Identification of a site-specific tyrosine recombinase that mediates promoter inversions of phase-variable mpl lipoprotein genes in *Mycoplasma penetrans*

Atsuko Horino,1,2 Tsuyoshi Kenri,1 Yuko Sasaki,1 Noboru Okamura2 and Tsuguo Sasaki1†

1Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Musashimurayama, Tokyo 208-0011, Japan
2Laboratory of Microbiology and Immunology, Graduate School of Health Sciences, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

*Mycoplasma penetrans* has the ability to change its surface lipoprotein profiles frequently. The P35 family lipoproteins encoded by the *mpl* genes are key players in this profile variation. The *M. penetrans* HF-2 genome has 38 *mpl* genes that form three gene clusters. Most of these *mpl* genes have an invertible promoter sequence that is responsible for the ON/OFF switching of individual *mpl* gene expression. Here, we identified the recombinase that catalyses inversions of the *mpl* gene promoters. We focused on two open reading frames of the *M. penetrans* HF-2 genome, namely MYPE2900 and MYPE8180, which show significant homology to the tyrosine site-specific recombinase (Tsr) family proteins. Since genetic tools for *M. penetrans* are still not developed, we cloned the MYPE2900 and MYPE8180 genes and expressed them in *Mycoplasma pneumoniae* and *Escherichia coli*. The promoter regions of the *mpl* genes [p35 (MYPE6810) or p42 (MYPE6630) genes] were also introduced into *M. pneumoniae* and *E. coli* cells expressing MYPE2900 or MYPE8180. Inversion of these promoters occurred in the presence of the MYPE2900 gene but not in the presence of the MYPE8180 gene, indicating that the MYPE2900 gene product is the recombinase that catalyses *mpl* gene promoter inversions. We used a PCR-based method to detect *mpl* promoter inversion. This method also enabled us to detect inversions of 10 *mpl* gene promoters in *M. penetrans* HF-2 cells. All these promoter inversions occurred at the 12 bp inverted repeat (IR) sequences flanking the promoter sequence. The consensus sequence of these IRs was proposed as TAAYNNNDATTA (Y=C or T; D=A, G or T).

**INTRODUCTION**

Mycoplasmas belong to a group of bacteria with no cell wall and have the minimum range of genome sizes that are necessary for self-replication. Their small genomes (580–1350 kb) sequenced to date lack numerous genes required for biosynthetic pathways, thus reflecting their parasitic lifestyle with a dependence on host organisms for nutrient acquisition. Mycoplasmas usually inhabit the mucosal tissues of specific host organisms. Almost 200 mycoplasma species have been isolated from a wide range of host organisms, including humans. Several of these species are well recognized as pathogens (Sasaki, 2006; Waites *et al.*, 2005). As parasitic bacteria, mycoplasmas can continue to colonize their host even in the presence of specific immune responses. The molecular mechanisms responsible for this immune evasion are not fully understood; however, a number of recent studies have suggested probable mechanisms for continuous infection of mycoplasmas; these include molecular mimicry of host cell components (Jacobs *et al.*, 1995), modulation of host immunity by mycoplasmal cell components (Rottem, 2003), invasion of host cells (Baseman *et al.*, 1996) and generation of surface antigen variants of mycoplasmas (Denison *et al.*, 2005; Rosengarten *et al.*, 2000). Among these strategies, surface antigenic variation is a commonly observed phenomenon in many mycoplasma species (Citti *et al.*, 2005; Yogev *et al.*, 2002). Surface variations may play important roles in interaction between mycoplasmas and host cells during
infection. In most of the known cases, the variable surface molecules of mycoplasmas are lipoproteins. Depending upon the species, these lipoproteins are encoded by single or multiple genes and undergo frequent phase and size variation during mycoplasma growth (Citti et al., 2005). Various genetic mechanisms are used to modulate the expression of these lipoprotein genes. These mechanisms include DNA rearrangements (Glew et al., 2002; Shen et al., 2000), nucleotide insertions and deletions in the gene regions (Glew et al., 1998; Yogev et al., 1991), gene conversions (Noormohammadi et al., 2000), and site-specific DNA recombinations (Chopra-Dewasthaly et al., 2008; Lysnyansky et al., 2001). Characterization of these genetic mechanisms may provide us with not only a detailed understanding of the surface antigenic variation mechanism of mycoplasmas but also an insight into the survival strategy of minimalist bacteria through utilization of their small genomes.

