R1 Region of P97 Mediates Adherence of Mycoplasma hyopneumoniae to Swine Cilia

F. CHRIS MINION,* CARY ADAMS, AND TSUNGDA HSU†

Department of Veterinary Microbiology and Preventive Medicine, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa 50011

Received 23 November 1999/Returned for modification 11 January 2000/Accepted 27 January 2000

Adherence of Mycoplasma hyopneumoniae to the swine respiratory tract is mediated by the membrane protein P97. This protein is located on the outer membrane surface, and its role in adherence has been firmly established. The general region of P97 that mediates adherence to swine cilia is thought to be the R1 region near the carboxy terminus of the protein, but it was not clear if this region could mediate adherence to swine cilia independently of other P97 sequences. To examine this in more detail, a series of R1 repeat sequences containing different numbers of repeating units cloned in frame with lacZ was used to produce R1–β-galactosidase fusion proteins. These proteins were then tested for adherence to swine cilia and for reactivity to the adherence-blocking monoclonal antibody F2G5 and convalescent-phase swine sera. In this way it was possible to accurately define the cilium binding epitope of P97 and the minimal epitope recognized by antibody. Our results indicate that eight R1 repeating units are required for cilium binding and that three repeating units are needed for antibody recognition. These results could lead to more effective therapeutic measures against this important swine pathogen.

Attachment to host tissue is essential for colonization by most mucosal pathogens, including mycoplasmas (1, 2). The best-defined mycoplasmal adhesins, those of Mycoplasma pneumoniae and Mycoplasma genitalium, are found in a specialized attachment organelle (8). It is clear from numerous studies that their adhesins work in concert with other membrane proteins and accessory factors in a coordinated fashion to form the attachment organelle structure (8). Thus, the process of adherence in these species is multifactorial, requiring both membrane proteins and cytoskeletal elements (8). Most mycoplasmas, however, lack specialized attachment organelles like M. pneumoniae, and consequently, their adherence mechanisms differ. How most mycoplasma species adhere to their respective host tissues is largely unknown, and the genetic mechanisms of adherence are poorly defined.

As the etiological agent of porcine mycoplasmal pneumonia and a major component of the porcine respiratory disease complex, Mycoplasma hyopneumoniae continues to present significant problems for the swine industry. As is the case for many other mycoplasmal species, it is host specific (for swine), it has no readily apparent external structures or organelles that could be used for attachment, there are no genetic systems for analyzing the adherence phenotype, and studies of adherence have been largely qualitative rather than quantitative in nature. For instance, M. hyopneumoniae closely adheres to swine respiratory epithelium both in vivo and in vitro (3, 9). It has also been demonstrated that M. hyopneumoniae can attach to pig and human lung fibroblasts, to pig kidney cells in culture (15), and to turkey red blood cells (12).

Our interests in adherence mechanisms as the prelude to colonization and disease led us to take advantage of the available adherence-blocking monoclonal antibodies (MAbs) (14) and a cilium adherence assay (CAA) (13) to address adherence mechanisms at the molecular level through a heterologous in vitro system. The cloning and analysis of the P97 structural gene (5) and analysis of nearby genes in the chromosome (7) have been accomplished. DNA sequence analysis revealed two repeat regions near the 3′ end of the P97 gene, the R1 and R2 regions (5). It was clear from previous studies using transposon mutagenesis and β-galactosidase fusion proteins that the R1 region participated in the adherence to swine cilia (6). In these studies, the R1 region was cloned upstream of lacZ, producing a recombinant R1–β-galactosidase fusion protein capable of binding to swine cilia with same specificity as intact cells. These studies demonstrated the potential of using fusion proteins to study the functional regions of P97. The R1 region PCR fragment used in these cloning experiments, however, contained an additional 78 bp upstream of the R1 region because of PCR primer design limitations. Transposon mutagenesis had completely ruled out the need for amino acids downstream of the R1 region (6), but it was not clear without more precise analysis if upstream sequences were important in the structure of a functional binding epitope. These additional 26 amino acids in the translation product could have an important role in the cilium binding epitope. Here we report studies using a different approach that further defines the binding epitope of P97 to swine cilia.

