A novel cell wall lipopeptide is important for biofilm formation and pathogenicity of Mycobacterium avium subspecies paratuberculosis

Chia-wei Wu, Shelly K. Schmoller, John P. Bannantine, Torsten M. Eckstein, Julia M. Inamine, Michael Livesey, Ralph Albrecht, Adel M. Talaat

Abstract

Biofilm formation by pathogenic bacteria plays a key role in their pathogenesis. Previously, the pstA gene was shown to be involved in the virulence of Mycobacterium avium subspecies paratuberculosis (M. ap), the causative agent of Johne's disease in cattle and a potential risk factor for Crohn's disease. Scanning electron microscopy and colonization levels of the M. ap mutant indicated that the pstA gene significantly contributes to the ability of M. ap to form biofilms. Digital measurements taken during electron microscopy identified a unique morphology for the ΔpstA mutant, which consisted of significantly shorter bacilli than the wild type. Analysis of the lipid profiles of the mycobacterial strains identified a novel lipopeptide that was present in the cell wall extracts of wild-type M. ap, but missing from the ΔpstA mutant. Interestingly, the calf infection model suggested that pstA contributes to intestinal invasion of M. ap. Furthermore, immunoblot analysis of peptides encoded by pstA identified a specific and significant level of immunogenicity. Taken together, our analysis revealed a novel cell wall component that could contribute to biofilm formation and to the virulence and immunogenicity of M. ap. Molecular tools to better control M. ap infections could be developed utilizing the presented findings.

1. Introduction

Mycobacterium avium subspecies paratuberculosis (M. ap) is the causative agent of Johne's disease (paratuberculosis), a disease that infects ruminants worldwide, most notably dairy cattle [17]. It is also possible that exposure to M. ap could play a role in the development of Crohn's disease in humans [4]. Experts estimate that Johne's disease results in a $220 million loss per year in the USA alone [22]. Currently, there is no effective control strategy for Johne's disease and infection with M. ap is hard to diagnose and monitor because of the chronic nature of the disease. Additionally, it is very difficult to remove M. ap from the environment, which threatens any effective control strategy. In recent studies, M. ap was shown to be ubiquitous in animal environments [11,23], especially among wildlife animals [6]. One reason M. ap may be difficult to eliminate from the environment and to treat with antibiotics is the possibility that the M. ap bacilli may form biofilm-like structures. Biofilm formation has been described before in Mycobacterium smegmatis [24,25], Mycobacterium tuberculosis [20], and M. avium subspecies avium (M. avium) [34]. In M. smegmatis and M. avium, biofilm formation is affected by glycopeptidolipids (GPLs) that are a major component of mycobacterial cell wall [3]. The biosynthesis of GPLs in M. avium is initiated by an operon containing two large genes encoding for non-ribosomal peptide synthetases (pstA and pstB) that incorporate four amino acids (Phe, Thr, Ala and alaninol) into the core lipopeptide molecules. In this report, we examined the contribution of the pstA gene in forming cell wall lipids and the impact of the disruption of the pstA gene product on biofilm formation and virulence of M. ap.

Biofilm formation is considered a virulence phenotype in both gram-negative (e.g. Vibrio cholera) [13] and gram-positive bacteria (e.g. Staphylococcus aureus) [5]. The main hypothesis behind this characterization is the ability of bacterial biofilms to induce a persistent source of infection and to resist antibiotics [8]. Recently, M. avium, a bacterium closely related to M. ap, was found to form biofilms especially in water pipe systems of large cities [9,18]. This phenotype can exacerbate the problems associated with M. avium infection in AIDS patients [29]. A similar situation could also exist in animal pastures where infected and naive animals...
come in close contact. Clinically-infected cows can shed $10^6$–$10^8$ CFU/g of fecal material that can easily contaminate animal surroundings for a long period of time. The ability of \textit{M. ap} to form a biofilm could increase the survival of this pathogen under stress conditions and could increase the infection rate among cattle herds. Understanding the genetic basis of biofilm formation in \textit{M. ap} will greatly enhance our knowledge of the pathogenesis of \textit{M. ap}.