*Mycoplasma penetrans* was first isolated from a urine sample of a human immunodeficiency virus (HIV)-infected patient (Lo et al., 1992). It was also isolated as a potential aetiological agent from a primary antiphospholipid syndrome patient without HIV infection (Yánez et al., 1999). Although the pathogenicity of *M. penetrans* to humans remains questionable, the characteristics of this bacterium (i.e. invasion of eukaryotic cells, toxicity to chick embryo, and haemolytic and haemoxidative activity) (Girón et al., 1996; Kannan & Baseman, 2000; Lo et al., 1993) suggest its potential pathogenicity to humans. *M. penetrans* also has the ability to change its surface antigenicity frequently by changing the expression pattern of the P35 family lipoproteins (Neyrolles et al., 1999; Röske et al., 2001). The P35 family lipoproteins are encoded by the *mpl* genes and, thus far, 38 *mpl* genes have been found in the *M. penetrans* HF-2 genome. Of these 38 genes, 30 form a large gene cluster in a 50 kb region of the genome (Horino et al., 2003; Sasaki et al., 2002). *M. penetrans* uses a unique mechanism to modulate the expression of these *mpl* genes. Most of the *mpl* genes have an independent promoter for their expression. These promoter sequences are present in approximately 135 bp DNA regions and are flanked by 12 bp inverted repeats (IRs). Inversion of these promoters causes ON/OFF switching of individual *mpl* genes. The invertible promoter elements also contain unique sequences that form a terminator-like structure depending on the direction of promoters (see Fig. 4a). This terminator-like structure may serve to prevent readthrough transcription from preceding genes or antisense transcription from OFF configuration promoters (Horino et al., 2003). Thus, the *mpl* genes have unique genetic switches, which possess promoter and terminator functions in the short DNA region. To further characterize these unique genetic switches and understand the nature of antigenic variation of *M. penetrans*, in this study we attempted to identify the factors involved in *mpl* gene promoter inversions. For this purpose, we reconstructed the *mpl* promoter inversion system in *Mycoplasma pneumoniae* and *Escherichia coli* cells.

### METHODS

**Bacterial strains and culture conditions.** *M. penetrans* strain HF-2 (Yánez et al., 1999) and *M. pneumoniae* strain M129 (Lipman et al., 1969) were cultured in PPLO medium at 37°C as described previously (Horino et al., 2003; Kenri et al., 2004). Transformation of *M. pneumoniae* M129 with the staphylococcal transposon Tn1000mod vectors was performed by the electroporation method (Hedreya et al., 1993). *M. pneumoniae* transformants were selected in PPLO liquid medium containing 18 μg gentamicin (Cm) ml⁻¹ or 15 μg chloramphenicol (Cm) ml⁻¹. Genomic DNAs of the mycoplasma strains were extracted by the QIAamp mini kit (Qiagen) and were used as templates for PCR to construct the plasmids. *E. coli* strains were grown in Luria–Bertani (LB) medium with or without 50 μg ml⁻¹ of ampicillin (Ap), kanamycin (Kn) or spectinomycin (Sp), or 15 μg Cm ml⁻¹ to select the plasmid markers.

