Analysis of putative ABC transporter genes in *Mycoplasma hyopneumoniae*

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A previously described DNA probe specific for *Mycoplasma hyopneumoniae* (I-141) was fully sequenced and found to consist of 1618 bp and to contain two tandemly repeated ORFs. The deduced amino acid sequence of the two ORFs showed significant homologies with ATP-binding cassette (ABC) transporter proteins, particularly those of the eukaryotic multidrug resistance (MDR) protein family (up to 21% identity and 47% similarity). A somewhat lower homology was evident with the secretion protein HlyB of the RTX-haemolysin from *Escherichia coli*. The location of the two ORFs on the *M. hyopneumoniae* chromosome was downstream of the *rrl* gene encoding the 23S rRNA, but transcribed in the opposite direction. PCR amplification and subsequent chromosomal analysis by Southern blot hybridization of several *M. hyopneumoniae* strains showed that all field strains contained the two putative ABC transporter genes. However, some culture collection strains derived from strain J had lost these genes as the result of a 2221 bp deletion.

Keywords: mollicutes, *Mycoplasma hyopneumoniae*, deletion, MDR proteins, ABC transporters

INTRODUCTION

Mollicutes are amongst the smallest autonomously replicating organisms, which differ structurally from other eubacteria in the lack of a cell wall, a small genome and a low G + C content (Bové, 1993). *Mycoplasma hyopneumoniae* colonizes the ciliated respiratory epithelium of piglets inducing loss of cilia (Blanchard *et al.*, 1992) and is the etiological agent of enzootic pneumonia (EP) (Ross, 1992). EP is one of the most common and economically important diseases that occur in swine. Recent studies also suggest a role for *M. hyopneumoniae* in the initiation of a variety of other bacterial and virus infections that occur under field conditions (Ross, 1992; Kobisch *et al.*, 1993), but the mechanism of its pathogenicity is poorly known.

Typically, detection of *M. hyopneumoniae* in pig lungs is based on culture or immunofluorescence (IF) assays (L'Ecuyer & Boulanger, 1970; Hovind-Hougen & Friis, 1991). However, identification by culture is laborious, and the specificity of the IF test is ambiguous, since *M. hyopneumoniae* and *Mycoplasma flocculare* (a frequently isolated non-pathogenic pig mycoplasma) share common

antigenic determinants (Bölske *et al.*, 1987). Thus, many attempts have been made to develop alternative diagnostic systems.

We have recently shown that a DNA fragment (I-141) from *M. hyopneumoniae* may be used as a specific probe for the detection of *M. hyopneumoniae* in tracheobronchiolar washings obtained from experimentally infected piglets (Abiven *et al.*, 1992). Here, we report the sequence of the I-141 DNA fragment and the DNA surrounding the fragment in the *M. hyopneumoniae* chromosome. The results show the presence of two ORFs with sequence similarities to genes encoding ABC (ATP-binding cassette) transporters. Some strains derived from strain J do not possess these genes.

METHODS

Bacterial strains, DNA and plasmids. *Escherichia coli* TG1 was used as the host for propagation of plasmids and viruses. *M. hyopneumoniae* strain J (ATCC 25934) was received directly from ATCC and cultivated for less than ten passages in modified Friis culture medium (Hovind-Hougen & Friis, 1991). We refer to this strain as J-LP (low passage J strain). Genomic DNA from this strain was used to construct the I-141 probe (Abiven *et al.*, 1992).

Purified DNA from other *M. hyopneumoniae* strains, field strains and strains derived from the original strain J were given by

Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance.

The GenBank accession numbers for the sequences reported in this paper are U02537, U02538 and U19889 (see text for details).

