Expression of *Mycoplasma pulmonis* Antigens in *Escherichia coli*

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The expression of *Mycoplasma pulmonis* antigen in *Escherichia coli* was investigated by cloning genomic DNA derived from *M. pulmonis* m 53, and the DNA fragment participating in antigen expression was identified. When the DNA library of *M. pulmonis* was screened by colony immunoassay using anti-*M. pulmonis* serum, 10 recombinant clones expressing seroreactive antigens were obtained. The recombinant plasmids isolated from these clones included 3.7~6.5 kilobase pair (kbp) DNA inserts, while all clones contained a common 2.3-kbp DNA fragment. Subcloning of initial DNA inserts showed that the common 2.3-kbp fragment is essential for antigen expression. Moreover, antiserum against the recombinant antigen generated from the 2.3-kbp DNA fragment recognized a native *M. pulmonis* antigen. The reactivity of this antiserum was absorbed specifically with *M. pulmonis*. These results suggest that the cloned 2.3-kbp DNA fragment codes an antigen specific to *M. pulmonis*.

In the previous study [5], we analyzed genomic DNA of typical rodent mycoplasmas, *Mycoplasma pulmonis*, *M. arthritidis* and *M. neurolyticum*, and demonstrated that the DNA sequences of these species possess a high degree of species specificity. In addition, it was revealed that genomes of *M. pulmonis* strains are closely related [5, 12]. These findings strongly suggest the existence of DNA fragments that are specific to *M. pulmonis* species. Therefore, the authors attempted to clone a DNA fragment which codes a species-specific antigen of *M. pulmonis* in order to isolate a DNA fragment specific to this species. In this paper, we report the characteristics of *M. pulmonis* antigen expressed in *Escherichia coli* by the recombinant DNA technique and the characteristics of the DNA fragment which codes this antigen.

Materials and Methods

Organisms and culture conditions: *M. pulmonis* m 53, *M. arthritidis* PG 6, and *M. neurolyticum* Type A were used for DNA extraction and antigen preparation. The sources and culture conditions have been described previously [5]. *E. coli* JM 109 was cultured in LB medium [7] at 37°C, and used as the recipient for the plasmid vector pUC 19 (Takara Shuzo Co., Kyoto, Japan) [15].

Preparation of antisera: Anti-*M. pulmonis* serum was produced in a rabbit (Japanese white, female) by biweekly intravenous injections of sonicated *M. pulmonis* antigens. Three hundred micrograms of protein was used for each injection. Antiserum taken after the third injection was absorbed for 12 hr at 4°C with intact *E. coli* JM 109 carrying pUC 19 at a concentration of 1×10^{11} *E. coli* cells per ml of

serum, before it was used for colony immunoassay and Western blot analysis.

Antiserum against recombinant antigens was produced in a rabbit (Japanese White, female) by immunization with an ammonium sulphate precipitate of a lysate from the recombinant *E. coli* clone. The *E. coli* cells were suspended in 0.1 M carbonate-bicarbonate buffer (pH 9.8), incubated at 37°C for 20 min, then sonicated. After precipitation with 67% ammonium sulphate, the precipitates were dissolved in phosphate buffered saline (PBS) and dialyzed with PBS. A rabbit was immunized with biweekly intravenous injections of 200 µg of protein. After the fourth injection, the antiserum was taken, purified with an affinity column prepared by coupling *M. pulmonis* lysate to Sepharose 4 B (Pharmacia, Sweden), then absorbed with intact *E. coli* carrying pUC 19 at a concentration of 1×10^{10} *E. coli* cells per ml of serum for 12 hr at 4°C.

All animals used for immunization were found to be free of antibodies against *M. pulmonis*, *M. arthritis*, and *M. neurolyticum* when their preimmune sera were examined by an enzyme-linked immunosorbent assay using protein A (P-ELISA) [10].

Construction of the DNA library : Genomic DNA from *M. pulmonis* was extracted and purified as previously described [5]. *M. pulmonis* DNA was partially digested with *Hind*III (Nippon Gene Co., Toyama, Japan) to an average size of 3 to 9 kilobase pairs (kbp), then ligated to plasmid vector pUC 19 with T4 DNA ligase (Takara Shuzo). Before the ligation, the vector DNA was digested with *Hind*III and dephosphorylated with bacterial alkaline phosphatase (Takara Shuzo). *E. coli* JM 109 cells were transformed with the ligation mixture by the CaCl_2 procedure [7]. Recombinant transformants were selected on LB agar plates containing 50 µg of ampicillin per ml, 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and 40 µg of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) per ml.

