Blood	Nosocomial	2	A7
	27, HT20020459	Rouen	1	F	Bronchopulmonary secretion	Nosocomial	3	G2
Osteoarthritis	28, HT20030749	Bondy	0	F	Blood	Unknown	2	A4
	29, HT20030095	Lyon	28	M	Ligament	Nosocomial	2	A3
	30, HT20020665	Marseille	14	F	Prosthesis	Nosocomial	3	G3

[a] Isolates 5, 13, and 14 from Switzerland were not among the 27 French *tst*-positive MRSA.
[b] M, male; F, female.
[c] Cases associated with TSS according to the reference criteria.
[d] Cases associated with TSS but that did not fulfill all criteria of TSST-1-mediated syndrome.
[e] ND, no data.

isolates were of capsular type 5, except for two isolates which could not be typed with this method. All but one were resistant to penicillin, oxacillin, kanamycin, and tobramycin and had intermediate resistance to fusidic acid; the remaining isolate was susceptible to kanamycin and tobramycin (Table 3). Seven isolates were resistant to other antimicrobial agents such as erythromycin, lincomycin, or tetracycline. All had an SCC*mec* element type IV, except for one isolate which had an SCC*mec* element type IVA and two isolates which were nontypeable (possibly new SCC*mec* variants). PFGE gave more diverse results: all but two of the isolates belonged to PFGE type A (14 subtypes), while the remaining isolates were of types D and E (Fig. 1). The main *spa* type was *spa* 2 (21 isolates). The other isolates had a related *spa* type that differed by one (*spa* 10 and *spa* 242) or two (*spa* 568) repeats. These isolates were all of ST5, as determined by MLST. Overall, the 25 *tst*-positive *agr2* MRSA isolates were highly clonally related. This clone was detected in 12 towns in France, and three isolates from Switzerland had similar characteristics. Five Japanese *tst*-positive *agr2* MRSA isolates from patients with NTED were related to this clone (Table 3).

Two *tst*-positive *agr3* MRSA isolates were identified. They possessed the *sea*, *sem*, *seo*, *hlg*, *clfA-B*, *cna*, and *ebpS* genes and were of capsular type 8. These two isolates were resistant to penicillin, oxacillin, kanamycin, tobramycin, and erythromycin and had intermediate resistance to fusidic acid. One isolate had the SCC*mec* IV element, whereas the other had the SCC*mec* IVA element. Their PFGE patterns differed by three bands, and both isolates belonged to PFGE type G (Fig. 1). Their *spa* types differed by only four repeats (*spa* 638 and *spa* 584), and both isolates were ST30, as determined by MLST. These two isolates were considered to be clonally related.

Comparison of the *tst*-positive *agr2* isolates with the New York/Japan clone and the Pediatric clone. The 25 *tst*-positive *agr2* MRSA isolates were ST5 and belonged to capsular type 5, like the New York/Japan and Pediatric clones. The New York/Japan clone contained the *tst* toxin gene, contrary to the Pediatric clone. The New York/Japan clone also did not contain the toxin gene (*sed*), contrary to the 25 *tst*-positive *agr2* MRSA isolates. The 25 *tst*-positive *agr2* MRSA isolates and the Pediatric clone harbored SCC*mec* element IV, whereas the New York/Japan clone harbored SCC*mec* element type II. The 25 *tst*-positive *agr2* MRSA isolates and the New York/Japan clone were *spa* type 2, while the Pediatric clone was *spa* type 311 (diverging by only one repeat).

Comparison of *tst*-positive MRSA isolates with MSSA isolates. The 25 *tst*-positive *agr2* MRSA isolates and the 5 *tst*-positive *agr2* MSSA isolates had similar virulence determinants, an identical capsular type (type 5) and sequence type (ST5), and a common PFGE type (A) which differed by a

TABLE 3. Microbiological characteristics of *tst*-positive *Staphylococcus aureus* isolates

Resistance group, country of origin, and no. of isolates	ST[a]	CC[b]	*spa* type	Capsular type	*tst* gene	Toxin genes	Adhesin genes	SCC*mec* type	Antibiotic resistance[c]	PFGE type(s)
MRSA isolates *agr2*										
France (n = 27)										
13	5	5	2	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, FU	A1, A2, A3, A7. E
1	5	5	568	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, FU	A3
1	5	5	242	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, FU	A4
4	5	5	2	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, E, FU	A3
1	5	5	2	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, E, L, FU	A8
1	5	5	2	NT[d]	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	NT	P, OX, K, T, TE, FU	A12
1	5	5	2	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, E, TE, FU	A3
1	5	5	10	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, FU	A7
1	5	5	10	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IVA	P, OX, K, T, FU	A1
1	5	5	2	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	NT	P, OX, K, T, FU	A12
Switzerland (n = 3)										
3	5	5	ND[e]	5	+	*sec, sed, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, FU	A3, A9, A10
Japan (n = 5)										
3	5	5	ND	5	+	*sec, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	II	P, OX, K, T, G, E, L, TE, F, PE	C4, C5, C2
1	5	5	ND	5	+	*sec, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	II	P, OX, K, T, G, E, L, TE, FU	C1
1	5	5	ND	5	+	*sec, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	II	P, OX, K, T, E, L, TE, F, PE	C3
Pediatric clone	5	5	311	5	−	*sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	IV	P, OX, K, T, G, E	B
New York/Japan clone	5	5	2	5	+	*sec, sel, sem, seo, lukED, hlgv*	*clfA-B, ebpS, eno, efb*	II	P, OX, K, T, G, E, L, TE, F, PE	A6
MSSA isolates *agr2*										
France (n = 5)										
1	5	5	105	5	+	*sec, sed, sel, seo, sem, lukED, hlgv*	ND	NA[f]	ND	A13
1	5	5	88	5	+	*sec, sed, sel, seo, sem, lukDE, hlgv*	ND	NA	ND	A11
1	5	5	572	5	+	*sec, sed, sel, seo, sem, lukDE, hlgv*	ND	NA	ND	A14
1	5	5	570	5	+	*sem, seo, lukDE, hlgv*	ND	NA	ND	A13
1	5	5	548	5	+	*sed, sel, seo, sem, lukDE, hlgv*	ND	NA	ND	A11
MRSA isolates *agr3*										
France (n = 2)										
1	30	30	584	8	+	*sea, seo, sem, hlg*	*clfA-B, cna, ebpS*	IV	P, OX, K, T, E, FU	G2
1	30	30	638	8	+	*sea, seo, sem, hlg*	*clfA-B, cna, ebpS*	IVA	P, OX, K, T, E, FU	G3
MSSA isolates *agr3*										
France (n = 70)										
15	30	30	ND	8	+	*sem, seo, hlg*	ND	NA	ND	ND
6	30	30	ND	8	+	*sem, seo, hlb, hlg*	ND	NA	ND	ND
15	34	30	ND	8	+	*seh, sem, seo, hlg*	ND	NA	ND	ND
3	34	30	ND	8	+	*seh, sem, seo, hlb, hlg*	ND	NA	ND	ND
28	30	30	17, 12[g]	8	+	*sea, sem, seo, hlg*	ND	NA	ND	G1
1	30	30	ND	8	+	*sea, sel, sem, seo, hlg*	ND	NA	ND	ND
1	1	Singleton	ND	8	+	*lukPV, sea, seh, sek, lukDE, hlgv*	ND	NA	ND	ND
1	30	30	ND	8	+	*lukPV, sec, sel, sem, seo, lukDE, hlgv*	ND	NA	ND	ND

[a] ST, sequence type.
[b] CC, clonal complex.
[c] P, penicillin; OX, oxacillin; K, kanamycin; T, tobramycin; G, gentamicin; E, erythromycin; L, lincomycin; TE, tetracycline; F, fosfomycin; PE, pefloxacin; Fu, fusidic acid.
[d] NT, nontypeable.
[e] ND, not determined.
[f] NA, not applicable.
[g] Determined on two isolates only.