The difficulty in cloning the region cleanly in the original studies was due to the nature of the AT-rich mycoplasmal genome and the lack of good PCR primer binding sites. Thus, it was decided to construct the region systematically, one repeat unit at a time, using the same β-galactosidase fusion strategy employed earlier (6). This strategy had several advantages over the previous approach of direct cloning. It would allow us to precisely identify the amino acids and the number of repeat units needed for cilium binding. It would unambiguously confirm the structure of the antigenic epitope recognized by the adherence-blocking MAbs. It would also provide a way to perform site-directed mutagenesis for a detailed in-depth analysis of the binding epitope.
Bacterial strains and growth. Escherichia coli strain GM124 (CGSC 6498) [lacZ118(0c) rpsL725 dam-4] was used throughout this study as both the cloning and expression host. This strain was Lac^- to allow for scoring in-frame lacZ fusions during plasmid constructions. It was also Dam^- so that plasmids isolated from this strain could be digested with the dam methylation-sensitive restriction enzyme BspEI.

Construction of R1-β-galactosidase gene fusions. Our overall strategy was to construct different numbers of R1 repeating units fused to β-galactosidase so that cilium binding experiments could be performed with the fusion proteins. The approach needed to construct the R1 region was not immediately apparent, however, because of the amino acid sequence variability in the R1 region expressed as a charged-noncharged alternating motif and the substitution of threonine for alanine in the fourth repeat unit (Fig. 1). Thus, it was necessary that the repeat region be constructed one unit at a time with the option of altering the sequence within each new unit while maintaining the exact amino acid sequence of the wild-type R1 region. Our approach combined construction of the two different repeat units found in the R1 region with ligation of purified blunt-ended digestion fragments to construct the R1 region systematically. Since it was necessary to allow for the use of the repeat unit both as an additive element in further plasmid constructions and as a final lacZ fusion, it was necessary to construct two derivatives of each repeat region subset. One of these contained an additional cytosine to bring the final gene fusion into frame with the lacZ reading frame.

The backbone for all plasmid constructions was plasmid pMLB1107, a unique cloning vector that provided the lacZ sequence for the gene fusions, an isopropyl thio-β-D-galactopyranoside inducible promoter, and lacF^ for controlling gene expression (6). The R1-β-galactosidase fusions were constructed with PCR products obtained using pMLB1107 template DNA. The PCR primers used in the construction of these plasmids are shown in Table 1. These primers were designed to accommodate the codon usage of E. coli rather than that of mycoplasmas. Initially, plasmids with one to four repeating units, i.e., plasmids pISM1301.1, pISM1302.1, pISM1303.1, pISM1304.1, and pISM1304.2, were constructed by generating left and right plasmid halves by PCR which were then ligated. Plasmids pISM1301.3, pISM1301.4, pISM1301.5, and pISM1301.6 were also constructed in this fashion, but they included additional restriction sites to facilitate the second round of cloning. This second set of four plasmids included the valine and glutamic acid codon-containing repeat units and corresponding constructs with an additional cytosine at the R1-lacZ junction to bring lacZ into the proper reading frame.

The PCR conditions used to generate the fragments were as follows. The primer pairs TH148-TH150 and TH154-TH157 were used to generate fragments representing the left and right halves, respectively, of the plasmid pISM1301.3 (Fig. 1B; Tables 1 and 2). The primer mixture for the left fragment contained 25 pmol of each phosphorylated primer, 2.5 mM MgCl2, 2.5 U of Pfu polymerase (Stratagene), 1× Stratagene Pfu polymerase buffer, 0.2 mM deoxynucleoside triphosphates, and 200 pg of template DNA in a total volume of 100 µl. The reaction conditions were denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 0.5 min, renaturation at 56°C for 0.5 min, and extension at 72°C for 8 min; and a final 14-min extension step at 72°C. The primer mixture for the right fragment was the same except that no additional MgCl2 was added. The reaction conditions for the right fragment were denaturation at 94°C 3 min; 5 cycles of denaturation at 94°C for 0.5 min, renaturation at 58°C for 0.45 min, and extension at 72°C for 7.5 min; 30 cycles of denaturation at 94°C for 0.5 min and extension at 68°C for 8 min; and a final extension at 72°C for 14 min. The fragment from each PCR was digested with restriction enzymes prior to gel purification, ligation, and transformation. For left fragments, the fragments were digested with SmaI and BspEI; for right fragments, the enzymes SnaBI (for plasmids with regions containing an initial valine codon) or BsrBI (for plasmids containing an initial glutamic acid codon) and BspEI were used.