The \textit{pstA} gene (~12 kb) participates in GPL biosynthesis and biofilm formation in \textit{M. avium} as a non-ribosomal peptide synthetase (\textit{nrp}) [34]. In some mycobacteria, the function of \textit{PstA} is a result of two different genes, \textit{pstA} and \textit{pstB} (also referred to as \textit{nrp} and \textit{mps}) [10]. A transposon insertion mutant in the \textit{pstA} coding sequence of \textit{M. avium} failed to form a biofilm in a water recirculation system [10,34]. Through screening a transposon mutant library of \textit{M. ap}, an insertion mutant of the \textit{pstA} gene was shown to be attenuated in a mouse model of paratuberculosis with significant reduction in tissue colonization of the mutant [26]. In this report, our analysis indicated that \textit{M. ap} is able to form biofilms while its isogenic mutant, \textit{ΔpstA}, is deficient in this phenotype, indicating the likely involvement of the \textit{pstA} gene in biofilm formation. On the cellular level, electron microscopy analysis displayed a significant reduction in extracellular matrix of biofilms formed by the \textit{ΔpstA} mutant with significantly shorter bacilli than both wild type and complemented strains. Further lipidomic analysis of the \textit{M. ap} strains identified a unique lipopeptide in the wild type and \textit{pstA} complemented strains that was absent from the \textit{ΔpstA} mutant. Moreover, studies in cattle indicated the involvement of \textit{pstA} in intestinal invasion and immunogenicity of \textit{M. ap}.

2. Results

2.1. Phylogenetic analysis of the \textit{pstA} gene

A large-scale screening strategy of a bank of \textit{M. ap} transposon mutants identified a mutant with an insertion in the \textit{pstA} gene to be attenuated in a murine model of paratuberculosis [26]. Sequence analysis indicated that the Tn5367 transposon was inserted at base 463 (3.8%) from the predicted start codon of the \textit{pstA} gene (Fig. 1a). Earlier reports indicated the involvement of orthologues of the \textit{pstA} gene in biofilm formation in \textit{M. avium} (\textit{pstB}) [34] and \textit{M. smegmatis} (\textit{mps}) [25]. BLASTp analysis of the \textit{pstA} sequences identified 14 orthologues with significant E-scores (<10^{-5} and >25% overlap) (GenBank release 147, April 2005). Alignment of the orthologues identified that \textit{pstA} sequence of \textit{M. ap} K-10 is closest to that of \textit{M. avium}, indicating that \textit{pstA} is conserved during mycobacterial evolution (Fig. 1b) and could play an important role in the pathobiology of \textit{M. ap}.

2.2. Biofilm formation by \textit{M. ap}

A recent analysis of the \textit{M. avium} \textit{ΔpstB} mutant suggested a role for this gene in biofilm formation [34]. Sequence analysis indicated that the \textit{pstB} coding sequence is not present in \textit{M. ap}; however, the \textit{pstB} gene in \textit{M. ap} and the \textit{pstB} gene in \textit{M. avium} are closely related and hence, may perform a similar function. To examine the contribution of the \textit{pstA} gene to biofilm formation in \textit{M. ap}, we adopted a standard method on PVC surfaces that was previously used to quantify biofilm formation in \textit{M. avium} [34]. In this assay, the extent of biofilm structure is measured by the level of bacterial adherence to the PVC surfaces with crystal violet, a bacterial staining dye. Repeated measurements (at least 3 times) of biofilm formation of both the wild type and \textit{ΔpstA} mutant showed a significant ($P < 0.05$) reduction in the ability of the \textit{ΔpstA} mutant to form a biofilm compared to its parent strain of \textit{M. ap} ATCC 19698, especially at 7 and 9 days post-inoculation (Fig. 2).