**Cloning of the p42 and p35 promoter regions.** PCR primers used for cloning are listed in Supplementary Table S1 (available with the online version of this paper). An approximately 3.2 kb region containing the p42 *mpl* gene (MYPE6630) and its invertible promoter region (see Figs 1a and 5a) was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers p42-F and p42-R. To minimize mutations caused by PCR amplification, a high-fidelity DNA polymerase (PyroBest; Takara) was used. The corresponding region of the p35 *mpl* gene (MYPE6810) was also amplified from *M. penetrans* HF-2 by PCR with primers p35-F and p35-R. The amplified p42 and p35 regions were inserted into the pENTR/D-TOPO plasmid by using the TOPO cloning system according to the manufacturer's instructions (Invitrogen), resulting in plasmids pAH501 and pAH502 (Table 1). The cloned p42 and p35 promoter sequences on these plasmids were in the ON direction. To introduce the p42 promoter sequence into *M. pneumoniae* cells, the cloned p42 region on plasmid pAH501 was transferred to the Smal site of the Tn4001mod vector plasmid pISM2062.2 (Knudtson & Minion, 1993) by using the Gateway cloning technique (Invitrogen). Briefly, pISM2062.2 was converted into the Gateway destination vector by inserting the Gateway vector conversion system (reading frame cassette A) at the Smal site. The p42 sequence of the pAH501 plasmid was transferred to the pSM2062.2 destination vector by the LR reaction of the Gateway system. The resulting plasmid was designated pAH511 (Table 1) and introduced into *M. pneumoniae* M129 by electroporation. The p42 and p35 promoter sequences on the pAH501 and pAH502 plasmids, respectively, were also transferred to the Smal site of the pCl.1920 plasmid, which has a pSC101 replicon (Lerner & Inouye, 1990), by using the Gateway cloning system for introduction into *E. coli* cells. These plasmids were designated pAH521 and pAH522, respectively (Table 1).

**Construction of the MYPE2900 and MYPE8180 expression clones.** PCR primers used for plasmid construction are listed in Table S1. MYPE2900 was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers 2900-F-TOPO and 2900-R-Asc. MYPE8180 was also amplified using primers 8180-F-TOPO and 8180-R-Asc. The amplified MYPE2900 and MYPE8180 fragments were inserted into the plasmid pENTR/D-TOPO by using the TOPO cloning system, resulting in plasmids pAH301 and pAH302 (Table 1). The cloned MYPE2900 and MYPE8180 on the pAH301 and pAH302 plasmids were sequenced to confirm that no mutations were incorporated during the PCR cloning. To express MYPE2900 and MYPE8180 in *M. pneumoniae* cells, the *tuf* promoter (P₅₉) sequence of *M. pneumoniae* was linked to MYPE2900 and MYPE8180. The P₅₉ sequence was amplified from *M. pneumoniae* M129 genomic DNA by PCR using primers tuf-F-Bam-TOPO and tuf-R-Afl. The amplified *tuf* promoter fragment was digested with BamHI and AflIII and ligated into the BamHI–NcoI sites of pAH301 and pAH302, resulting in plasmids pAH303 and pAH304 (Table 1). The MYPE2900 and
**Table 1.** Plasmids constructed in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Marker</th>
<th>Gene</th>
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<tr>
<td>pAH301</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>MYPE2900</td>
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<tr>
<td>pAH302</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>MYPE8180</td>
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<tr>
<td>pAH303</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P&lt;sub&gt;nf&lt;/sub&gt;-MYPE2900</td>
</tr>
<tr>
<td>pAH304</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P&lt;sub&gt;nf&lt;/sub&gt;-MYPE8180</td>
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<tr>
<td>pAH311</td>
<td>pKV104 (Tn&lt;sup&gt;4001&lt;/sup&gt;mod)</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>MYPE2900</td>
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<tr>
<td>pAH312</td>
<td>pKV104 (Tn&lt;sup&gt;4001&lt;/sup&gt;mod)</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P&lt;sub&gt;nf&lt;/sub&gt;-MYPE8180</td>
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<tr>
<td>pAH331mut</td>
<td>pColdI</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P&lt;sub&gt;Op&lt;/sub&gt;-MYPE2900mut</td>
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<tr>
<td>pAH332</td>
<td>pColdI</td>
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<td>pAH332mut</td>
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<tr>
<td>pAH501</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>p42 and promoter region</td>
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<tr>
<td>pAH502</td>
<td>pENTR/D-TOPO</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>p35 and promoter region</td>
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<tr>
<td>pAH511</td>
<td>pSM2062.2 (Tn&lt;sup&gt;4001&lt;/sup&gt;mod)</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>p42 and promoter region</td>
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<td>pAH521</td>
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MYPE8180 genes on the pAH303 and pAH304 (P<sub>nf</sub>-MYPE2900 and P<sub>nf</sub>-MYPE8180, respectively) were then transferred to the SmaI site of the Tn<sup>4001</sup>mod vector plasmid pKV104 (Hahn et al., 1999) by using the Gateway cloning technique. The resulting Tn<sup>4001</sup>mod plasmids pAH311 and pAH312 (Table 1) were used to transform M. pneumoniae. To express the MYPE2900 and MYPE8180 genes in E. coli, these genes were amplified from M. penetrans HF-2 genomic DNA by PCR with the primer pairs 2900-F-SacTGG and 2900-R-XhoI and inserted into the SacI–XhoI site of plasmid pColdI (TaKaRa), resulting in plasmid pAH331mut (Table 1). The MYPE2900mut gene on plasmid pAH331mut was sequenced, and the conversion of the codon UGA to UGG was confirmed (nt position 28–30 of the MYPE2900 gene). The pAH331mut was sequenced, and the conversion of the codon UGA to UGG by using the GeneTailor site-directed mutagenesis system (Invitrogen) was confirmed. Two UGA codons present in MYPE8180 (nt positions 145–147 and 517–519) were converted to CAG by PCR amplification. MYPE8180 fragments were digested with SacI and XhoI and inserted into the SacI–XhoI site of plasmid pColdI, resulting in plasmid pAH332mut (Table 1). Two UGA codons present in MYPE8180 were digested with NdeI and EcoRI and inserted into the NdeI–EcoRI site of plasmid pColdI, resulting in pAH332 (Table 1). The amplified MYPE2900 and MYPE8180 were digested with SacI and XhoI and inserted into the SacI–XhoI site of plasmid pColdI (TaKaRa), resulting in plasmid pAH331mut (Table 1). The plasmids pAH331mut and pAH332mut were introduced into E. coli BL21(DE3) to express MYPE2900 and MYPE8180.