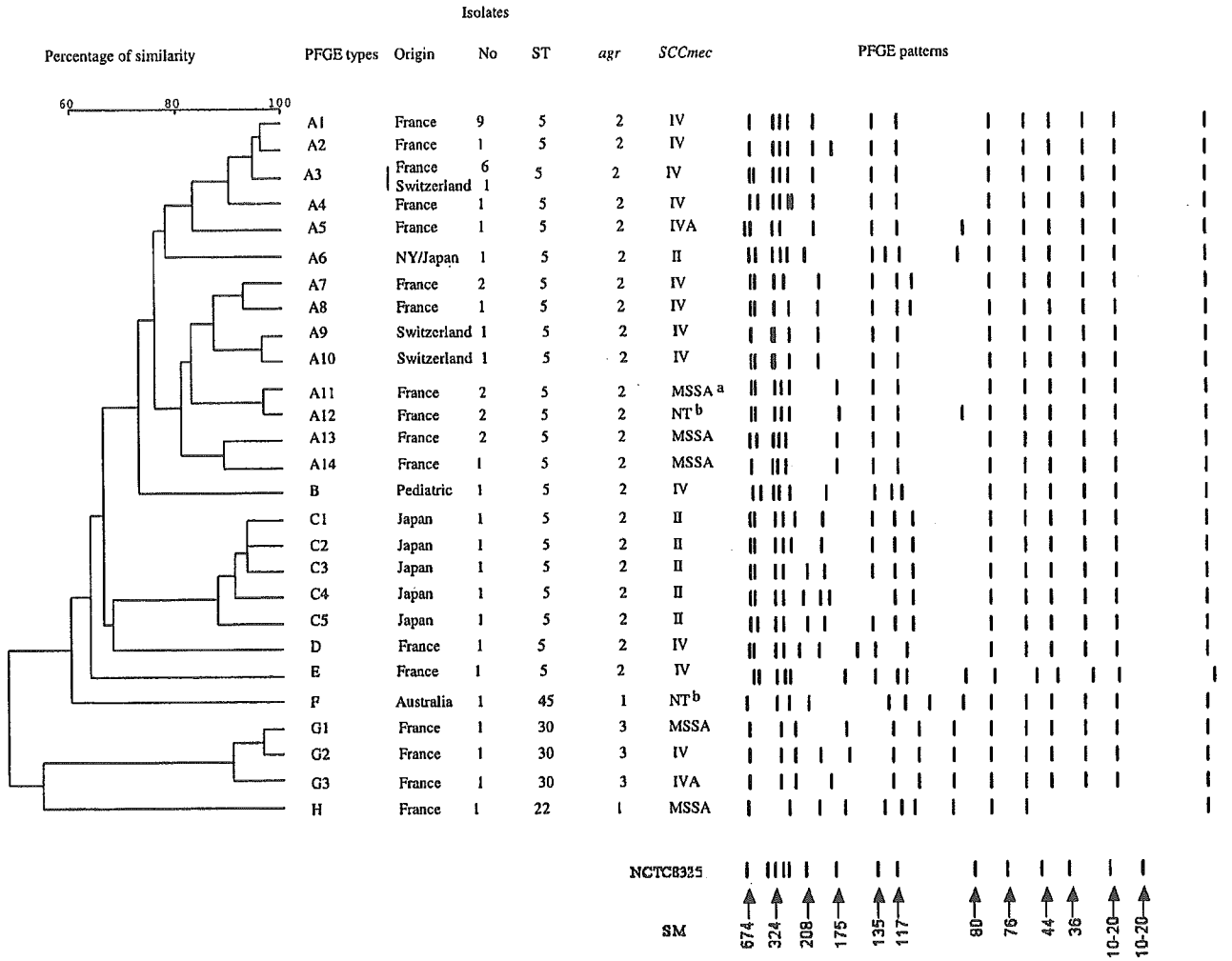

FIG. 1. Unweighted pair group method with averages dendrogram of PFGE results based on the Dice matrix and schematic representation of the pulsotype (SmaI restriction enzyme) of *tst*-positive MRSA isolates; *S. aureus* NCTC 8325 is the reference strain for the size marker (SM), expressed in kilobases. Isolates differing by more than six fragments were considered to be subtypes of a given clonal type. The *agr2* MRSA clone belonged to the PFGE type A except two isolates of PFGE types D and E. NT[b], nontypeable.

single band between the MRSA isolates (192 kb) and the MSSA isolates (151 kb). We concluded that the *tst*-positive *agr2* MRSA and MSSA clones were closely related. The presence of the SCC*mec* IV element in the 192-kb band of MRSA isolates was demonstrated after excising this band from the gel and amplifying *mecA* and the elements characteristic of SCC*mec* type IV. *mecA* and SCC*mec* PCR were negative for the 151-kb band.

The *spa* types of the MSSA isolates (*spa* 88, *spa* 105, *spa* 548, *spa* 570, and *spa* 572) were very similar to the *spa* type 2 of the MRSA isolates, diverging by only one or three repeats.

The two *tst*-positive *agr3* MRSA isolates shared characteristics with 25 of the 70 *tst*-positive *agr3* MSSA isolates; their PFGE patterns differed by a single band containing *mecA* in MRSA isolates. The *spa* types of two *tst*-positive *agr3* MSSA isolates (*spa* 12 and *spa* 17) were closely related to *spa* types 638 and 584 of the *tst*-positive *agr3* MRSA strain.

DISCUSSION

A passive survey of *S. aureus* infections in France during 2002 and 2003 identified *tst*-positive MRSA clones. We identified a major ST5 *agr2* clone which included 25 of the 27 *tst*-positive MRSA isolates, and a minor ST30 *agr3* clone accounted for the remaining two isolates. Both clones mainly caused hospital-acquired infections (12 cases), but the ST5 *agr2* clone was also sometimes acquired in the community (8 cases). These infections mainly affected children (overall median age, 3 years) and corresponded to both TSS and suppurative infections. The clones were widely disseminated throughout France, as *tst*-positive MRSA isolates were recovered from 12 French towns between 2002 and 2003. In our database, this clone was also detected in 2000 (one case) and in 2001 (two cases); these three cases were hospital acquired. The ST5 *agr2* clone was also detected in Switzerland.

The two French *tst*-positive MRSA clones had similar antibiotic resistance profiles: they were usually resistant to oxacillin, kanamycin, and tobramycin and had intermediate resistance to fusidic acid, while resistance to erythromycin was more variable. This antibiotic resistance profile is uncommon among French hospital MRSA isolates. Intermediate fusidic acid resistance is rare in French hospital MRSA clones, which are usually susceptible to fusidic acid and resistant to quinolones (39). It is noteworthy that the antibiotic resistance profiles of the two French *tst*-positive MRSA clones are very similar to that of the major community-acquired ST80 MRSA clone harboring the PVL genes, which is currently spreading throughout Europe (37). For instance, the ST80 clone is also resistant to oxacillin and kanamycin and has intermediate resistance to fusidic acid, whereas it is susceptible to tobramycin and resistant to tetracycline. These differences in antibiotic resistance profiles may help to identify the PVL-positive clone ST80 and the *tst*-positive clones ST5 and ST30 in the clinical setting. It is surprising that these emerging clones, which are either *tst* or PVL positive, share certain genetic determinants encoding resistance to antibiotics despite their very different genetic backgrounds. This may reflect a peculiar pattern of antibiotic usage in France, notably in the community.

Two categories of MRSA had previously been recognized in France. The first comprises hospital strains (H-MRSA) that can potentially spread into the community, giving rise to infections in patients with risk factors such as recent hospitalization or surgery, chronic underlying diseases, immunosuppression, or intravenous drug use. The second category corresponds to MRSA strains arising de novo in the community (C-MRSA), which infect patients with no established risk factors. H-MRSA infections differ from C-MRSA infections in their epidemiological, clinical, and microbiological characteristics: C-MRSA infects younger subjects and mainly causes skin infections, whereas H-MRSA is associated with a wider range of infections (urinary tract, respiratory tract, skin, etc.). C-MRSA usually harbors the PVL genes, which are associated with skin and soft tissue infections (37), and occasionally the exfoliative toxin genes (17). The epidemiology of the *tst*-positive MRSA clones is atypical. Like C-MRSA, *tst*-positive MRSA generally infects children in the community, but 12 of our cases were strictly hospital acquired. However, it is not known whether the patients with "hospital-acquired" infections were nasal carriers of *tst*-positive MRSA or whether they actually acquired the strain in the hospital. None of the hospital-acquired *tst*-positive MRSA infections was associated with hospital outbreaks or with documented horizontal transmission. The known prevalence of H-MRSA in French pediatric units is low (9.8% in our hospital in Lyon [J. Etienne, personal communication]), as is the overall prevalence of *tst*-positive MRSA in France (27 isolates from 12 different hospitals in a 2-year period). This suggests that these *tst*-positive MRSA strains are being imported into hospitals from the community. *tst*-positive MRSA strains appear to be highly virulent and to cause a variety of illnesses, ranging from toxic shock syndrome to various suppurative infections.