Table 1. Source and reference of *M. hyopneumoniae* strains tested and size of DNA fragments obtained by PCR with HP1 or HP3 primers

Strain	Reference or source*	Obtained from:	Size of fragment (kbp)
J-LP	ATCC 25934	Our laboratory	1.6
BQ14	Field isolate	Our laboratory	1.6
VL4	Field isolate	Our laboratory	1.6
39A	Field isolate	Our laboratory	1.6
J	ATCC 25934	J. Frey	1.8
J	NCTC 10110	J. Frey	1.8
JF 184	NCTC 10110	J. Frey	1.8
EP 29	Field isolate	J. Frey	1.6
EP-S 946	Field isolate	J. Frey	1.6
EP-S 924	Field isolate	J. Frey	1.6
EP-S 938	Field isolate	J. Frey	1.6
EP-S 939	Field isolate	J. Frey	1.6
VPP11	Field isolate	R. Ross	1.6
232	Field isolate	R. Ross	1.6
1361	Field isolate	R. Ross	1.6
1424	Field isolate	R. Ross	1.6
J	ATCC 25934	R. Ross	1.8
J	ATCC 25934	P. Ahrens	1.8
15 unnamed strains	Field isolates	P. Ahrens	1.6

* ATCC, American Type Culture Collection, Rockville, MD, USA; NCTC, National Collection of Type Cultures, London, UK.

various laboratories. The sources of these strains are presented in Table 1.

Plasmid pMR7 containing the complete 23S rDNA of *M. hyopneumoniae* (strain J ATCC 27719) was kindly provided by R. Hermann (ZMBH, Heidelberg University, Germany) (Taschke *et al.*, 1986).

Primers. Primers HP1 and HP3 were designed from the nucleotide sequence of I-141 and synthesized on an Applied Biosystems DNA synthesizer. The HP1 primer sequence was 5'-TTCAAATTATAACCTCGGTC-3' and the HP3 primer sequence was 5'-AGCAAATTTAGTCTCTCTGC-3'.

PCR amplification. Amplification was performed in a final volume of 50 µl in a DNA thermal cycler (Perkin-Elmer Cetus). Each reaction mixture contained: 2.5 units *Taq* polymerase; 1 µM of each of the primers HP1 and HP3; 200 µM of each of the deoxynucleoside triphosphates (dNTPs); assay buffer (Tris/HCl, 70 mM, pH 8); MgCl₂, 2 mM; (NH₄)₂SO₄, 17 mM; β-mercaptoethanol, 10 mM; BSA, 200 µg ml⁻¹ and dimethyl sulfoxide (DMSO), 5% (v/v). Reaction mixtures were overlaid with mineral oil, heated at 92 °C for 10 min and submitted to 40 PCR cycles. Each cycle consisted of 1 min at 92 °C, 1 min at 57 °C and 2 min at 72 °C. After 40 cycles an extension step for 10 min at 72 °C was performed.

DNA cloning and sequencing. The amplified DNA fragments of 1.8 kbp obtained from strain J (NCTC 10110) and used for cloning were phenol-extracted, after removal of oligonucleotides by spin dialysis on a Centricon-30 concentrator (Amicon) as recommended by the manufacturer. The fragments were phosphorylated and ligated into the *Hinc*II site of either pUC18 or M13 vector as described by Sambrook *et al.* (1989). The resulting plasmids were used to transform *E. coli* TG1 cells by the method of Hanahan (1983).

The preparation of single-stranded DNA templates and performance of annealing reactions were as described in the Sequenase version 2.0 kit handbook (United States Biochemical).

Double-stranded plasmid DNA was prepared as described by Mierendorf & Pfeffer (1987) and sequenced according to the procedure described in the Sequenase version 2.0 kit handbook using synthesized oligonucleotides as primers.

Initial comparisons between proteins of *M. hyopneumoniae* and those in the GenBank/EMBL databases were performed using the program BLAST. Computer-assisted analysis of nucleotide and protein sequence data were performed with PC gene software. The CLUSTAL and Genetics Computer Group (GCG) programs were used to calculate percentage identity (identical amino acids) and similarity (identical and equivalent amino acids) between aligned sequences.