Colony immunoassay : To screen the clones producing *M. pulmonis* antigens, colony immunoassay was performed by a modification of the method of Helfman et al. [2]. White colonies carrying recombinant plasmids were replica plated on LB agar containing ampicillin, IPTG and X-gal. After incubation overnight at 37°C, the colonies were transferred to nitrocellulose

filters (Toyo Co., Tokyo, Japan) and lysed with chloroform vapor. These filters were immersed in a blocking solution [50 mM Tris-HCl, pH 7.5/150 mM NaCl/5 mM MgCl_2 /1% bovine serum albumin (BSA)/1 µg of deoxyribonuclease I (Sigma Chemical Co., Louis, Mo.) per ml/40 µg of lysozyme (Sigma Chemical Co.) per ml] for 1 hr at 37°C, rinsed in PBS, and incubated for 1 hr at 37°C with anti-*M. pulmonis* serum (1 : 200 dilution in PBS containing 0.1% BSA). The filters were washed 3 times with PBS and reacted with horseradish peroxidase-conjugated protein A (HRPO-protein A) for 1 hr at 37°C. After being washed 3 times with PBS, the filters were developed in substrate buffer (50 mM Tris-HCl, pH 7.2/0.3 mg of 3,3'-diaminobenzidine per ml/0.03% H_2O_2).

Western blot analysis : Transformants and mycoplasmas were solubilized by boiling for 5 min in 2% sodium dodecyl sulfate (SDS)/5% 2-mercaptoethanol/10% glycerol/62.5 mM Tris-HCl, pH 6.8. Samples containing 15 µg of proteins were electrophoresed (20 mA/gel, 90 min) on a 12% polyacrylamide gel according to the method of Laemmli [6]. Proteins separated on the gel were transferred electrophoretically to a nitrocellulose filter (42 V, 2 hr) by the method of Towbin et al. [11]. After treatment with PBS containing 3% BSA overnight at 4°C, the blots were reacted with anti-*M. pulmonis* serum or anti-recombinant antigen serum. Antibody binding was detected with HRPO-protein A as described for colony immunoassay.

DNA analysis of recombinant clones : The restriction fragments of the recombinant plasmids were analyzed by agarose gel electrophoresis. Plasmid DNA was isolated from the recombinant *E. coli* by the alkaline lysis method [1] and digested with the appropriate restriction endonuclease. The digested DNA was electrophoresed on a 1% agarose gel in 40 mM Tris-acetate/1 mM ethylene diamine tetraacetate, pH 8.0. DNA fragments in the agarose gel were visualized by ethidium bromide staining. *Hind*III-digests of λ phage DNA (Pharmacia) were used for DNA size markers.

The 2.3-kbp *Hind*III-fragment isolated from a recombinant plasmid was used as a probe for Southern hybridization. The probe was recovered from a low-meltingpoint gel by the method of Wieslander [13] and labeled with [α - ^{32}P]

deoxy cytidine triphosphate by the multiprime DNA labeling system (Amersham, England). Labeled probe was denatured by boiling for 5 min and quenching on ice, then used immediately for hybridization. *Hind*III-digests of recombinant plasmid DNA separated in an agarose gel were transferred to a nitrocellulose filter by the method of Southern [9]. The filter was hybridized with 5 ng/ml of 32 P-labeled probe, washed, and autoradiographed as described previously [5].

Subcloning was carried out by *Hind*III-digestion of the initial clones, followed by self-ligation. The subclones were analyzed for DNA inserts by agarose gel electrophoresis and for *M. pulmonis* antigen expression by Western blotting as described above.

Absorption of anti-recombinant antigen serum: Anti-recombinant antigen serum was absorbed with intact organisms of *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, recombinant *E. coli*, and control *E. coli* carrying pUC 19 at a concentration of 2 mg (wet weight) of organisms per ml of serum for 12 hr at 4°C. After incubation, the supernatant was tested by P-ELISA using lysed *M. pulmonis* as an antigen for detection.