The two *tst*-positive MRSA clones, with *agr2* or *agr3* genetic backgrounds, seem to be clonally related to their respective *agr2* or *agr3* *tst*-positive MSSA counterparts. A single PFGE band difference, corresponding to an SCC*mec* IV element, distinguished the MRSA isolates from the MSSA isolates. It is unclear whether insertion of a *mecA* element can occur in such MSSA strains. The *tst*-positive *agr2* MSSA clone has rarely been detected in France (only 5 isolates in our collection), contrary to the *tst*-positive *agr3* MSSA clone (70 isolates in our collection). It is surprising that the major *tst*-positive MRSA clone (*agr2*) should have emerged from an infrequently detected *tst*-positive MSSA background. We compared our *tst*-positive *agr2* MRSA clone with the well-described New York/Japan and Pediatric MRSA clones that have spread worldwide. Our *tst*-positive *agr2* MRSA clone has the same genetic background as the New York/Japan clone. Even if SCC*mec* acquisition by MSSA clones was four times more common than the replacement of one SCC*mec* by another, we cannot exclude the possibility that our clone arose from the New York/Japan clone through SCC*mec* II substitution by SCC*mec* IV (28). Further phylogenetic studies are needed to determine the precise origin of our clone, and these studies may help to identify factors that tend to promote the spread of *tst*-positive *agr2* MRSA rather than *tst*-positive *agr3* MRSA.

Most emerging C-MRSA isolates with heightened virulence have been found to harbor the PVL genes and, less frequently, exfoliative toxin genes (17). The emergence and spread of virulent C-MRSA isolates harboring the *tst* gene is of major concern, as they appear to share certain characteristics with PVL-positive C-MRSA, including a predilection for children. Prospective studies are needed to determine the incidence of infections due to these different clones, in order to bolster measures aimed at limiting the spread of C-MRSA.

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TODAY'S THERAPY

2006 今日の治療指針

私はこう治療している

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北原　光夫
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山脇　成人

〈五十音順〉

医学書院

■患者説明のポイント
・重症で後遺症がある，細菌感染症の中では最も重篤な疾患の1つで，中枢神経系の後遺症がありうることを説明する．
・治療薬の副作用がある．抗菌薬の量が多く，投与期間が長いので，薬剤アレルギーを起こす可能性があり，原疾患の経過が良好でも注意深い観察が必要である．
・繰り返すことがある．繰り返し発症する場合には髄液漏などの基礎疾患を検討する必要がある．

■5類感染症 - 定点把握

マイコプラズマ感染症
Mycoplasmal Infection

成田光生　JR札幌鉄道病院・小児科医長

病態と診断
肺炎マイコプラズマ（*Mycoplasma pneumoniae*）は遺伝子量が通常細菌の1/5程度の小型細菌である．細菌壁をもたないため，壁合成阻害薬は無効である．またそれ自体には強力な細胞傷害性はなく，肺炎の発症には宿主の免疫応答が強く関与している．飛沫にて感染するため，院内感染対策は飛沫感染症に対する標準的予防対策で十分である．マクロライド耐性菌が増加しつつあり，平成16年までの状況ではマイコプラズマ野生株の15%がマクロライド耐性である．長く続く，乾いた咳が本感染症を疑わせるが，とりわけ特徴的な身体所見，X線所見はない．PCR法による迅速診断も普及しつつあるが，確定診断はペア血清を用いた抗体価測定によらざるを得ない．

治療方針
通常のマイコプラズマ肺炎には年齢を問わずマクロライド薬が第1選択であるが，14員環マクロライドは薬物相互作用を起こす薬剤（テオフィリンなど）が多いので注意を要する．経口不可の場合にはクリンダマイシンあるいはミノサイクリンを静注する．呼吸窮迫症候群など劇症化例では抗菌薬使用のもとにステロイド薬の併用を考慮すべき場合もある．マクロライド耐性菌に対してはテトラサイクリン系あるいは一部のニューキノロン系薬剤が有効である．

A. 成人

℞処方例　下記のいずれかを用いる
1) クラリス錠（200 mg）　2錠　分2　7日間
2) ジスロマック錠（250 mg）　2錠　分1　3日間
3) ミノマイシン注（100 mg）　1回100 mg　1日2回　点滴静注

B. 小児，体重20kg

℞処方例　下記のいずれかを用いる
1) クラリス錠（50 mg）　4錠　分2　7日間
2) ジスロマック細粒（100 mg/g）　200 mg（成分量として）　分1　3日間
3) ダラシンS注　1回100 mg　1日3回　点滴静注

C. マクロライド耐性菌による感染が疑われる場合

℞処方例　下記のいずれかを用いる
1) クラビット錠（100 mg）　3錠　分3　7日間
2) シプロキサン注　1回300 mg　1日2回　点滴静注

■5類感染症 - 定点把握

MRSA感染症
Methicillin - Resistant *Staphylococcus aureus* Infection

二木芳人　川崎医科大学講師・呼吸器内科

病態と診断
MRSA（メチシリン耐性黄色ブドウ球菌）は，さまざまなリスクファクターを有する患者に多彩な感染症を生ずるが，通常の黄色ブドウ球菌同様，ヒトの皮膚，粘膜，腸内の常在菌的な存在でもある．したがって，その分離・同定が直ちにMRSA感染症を意味せず，いわゆる保菌や定着であることも少なくない．これらの状態では当然抗菌薬療法は不要である．感染症状や所見がある場合は，ほかの原因菌による感染症であることも多く，MRSAのみに絞った抗菌薬療法は，治療の失敗に結びつくことがある．他方，MRSAの保菌状態などでも，抗MRSA活性を有さない抗菌薬の頻用や長期使用は，菌交代症によるMRSA感染症を招くこととなりかねない．すなわち，MRSA感染症の治療においては，患者状態を理解し，さらに適切な感染症と原因菌の把握のうえで行うことが重要である．なお，近年では院内感染のみならず，市中感染でもMRSAの分離頻度が増加傾向にある．

治療方針
MRSA感染症は種々のリスクファクターを有する患者に発症しやすいので，治療が必要と考えられれば，以下の抗MRSA薬を積極的かつ効果的に用いるようにする．静注薬はいずれも血中濃度モニタリング（TDM：therapeutic drug monitoring）が可能なので活用し，有効かつ安全な治療を心がける．

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図　説

呼吸器系細菌感染症：疫学、診断、治療

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序　文

本書は，新興・再興感染症研究事業「百日咳菌，ジフテリア菌，マイコプラズマ等の臨床分離菌の収集と分子疫学的解析に関する研究」班（平成15～17年度）における研究成果をベースに，更に国立感染症研究所細菌第一部並びに第二部で取り扱っております呼吸器系細菌について，それらの感染機構，疫学，診断，治療法等につきまして写真，図表を中心に，図説版として纏めたものです．

厚生労働科学研究費補助金事業とは，厚生労働科学研究の振興を促し，もって，国民の保健医療，福祉，生活衛生，労働安全衛生等に関し，行政施策の科学的な推進を確保し，技術水準の向上を図ることを目的としており，現在３４事業を有しています．新興・再興感染症の予防，診断，治療の向上その他新興・再興感染症対策の推進に資することを目的とする「新興・再興感染症研究事業」もその一つです．

本書では，「感染症の予防及び感染症の患者に対する医療に関する法律（ 感染症法 ）」に指定する細菌による呼吸器系感染症の起炎菌として，２類（ジフテリア菌），新４類（レジオネラ菌），新５類（肺炎マイコプラズマ，インフルエンザ菌，肺炎クラミジア，肺炎球菌，A群溶血性連鎖球菌，モラキセラ・カタラリス，百日咳菌，髄膜炎菌）及び結核予防法で指定する結核菌について取り上げました．これらの病原体による呼吸器系疾患は，咽頭炎，上気道炎，気管支炎，肺炎など多様であり，またこれらの病原体における薬剤耐性の獲得も深刻で，耐性菌の出現と蔓延に伴い，肺炎や敗血症，髄膜炎に発展すると予後が極めて悪くなり，国内外で問題となっております．これら社会的に関心の高い病原体につきまして，日頃，各病原体を取り扱っております専門家にその感染機構，疫学，診断，治療法等をできるだけ分かりやすくまとめていただきました．

執筆者はいずれもそれぞれの病原体における第一線の研究者であり，最新の疫学データ及び知見をベースに良くまとめられております．

ご執筆いただきました先生方に感謝するとともに本書が臨床の先生方，研究者，医学生並びに臨床検査に係わる諸兄に活用していただければ幸いです．尚，本書に示しました診断・治療法は現在の技術・知識におけるその一端を紹介しておりますが，実際の医療現場における責任を負うものではありません．

2006年1月15日
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付属資料

病原体検出情報システム

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病原体の入手方法

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Multiple Promoter Inversions Generate Surface Antigenic Variation in *Mycoplasma penetrans*