**Protein analysis.** E. coli BL21(DE3) strains harbouring plasmids pAH331mut, pAH332mut or pColdI were grown at 37°C until the mid-exponential phase. The cultures were maintained at 15°C for 30 min, and 0.2 mM final concentration of IPTG was added. After the addition of IPTG, the cultures were maintained at 15°C with shaking for 24 h. E. coli cells were collected from a 1 ml volume of the culture by centrifugation at 20,000 g for 20 min at 4°C. The pellets were visualized by Coomasie brilliant blue staining. For Western blot analysis, the proteins, separated by SDS-PAGE, were transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal antibody specific for His<sub>6</sub>-tag (Cell Signaling Technology) was used at a 1:1000 dilution to detect His<sub>6</sub>-tagged MYPE2900 and MYPE8180 proteins. The reacting antibodies were detected with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulin G; Promega) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) Colour Development Substrate (Promega) according to the manufacturer’s instructions.

**RESULTS**

**MYPE2900 belongs to the tyrosine site-specific recombinase (Tsr) family and catalyses p42 promoter inversion in M. pneumoniae cells**

The gene annotation process for the complete genome sequence of M. penetrans HF-2 indicated that two open...
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**Fig. 1.** Promoter inversion of the p42 (MYPE6630) gene in *M. pneumoniae* cells. (a) The PCR method for detecting promoter inversion. The gene region containing p42 and its invertible promoter is illustrated schematically. Three arrowed boxes represent the MYPE6640, p42 and MYPE6620 genes (also see Fig. 5a). The triangle indicates the invertible promoter of the p42 gene (in the ON direction). Filled boxes in the MYPE6640 and p42 genes indicate the signal peptide region of lipoproteins. The positions of the three primers, namely, F (forward), R (reverse) and P (promoter), are shown by arrows. The ON and OFF products (approx. 2 and 1.2 kb, respectively) are amplified by two PCRs with primer sets FP or RP, depending on the direction of the p42 promoter. The primer sequences are listed in Supplementary Table S2. (b) Agarose gel (0.8%, w/v) electrophoresis pattern of the PCR products. Genomic DNAs of M129-p42 cells carrying P_{nr}-MYPE2900 (lanes 1 and 2), P_{nr}-MYPE8180 (lanes 3 and 4) or pKV104 vector alone (lanes 5 and 6) were analysed by the PCR method. Primer pairs used for the PCR analysis are shown below. Arrows indicate positions of the ON and OFF products.