Results

Isolation of recombinant clones: It was shown by preliminary experiments that *M. pulmonis* DNA contained numerous restriction sites for *Hind*III. Therefore, the DNA library was constructed by inserting *M. pulmonis* DNA partially digested with *Hind*III into the plasmid vector pUC 19. Approximately 3×10^3 transformants were obtained, and about 85% of the clones were found to be recombinants by inactivation of β -galactosidase. After screening for the expression of *M. pulmonis* antigens by colony immunoassay using anti-*M. pulmonis* serum, 10 clones which showed a strong positive signal were selected.

Characterization of *M. pulmonis* antigens produced in recombinants: Western blot analysis was employed to characterize the *M. pulmonis* antigens produced in recombinant clones. The antigens reacting with anti-*M. pulmonis* serum were detected in the lysates of all, 10 recombinants (Fig. 1). Seven clones (clones 1, 2, 3, 6, 7, 9, and 10) produced the 45-kilodalton(kDa) and 42-kDa antigens, 2 clones

(clones 4 and 5) produced the 42-kDa and 37-kDa antigens, and 1 clone (clone 8) produced the 43-kDa and 40-kDa antigens reacting strongly. None of the components that react with this antiserum was detected in the lysate of control *E. coli* carrying nonrecombinant pUC 19. These recombinant antigens were also produced in the absence of IPTG.

Analysis of cloned *M. pulmonis* DNA fragments: The plasmid DNA isolated from the 10 clones producing the *M. pulmonis* antigens were digested with *Hind*III, and the size of DNA inserts was determined by agarose gel electrophoresis (Fig. 2). These clones contained the 3.7 to 6.5-kbp DNA inserts and all clones carried the 2.3-kbp *Hind*III-fragment. The 1.4-kbp *Hind*III-fragment was also included in the

Fig. 1. Western blot analysis of recombinant antigens expressed in *E. coli*. The blots of whole cell lysates from control *E. coli* carrying pUC 19 (lane B), recombinant *E. coli* clones 1-10 (lane C-L), and *M. pulmonis* (lane M) were reacted with anti-*M. pulmonis* serum. Lane A shows the molecular weight standards.

7 clones that produced the 45-kDa and 42-kDa recombinant antigens, and the 1.6-kbp *Hind*III-fragment was inserted into the 3 clones that produced recombinant antigens of a slightly smaller size than the above-mentioned 7 clones.

Partial restriction mapping and subcloning: Partial restriction mapping was conducted on the 3 clones (clones 3, 5, and 8) that produced recombinant antigens of different molecular weights (Fig. 3-1, 2 and 3). *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, and *Xba*I were used for restriction endonuclease digestion, but DNA inserts of these 3 clones did not contain *Bam*HI, *Kpn*I, *Pst*I, *Sal*I, and *Sma*I sites. The 2.3-kbp *Hind*III-fragments in all 3

Fig. 2. *Hind*III restriction analysis of recombinant plasmid DNA Lane A: *Hind*III-digests of λ phage DNA as size markers Lane B: pUC 19 DNA digested with *Hind*III Lanes C-L, recombinant plasmid DNA from clones 1-10 digested with *Hind*III. Each sample was electrophoresed on a 1 % agarose gel and stained with ethidium bromide.

Fig. 3. Partial restriction maps of original clones and subclones expressing recombinant antigens. 1, clone 3; 2, clone 5; 3, clone 8; 4 and 5, subclones inserted the same 2.3-kbp DNA fragment in opposite orientation. E, *Eco*RI; H, *Hind*III; S, *Sac*I; X, *Xba*I. Restriction sites present in the vector DNA are shown within boxes. The molecular weights of recombinant antigens expressed by each clone are shown in the right column.

Fig. 4. Southern hybridization of the 2.3-kbp DNA insert (A): The plasmid DNA isolated from recombinant clones 3, 5 and 8 (lanes 1, 2, and 3, respectively) was digested with *Hind*III and electrophoresed on a 1 % agarose gel. Lane M contains *Hind*III-digests of λ phage DNA as size markers. (B): The DNA bands were transferred to a nitrocellulose filter and probed with 32 P-labeled 2.3-kbp DNA insert from clone 3.

clones contained the identical *Eco*RI site. In clones 5 and 8, a 3.9-kp DNA fragment containing the same restriction sites was inserted in the opposite orientation. The 2.3-kbp *Hind*III-fragment from clone 3 was used as a probe for Southern hybridization against the *Hind*III-digested plasmid DNA from clones 3, 5, and 8. It was found that the 2.3-kbp inserts in these clones were identical (Fig. 4).