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***Mycoplasma penetrans* is a newly identified species of the genus *Mycoplasma*. It was first isolated from a urine sample from a human immunodeficiency virus (HIV)-infected patient. *M. penetrans* changes its surface antigen profile with high frequency. The changes originate from ON↔OFF phase variations of the P35 family of surface membrane lipoproteins. The P35 family lipoproteins are major antigens recognized by the human immune system during *M. penetrans* infection and are encoded by the *mpl* genes. Phase variations of P35 family lipoproteins occur at the transcriptional level of *mpl* genes; however, the precise genetic mechanisms are unknown. In this study, the molecular mechanisms of surface antigen profile change in *M. penetrans* were investigated. The focus was on the 46-kDa protein that is present in *M. penetrans* strain HF-2 but not in the type strain, GTU. The 46-kDa protein was the product of a previously reported *mpl* gene, pepIMP13, with an amino-terminal sequence identical to that of the P35 family lipoproteins. Nucleotide sequencing analysis of the pepIMP13 gene region revealed that the promoter-containing 135-bp DNA of this gene had the structure of an invertible element that functioned as a switch for gene expression. In addition, all of the *mpl* genes of *M. penetrans* HF-2 were identified using the whole-genome sequence data that has recently become available for this bacterium. There are at least 38 *mpl* genes in the *M. penetrans* HF-2 genome. Interestingly, most of these *mpl* genes possess invertible promoter-like sequences, similar to those of the pepIMP13 gene promoter. A model for the generation of surface antigenic variation by multiple promoter inversions is proposed.**

Mycoplasmas are bacteria with no cell wall and the minimum range of genome sizes necessary for self-replication. They lack most of the genes required for nutrient metabolism and adopt a parasitic lifestyle in host organisms. Over 100 mycoplasma species have been isolated from a wide range of host organisms. Several of these species are well recognized as pathogens (29, 30). As parasitic bacteria, mycoplasmas can continue to colonize the host even in the presence of a specific immune response. This property of mycoplasmas may explain the slowly progressive chronic manifestations of mycoplasma-associated diseases. The mechanisms for evasion of host immune responses in mycoplasmas are poorly understood. However, a number of recent studies have demonstrated that many mycoplasma species can modify their surface antigenic molecules with high frequency (31, 32), which might play a key role in circumventing the host immune system. The rapid change of surface antigenic molecules may generate phenotypic heterogeneity in the propagating mycoplasma population and provide advantages not only for evasion of host immune responses but also for other aspects of mycoplasma survival, such as adaptation to environmental changes.

Most of the variable surface antigenic molecules of mycoplasmas are lipoproteins (5, 45). These lipoproteins, depending upon the species, are encoded by single or multiple genes and undergo frequent phase and size variation during mycoplasma growth (31, 32, 46). A variety of genetic mechanisms are used to modulate the expression of these lipoprotein genes, including DNA rearrangements, nucleotide insertions and deletions, gene conversions, and site-specific recombination (3, 7, 12, 26, 37). The characterization of these mechanisms may provide a detailed understanding not only of mycoplasma antigenic variation but also of bacterial gene regulation systems.

Mycoplasma penetrans is a newly identified species of mycoplasma that infects humans. It was first isolated from a urine sample from a human immunodeficiency virus (HIV)-infected patient (21). Epidemiological studies have demonstrated that *M. penetrans* detection is mainly associated with HIV infection (14, 42, 43); however, *M. penetrans* has also been isolated from a patient with a case of primary antiphospholipid syndrome without HIV infection, suggesting that *M. penetrans* may be pathogenic for humans without HIV (47). The morphology of this mycoplasma is that of an elongated flask with a tip-like structure at one pole of the cell (10, 20).

M. penetrans also has the ability to change its surface antigenicity (24, 33). The surface-exposed lipid-associated membrane proteins (LAMPs) of *M. penetrans* frequently change their profiles. The most abundant LAMP is the P35 lipoprotein, a major antigen recognized by the human immune system during *M. penetrans* infection (25). P35 undergoes high-frequency ON↔OFF phase variation, causing the change of LAMP profile (24). In addition to P35, LAMPs contain a considerable number of the P35 family lipoproteins that are encoded by the *mpl* genes (for *M. penetrans* lipoprotein). These lipoproteins also independently undergo ON↔OFF phase variation (24, 33). Although the phase variation of the P35 family lipoproteins seems to occur at the transcriptional level of *mpl* genes, the precise mechanism remains unclear.

In this study, we investigated the molecular mechanisms of LAMP profile change in *M. penetrans*, focusing on the anti-

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genic variation of the 46-kDa protein, which is present in the *M. penetrans* isolate HF-2 but not in the GTU type strain. We established that the 46-kDa protein is the product of the previously reported *mpl* gene pepIMP13 and demonstrated that the antigenic variation was caused by promoter inversion.

Furthermore, the whole genome sequence of *M. penetrans* strain HF-2 has been determined recently. This information has enabled us to identify all of the *mpl* genes and their localization in the genome. The *M. penetrans* HF-2 genome contains at least 38 *mpl* genes, and most of them possess independent invertible promoter-like sequences. We propose a novel system for generating antigenic variations by multiple promoter inversions.

MATERIALS AND METHODS

Mycoplasma strains and culture conditions. *M. penetrans* GTU-54 was the original isolate from a urine sample of an HIV-infected patient (21) and was kindly provided by S.-C. Lo (Armed Forces Institute of Pathology, Bethesda, Md.). *M. penetrans* HF-2 was isolated from an HIV-negative patient with primary antiphospholipid syndrome (47) and was kindly provided by L. Cedillo and A. Yáñez (Centro de Investigación Biomédica de oriente-IMSS and Benemérita Universidad Autónoma de Puebla, Puebla City, Mexico). *M. penetrans* strains were cultured in PPLO medium (2.1% PPLO broth [Difco Laboratories, Detroit, Mich.], 0.25% glucose, 0.002% phenol red, 5% yeast extract [Difco Laboratories], 10% horse serum [Gibco BRL, Rockville, Md.], 50 μg of ampicillin per ml) at 37°C.

Antibodies. A murine monoclonal antibody (MAb) specific to P35 (MAb 7) was established by T. Sasaki and was previously shown to react specifically with the P35 protein (24, 33). MAb 7 was used in this study at a 1:5,000 dilution for immunoblot analysis. Serum 6 (anti-HF-2) was produced by immunization of mice with total cell lysate of *M. penetrans* HF-2 and was used at a 1:500 dilution for immunoblot analysis.

Protein analysis. *M. penetrans* proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under the conditions described by Laemmli (19). In most cases, 12% gels were used. Fractionation of LAMPs was done by the Triton X-114 (TX-114) phase-partitioning method (9). Membrane and cytosolic protein fractionation and TX-100 partitioning were also performed using published methods (28). For immunoblot analysis, proteins were transferred to nitrocellulose membranes after electrophoresis (38) and were detected by antibodies.

Peptide sequencing of the 46-kDa protein. The 46-kDa protein was extracted from strain HF-2 by TX-114 phase partitioning and was purified by SDS-PAGE. The 46-kDa protein band was excised from the gel and treated with *Staphylococcus aureus* V8 protease (Sigma-Aldrich, Steinheim, Germany). The digested peptide fragments were separated using SDS–15% PAGE gels, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.), and stained with Coomassie blue. Two major peptide fragment bands (approximately 15 and 8 kDa) were excised from the membrane, and the N-terminal sequences were analyzed by Edman degradation. Peptide sequencing was performed at APRO Life Science Institute (Tokushima, Japan) using the Procise 494 HT protein-sequencing system (Applied Biosystems, Foster City, Calif.).

DNA-sequencing analysis of pepIMP13 gene region of strain GTU. Genomic DNA was isolated from a 5-ml culture of *M. penetrans* strain GTU (QIAmp DNA Mini Kit; Qiagen, Hilden, Germany) and was used as a template for PCR amplification. The pepIMP13 gene region was amplified by PCR with the oligonucleotide primers IMP13-CF (GCAACTGCAGATGGCAACAA) and IMP13-CR (ATGGCACCGCCTGATAACAT) using a high-fidelity DNA polymerase, Pyrobest (Takara, Tokyo, Japan). The amplified fragments were ligated into the *Sma*I site of the pUC19 plasmid. Sequencing of cloned PCR fragments was performed by a primer-walking method with the Big Dye terminator cycle-sequencing kit and the DNA sequencer PRISM 310 (Applied Biosystems). To avoid the artificial factor of mutations generated in the course of PCR, three independent plasmid clones were sequenced, and the data were integrated.