reading frames, namely MYPE2900 and MYPE8180, show significant homology to the Tsr family genes (Horino et al., 2003; Sasaki et al., 2002). The amino acid sequences of MYPE2900 and MYPE8180 share relatively high homologies to the Tsr of *Mycoplasma pulmonis* and *Mycoplasma agalactiae*, which were previously shown to mediate site-specific DNA inversions (Chopra-Dewasthaly et al., 2008; Sitaraman et al., 2002). We designed experiments to define whether the MYPE2900 or MYPE8180 products can mediate promoter inversion of mpl genes. Since genetic tools are still unavailable for *M. penetrans*, an mpl promoter inversion system was reconstructed in *M. pneumoniae* cells. For this purpose, a 3.2 kb DNA fragment containing the p42 mpl gene and its promoter region of *M. pneumoniae* Hf-2 was introduced into *M. pneumoniae* strain M129 by using the staphylococcal transposon Tn4001 mod vector, which carries a Gm<sup>R</sup> marker (see Methods). Genomic DNA was extracted from the whole transformed population (M129-p42; the p42 promoter sequence is inserted at random in the chromosome) and was analysed by PCR to examine the direction of the p42 promoter. This PCR analysis (Fig. 1a) gave only the 2 kb product from the genomic DNA of M129-p42 (data not shown), indicating that the direction of the p42 promoter in M129-p42 cells was in the ON direction and that p42 promoter inversion does not occur in *M. pneumoniae* cells, regardless of the position of the p42 promoter in the *M. pneumoniae* chromosome.

We then introduced recombinase genes into the M129-p42 cells. The MYPE2900 and MYPE8180 clones linked with *M. pneumoniae* P_{nr} promoter were introduced into M129-p42 cells by using the Tn4001 mod derivative vector that carries a Cm<sup>R</sup> marker (see Methods). Thus transformants of M129-p42 (Gm<sup>R</sup>-Cm<sup>R</sup>) having the p42 promoter sequence and the recombinase genes were obtained. We then extracted genomic DNA from these transformants (whole transformed population) and examined the direction of the p42 promoter by PCR. This analysis showed that both 2 and 1.2 kb products were amplified from the genomic DNA carrying the MYPE2900 gene (Fig. 1b, lanes 1 and 2). However, only 2 kb product was obtained from the genomic DNAs carrying the MYPE8180 gene (Fig. 1b, lanes 3 and 4) or Tn4001 mod vector alone (Fig. 1b, lanes 5 and 6). These results suggested that the MYPE2900 protein is the recombinase that has an activity to invert the p42 promoter sequence.

**Reconstruction of the mpl promoter inversion system in E. coli**

To further characterize mpl promoter inversion and to develop a simple experimental system, we reconstructed the mpl promoter inversion system in *E. coli*. Similar to standard mycoplasmal genes, MYPE2900 and MYPE8180 have UGA codons, which are translated into tryptophan in mycoplasma cells. MYPE2900 has a single UGA codon, and MYPE8180 has two UGA codons. Since these UGA codons block full translation of MYPE2900 and MYPE8180 in *E. coli*, we converted these UGA codons into UGG. The converted MYPE2900 and MYPE8180 genes (MYPE2900<sub>mut</sub> and MYPE8180<sub>mut</sub>) were linked with the *E. coli* cspA promoter and His<sub>6</sub>-tag sequence at the 5’ end on plasmid pAH331<sub>mut</sub> and pAH332<sub>mut</sub>, respectively (see Methods and Table 1). Plasmids pAH331<sub>mut</sub> and pAH332<sub>mut</sub> were introduced into *E. coli* BL21(DE3), and the strain was cultured under appropriate conditions to activate the cspA promoter (see Methods). After 24 h culture at 15 °C with or without IPTG, the expression of MYPE2900<sub>mut</sub> and MYPE8180<sub>mut</sub> proteins was detected by immunoblot analysis specific for His<sub>6</sub>-tag (Fig. 2). The
MYPE2900 and MYPE8180 proteins were produced apparently in the presence of IPTG (Fig. 2, lanes 10 and 12), although the reason for the low expression level of MYPE8180 compared to MYPE2900 is not clear. In addition, a weak expression of MYPE2900 was observed in the absence of IPTG (Fig. 2, lane 9).