DNA isolation and hybridization. Mycoplasma chromosomal DNA was isolated as described by Marmur (1961). Restriction enzyme digestions were performed according to the manufacturer's protocols (Life Technologie). DNA fragments were resolved on 0.8% agarose gels and transferred to nylon N+ membranes (Amersham). Labelling and hybridization procedures using the I-141 probe were as previously described (Abiven *et al.*, 1992).

RESULTS

Sequencing of the I-141 DNA fragment

The *Hind*III I-141 DNA fragment of *M. hyopneumoniae* strain J-LP (obtained directly from ATCC, see Methods) was sequenced (GenBank accession number U02537) and

Fig. 1. Organization of *M. hyopneumoniae* DNA containing the *Hind*III I-141 fragment and ORFs 1 and 2. (a) Restriction map of the pMR7 plasmid insert containing the 23S rRNA gene as described by Taschke *et al.* (1986). (b) Organization of genomic DNA of J-LP (low passage) and field strains; the position of the *Hind*III I-141 DNA fragment is shown. (c) Genomic organization of other J strains; the 2221 bp deletion is shown as a dotted line. (d) Location and direction of transcription of the 23S rRNA gene and ORF1 and ORF2. E, *Eco*RI; H, *Hind*III; B, *Bgl*III; T, terminator of the 23S rRNA gene (Taschke *et al.*, 1986). The positions of primers HP1 and HP3 are indicated by arrows.

Fig. 2. Southern blot analysis of genomic DNA of *M. hyopneumoniae* J-LP strain (a) and a representative J strain (b) with the radiolabelled I-141 probe. DNA was restricted by *Eco*RI/*Bgl*III (lane 1), *Eco*RI (lane 2) or *Hind*III (lane 3). Lane M contains molecular mass standards (1 kbp DNA ladder, BRL). Sizes are indicated in kbp.

found to be 1618 bp long. Its base composition was 37.1% A, 36.9% T, 13.6% G and 12.4% C. Taking UGA as a tryptophan codon (Yamamoto *et al.*, 1985), two partial putative ORFs overlapping over eight nucleotides were identified. One ORF comprised nucleotides 1–592 and the other, nucleotides 585–1618.

To identify the two entire ORFs, the location of the I-141 DNA fragment on the *M. hyopneumoniae* genome was determined and the adjacent DNA region sequenced.

Localization of the I-141 fragment on *M. hyopneumoniae* genomic DNA

The position of the I-141 DNA fragment on the *M. hyopneumoniae* genome was mapped by field inversion gel electrophoresis (FIGE) and Southern blot hybridizations (data not shown). I-141 was found in the vicinity of *rrl* and *rrs* encoding, respectively, the 23S rRNA and 16S rRNA genes, and more precisely the region near the terminator of the 23S rRNA gene (J. Frey, unpublished data).

Taschke *et al.* (1986) showed that *rrs* and *rrl* were single-copy genes with a short intergenic region. The 23S rRNA gene, including the terminator, was located on a 7 kbp *Eco*RI DNA fragment which was subsequently cloned (Taschke *et al.*, 1986) in a plasmid named pMR7 (Fig. 1a). In the present study, the use of the *Hind*III I-141 DNA fragment as a probe in Southern blot hybridizations with restricted genomic DNA of *M. hyopneumoniae* J-LP showed that I-141 hybridized to the 7 kbp *Eco*RI DNA fragment

(Fig. 2a, lane 2) and to a 1.6 kbp *Hind*III (lane 3) and a 3 kbp *Eco*RI/*Bgl*III (lane 1) fragment. Hybridizations between *Hind*III I-141 DNA as probe and the pMR7 insert digested by the same enzymes gave the same result (data not shown). Thus, the results indicate that the *Hind*III I-141 DNA fragment hybridizes with the *Hind*III fragment of the pMR7 DNA insert described by Taschke *et al.* (1986).