The second set of four plasmids was used in other plasmid constructions by ligating left and right fragments from these and subsequent plasmid constructions to form functional R1-lacZ fusions with differing numbers of repeating units (Table 2). To streamline the cloning procedure, most of the plasmids required SnaBI digestion of the PCR fragment to generate a blunt end (Table 2). The BsrBI digestion, filling in, and mung bean digestion were used only in construction of plasmids 1301.5 and 1301.6. Because of the sensitivity of restriction enzyme BspEI to dam methylation, the ligation mixtures were transformed into E. coli GM124, which is phenotypically Lac^- Dam^- . In this way, plasmids could be isolated from this background and used directly for the next round of cloning or scored for the Lac^- phenotype on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing media. The left and right fragments were obtained by restriction digestion of each plasmid shown in Table 2 followed by purification of the appropriate fragment. The variation in the fourth repeat unit in pISM1304.1 and pISM1304.2 was introduced by producing the right fragment by PCR. Cloning of the repeat regions required joining of blunt ends within the repeat region and a BsrBI site within the vector region of the plasmid (Fig. 1C). The addition of each repeat unit was monitored by the size of the HindIII-EcoRV digestion product, and each final construct was sequenced through the repeat region to ensure that no errors had been introduced during the cloning process.

CAA. The CAA has been described previously (6, 13). Lysates from induced E. coli cultures containing different R1-β-galactosidase fusions were prepared, and the β-galactosidase activity and protein content were measured. The assay was modified to normalize the units of β-galactosidase activity added to each well (20 units per well) as determined by the method of Miller (10). In this way the assay was more reproducible than it would have been by adding a standard amount of protein as in previous studies (6) (data not shown). Each lysate was tested in triplicate, and the experiment was repeated twice. Figure 2 shows the results of two cilium binding experiments using the series of R1-lacZ fusion plasmids. β-Galactosidase had no affinity for swine cilia, as demonstrated by its lack of binding in the Lac^- control. This was also observed in previous studies (6). There was also little cilia binding of the R1-β-galactosidase fusions with seven repeating units or fewer. Binding was not significant until there were at least eight repeating units. This was reproducible between the two experiments shown and in other experiments not shown.

Immunoblot analysis. In addition to the need to define the repeat structure necessary for swine cilia adherence, it was also useful to determine the size of the R1 repeat sequence required to form the antigenic epitope recognized by adherence-blocking MAbs. This was accomplished by developing immunoblots of the E. coli lysates containing the different R1–β-galactosidase fusion proteins with MAb F2G5 (14) and with anti-β-galactosidase antibodies. The results of the immunoblot analysis are shown in Fig. 3. F2G5-immunoreactive bands were observed with three, four, and six repeating units but not with one or two repeating units (Fig. 3A). To confirm that β-galactosidase fusion protein was present in each lane, an identical blot was reacted with anti-β-galactosidase antibodies.
FIG. 1. Strategy for the construction of R1-lacZ fusion plasmids. (A) Amino acid sequence of the R1 region of P97. The underline indicates the substitution of threonine for alanine in the fourth repeat unit. (B) Construction of plasmids pISM1301.1, pISM1301.3, pISM1301.4, pISM1301.5, and pISM1301.6 using PCR-generated fragments. These plasmids served as sources for the left and right fragments for other plasmid constructions. Where appropriate, plasmid designations are given as examples. In step 1, using pMLB1107 template DNA, PCR was performed with different primer sets (Tables 1 and 2) to obtain left and right fragments for cloning. Two different primers were used at the R1-lacZ junction site; one primer (GGG) maintained the reading frame within the repeating units. These plasmids were used only in the construction of the R1 region with various numbers of repeating units. During the addition of the last repeating unit for final Lac<sup>+</sup> constructs, the second primer (CGGG) altered the final frame of the lacZ coding sequence to align it with the upstream R1 region. In step 2, the fragments were digested and purified. In step 3, the purified fragments were ligated to form the starting plasmids for the R1-lacZ fusions. (C) Construction of multiple repeating units fused to β-galactosidase (β-gal). The plasmids were constructed consecutively, a repeat at a time, in order to have the starting fragments for the next plasmid construction. In step 4, for each step, the appropriate plasmids (Table 2) were digested, and the individual fragments were purified. In some cases, a PCR step as described for panel B was performed for one of the fragments, such as in the construction of pISM1302.1 (Table 2). In step 5, the fragments were blunt-end ligated to form the desired repeat. This resulted in loss of the SmaI and SmaBI or BstBI cloning sites at the cloning junction. In step 6, steps 4 and 5 were then repeated consecutively with the plasmids described in Table 2 until the series of R1-lacZ fusions was constructed.
(Fig. 3A). In this experiment, 10 μg of protein was loaded per lane, except for lane 1, which contained 20 μg of protein to control for nonspecific binding of antibodies to the fusion protein. Thus, it appeared that three repeating units formed an antigenic epitope recognized by the MAb. Convalescent-phase swine sera also recognized the R1 repeat structure (Fig. 3B).