Furthermore, to identify the DNA fragments coding the recombinant antigens, *Hind*III-digested initial inserts of clones 3, 5, and 8 were subcloned. All of the subclones containing a 2.3-kbp *Hind*III-fragment from each clone produced recombinant antigens, and the molecular weights of these antigens were the same as those produced in clone 5 or 8, depending on the direction of the DNA insert (Fig. 3-4 and

Fig. 5. Western blot analysis of a native *M. pulmonis* antigen using anti-recombinant antigen serum. The blots of whole cell lysates from control *E. coli* carrying pUC19 (lane 2), recombinant *E. coli* carrying the 2.3-kbp *M. pulmonis* DNA (lane 3), and *M. pulmonis* (lane 4) were reacted with antiserum against the recombinant antigens generated from the 2.3-kbp *M. pulmonis* DNA. Lane 1 shows the molecular weight standards.

5). On the other hand, in the subclones in which a 1.4-kbp or 1.6-kbp *Hind*III-fragment was inserted, the expression of recombinant antigens was not detected.

Identification of native *M. pulmonis* antigens related to recombinant antigens: In order to identify the native *M. pulmonis* antigens related to the recombinant antigens generated from the 2.3-kbp *Hind*III-fragment, Western blot analysis was conducted by using antiserum against the recombinant antigens expressed in *E. coli* carrying the 2.3-kbp insert (Fig. 5). This antiserum reacted specifically with the recombinant antigens, but did not react with the control *E. coli* carrying nonrecombinant pUC 19. A 70-kDa antigen of *M. pulmonis* was recognized by this antiserum.

Further, in order to confirm the specificity of the recombinant antigens, an absorption test was conducted. As shown in Table 1, the reactivity of anti-recombinant antigen serum was significantly reduced after absorption with *M. pulmonis* and recombinant *E. coli* carrying the 2.3-kbp insert, but after absorption with *M. arthritidis*, *M. neurolyticum* and control *E. coli* carrying pUC 19, there was no change in this reactivity.

Discussion

To obtain the DNA fragment which codes an antigen specific for *M. pulmonis*, genomic DNA derived from *M. pulmonis* m 53 was cloned and screened for antigen expression in *E. coli*. As a result, recombinant clones which reacted specifically with anti-*M. pulmonis* serum were obtained, and in these clones, the seroreactive antigens seemed to be expressed from *M. pulmonis* DNA fragments inserted in

Table 1. Effect of absorbing anti-recombinant antigen serum with intact organisms on ELISA¹⁾

Treatment of serum	Inhibition (%) ²⁾
(-)	0
<i>M. pulmonis</i>	77.6
<i>M. arthritidis</i>	3.9
<i>M. neurolyticum</i>	1.1
Recombinant <i>E. coli</i> ³⁾	74.5
Control <i>E. coli</i> ⁴⁾	3.4

1) Lysed *M. pulmonis* (10 μ g of protein per ml) was used as ELISA antigen

2) Inhibition(%) = $\frac{\text{OD value of unabsorbed serum} - \text{OD value of absorbed serum}}{\text{OD value of unabsorbed serum}} \times 100$

3) *E. coli* carrying the 2.3-kbp *M. pulmonis* DNA

4) *E. coli* carrying nonrecombinant pUC19

a plasmid vector. Regarding the expression of mycoplasmal antigens in *E. coli*, both successes and failures in autonomous expression by mycoplasmal promoters have been reported [3, 4, 14]. In this study, *M. pulmonis* antigens were expressed in *E. coli* even without a lac inducer, IPTG. This finding indicates that the promoters of *M. pulmonis* can also function in *E. coli*.

In order to identify the DNA fragment coding the *M. pulmonis* antigens expressed in *E. coli*, DNA inserts of the recombinant plasmids were analyzed. The results of restriction digestion and Southern hybridization indicated that the recombinant plasmids contained a 2.3-kbp DNA fragment as a common sequence. In addition, the examination for *M. pulmonis* antigen expression in subclones showed that recombinant antigens were produced only in *E. coli* carrying the 2.3-kbp DNA fragment, which seems to be an essential fragment for antigen expression.