We then constructed pSC101 derivative plasmids carrying the p42 or p35 mpl genes and their promoter regions from the M. penetrans HF-2 genome (approx. 3.2 kb DNA region). These plasmids, namely pAH521 and pAH522 (Table 1), were introduced into E. coli BL21(DE3) harbouring plasmid pAH321mut or pAH322mut. After culturing these E. coli strains under appropriate conditions to activate the cspA promoter, the plasmids were recovered from the cells and analysed by PCR to determine the directions of the p42 and p35 promoter sequence (see Methods). As shown in Fig. 3, the PCR analysis revealed ON and OFF direction products of the p42 and p35 promoter sequences in the presence of the MYPE2900mut gene (Fig. 3, lanes 1, 2, 3, 4, 9, 10, 11 and 12). The absence of 0.2 mM IPTG did not affect this inversion event (Fig. 3, lanes 1, 2, 9 and 10), suggesting that a small amount of MYPE2900 recombinase was sufficient to catalyse p42 and p35 promoter inversions. On the other hand, only ON direction products were obtained in the presence of the MYPE8180mut gene (Fig. 3, lanes 5, 7, 13 and 15), indicating that the promoters p42 and p35 were locked in the ON direction in these conditions. These results strongly support the conclusion that the MYPE2900 protein is the recombinase that mediates DNA inversions of the mpl gene promoters. We also performed the same experiments in E. coli strain YK1340 (Wada et al., 1988), which lacks the histone-like protein HU. Even in the YK1340 background, inversion of the p42 and p35 promoters occurred only in the presence of the MYPE2900mut gene (data not shown). The recombination sites of DNA inversion (IR sequences) of the p42 and p35 promoters are TAACATAAATTA and TAACAAGAATTA, respectively. The MYPE2900 recombinase can probably recognize both these sequences as a recombination site.

Fig. 2. Production of MYPE2900 and MYPE8180 proteins in E. coli. SDS-PAGE gradient gel (5–20%, w/v, lanes 1–6) and immunoblot analysis (lanes 7–12) patterns of proteins from E. coli transformants. E. coli strains carrying pAH331mut (lanes 3, 4, 9 and 10), pAH332mut (lanes 5, 6, 11 and 12) or pColdI vector plasmids (lanes 1, 2, 7 and 8) were analysed. Addition (+) or no addition (−) of 0.2 mM IPTG during culture is indicated below. His-tagged MYPE2900 and MYPE8180 proteins (MYPE2900mut and MYPE8180mut gene products) were detected using a monoclonal antibody against His-tags on a blotted nitrocellulose membrane (lanes 7–12). The MYPE2900 and MYPE8180 proteins detected are indicated by an arrow.

Fig. 3. Electrophoresis patterns of PCR analysis of p42 and p35 promoter inversions in E. coli cells. The PCR products were electrophoresed on a 0.8% (w/v) agarose gel. PCR analysis results of the p42 promoter (lanes 1–8) and p35 promoter (lanes 9–16) are shown. The recombinase genes present in E. coli cells (MYPE2900mut or MYPE8180mut) are indicated. Addition (+) or no addition (−) of 0.2 mM IPTG during culture is indicated. Primer sets used for PCR analysis (FP or RP) are indicated below. The positions of PCR products from the ON and OFF direction promoters are indicated on the right.
Detection of inversions of mpl gene promoters in M. penetrans