Nucleotide sequence analysis of the pMR7 insert

To obtain the full sequence of the two ORFs on I-141, we sequenced the pMR7 insert from the terminator of the 23S rRNA gene to the I-141 fragment with internal primers; we also sequenced the 630 nucleotides upstream of the *Hind*III site of the I-141 fragment (Fig. 1 line a). The entire sequence (GenBank accession number U19889) comprised 3788 bp (Fig. 3).

No coding sequence was found on the strand harbouring the 23S rRNA gene. However, the complementary strand showed two overlapping ORFs (Fig. 3). The first one, designated ORF1, extended from nucleotides 62 to 1661 and the other, from nucleotides 1656 to 3266. The two ORFs each had an ATG initiation codon and a TAA stop codon.

The 3'OH end of the 16S rRNA of *M. hyopneumoniae* had the sequence ACCUCCUUUCUA-3'OH (Stemke *et al.*, 1992). Preceding the ATG codon of ORF1 (nucleotide 62) was a Shine–Dalgarno ribosome-binding sequence (AGGAGGT) complementary to the sequence of the

1
ATTAGAAATCTCAAAAATTTTGGCTTAAATTTAAATCAAAAATTTTACCTAGGAGTTTCATATGCGTTTTAACAGGCTTTTACTCTAGCAAACTTAAATTTACAATTATTTTGTAGT
-10 RBS
ORF1-M R F N R L F S L A K L K P T I I P L V

121
AATCTTTATAAAGAGCGGGGATTCTAACCCATATTTTAGCTACAAGTTATTTTGGAGTATTTTCAGAGAAAAACCTACTTTTAACTAACTTGGTCCCTAATTTTGTGTGTAAT
L F I K E A G P L T H I F S Y K F I F E Y F S E K N P T F N L T W S L I L L L L M

241
GCCGCTTCCATTTTATCAITTTTACTTTTTGAAAGGATGAATTTTAGTTATTTAGAAATGGAAGTACGCAATAAACTAAGCGATATTATAATCAAAAAATTCAAAAAAGTAATTA
P L A I F I I P T F L K G W I F S Y L E N E V R N K L S D I I I K K I Q K S N Y

361
TTCTGACCTAAATACAAATAAAATGCAATGCTTTCTGATTTAATTATGATCTCAAACTCGGTTGAATGCAATTGAAAACTTTTAAATATTATTTACCGCTTGTTCATTATCAC
S D L K Y N K N A M L S W F N Y D L K L G L N A I E N L L N I I S P L V S I I T

481
TGGAAITCTTTGATGATAGTTTATCACCAGCAATGAGGTGAATTTAATTGTTACTACCTTAATCTAAGTTAATTTTACTAATTTTCAAGGGAAAAATTAGTAATGCTGCTTCAAA
G I S L N I V L S P Q W G W I L I V T T L I L S L I L L I P Q G K I S N A A S K

601
ACCAAGTGAAGAACTATCAAGTGATAGTGAAGCTTTTCAAATTATAACCTCGGTCTTCTTAACCTTTTAAATCATTTTCTTTCATACAAAAAATGAAAAATTAAGCAACTTTTTTA
P V M K L S S D S E A F S N Y N L G L L N S F K S P F F H N K N E K F K Q L F Y

721
TAAAGTTATGAAAAATTTATCCAAAGATCAATAAAACAATATAAGAAATTAACATAAATTAATGTAATCTGACTGTCTTATTAGTATTTCGATTGTGATTGTTTTAACTGAATTAAC
K S Y E N L S E D Q I K Q Y K K L T K L N V I L T V L P S I S I V I V L T S L A

841
AGTGTTAACTTCATTTAAATTTTATAGTTTAGCTATTTTATCCCTACTTTTATCTCAAAATCGCTTAGTACCGATATGGGAAACATTGTTCTTGGTTTATTGGATATAAGCAATC
V L T S F K F Y S L A I F L S L L L Y S N R F S T D M G N I V L G L F G Y K Q S