To quantify the contribution of the \textit{pstA} gene to biofilm formation in \textit{M. ap}, we estimated the number of bacterial colonies participating in forming biofilms in the wild type, its isogenic \textit{pstA} mutant and the complemented strain of \textit{M. ap} \textit{ΔpstA}::\textit{pstA}. Cultures...
of all strains were allowed to grow in falcon tubes in the presence of a PVC tube strip. Following 10 days of incubation, a larger biofilm structure was formed on the sides of PVC strips of M. ap wild type compared to the ΔpstA mutant. Further counting of the bacterial cells forming biofilms compared to the planktonic cells indicated that 51.6% of ATCC 19698 participated in biofilm formation while only 31.4% of the ΔpstA participated in biofilm formation. On the other hand, 54.1% of cells of the complemented strain, ΔpstA::pstA, participated in biofilm formation, an indication of the ability of the complemented strain to restore biofilm formation in M. ap. Taken together, binding to PVC (plates and strips) indicated the ability of M. ap to form a biofilm was significantly reduced in the absence of pstA.

2.3. Visualization of M. ap biofilms

To further characterize biofilm formation on the cellular level, we examined mycobacterial bacilli with different genetic backgrounds following attachment to glass coverslips at 5, 10 and 24 h post-incubation. Cultures of the wild type, mutant and complemented strains of M. ap were analyzed using scanning electron microscopy (SEM) following incubation in the presence of HBSS [34] or Middlebrook 7H9 broth that is usually used to grow M. ap cultures. As expected, all characteristics of biofilm communities (presence of aggregates with extracellular matrix) were clearly evident in all cultures of M. ap ATCC 19698, ΔpstA::pstA and to a lesser extent in the ΔpstA mutant (Fig. 3). A clear difference observed in the ΔpstA mutant is the lack of extensive extracellular matrix materials that were present in both M. ap ATCC 19698 and the ΔpstA::pstA strains. Similar biofilm characteristics were observed in samples analyzed from HBSS (Fig. 3) or 7H9 broth cultures (data not shown), suggesting the ability of M. ap to form biofilms, regardless of the culture medium. Additionally, biofilm structures were clearly developed as early as 5 h post-incubation in all examined strains as well as at 10 and 24 h post-incubation. Notably, all formed structures contained extracellular matrix, a clear distinction from bacterial aggregates.

Further analysis of SEM images revealed an interesting phenotype characteristic for the ΔpstA mutant that was shorter in length compared to the other strains. To examine this phenotype further, we measured the dimensions of individual, 90 mycobacterial bacilli collected from different sample preparations of all strains. Interestingly, bacilli of the complemented strain, ΔpstA::pstA were significantly longer (average length 2.1 μm) than the ΔpstA mutant (1.2 μm) or the wild type (1.7 μm) strains (Fig. 4). However, all bacilli showed similar width (~0.5 μm), suggesting a role for the pstA-encoded protein in bacilli elongation, a fact that was supported by the analysis of length/width (L/W) ratio. The L/W ratio maintained the same relationship in length between strains, suggesting that cell length but not width is the reason for the observed phenotype. Overall, biofilm visualization confirmed the ability of M. ap to form a biofilm and that biofilm formation and perhaps cell size appear to be significantly affected by the disruption of the pstA gene.

2.4. The lipid profile of the ΔpstA mutant

The biosynthesis of GPLs in M. avium is initiated by an operon encoding for non-ribosomal peptide synthetases (pstA and pstB) [3]. It is possible that disruption of the pstA gene in M. ap could disrupt the synthesis of this or a similar lipopeptide. To test this hypothesis, the whole lipid profile of the ΔpstA mutant was compared to that of the wild-type M. ap ATCC 19698. Using two-dimensional thin-layer chromatography (2D-TLC) in the five standard solvent systems (A through E) spanning the whole polarity, one difference between the two lipid profiles was identified within the polar system A. This lipid, termed WC-A-02, was found only in the wild-type ATCC 19698 but not in the transposon mutant, ΔpstA (Fig. 5a and b). No other differences were identified within the lipid composition of the parental strain and the transposon mutant strain. Moreover, when the lipid profile of the complemented strain

![Fig. 3. Visualization of M. ap biofilms. Scanning electron microscopy of biofilms formed by wild-type M. ap ATCC 19698, ΔpstA mutant and complemented strain, ΔpstA::pstA. Samples were analyzed at 5 h post-inoculation of M. ap grown on glass coverslips in the presence of HBSS buffer at 10,000× (a, b, c) or 20,000× (d, e, f). Arrows point to extracellular matrix in different images. All analyses were repeated twice from independent cultures. Representative images are shown here.](image-url)
(ΔpstA::pstA) was examined, the presence of the WC-A-02 lipid was partially restored (Fig. 5c).