To characterize the consensus sequence of the recombination site of the mpl gene promoters, we aligned 30 IR sequences found in the promoter regions of the mpl genes in the M. penetrans HF-2 genome. The alignment shown in Fig. 4(b) revealed that nucleotides TAA and ATTA at both ends of 12 bp IRs were conserved; however, five nucleotides in the middle of the repeats differed from each other. The consensus sequence was proposed as TAAYNNNDATTA. To determine whether mpl promoter inversions actually occur at these various IR sequences in M. penetrans cells, we extended the PCR-based inversion detection method. In addition to the primer sets for p42 and p35 promoter regions, we designed eight sets of PCR primers for MYPE6840, 6780, 6740, 6690, 6660, 6560, 6530 and 6490 gene promoters (Supplementary Table S2 and Fig. 5a). Of these 10 promoters, the p42 and p35 promoters were in the ON direction, and the others were in the OFF direction in the consensus genome sequence data of M. penetrans HF-2 (Fig. 5a). To detect the inverted form of these promoters, we analysed M. penetrans HF-2 genomic DNA (1 ng template) by the PCR method with 10 sets of primers (Fig. 5a). This PCR analysis showed that amplification products from both the ON and the OFF direction promoters (approx. 2 and 1 kb products) were obtained from all the 10 promoters examined (Fig. 5b), although the amounts of the ON direction products from MYPE6840 and MYPE6660 promoters and the OFF direction product from MYPE6810 (p35) were low (Fig. 5b, lanes 1, 4 and 11). We analysed the nucleotide sequences of the PCR products of ON and OFF directions and confirmed that inversion of these promoter sequences occurred at the 12 bp IR sequences listed in Fig. 4(b) (data not shown).

DISCUSSION

In this study, we identified the recombinase that catalyses inversion of the mpl gene promoters. Reconstruction of the mpl promoter inversion system (recombinase and recombination sites) in M. pneumoniae and E. coli cells clearly showed that the MYPE2900 gene product has an activity to invert the p35 and p42 promoters. These experiments indicated that mpl promoter inversion is catalysed by a single factor of M. penetrans. In some other site-specific recombination systems, additional factors such as nucleoid-associated proteins are required for DNA recombination together with recombinase (Esposito et al., 2001; Wada et al., 1989). However, these factors may not be essential in mpl promoter inversions. The recombination sites (12 bp IR) of the p42 and p35 genes are similar but slightly different, suggesting that MYPE2900 recombinase has flexibility in recognizing the recombination site sequences. The mpl promoters controlled by MYPE2900 are one of the smallest genetic switches in bacteria (Fig. 4a). MYPE2900 recombinase and its short recombination site may be utilized as a tool for genetic engineering such as modulation of recombinant gene expressions, inversion or excision of specific chromosomal DNA regions, or construction of gene clones by DNA recombination. To develop these tools, the mpl inversion systems reconstructed in E. coli (with
His6-tagged MYPE2900) are useful for detailed characterization of the recombination mechanism and designing of genetic tools.

In our previous study, we did not perform direct detection of mpl promoter inversions in M. penetrans cells (Horino et al., 2003). In the present study, we used a PCR-based method and found the presence of an inverted form of 10 mpl gene promoters (including the p35 and p42 promoters) in M. penetrans genomic DNA. Inversions of these mpl gene promoters occurred at the 12 bp IR sequences that are similar to those of the p35 and p42 promoters (Fig. 4b), suggesting that all these promoter inversions may be catalysed by MYPE2900 recombinase in M. penetrans cells.

The 12 bp IR sequences of all mpl genes slightly differ from each other and show the consensus sequence TAAYNNNDATTA. This type of dyad motif is commonly present as a recognition site of site-specific DNA recombinases. However, the 12 bp sequence is considerably shorter than that of the other known recombination sites recognized by site-specific DNA recombinases (Glew et al., 2002; Honarvar et al., 2003; Li et al., 2002; Lysnyansky et al., 2001; Sitaraman et al., 2002). The variation in 12 bp IRs of the mpl gene promoters may contribute to the prevention of inappropriate recombination between different mpl gene promoters and to maintaining the structure of the mpl gene cluster (Fig. 5a). The variation in recombination sites may also affect the frequency of inversion of individual promoters. In this study, we did not examine the inversion frequency of each promoter. This could be evaluated by a real-time PCR method or by using reconstruction systems in M. pneumoniae and E. coli cells in further studies.