961
ATCAGTTTCTGCTGATAAAATTTGGAAAAAGAAAGATTGACTGAACAAAAACAGTTTGAAGAGCCAAATAATAAATAAATGATTTTGAATGTTTCTTTTAGTTATAAGGATAT
S V L L D K I L E K K D L T E Q K T V L E E P I I N K I D F E N V S F S Y K D I

1081
TAAAGTACTTGAAAAATTTAATTTTCTTTCGAAAAAATAAAAAATTTTAGTAAGTGCCCAATGGAACAGGAAAAATCAACCTTAATTAATTAATATCCGGGTTTATGATAATTA
K V L E N F N F S P E K N K K Y L V S A P N G T G K S T L I K L I S G V Y D N Y

1201
TCTTGGAAAAATTTAATTAATGATAGTTATAACCAAAAAGAAATGGATCAAAAAATTTAAGAAAACAAAATGGTTTATCGACAATCAGAATTAATTTTGAAGCAAGTCTCAAAA
L G K I L I N D S Y N Q K E L D Q K Y L R K Q I G F I D N Q N L I P E A S L E N

1321
TAACATCACTTGTGATGAAAAATCTGATTATAAAAAATTAATTAATCAATCTCAAAATCACTAAAAATGAATTTGATATTGAAGAAGAAATAGTCTAGCAAACTCTCAGAAGSTCA
H I T L F D E N P D Y K K L N S I L K S L K I E F D I E E E I S A S K L S E G Q

1441
AAAACAAAAAATGTTTTCGCCAGGCTTAGATATTCGCCGATCAAAATTTGACTAATCGATGAGGCTTTGATAATTAATAAAGAAATATTCACAAATTTAATTAGTACGCTGCTAGA
K Q K I V F A R L R Y S P I K F W L I D E A F D N I Q K E Y S Q I L I S Q L L S

1561
AAACCCCGAATTTACCGTGTGTTTTATTAGTCACCATATAAGCGAATGCAAAAAATTAATTTGATCAAAATTAATGAATTAAGCGGAAAAAATGAATAAACTAATTAATTACATC
RBS
H P E F T V V F I S H H I S E W Q K L K P D Q I I E L G G K K Y E STOP

ORF11-M N K L I K L N L

1681
TTTTAATGTTATCTACTCGGTTTTATTGTTTGTAGTAACTCTCTCAGTTATGCTAATAAATCTTATATCAGTCTTTGTTGAAAACGACCAAAATCAAAATGATAATTTGACTTT
F N V I Y S V F I F G L V T L S V N A N K F L Y Q S L V E N D Q I Q N I I W L S

1801
CAGCCGAAGCAGGCGCAATTTGATTAGTCTTTTTTGTCCGTAATCCATAATCTTTACTAACTCGAATTTTGGGCGAGGCGGCTTAAATTAATTAAGAAAAAATACAAATTT
A E A G A I L I S S F L S V I H N S L L T R I F G Q A G A L K L I K E K I Q I F

1921
TTAGTAATTTGACATATAAAGATATGTTAAAAATACACCAGGAAATTTTTCCTTATTTTCAATGAAGGTTTTAATACCGTGAATCATTTTTCCTTTTTTATTTTCATGTAATCT
S N L T Y K E Y V K N T P G N Y F S L P F N E G F N N G E S P F A F L F N V I S

2041
CGCTAATTTTCCAACTACTGCGATTTTAGGAATCTTTTTATATAAACCAATAATTTGGAGCTGTTATTATCGGGCTCAGACTGATTGGATCACTTTTCCGGTATTTTATAGAAAA
L I F P I T A I L G T I F Y I N P I I G A V I I G L T L I W I T P P V F F R K F

2161
TTTTAGTAGAAAAATAGCAGAGAGACTAAATTTGCTTGAAAAATTTCAATGCGGAATTTAGTGAAAAATTAATAAATTTGAAGCTTTTTTATTTTGGAAAAATTAATTTATTAATAA
F S R K I A E R L N L L E N F N G E F S E K L N K F E A F L P F G K I N L L E N

Fig. 3. For legend see facing page.