Additional structural analyses of the nature of the unique WC-A-02 lipid by a differential staining protocol were employed before [7]. The generated staining profile of WC-A-03 suggested the presence of a lipopeptide without a sugar moiety (Fig. 5d) or a free primary amino group (Fig. 5e). Furthermore, this lipid is not a phospholipid (Fig. 5f). Since no primary amino group could be detected a peptide should be N-terminal linked to another moiety such as a fatty acyl chain. Thus, the most likely structure of WC-A-02 should be a lipopeptide. In general, the lipidomic analysis indicated that thepstA gene is involved in the generation of a cell wall lipid with the strong indication that it is involved in the biosynthesis of a lipotripeptide since the non-ribosomal peptide synthetase contains three amino acid binding modules.

2.5. Virulence of the M. ap ΔpstA mutant in cattle

Previously, we showed that a pstA insertion mutant of M. ap is not able to efficiently colonize the intestines and livers of infected mice [26]. Because of the sampling strategy used in that study, the early stages of tissue colonization were not analyzed. To examine the role of pstA in M. ap virulence in cattle, we examined the ability of the M. ap ΔpstA mutant to transverse the intestinal epithelial cells using a recently described bovine model for Johne’s disease [33]. Both the wild type and its isogenic mutant, ΔpstA, were surgically inoculated directly to the ileum of a paratuberculosis-free calf. Infection was allowed to proceed for 2 h before samples from mesenteric lymph nodes and liver were harvested for colony counts. Typically, the competitive index of virulence (CIV) of virulent strains should be 1. In the case of the ΔpstA mutant, the CIV was <0.4 compared to the wild-type M. ap ATCC 19698 (Fig. 6) indicating that the ΔpstA had a reduced ability to transverse the intestine to the liver and mesenteric lymph nodes. The low CIV suggested that the pstA gene product can play a key role in intestinal invasion, particularly during early stages of infection.

2.6. Immunogenicity of PstA

The large pstA coding sequence was divided up into 9 segments for cloning and expression in E. coli. Seven of the 9 segments were successfully cloned, expressed and purified (Fig. 7a). All 7 purified proteins were detected by immunoblot analysis using monoclonal antibodies directed against the protein tag, MBP (data not shown). However, when identical immunoblots were probed with sera from rabbits exposed to M. ap, strong signals were detected only for PstA-6, PstA-8 and PstA-9 peptides, which encompass the C-terminal half of this large protein (Fig. 7b). These data suggest that these regions of the C-terminus of PstA might be surface exposed and hence, the most immunogenic. To examine the immunogenicity of PstA peptides in naturally infected animals, the PstA peptides were analyzed by immunoblot using sera from a total of 6 Johne’s disease infected cows and 7 disease-free animals. Consistently, sera from infected animals (N = 6) gave more bands with PstA peptides than when rabbit sera were used, including the peptides numbered 6, 7 and 8 (Fig. 7c). Interestingly, more PstA fragments with higher intensities were detected with a serum sample from a cow suffering from sub-clinical infection (cow 253) compared to clinically-infected cows of Johne’s disease. As expected, sera from control cows (N = 7) did not yield any reactivity with PstA peptides suggesting the specificity of the PstA (data not shown).
3. Discussion