RNA isolation and slot blot analysis. Total cellular RNA was isolated from mid-logarithmic-phase cultures of both *M. penetrans* GTU and HF-2 (RNeasy Mini Kit; Qiagen). Twenty micrograms of total RNA were treated with 10 U of RNase-free DNase (Takara) for 1 h at 37°C. One microgram of RNA was diluted in RNase-free denaturation solution containing formamide (66%), formaldehyde (8%), and MOPS (morpholinepropanesulfonic acid) buffer and heated at 65°C for 5 min. The RNAs were blotted onto a GeneScreen Plus membrane (NEN, Boston, Mass.) by vacuum, using the Convertible Filtration Manifold System (Invitrogen, Carlsbad, Calif.). The oligonucleotide probes P35-P (CCC TTAATTGCAGCAGAATCACC) for the *p35* gene transcript and P42-P (TTA AATCTGTTTCAGCTGTAATTT) for the *p42* gene transcript were enzymatically labeled with digoxigenin (DIG)-labeled ddUTP by using the DIG oligonucleotide 3′-end labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The blots were hybridized with the probes at 37°C for 16 h in DIG Easy Hyb solution (Roche Diagnostics). After incubation, the hybridization signals were detected with the DIG luminescence detection kit (Roche Diagnostics) and visualized by exposure to medical X-ray film (Fuji Film, Tokyo, Japan).

Primer extension analysis. Two oligonucleotide primers, P42-EXT3 (GCAA CAATCCCAAAAGCT) and P42-EXT4 (TCCATTTCCATTATTGTTAT), were used for primer extension analysis to identify the 5′ end of the *p42* gene transcript. The primer extension reaction was performed using the primer extension system-avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wis.). Briefly, the primers were end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Ten micrograms of total RNA prepared from *M. penetrans* HF-2 was mixed with each of the labeled primers in the AMV primer extension buffer and heated at 58°C for 20 min. The mixtures were cooled at room temperature for 10 min, and the AMV reverse transcriptase mix solution, containing 1 U of enzyme, was added. The extension reaction mixtures were incubated at 42°C for 30 min, 20 μl of loading dye was added, and the extension was terminated by heat inactivation at 90°C for 10 min.

The *p42* gene region was cloned from *M. penetrans* HF-2 genomic DNA by PCR with primers IMP13-CF and IMP13-CR. The amplified *p42* gene fragment was purified with the QIAquick PCR purification kit (Qiagen) for use as a template for DNA-sequencing reactions. The same primers that were used for primer extension were also used for sequencing. The *fmol* DNA-sequencing system (Promega) was used for DNA-sequencing reactions. The products of primer extension and sequencing reactions were analyzed by electrophoresis, using a 5% polyacrylamide sequencing gel. After electrophoresis, the gel was dried and exposed to X-ray film overnight to visualize the products.

Nucleotide sequence accession numbers. The complete genome sequence of *M. penetrans* strain HF-2 was recently determined by our group in collaboration with Kitasato University. The detail of the genome sequence analysis will be described in another paper (33a). The whole-genome sequence data for *M. penetrans* HF-2 will appear in the DDBJ, EMBL, and GenBank databases under accession numbers AP004170, AP004171, AP004172, AP004173, and AP004174. The nucleotide sequence data for the *p42* gene region of *M. penetrans* GTU will also appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB084070.

RESULTS

Comparison of protein profiles between *M. penetrans* strains. The protein electrophoresis patterns of the *M. penetrans* type strain, GTU, and isolate HF-2 were compared to identify molecular polymorphisms between *M. penetrans* strains (Fig. 1A). The major surface lipoprotein P35 of *M. penetrans* was present in both strains. However, a 34-kDa protein was present only in GTU, and a 46-kDa protein was found only in HF-2. The electrophoresis patterns were reproducible when the protein samples were prepared from cultures of other single colonies of each strain. Immunoblotting analysis gave a similar result (Fig. 1B and C). MAb 7 detected the P35 protein in both strains (Fig. 1B), while serum 6 (anti-HF-2) reacted with many proteins of both strains (Fig. 1C). Although the strongest reaction was observed against the P35 protein, serum 6 clearly detected a 46-kDa protein only in HF-2.

Characterization of 34- and 46-kDa proteins. We thought that the 34- and 46-kDa proteins were variable surface lipoproteins of the P35 family (products of *mpl* genes) because of their different expression patterns among *M. penetrans* strains. To confirm this, we characterized the properties of the 34- and 46-kDa proteins. First, we fractionated the proteins of *M. penetrans* strains GTU and HF-2 by a TX-114 extraction proce-

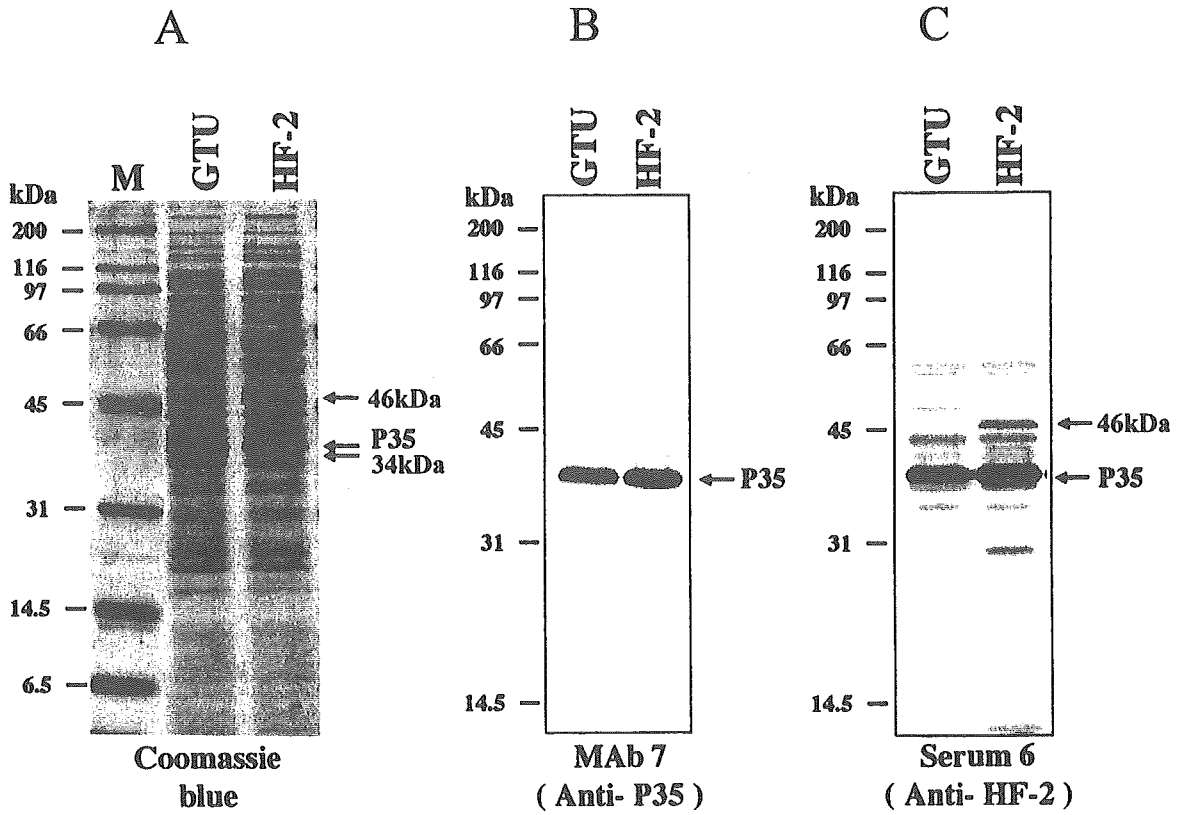

FIG. 1. Analysis of protein profiles of *M. penetrans* strains GTU and HF-2. (A) Coomassie blue-stained SDS–12% PAGE of total proteins of *M. penetrans*. A protein molecular mass marker (Bio-Rad, Hercules, Calif.) is in lane M, and the masses are shown on the left. The P35 and 34- and 46-kDa proteins are indicated. (B) Immunoblot analysis of *M. penetrans* proteins. Anti-P35 MAb (MAb 7) was used for detection. The P35 protein is indicated. (C) Immunoblot analysis with the polyclonal serum 6 (anti-HF-2). The positions of the P35 and 46-kDa proteins are indicated.

dure that is used to separate LAMPs of *M. penetrans* (9, 33). The 34- and 46-kDa proteins were found in the TX-114 detergent phase together with the P35 protein (Fig. 2). Differences between the TX-114 extraction profiles of strains GTU and HF-2, in addition to the 34- and 46-kDa proteins, were also observed (Fig. 2). The GTU strain had at least two distinct bands, between 36 and 40 kDa, that were missing in the HF-2 profiles. One of these bands might be the P38 lipoprotein previously reported to be present in strain GTU (24, 33). We then analyzed the GTU and HF-2 proteins by membrane and cytosolic separation or by the TX-100 extraction method (28). In these analyses, the 34- and 46-kDa proteins were separated into membrane or TX-100-soluble fractions, along with the P35 protein (data not shown). These results suggested that the 34- and 46-kDa proteins are hydrophobic proteins with properties similar to those of the P35 lipoprotein.