Fig. 3. Nucleotide sequence of 3788 bp of pMR7 located downstream of the 23S rRNA gene and predicted amino acids of the two ORFs. Potential -10 sequence promoters and ribosome-binding sites (RBS) are shown. Transcription terminators are indicated by stars. Underlined amino acid sequences represent putative membrane-spanning domains. Amino acid sequences marked by triangles represent binding motifs characteristic of ATP/GTP-binding sites.

above 3'OH end of the 16S rRNA. Similarly, the sequence AGGGGG, which precedes the ATG codon at nucleotide 1656 (ORF2), could pair with five bases at the 3' end of the 16S rRNA. Upstream of ORF1, two consensus sequences for -10 boxes of transcription promoters were located at nucleotides 26-31 and 31-36 (Fig. 3). They differed by only one nucleotide from the consensus sequence (TATAAT) characteristic of promoters recognized by eubacterial RNA polymerase functioning with the general σ factor. No classic consensus sequence for a -35 region was found.

An inverted repeat beginning at nucleotide 3308 may represent a Rho-independent terminator (Fig. 3). The terminator of the 23S rRNA gene, determined by Taschke & Hermann (1986) and located between two *Hind*III sites, was found between nucleotides 3364 and 3403 on the complementary strand (Fig. 3).

Analysis of the amino acid sequence encoded by ORF1 and ORF2

The putative protein product encoded by ORF1 (1661 bp) comprised 532 amino acid residues (PR1) and that by ORF2 (1613 bp) 536 residues (PR2). The predicted amino acid sequences of the two proteins were 26% identical and 55% similar. Database comparisons of the deduced amino acid sequences of PR1 and PR2 revealed significant similarities with proteins belonging to the ABC transporter family, and particularly with the eukaryotic multi drug resistance (MDR) proteins.

The amino acid sequence of PR1 protein showed 21% identity and 47% similarity with amino acid residues 701-1280 of mouse MDR3 protein (accession number P21447), and the PR2 peptide showed 23% identity and 50% similarity to amino acids 1-635 of MDR3. Amino acid residues 340-393 of the PR1 protein showed 30%

Fig. 4. Analysis of the PCR products obtained using HP1 or HP3 primers with different *M. hyopneumoniae* strains. Lanes: 1–9, amplified DNA fragments from strains J (ATCC 25934), J (NCTC 10110), JF 184, EP 29, BQ 14, EP-S 924, EP-S 938, EP-S 939 and J-LP (ATCC 25934), respectively; M, molecular mass standards (1 kbp DNA ladder). Sizes are indicated on the left in kbp.

identity and 60 % similarity with two regions of the MDR sequence comprising residues 400–486 and 1040–1200. Similarly, residues 339–431 of PR2 were 26 % identical and 56 % similar to the MDR sequences between amino acid residues 399–450 and 1093–1133. Two putative nucleotide-binding motifs characteristic of ATP/GTP-binding sites are present in the PR1 and PR2 proteins (shown by black triangles in Fig. 3), at amino acid positions 358–365 and 382–386, and 357–364 and 475–479 respectively (Fath & Kolter, 1993).

Comparison of PR1 and PR2 hydropathy plots with those of MDR proteins also revealed some similarities. The MDR proteins are characterized by several membrane-spanning α -helices present within hydrophobic domains (Gros *et al.*, 1986). Six and five putative membrane-spanning domains were found in the N-terminal halves of proteins PR1 and PR2, respectively (Fig. 3, amino acids underlined). All comprised 17 consecutive hydrophobic or uncharged residues. No signal peptide was found at the N-terminal end of the protein. The amino acid sequences at the C-terminal region of PR1 and PR2 were strongly conserved and correspond to ATP-binding sites.