Another characteristic feature of the mpl promoter inversion system is the location of the recombinase gene. In many site-specific recombination systems, which control antigenic variations, the Tsr family genes tend to be located near the recombination sites (Chopra-Dewasthaly et al., 2008; Komano, 1999; Kuwahara et al., 2004; Ron et al., 2002). However, in the mpl promoter inversion system, the MYPE2900 gene is located approximately 500 kb apart from the major mpl gene cluster. The other two minor mpl gene clusters are also separated by approximately 600 or 40 kb from the MYPE2900 gene (Horino et al., 2003). In addition, MYPE2900 recombinase efficiently catalysed the

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**Fig. 5.** Detection of promoter inversion of selected mpl genes in M. penetrans cells by PCR. (a) Schematic illustration of the largest mpl gene cluster of the M. penetrans HF-2 genome (Horino et al., 2003). The figure was prepared on the basis of the whole genome sequencing data of M. penetrans HF-2 (Sasaki et al., 2002; GenBank accession no. BA000026). Grey arrowed boxes represent the mpl genes in this region. White arrowed boxes represent the non-mpl genes. Filled boxes in the mpl genes represent the signal peptide region of these genes. Open triangles indicate invertible promoters and their ON and OFF directions. Squares represent terminator-like sequences. The terminator-like sequences in inactive form are marked with an asterisk (p35 and p42 genes, see Fig. 4a). Small arrows below the genes indicate the relative positions of PCR primers used for the detection of promoter inversions. (b) PCR analysis of promoter inversions of 10 mpl genes. The PCR products were electrophoresed on a 0.8 % (w/v) agarose gel. The promoters analysed are indicated above. P and R primer sets were used to detect the ON direction promoters (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19). P and F primer sets were used to detect the OFF direction promoters (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20). The sequences of each primer set are listed in Supplementary Table S2. The positions of ON and OFF products are indicated on the right.
inversions of the p42 and p35 promoters in a trans-acting manner in E. coli cells (the MYPE2900 gene and the p42 or p35 promoters were present in different plasmids). Probably, there is no cis-acting effect between the MYPE2900 gene and mpl promoter recombination sites.

The amino acid sequence of MYPE8180 strongly suggests that it is also a Tsr. However, in our experimental conditions, the MYPE8180 product did not show recombinase activity in p42 and p35 promoter inversions. The reconstruction systems in this study are not identical to the natural context in M. penetrans cells, so that there are still possibilities that MYPE8180 recombinase takes part in the mpl system with additional factors in M. penetrans cells or that the His6-tag fused to MYPE8180 in this study disturbed the recombinase activity. However, it is also possible that MYPE8180 has no activity in mpl promoter inversions, but is involved in another site-specific recombination system of the M. penetrans genome. It is known that several mycoplasma species such as Mycoplasma genitalium and M. pneumoniae lack xer-like site-specific recombinase genes, possibly because of reductive evolution of their minimum genomes (approx. 580 and 816 kb in size) (Himmelreich et al., 1997). This fact suggests that these mycoplasma species, as a result of reductive evolution, do not require Xer-like recombinase for resolving their dimer chromosomes during cell division (Glew et al., 2002; Recchia & Sherratt, 1999). However, the genome of M. penetrans (1.36 Mb) is approximately twice as large as that of these species. Furthermore, MYPE8180 shows strong homology to the XerC/D group of the Tsr family proteins. Thus, there is a possibility that MYPE8180 is involved in a Xer-like site-specific recombination system for chromosome segregation in M. penetrans. There is a further possibility that MYPE8180 is involved in another phase or antigenic variation system. To examine these possibilities, identification of the target sequence of MYPE8180 would be an interesting future study.

Identification of MYPE2900 recombinase in this study may lead to further research on disruption of the MYPE2900 gene and development of phase-variation-deficient (phase-locked) strains of M. penetrans. Such phase-locked strains were recently developed in M. agalactiae, a ruminant pathogen that has a multiple site-specific recombination system responsible for its surface lipoprotein variation (Chopra-Dewasthaly et al., 2008). Phase-locked strains are useful for investigating host–pathogen interactions and for determining the functions of each lipoprotein variant. Although a gene disruption method needs to be developed in M. penetrans, an MYPE2900-deficient mutant might be developed in future studies. These attempts may provide an insight into the extent to which surface antigenic variations of mycoplasmas are involved in host immune evasion and help to establish their parasitic lifestyle. An understanding of these pathogenic mechanisms of mycoplasmas may be helpful for designing strategies to eradicate chronic mycoplasma infection.

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Promoter inversion in *M. penetrans*