PstA is the second largest gene in the *M. ap* genome with 12 kb [15] containing three modules, suggesting that this non-ribosomal peptide synthetase encodes for the biosynthesis of a lipoteptide, which makes it different from the *pstA* gene in *M. avium* (2 modules) or in *M. smegmatis* (4 modules). Importantly, the identification of a transposon mutant [26] and construction of the complemented mutant for this very large gene have enabled a thorough examination of the role PstA plays in biofilm formation and pathogenicity. Biofilm formation is an important phenotype for pathogens that could be transmitted through environmental sources. Under sub-optimal manure processing for dairy herds, *M. ap* was shown to persist for long periods of time [12]. It is possible that *M. ap* bacilli contaminating animal environments [11,23] persist in biofilms or biofilm-like structures. Experiments described here represent our attempt to characterize the ability of *M. ap* to form a biofilm and begin to dissect the genetic factors involved in biofilm formation of this important pathogen. Our analysis indicated that *M. ap* bacilli are able to form biofilms with a significant role played by the *pstA* gene product. Similar to *M. avium* [10], biofilm formation in *M. ap* was not completely dependent on the activity of PstA, a conclusion that was supported by the presence of a small level of biofilm structures in the Δ*pstA* mutant when biofilms were visualized by electron microscopy. Interestingly, SEM revealed a significant difference in bacilli length, depending on the presence of a fully active *pstA* gene, suggesting a role for *pstA* in elongation of mycobacterial cells. However, whether cell elongation/division has a causal relationship with biofilm formation that is mediated by *pstA* needs further studies. Interestingly, the *pstA* gene was over-expressed in the Δ*pstA::pstA* complemented strain and their bacilli were the longest among other bacilli. It is possible that *pstA* plays

![Fig. 5. Lipidomic analysis of *M. ap* strains used in this study. Thin-layer chromatography (TLC) of lipid extracts from the wild-type ATCC 19698 (a), the Δ*pstA* mutant (b) and the *pstA* complemented strain (Δ*pstA::pstA*) (c). TLC plates stained with α-naphthol (d), ninhydrin (e) or Dittmer–Lester (f) of *M. ap* ATCC 19698 samples are also shown. Key lipopeptides in different TLC runs are encircled.](image-url)

![Fig. 6. Contribution of *pstA* to the virulence of *M. ap* in cattle. Competitive index of the calf intestinal invasion assay for the liver and lymph node samples collected at 2 h following intra-intestinal inoculation of an equal mix of the wild-type ATCC 19698 and Δ*pstA* mutant.](image-url)
several roles important for the biology of *M. ap* bacilli growth and biofilm formation.

In *M. smegmatis*, the presence of active *groEL1* gene and both short-chain fatty acids and the available iron play a role in biofilm maturation [19,21]. In Fig. 2, biofilm formation of the ΔpstA mutant was significantly less than that of the wild type on days 7 and 9, but not in earlier time points. This result suggests pstA may contribute to biofilm formation in *M. ap* during the maturation stage, similar to the role played by *groEL1* in *M. smegmatis*. The lipidome analysis of the wild-type *M. ap* and its isogenic, ΔpstA mutant identified a particular cell wall lipid (WC-A-02) that was missing in the lipidome of the ΔpstA mutant. The employed lipid extraction protocol suggested that the WC-A-02 could be a component of cell wall of *M. ap*, similar to the lipopeptide component of glycopeptidolipids (GPLs) present in *M. avium* [28]. The identified lipid spot does not represent a glyco- or glycopeptidolipids (negative with a-naphthol staining) nor a phospholipids (negative Lester–Dittmer staining). Furthermore, it does not contain amino acids with NH2 groups nor does it have a free N-terminus. Since the fatty acyl chain within the GPLs of *M. avium* is a long β-hydroxy fatty acyl attached to a phenylalanine, this might be the case for *M. ap* as well, especially, after comparative genomics reveals a high similarity between the *pstA* of *M. avium* and *M. ap*. It is possible that the synthetase encoded by the *pstA* gene participates in the pathway that leads to the formation of this particular lipopeptide.