Our results indicate that the cilium binding domain of P97 is found exclusively within the R1 region. The amino acids upstream of the R1 region are not necessary for cilium binding.

![Image](https://via.placeholder.com/150)

FIG. 2. Detection of adherence of R1–β-galactosidase fusion proteins to swine cilia. R1–β-galactosidase fusion proteins in E. coli lysates were examined for adherence to purified swine cilia using the microtiter plate CAA as described in the text. Data are represented as the means plus standard deviations of the optical densities at 412 nm of triplicate wells. White and gray bars represent separate experiments on different days. C (−), Lac− control; C (+), Lac+ control.

The functional site required a minimum of eight repeating units, supporting our previous transposon mutagenesis study (6). This gives a good idea of the size of the functional binding domain but leaves a number of unresolved issues. For instance,
we could not determine with our existing fusions if the first eight repeating units, including the modified fourth unit, are required for binding or if any of the repeating units would suffice as long as eight units were involved. It was also not possible to determine the role, if any, of the charged-noncharged motif of the R1 region in cilium binding. There was also the possibility that cellular proteases could be degrading the fusion product by cleaving the product at the fusion junction. Immunoblot analysis of *E. coli* lysates with anti-β-galactosidase antibodies and the F2G5 MAb with a large-format gel system gave no evidence of product degradation (data not shown). Since these lysates could be stored for at least 24 h at 4°C without loss of binding activity, and since the data fit nicely with previous studies (6), it seemed unlikely that degradation of the fusion product was occurring.

Finally, the sequence of the antigenic epitope of P97 is now better understood. It is clear from these studies that three repeat units (15 amino acids) are needed for the proper antigenic structure. It seems logical that the epitope is conformational in nature because of its size. Interestingly, this epitope appears to be the major P97 epitope in mice, because attempts to produce MAbs against a non-R1 epitope of P97 have all failed (R. F. Ross and T. F. Young, unpublished data). Likewise in swine, serum antibodies are produced against this epitope (Fig. 3B), but it is not known if antibodies of this specificity are found in significant levels in the respiratory tract during disease. Since the role of this region in adherence is now known, it is possible that development of mucosal vaccines using four or more R1 repeating units might prove effective in preventing disease or possibly even colonization. If high anti-R1 antibody levels could be produced at the respiratory surface, perhaps by fusion with the cholera toxin A2/B subunit as described by Hajishengallis et al. (4), protection against colonization might be possible.

**REFERENCES**


**Editor:** J. T. Barbieri

---

**FIG. 3.** Immunoblot analysis of R1-β-galactosidase fusion proteins. Induced *E. coli* lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and developed as follows. Each well contained 10 μg of protein, except for lane 1, which contained 20 μg of protein. (A) β-gal, blot developed with antibodies against *E. coli* β-galactosidase (Sigma); F2G5, blot developed with MAb F2G5 (14). (B) Blot developed with convalescent-phase swine serum S27 (11). Lane numbers indicate the number of repeating units in the β-galactosidase fusion protein. Lane +: Lac⁺ *E. coli* control containing pMLB1107 with no R1 repeat unit.