Search for PR1 and PR2 proteins in various *M. hyopneumoniae* strains

PCR analysis was used to investigate whether all *M. hyopneumoniae* strains contain DNA coding for the PR1 and PR2 proteins. Two primers, HP1 and HP3, were selected using the I-141 DNA sequence to amplify a 1.6 kbp DNA fragment. PCR products from the DNA of different *M. hyopneumoniae* strains were then analysed on agarose gels (Fig. 4). According to the strain used an amplified DNA fragment of 1.6 or 1.8 kbp was obtained (Table 1).

With all field strains tested and one strain (ATCC 25934) obtained directly from ATCC and passaged less than 10 times in our laboratory (J-LP), a 1.6 kbp amplified DNA fragment of the same size as the I-141 DNA fragment was obtained (Fig. 4, lanes 4–9). However, DNA of strain J obtained from some other laboratories gave a 1.8 kbp amplified DNA fragment (Fig. 4, lanes 1 to 3).

Southern hybridization conducted with the restricted DNA from J strain ATCC 25934 provided by J. Frey (Fig. 2b) revealed that the I-141 probe hybridized with a 0.25 kbp *Hind*III DNA fragment instead of the 1.6 kbp fragment expected (lane 3). The I-141 probe also hybridized with a 4.8 kbp *Eco*RI DNA fragment (lane 2) and a 3.8 kbp *Eco*RI/*Bgl*II DNA fragment (lane 1). The 4.8 kbp *Eco*RI DNA fragment was replaced by a 7 kbp fragment in the J-LP strain (Fig. 2a) and in the field strains.

In view of the different sizes of the amplified DNA fragments, we cloned and sequenced the 1.8 kbp amplified DNA fragment.

Nucleotide sequence analysis of the 1.8 kbp amplified DNA fragment

Sequencing of the 1.8 kbp amplified DNA fragment (GenBank accession number U02538) revealed that the DNA region comprising nucleotides 1620 to 1824 was 100 % homologous to the 3' end of fragment I-141 (nucleotides 1407–1611). The other 1620 nucleotides were 100 % homologous to the 3' end of the 23S rRNA gene, including the terminator of the gene located between two *Hind*III sites. Surprisingly, sequence analysis also revealed that primer HP3 hybridized with a DNA fragment present on the 23S rRNA gene with only 55 % homology. This sequence (5'-AGTTTTTGTGTCGAGAGACG-3') is located 1280 nucleotides from the 23S terminator determined by Taschke & Hermann (1986). Nucleotide sequence alignment of the 1.8 kbp amplified DNA fragment, the I-141 DNA fragment and pMR7 of Taschke *et al.* (1986) containing *rrl*, indicates that 2221 bp have been deleted from the DNA of *M. hyopneumoniae* J strains provided by other laboratories (Fig. 1c). These *M. hyopneumoniae* strains lack genes coding for the putative ABC transporter proteins.

DISCUSSION

Intraspecies genomic polymorphism among field and reference strains of *M. hyopneumoniae* has been described previously (Frey *et al.*, 1992). Our studies have revealed genomic heterogeneity among *M. hyopneumoniae* field and culture collection strains. Differences have also been noted between strain J (ATCC 25934) received from ATCC and cultured for only a few passages in Friis medium in our laboratory (strain J-LP) and the same strain cultured elsewhere. Our results showed that some of the latter strains have lost a DNA fragment of 2221 bp in a region close to the terminator of the 23S rRNA gene. For *M. hyopneumoniae* J-LP, the DNA fragment upstream of the terminator of 23S rRNA had no ORF on the DNA strand coding for the 23S rRNA gene, but two over-

lapping ORFs on the complementary strand. These two ORFs were part of a single transcription unit.

The amino acid sequences of the two proteins, PR1 and PR2, encoded by the two ORFs, showed significant homology with eukaryotic and prokaryotic ABC transporters (Fath & Kolter, 1993). All ABC transporters have an ATP-binding domain and membrane-spanning domains (MSDs). Generally, bacterial ABC importers have the ATP-binding domain and MSDs on separate polypeptides, whereas bacterial ABC exporters can have these two components on the same or on different polypeptides. In the case of eukaryotic ABC transporters, the ATP-binding domain and MSDs are located on the same protein (Fath & Kolter, 1993).