In addition, the specificity of the antigen coded by the 2.3-kbp DNA fragment was analyzed by using antiserum against the recombinant antigen. Antiserum which reacted specifically with the recombinant antigen expressed in *E. coli* also reacted with the 70-kDa component in *M. pulmonis* lysate. Therefore, the recombinant antigen was considered to be related to a native *M. pulmonis* antigen. Moreover, although serological cross-reactivity between *M. pulmonis* and *M. arthritidis* has been noted [8], antibodies to the recombinant antigen were specifically absorbed by *M. pulmonis* but were not absorbed by *M. arthritidis* and *M. neurolyticum*. These results suggest that the recombinant antigen expressed from the 2.3-kbp fragment of *M. pulmonis* DNA has specificity for *M. pulmonis*.

In conclusion, we succeeded in obtaining a 2.3-kbp DNA fragment coding the species-specific antigen of *M. pulmonis*. This DNA fragment may be used by itself as a DNA probe for the detection and identification of *M. pulmonis*.

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References

- [1] Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, **7**, 1513-1523.
- [2] Helfman, D. M., Feramisco, J. R., Fiddes, J. C., Thomas, G. P., and Hughes, S. H. (1983). Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. *Proc. Natl. Acad. Sci. USA* **80**, 31-35.
- [3] Kawauchi, Y., Muto, A., Yamao, F., and Osawa, S. (1984). Molecular cloning of ribosomal protein genes from *Mycoplasma capricolum*. *Mol. Gen. Genet.*, **196**, 521-525.
- [4] Klinkert, M.-Q., Herrmann, R., and Schaller, H. (1985). Surface proteins of *Mycoplasma hyopneumoniae* identified from an *Escherichia coli* expression plasmid library. *Infect. Immun.*, **49**, 329-335.
- [5] Kunita, S., Terada, E., Ghoda, A., Takakura, A., and Kagiya, N. (1989). Genomic DNA analysis of rodent mycoplasmas. *Exp. Anim.*, **38**, 201-206.
- [6] Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680-685.
- [7] Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning, In a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- [8] Minion, F. C., Brown, M. B., and Cassell, G. H. (1984). Identification of cross-reactive antigens between *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. *Infect. Immun.*, **43**, 115-121.
- [9] Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503-517.
- [10] Takakura, A., Kagiya, N., Sakurai, Y., Suzuki, H., Kobayashi, N., and Terada, E. (1988). Detection of Sendai virus antibody in mouse and guinea pig sera by an enzyme-linked immunosorbent assay with protein A. *Exp. Anim.*, **37**, 279-283.
- [11] Towbin, H., Starhelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
- [12] Watson, H. L., Davidson, M. K., Cox, N. R., Davis, J. K., Dybvig, K., and Cassell, G. H. (1987). Protein variability among strains of *Mycoplasma pulmonis*. *Infect. Immun.*, **55**, 2838-2840.
- [13] Weislander, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal. Biochem.*, **98**, 305-309.
- [14] Wise, K. S. (1984). Antigen expression from cloned genes of *Mycoplasma hyorhinis*: an approach to mycoplasma genomic analysis. *Isr. J. Med. Sci.*, **20**, 754-757.
- [15] Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. *Gene*, **33**, 103-119.

Mycoplasma pulmonis 抗原の大腸菌における発現

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Mycoplasma pulmonis m53 株由来のゲノム DNA をクローニングし、大腸菌における *M. pulmonis* 抗原の発現ならびに抗原発現に關与する DNA 断片の同定を行った。抗 *M. pulmonis* 血清を用いたコロニー・イムノアッセイにより *M. pulmonis* の DNA ライブラリーをスクリーニングしたところ、本抗血清と反応する抗原を発現している10個のクローンが得られた。これらのクローンから分離された組換えプラスミドには3.7-6.5 キロベースペア (kbp) の DNA が挿入されており、す

べてのクローンが2.3-kbp の DNA 断片を有していた。挿入 DNA をサブクローニングしたところ、共通に存在する2.3-kbp の DNA 断片が抗原発現には不可欠であることが判明した。また、2.3-kbp の DNA から発現した組換え抗原に対する抗血清は *M. pulmonis* 由来の抗原を認識し、その反応性は *M. pulmonis* によって特異的に吸収された。以上の成績より、クローン化された2.3-kbp の DNA 断片は *M. pulmonis* に特異的な抗原をコードしていることが示唆された。