We also employed a standard protocol for colony counting to quantify the contribution of the *pstA* gene to biofilm formation on solid surfaces, a key phenotype that cannot be quantified by SEM analysis. Our colony counting protocol showed that inactivation of the *pstA* gene yielded a mutant that was still able to form biofilm but to a lesser extent compared to the wild-type strain, suggesting the presence of other genes that could participate in the *M. ap* biofilms. In *M. avium*, several genes were shown to contribute to biofilm formation [34]. It is possible to identify the whole set of genes important for biofilm formation by screening the *M. ap* mutant library that was screened before for virulence genes [26]. In this screen, the *pstA* gene was shown to be involved in virulence. Whether biofilm-related genes other than *pstA* are also involved in *M. ap* virulence remains to be investigated.

An important goal in this study was to analyze the role played by the *pstA* gene on the virulence of *M. ap*, especially during early stages of Johne’s disease. We demonstrated that the gene was expressed during infection, as segments of the protein were detected by sera from Johne’s disease cows indicating its immunogenicity. Previously, GPLs of *M. avium* were shown to induce proinflammatory responses [2]. Furthermore, the calf model of infection suggested a role for *pstA* in early stages of *M. ap* invasion across intestinal barriers. It is possible that the early translocation event tested in the calf model is dependent on the ability of the *pstA* gene product to interact with intestinal cells which can lead to rapid spreading from the site of primary infection (intestine) to other organs such as spleen and liver. A role for interacting with host cells was suggested before for GPLs in *M. avium* [28] and could explain the inefficiency of intestinal invasion by the ΔpstA mutant observed in the calf model of invasion employed here. However, the virulence of ΔpstA mutant should be tested on more calves in the future.

In summary, our results and others analyzing *M. smegmatis* [25] and *M. avium* [34] strains indicate that the *pstA* gene could be involved in biofilm formation in both virulent and avirulent strains of mycobacteria. More experiments are needed to focus on the role played by *pstA* and host cells in *M. ap* pathogenesis and to further decipher the molecular interactions activated between host cells and mycobacterial cell wall components, in general. Such analysis can also improve our understanding of the pathogenesis of the inflammatory bowel diseases including Johne’s and Crohn’s diseases. Peptides derived from *pstA* sequence could be further developed to a diagnostic tool to differentiate infected from healthy animals. In general, findings presented here will facilitate the analysis of other mycobacterial species and can provide a model for the analysis of other biofilm-forming pathogen.
4. Materials and methods

4.1. Bacteria and plasmids

*M. ap* strains used in this study include the type strain ATCC 19698, its isogenic mutant Δ*pstA* and the complemented strain Δ*pstA::pstA*. All strains used in this study were grown in 7H9 broth (Difco) supplemented with Mycobactin J, 10% ADC and 0.05% Tween 80 [32]. The Δ*pstA* mutant was generated using transposon mutagenesis of ATCC 19698, as described previously [26]. The Δ*pstA* mutant was grown in Middlebrook media supplemented with kanamycin (30 μg/ml). The complemented strain (Δ*pstA::pstA*) was grown in media supplemented with kanamycin (30 μg/ml) in addition to hygromycin (100 μg/ml). To construct the complementation vector (pVV16–MAP1242), the sequence of the MAP1242 vector was amplified with High Fidelity Taq/Tgo Polymerase in triplicate. Plates were incubated at 37°C for 48 h. Transformants were selected on 7H11 agar plates incubated at 37°C for 48 h.

4.2. Biofilm formation assays

Cultures of *M. ap* ATCC 19698 and its isogenic Δ*pstA* mutant grown to log phase (OD$_{600}$ = 1.0) were centrifuged and resuspended to OD$_{600}$ = 1.0 using Hanks’ Balanced Salt Solution (HBSS) (BioWhittaker/Cambrex). Aliquots of 200 µl of bacterial suspensions were added to each well and incubated for 15 min. Plates were then washed 3 times to remove bacteria that had not adhered to the PVC plate. Ethanol (95%) was added to each well and the absorbance of wells was recorded at a 540 nm wavelength using a Thermodynamic plate reader (Fisher Scientific). The net absorbency for each sample is recorded after subtracting out the absorbency of sterile culture media used as a control. All assays were performed in triplicates and repeated 3 times starting from new *M. ap* cultures.