The highest homology was found between PR1 or PR2 and the mouse multidrug resistance protein MDR3. Homology was also evident with the secretion protein HlyB of the RTX haemolysin of *E. coli* (Holland *et al.*, 1990) and the SpaB protein of *Bacillus subtilis* (Klein *et al.*, 1992). The homology between eukaryotic multidrug resistance proteins (P-glycoprotein or MDR) and bacterial transport proteins has been described previously (Gros *et al.*, 1986). The P-glycoprotein of eukaryotic cells is a large polypeptide consisting of four domains equivalent to the two hydrophobic and the two ATP-binding components of a bacterial periplasmic transport system (Higgins *et al.*, 1990). Gros *et al.* (1986) reported that the homology between MDR proteins and the bacterial transport proteins occurred within the internal duplication of the MDR in the region of greatest conserved homology (residues 400–600 and 1000–1250 of MDR). These sequences include the highly conserved ATP-binding cassette which is the most characteristic feature of the ABC transporter superfamily. Each half of a MDR protein is similar to a single bacterial transport protein, suggesting that the gene has evolved from a common ancestor by gene duplication or gene fusion events (Lincke *et al.*, 1991; Croop, 1993).

In PR1 or PR2, the ATP-binding domain and MSDs are present on the same polypeptide. Thus, PR1 together with PR2 could act as the entire MDR protein or as two bacterial transporters.

Homology between proteins of ABC transporter systems and mollicute proteins has been described previously, in particular for *Mycoplasma hyorhinis*, a frequently isolated pathogenic mycoplasma (Dudler *et al.*, 1988; Gilson *et al.*, 1988). Three proteins encoded by a single operon, showed similarities with proteins of periplasmic binding-protein-dependent transport systems of Gram-negative bacteria. In our case, PR1 and PR2 also showed homology with the *E. coli* haemolysin export protein, HlyB.

These results were unexpected because mycoplasmas are known to be more related to Gram-positive bacteria than to Gram-negative bacteria (Weisburg *et al.*, 1989). Secretion by mycoplasmas involves passage through only one membrane, the plasma membrane, and resembles more closely the process in Gram-positive bacteria and eukaryotic cells than in Gram-negative bacteria where two membranes are involved. When high levels of

homology are observed over large evolutionary distances, strong functional relationships can usually be identified.

ABC transporter proteins are involved in import or export functions of a large variety of substrates including sugars, peptides, proteins and toxins (Higgins, 1992). The MDR proteins are often involved in the export of drugs and detoxification processes. The HlyB protein of *E. coli* (together with HlyD) is involved in the export of a haemolysin protein. Haemolysin activity has been examined in a variety of mycoplasmas but in comparison to species such as *Mycoplasma pulmonis* and *Acholeplasma laidlawii*, activity in *M. hyopneumoniae* was insignificant (Minion & Jarvill-Taylor, 1994).

The fact that some culture collection strains do not possess the gene coding for PR1 and PR2 is interesting. These strains were not grown in our laboratory, but such strains are routinely cultivated and often subcloned. Thus, the number of passages, or selection of clones by subtyping could explain such difference amongst strains. Strain J maintained *in vitro* for 60 passages was unable to cause pneumonia in pigs (Zielinsky & Ross, 1990). Moreover, the capacity of *M. hyopneumoniae* to induce epithelial damage in porcine tracheal organ culture diminished with *in vitro* passage (Debey & Ross, 1994). Attenuation of virulence through successive *in vitro* passage has similarly been described for *M. pulmonis* (Taylor-Robinson *et al.*, 1981) and *M. pneumoniae* (Collier *et al.*, 1985). Such loss of virulence arising from a lack of selective pressure (Ott, 1993) may lead to the identification of the genes involved in pathogenicity. Further experiments are needed to determine the role of PR1 and PR2 and whether their absence is correlated with lack of virulence.

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