The 46-kDa protein was chosen for amino acid-sequencing analysis. Initial attempts to analyze the intact 46-kDa protein by Edman degradation were unsuccessful, probably because of N-terminal blockage. Therefore, the 46-kDa protein was digested with protease V8 and peptide fragments of ~15 and 8 kDa were sequenced by Edman degradation. The sequence LNDKVSLAGS was obtained from the N terminus of the 15-kDa fragment. Two sequences, TDLKITVDGG and FNFNIG IDST, were obtained from the analysis of the 8-kDa fragment, suggesting that it was a mixture of two peptides. These partial amino acid sequences of the 46-kDa protein matched the de-

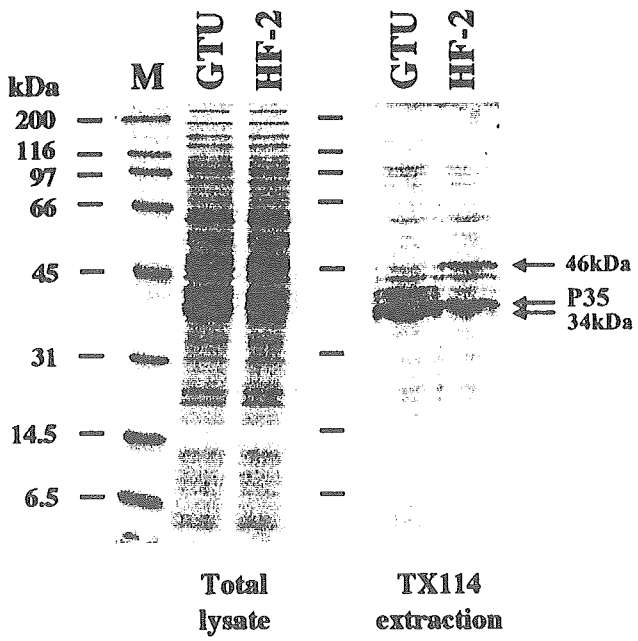

FIG. 2. Analysis of LAMP profiles of *M. penetrans*. The total cell lysate and TX-114 phase-fractionated proteins of *M. penetrans* strains GTU and HF-2 were analyzed by SDS–12% PAGE. The proteins were stained with Coomassie blue. The positions of the P35 and 34- and 46-kDa proteins are indicated. A protein molecular mass marker is in lane M, and molecular masses are on the left.

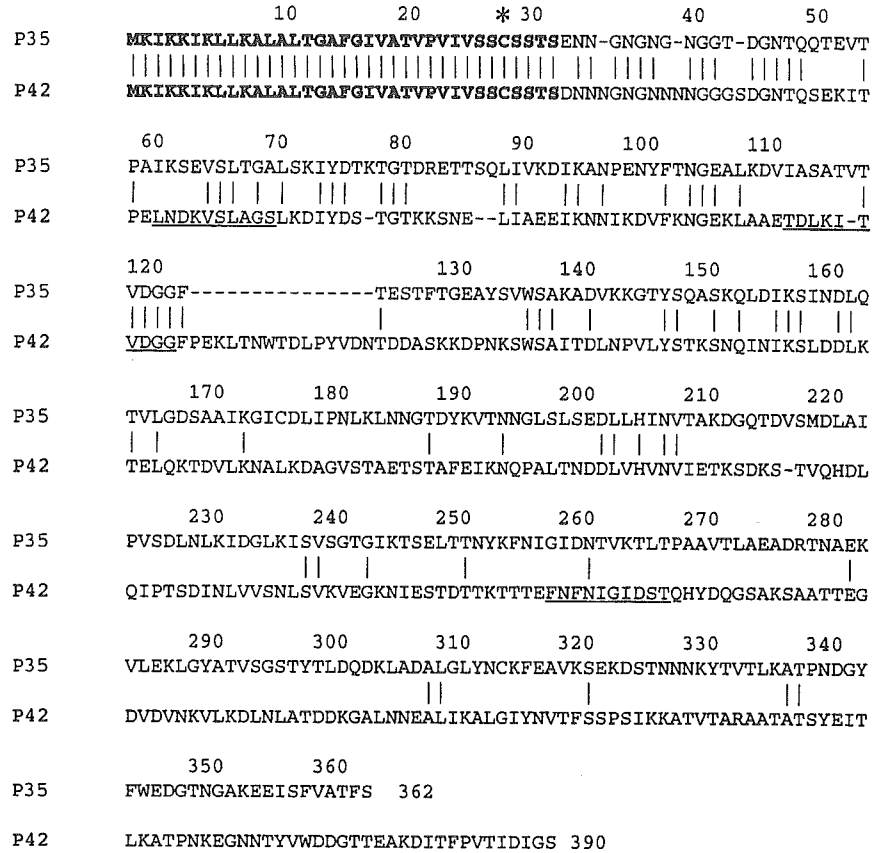

FIG. 3. Alignment of amino acid sequences of P35 and 46-kDa (pepIMP13) proteins. The 46-kDa protein was designated P42 based on the calculated molecular mass. The three partial amino acid sequences of P42 determined by Edman degradation of digested peptide are underlined. The signal sequence of the P35 family lipoprotein is shown in boldface characters. The cysteine residue marked with an asterisk is the potential binding site of fatty acid chains.

duced amino acid sequence of a previously reported putative *mpl* gene, pepIMP13 (24). The previously reported pepIMP13 sequence is derived from strain GTU (GenBank accession no. AJ006698) and is truncated at the N terminus. To obtain the full-length pepIMP13 gene sequence, the complete genome sequence of strain HF-2 (see Materials and Methods) was searched. The pepIMP13 gene was found in the *M. penetrans* HF-2 genome as an open reading frame (ORF), designated MYPE6630 in accordance with the nomenclature system of the genome project. The ORF MYPE6630 was located at nucleotide positions 850970 to 852142 of the genome. The amino acid sequence of the 46-kDa protein was deduced from the MYPE6630 sequence and was compared to that of the P35 protein (Fig. 3). The full-length 46-kDa protein (the pepIMP13 protein) consists of 390 amino acids (aa) and possesses an N-terminal sequence identical to that of the P35 protein. This 30-aa N-terminal sequence is thought to be the signal peptide of the P35 family lipoproteins (24). The calculated molecular mass of the 46-kDa protein was 41,814 Da, somewhat smaller than that estimated by SDS-PAGE. We designated the 46-kDa protein P42, based on the calculated molecular mass.

Analysis of the nucleotide sequence of the *p42* gene and flanking region. To investigate the mechanism that underlies the antigenic variation of the 46-kDa protein (P42), we analyzed the nucleotide sequences of the *p42* gene of strains HF-2 and GTU. The nucleotide sequence containing the *p42* gene and flanking regions of HF-2 was obtained from complete genome sequence data. Using this sequence, we designed PCR primers to amplify the corresponding region from strain GTU. The PCR-cloned *p42* gene region from GTU was sequenced by the primer-walking method and was compared to the sequence from HF-2. There were no differences in either the *p42* structural gene itself or the downstream region between the two strains. However, sequence differences were found in the upstream region of the *p42* gene (Fig. 4). The difference began 238 bp upstream from the ATG start codon of the *p42* gene and was 135 bp in length (Fig. 4A). It was found that the 135-bp DNA sequence was inverted between the GTU and HF-2 strains and was flanked by 12-bp inverted-repeat sequences (Fig. 4A), suggesting that the inversion might be produced by site-specific recombination between 12-bp inverted-repeat sequences. We hypothesized that this inversion was the cause of the antigenic variation of the P42 protein and would affect the structure of promoter or other regulatory sequences. As expected, inspection of the 135-bp inverted DNA sequences revealed the presence of a promoter-like sequence in this region. The promoter-like sequence was very similar to the experimentally characterized promoter of the *p35* gene (24). In the region containing a −10-like consensus sequence and a +1 transcription start site, the *p42* promoter-like sequence and the *p35* promoter were identical (Fig. 4B). In strain HF-2, the