To quantify the percentage of biofilm formation in *M. ap* strains, cultures grown to OD$_{600}$ = 1.0 were transferred to 50 ml Falcon tubes in which a 5 x 2 cm² strip of PVC tube was placed inside and incubated at 37°C with slow shaking (50 rpm). At 10 days post-incubation, PVC strips were washed and *M. ap* cells adhering to PVC tubes were scrapped thoroughly, until no film was visible, and rinsed with HBSS for colony counting on Middlebrook 7H10 agar plates. Aliquots were also collected from planktonic cultures (cultures in Falcon tubes not attached to the PVC strips) of each examined strain. The number of colony forming units (CFU) retrieved from the PVC tube was divided by the CFU from the 50-ml tube to represent the percentage of cells forming biofilms (biofilm percentage).

4.3. Scanning electron microscopy

Bacterial cultures, when OD$_{600}$ reached 1.0, were sonicated at 50 W for 2 min or until well-dispersed in a water-filled cup horn sonicator (Fisher Scientific), washed once with HBSS and resuspended in HBSS or Middlebrook 7H9 broth. On a 24-well cell culture plate, 500 µl of the bacterial suspensions were added onto an autoclaved 12-mm circular coverslip placed in the wells. The plate was sealed with parafilm to prevent liquid evaporation. After 24 h of incubation at 37°C, each coverslip was washed once with 1 ml HBSS and fixed by incubating in 500 µl of 2.0% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The samples were dehydrated through an alcohol series with 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol followed by drying with molecular sieve for 5 min. Dehydrated specimens were dried by the critical point drying procedure using 100% sieved-dried ethanol (Samdri 780-A, Tousimi) as the intermediate fluid and liquid CO$_2$ as the transitional fluid. All samples were then splatter coated with gold–palladium to a thickness of approximately 20 nm and observed with a Hitachi S-570 scanning electron microscope at 10 kV accelerating voltage [31].

4.4. Lipid extraction and thin-layer chromatography (TLC)

Bacterial cultures from *M. ap* cultures were harvested after 8 weeks of growth by scraping from Middlebrook 7H11 agar plates with supplements as described above. Cells were lyophilized prior to lipid extraction and total cell lipids were extracted twice with chloroform/methanol (2:1) (30 ml/g of lyophilized cells) at 55°C for 3 h. Crude lipid extracts were dried down under nitrogen and subjected to a Folch wash (chloroform/methanol/water 4:2:1 ml). The organic phase was transferred to a new tube and dried under nitrogen. Whole cell lipids were resuspended in chloroform/methanol (2:1) at a concentration of 10 mg/ml. Two-dimensional thin-layer chromatography was performed on 10 x 10 cm aluminum-backed silica gels 60 F$_{254}$. Samples of a 200 µg each of total cell lipids were separated in 5 systems with different polarities (A → E) using standard TLC protocols [7]. For example, system A was composed of chloroform/methanol/water 100:14:0.8 for dimension 1 and chloroform/acetonewater 50:60:2.5:3 for dimension 2. Separated lipids were visualized by spraying with 10% CuSO$_4$ in 8% H$_3$PO$_4$ followed by heating until spots appeared. Differential staining for structural analyses of the lipid WC-A-02 was performed with naphthol (sugar moieties), ninhydrin (free

Table 1

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<tr>
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<td>GTTGCCTGCTGGAGAGCTAG</td>
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<tr>
<td>pstA-P9F</td>
<td>Peptide production</td>
<td>ATTCGCACGCTGGAGAGCTAG</td>
</tr>
<tr>
<td>pstA-P9R</td>
<td>Peptide production</td>
<td>GTTGCCTGCTGGAGAGCTAG</td>
</tr>
</tbody>
</table>
primary amino groups), and Dittmer–Lester (phospholipids) followed by heating for naphthol and ninhydrin as described before [7].