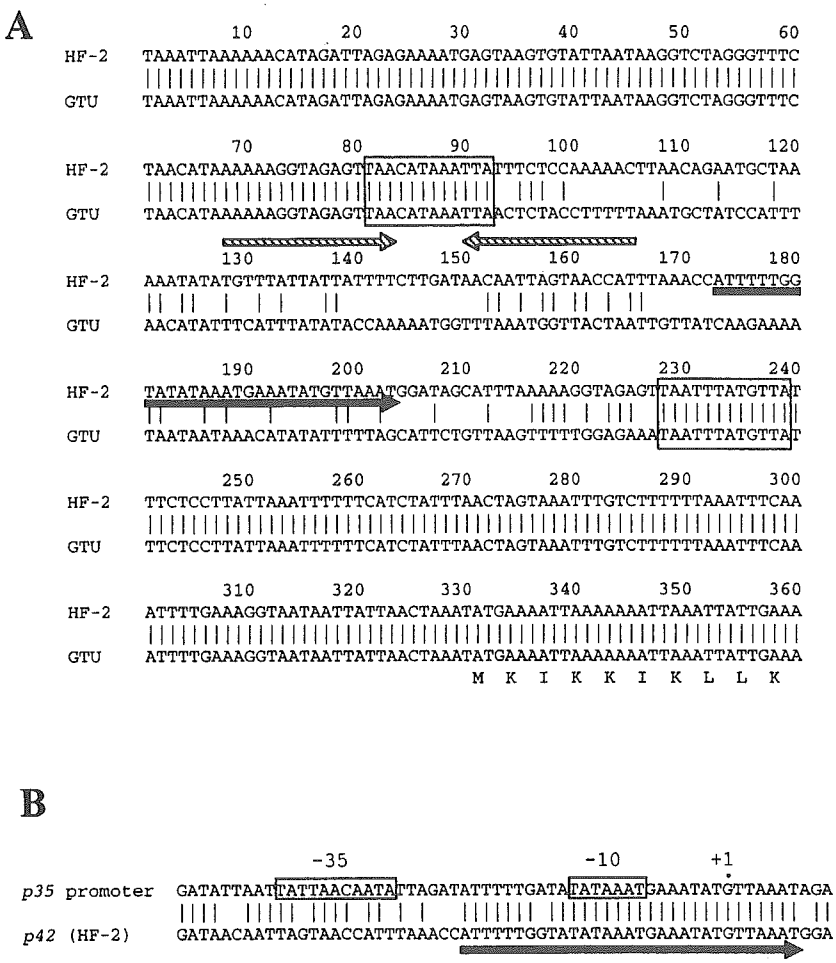

FIG. 4. Nucleotide sequence of *p42* gene upstream region. (A) Comparison of *p42* gene upstream regions from strains GTU and HF-2. The 12-bp inverted-repeat sequences adjacent to the 135-bp inverted DNA region (see the text) are boxed. The solid arrow indicates the core of the promoter sequence in the HF-2 sequence (panel B). The hatched arrows indicate a 16-bp inverted repeat in the GTU sequence. The deduced amino acid sequence of the P42 protein (the first 10 aa) is shown. The nucleotides are counted from 330 bp upstream from the ATG start codon. (B) Comparison of *p42* gene upstream sequence with promoter from *p35* gene. The −10 and − 35 consensus sequences of the *p35* promoter are boxed (24). The probable start site of *p35* transcription is also marked +1. The highly conserved region between the *p35* and *p42* promoter sequences is underlined with an arrow.

promoter-like sequence was oriented to transcribe the *p42* gene, but this orientation was inverted in strain GTU (Fig. 4A). The directions of the promoter-like sequence (toward the *p42* structural gene or not) were consistent with the expression patterns of the P42 protein in strains GTU and HF-2 (Fig. 1 and 2).

The existence of the 12-bp inverted repeat that flanked the promoter-like sequence raised the possibility that inversion of the promoter-like sequence would be reversible. Therefore, we analyzed the shotgun clones that were used in the whole-genome sequencing of strain HF-2 and found a clone that carried the *p42* promoter region in the inverted orientation that was identical to the promoter region found in strain GTU (data not shown). This finding strongly suggests that the *p42* promoter region is inverted in the strain HF-2 population during culture.

A characteristic 16-bp inverted repeat that partly overlapped one of the 12-bp inverted-repeat sequences adjacent to the 135-bp DNA was also found (Fig. 4A and 5A). This 16-bp inverted repeat seems to form a hairpin structure that resembles a terminator sequence (Fig. 5B). The calculated ΔG of this structure is −22.5 kcal/mol. The 16-bp inverted repeat can be formed only in the promoter orientation that is characteristic of GTU. In HF-2, DNA inversion between 12-bp inverted-repeat sequences disrupts the formation of the 16-bp inverted repeat and the hairpin structure (Fig. 5).

Transcriptional analysis of *p42* gene. To confirm whether the inversion of promoter-like sequence actually affects the expression of the *p42* gene, we analyzed the transcription of the *p42* gene. Total RNAs were isolated from strains GTU and HF-2 and analyzed by slot blot hybridization (Fig. 6). Using the oligonucleotide probe for the *p35* gene transcript, the hybridization signals from both strains were observed. In contrast, a hybridization signal was obtained only from HF-2 with the probe for the *p42* transcript. These results indicate that *p42* transcription occurs in HF-2 but not in GTU. To further characterize the *p42* promoter-like region, we performed primer extension analysis. Using the oligonucleotide primer P42-

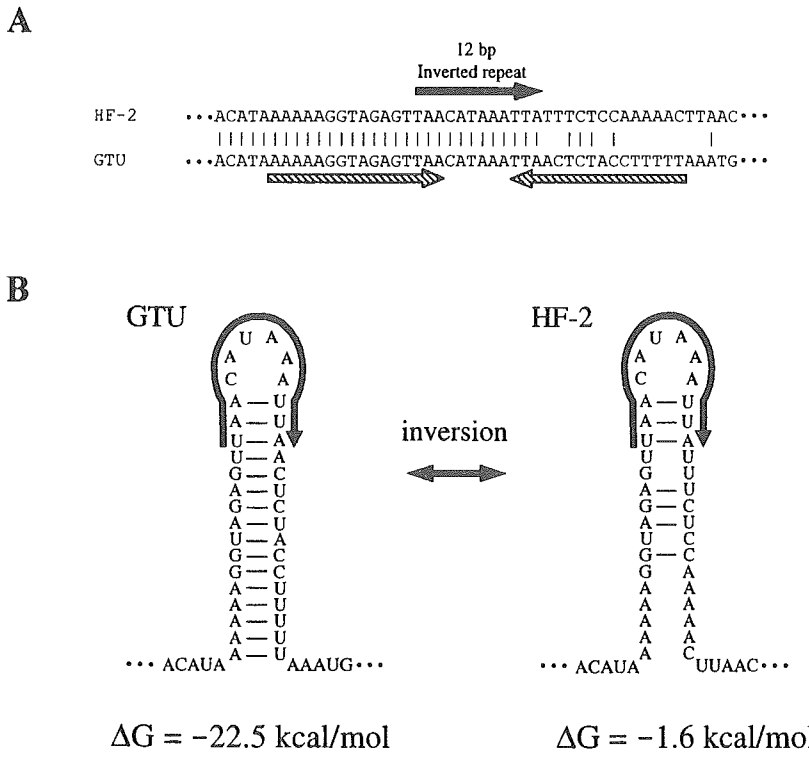

FIG. 5. Structure of 16-bp inverted-repeat sequence located upstream of *p42* gene. (A) Nucleotide sequences of 16-bp inverted-repeat regions from strains HF-2 and GTU. The nucleotide sequence corresponds to nucleotides 63 to 110 in Fig. 4A. The solid arrow indicates a 12-bp inverted-repeat sequence adjacent to a 135-bp inverted DNA sequence. The hatched arrows indicate the 16-bp inverted repeat that is formed only in the GTU sequence. (B) Hairpin structures of the 16-bp inverted repeat. Formation of the hairpin structure is disrupted in the HF-2 sequence by DNA inversion between 12-bp inverted-repeat sequences. The hairpin structures are shown as RNA sequences. The calculated Δ*G*s of these hairpin structures are shown at the bottom.

EXT3, the extension product was obtained with RNA from the HF-2 strain (Fig. 7A). The probable transcriptional start site was identified 137 bp upstream from the ATG start codon. This position corresponds to 3 bp upstream of the previously reported transcriptional start site of the *p35* gene (24) (Fig. 7B). The primer extension product was also obtained with the other oligonucleotide primer, P42-EXT4, and the same start site was identified (data not shown). These results indicated that the *p42* promoter-like sequence is an active promoter in *M. penetrans* cells.

Organization of *mpl* genes in *M. penetrans* HF-2 genome. It was demonstrated that the promoter inversion was the cause of antigenic variation of P42 protein. The involvement of a promoter inversion mechanism in *p42* gene expression raised the question of whether other *mpl* gene expression is regulated by a similar mechanism. To answer this question, we searched for all of the *mpl* genes in the whole-genome sequence of *M. penetrans* HF-2, using the *p35* gene sequence as a query. Homology search revealed that the *M. penetrans* HF-2 genome contains at least 38 *mpl* genes, including *p35* and *p42*. The deduced amino acid sequences of these *mpl* genes showed homology to that of the P35 protein (34 to 70% identity) and had almost identical *mpl* signal sequences in the N termini (data not shown). The signal sequences contain one cysteine residue (Fig. 3) that is thought to be the site which is modified with fatty acids, as are other known surface lipoproteins (5, 45). In contrast to the signal peptide sequences, the rest of the amino acid sequences of the *mpl* genes were of low homology. The homology search also detected six other genes (ORFs MYPE7020, -7030, -7040, -7050, -7060, and -7070) that were similar to the *p35* gene, with homology ranging from 28 to 44% identity (data not shown). However, the deduced amino acid

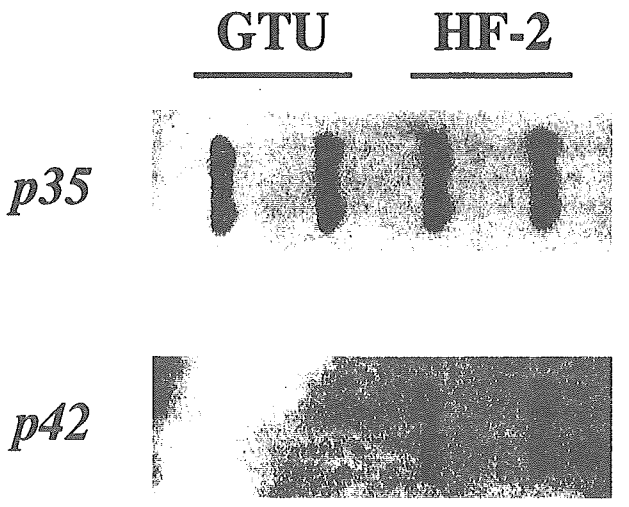

FIG. 6. RNA slot blot analysis of *p35* and *p42* gene transcription in *M. penetrans* strains GTU and HF-2. RNA samples from *M. penetrans* strains GTU and HF-2 were blotted in duplicate. The blots were hybridized with the oligonucleotide probes for *p35* and *p42* transcripts as indicated on the left.

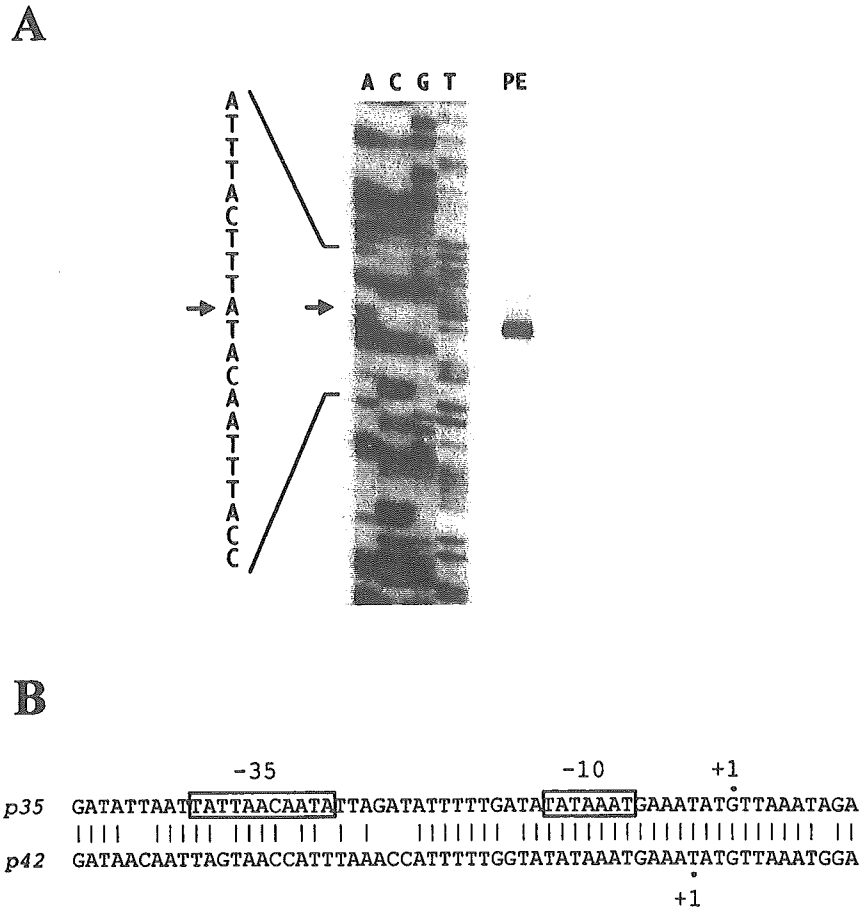

FIG. 7. Primer extension analysis of *p42* gene transcription. (A) Autoradiogram of sequencing gel used to analyze the primer extension product. PE indicates the primer extension product obtained with *M. penetrans* HF-2 RNA as a template. The corresponding nucleotide sequence is shown on the left, and the probable start site is indicated by arrows. (B) Comparison of the transcriptional start sites in *p35* and *p42* promoters. Probable start sites determined by primer extension of the *p35* promoter (24) and the *p42* promoter (panel A) are indicated as +1. The putative −10 and −35 consensus regions are boxed.

sequences of these six ORFs did not have the P35 signal peptide sequence in their N termini, so we did not include these genes among the members of the *mpl* gene family.

The 38 *mpl* genes clustered at three positions of the genome (Fig. 8). The largest cluster was located at nucleotide positions 830000 to 882000 of the genome (Fig. 8A). This 50-kb region contained 30 *mpl* genes and 6 non-*mpl* genes. The *p35* and *p42* genes were found in this cluster (MYPE6810 and -6630). The second cluster was found in a 20-kb DNA region at nucleotide positions 335500 to 355500 (Fig. 8B). In this region, two pairs of *mpl* genes are separated by five non-*mpl* genes. The last *mpl* gene cluster was located at nucleotide positions 966000 to 975000 (Fig. 8C). In this region, four *mpl* genes were found within 9 kb of DNA. In all three clusters, all the identified *mpl* genes were oriented in the same direction. The previously identified *mpl* genes, *p34A* (pepIMP14), *p30* (*p33*), and *p38* from strain GTU (24, 33), were also found in the largest cluster (Fig. 8). However, the previously reported pepIMP12 gene of GTU (24) (GenBank accession no. AJ006697) was not found in the HF-2 genome sequence. To determine whether the missing pepIMP12 gene in the HF-2 genome was the result of genomic polymorphism between strains GTU and HF-2, we used PCR to amplify the pepIMP12 gene sequence with primers IMP12-F (TAATATTAAATCTTTAGATG) and IMP12-R (AATTAAATGATAAAGTTAGC). Unexpectedly, the pepIMP12 sequence was not amplified from our GTU and HF-2 strains (data not shown). The reason is as yet unknown. It was also found that three *mpl* genes (MYPE6520, -6500, and -7380) possessed frameshift mutations in their sequences and were disrupted by internal stop codons. Of 14 non-*mpl* genes that exist in the three *mpl* gene clusters, MYPE6600, -6610, and -6620 showed relatively high homology to the transport system permease protein P69 (*Mycoplasma hyorhinis*), the ABC transporter ATP-binding protein (*Mycoplasma pulmonis*), and the high-affinity transport system protein P37 (*M. hyorhinis*), respectively. However, the other non-*mpl* genes did not show any significant homology to other known proteins (data not shown).

To investigate the mechanism involved in the expression of these *mpl* genes, we analyzed the intergenic sequences of these clusters. This showed that the intergenic sequences are well conserved and have structures similar to that of the *p42* promoter region, namely, 133- to 138-bp DNA sequences were flanked by 12- to 14-bp inverted-repeat sequences, suggesting that these regions are also invertible DNA (data not shown). Specifically, it was also noted that the 133- to 138-bp DNA