4.5. Calf intestinal invasion assay

Both the wild-type M. ap ATCC 19698 and its isogenic mutant, ΔpstA mutant were grown to OD_{600} = 1.0. Before animal inoculation, cultures were centrifuged, resuspended in PBS and equal volumes of cultures were mixed. A male Holstein calf, 14 days of age, was purchased from a dairy herd that has been Johne’s disease-free for the last 5 years and that is maintained by the School of Veterinary Medicine, University of Wisconsin-Madison. Sera and fecal samples collected from the calf and the dam were examined and demonstrated to be negative for evidence of M. ap infection by means of an IDEXX ELISA test [16] before inclusion in the study. All animal waste and disposable utensils used throughout the experiment were autoclaved before disposal. A total of 10 ml of the culture mixture was inoculated into 10 cm of occluded ileum of the anaesthetized calf as described before [33]. Following infection (1–2 h), samples were taken from mesenteric lymph nodes, small intestine, liver and spleen. Duplicate tissue homogenates were processed for bacterial counting using Middlebrook 7H10 agar in the presence or absence of kanamycin supplement. The identity of colonies retrieved from animals was verified using PCR. The percentage of wild type and mutant counts was calculated in the following formula to calculate the competitive index of virulence [33].

\[
CIV = \frac{(\text{CFU mutant}_{\text{output}}/\text{CFU wild type}_{\text{output}})}{(\text{CFU mutant}_{\text{input}}/\text{CFU wild type}_{\text{input}})}
\]

4.6. Production of PstA peptides and antibodies

The 12 kb coding sequence of pstA was too large to clone the entire open reading frame (ORF) into a protein expression vector. Therefore, the coding sequence was divided into 9 segments for cloning and protein production. A maltose binding protein (MBP) fusion for each of these PstA segments was constructed using the cloning and protein production. A maltose binding protein (MBP) Therefore, the coding sequence was divided into 9 segments for entire open reading frame (ORF) into a protein expression vector.

4.7. Immunoblot analysis

Polyacrylamide gel electrophoresis was performed using 12% (w/v) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose (Schleicher and Schuell, Keene, NH, USA) was accomplished with the Bio–Rad Trans Blot Cell (Bio–Rad Laboratories, Richmond, CA, USA) with sodium phosphate buffer (25 mM, pH 7.8) at 0.8 A for 90 min. After transfer, filters were blocked with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaPO4, pH 7.4) plus 2% bovine serum albumin (BSA) and 0.1% Tween 20, referred to hereafter as PBS–BSA. Rabbit antisera were diluted 1:1000 in PBS–BSA and all cattle sera were diluted 1:300. All cattle serum samples (N = 13) were obtained from the National Animal and Disease Center’s (NADC) herd where their Johne’s disease status is well-documented. Also, the status of the source animals for sera was blinded until the end of the experiment. The primary sera were incubated on the blot at room temperature for 2 h. After three washes in PBS plus 0.1% Tween 20, blots were incubated for 1.5 h in goat anti-bovine-peroxidase (ThermoScientific-Pierce) diluted 1:20,000 in PBS–BSA. The blots were again washed three times as described above and developed for chemiluminesence using Supersignal detection reagents (ThermoScientific-Pierce).

4.8. Sequence and statistical analyses

The sequence of the pstA gene in M. ap and its orthologues in other bacteria were downloaded from the GenBank database and prepared for BLASTp analysis [1]. Only sequences with E-value < 10^{-5} within at least 25% of the overall sequence were included in constructing the phylogenetic tree of orthologues sequences. Alignments of sequences were generated by the CLUSTALW program implemented in the MEGA 3.1 Software [14]. Phylogenetic trees were generated from the aligned sequences using Neighbor-Joining algorithm followed by bootstrapping (1000 x). For statistical analysis, Student’s t-test implemented in Microsoft Excel program was used to evaluate differences in the bacterial colonization and the ability to form a biofilm between M. ap strains used in this study. A level of P-value < 0.05 is considered statistically significance among paired samples.

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References

Mycobacterium avium


