Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae*

Yamei Zhang *, Jan M. Tennent, Aaron Ingham, Gary Beddome, Christopher Prideaux, Wojtek P. Michalski

CSIRO Animal Health, Australian Animal Health Laboratory, Private Bag 24, Geelong, Vic. 3220, Australia

Received 26 May 2000; accepted 31 May 2000

Abstract

Type 4 fimbriae have been identified on the cell surface of *Actinobacillus pleuropneumoniae* by electron microscopy and N-terminal sequencing analysis. *A. pleuropneumoniae* type 4 fimbrial subunit protein, purified from cell cultures and from outer membrane preparations, reacted with polyclonal antibody raised against type 4 fimbriae of *Moraxella bovis* on Western blots. N-terminal sequence analysis of the purified 17 kDa type 4 fimbrial subunit protein, named ApfA, revealed the first 12 amino acids to be identical to those of other type 4 fimbrial subunit proteins. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Type 4 fimbria; Fimbrial protein; Protein purification and sequencing; *Actinobacillus pleuropneumoniae*

1. Introduction

*Actinobacillus pleuropneumoniae* is the etiological agent of porcine pleuropneumonia, which is an acute or chronic infection characterized by hemorrhagic, fibrinous and necrotic lung lesions affecting pigs of all ages [1]. The disease is highly contagious and causes significant economic losses to the swine industry worldwide. To date, 12 serotypes of *A. pleuropneumoniae* have been identified based on the presence of capsular antigens [2]. Serotypes 1, 7 and 12 are geographically dominant in Australia and comprise approximately 90% of isolates [1]. *A. pleuropneumoniae* displays several virulence factors, including RTX toxins, lipopolysaccharide and other cell surface components such as outer membrane proteins, capsule and adhesins [3].

Bacterial colonization depends upon attachment of bacteria to host cells and is often mediated by filamentous protein structures, such as fimbriae (also referred to as pili) [4]. Type 4 fimbriae are found on many Gram-negative pathogenic bacteria including *Moraxella bovis*, *Moraxella nonliquefaciens*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Dichelobacter* (previously *Bacteroides*) *nodosus*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. Type 4 fimbrial subunits contain a highly conserved and easily recognizable N-terminus with methylated phenylalanine (N-methyl-Phe) as the first amino acid of the processed subunit [5,6].

The presence of fimbriae has been previously observed in some field isolates of *A. pleuropneumoniae* by transmission electron microscopy [7,8]. These isolates were also shown to adhere to porcine tracheal rings maintained in culture or to frozen porcine tracheal and lung sections [9,10]. The purpose of this study was to determine whether *A. pleuropneumoniae* expresses surface located virulence factors, such as type 4 fimbriae, that may play a role in adherence to target tissues.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains of *A. pleuropneumoniae* isolated in Australia, HS25 (serotype 1), HS134 (serotype 2), HS93 (serotype 7) and HS56 (serotype 12), were kindly supplied by Dr. Pat Blackall (Animal Research Institute, Moorooka, Qld., Australia). Strains were cultured in brain heart infusion (BHI) broth supplemented with 10 µg ml⁻¹ of nicotinamide adenine dinucleotide (NAD). Blood agar plates were made by adding 5% (v/v) defibrinated horse blood and 10 µg ml⁻¹ of NAD to the BHI agar (BHA) (Bacto agar, 1.2% w/v). Chemically defined media (CDM) were
made according to the method described previously [11]. Bacteria were incubated at 37°C in either aerobic or microaerophilic atmospheres for 16 to 18 h.

2.2. Preparation of membrane proteins and purification of fimbriae

A total membrane preparation was obtained from HS25 grown on 20 BHIA plates (9-cm petri dishes) as described previously [12]. The type 4 fimbrial subunit protein was then purified from the total membrane preparations by reverse-phase HPLC. Intact fimbriae were purified from HS25 cells grown in CDM by homogenization and MgCl₂ precipitation as described previously [13].

2.3. SDS–PAGE and Western blot analysis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were carried out as described previously [14]. Rabbit polyclonal antibodies raised against the type 4 fimbriae of M. bovis [15] were used at 1:500 dilution.

2.4. Sequencing of fimbriae

Purified type 4 fimbriae (MgCl₂ precipitation) and type 4 fimbrial subunit protein (HPLC purification) were dissolved in PBS and subjected to automated (Edman degradation) amino acid sequence analysis [16] with vapor-phase delivery of critical reagents [17] in an automated sequencer in conjunction with a PTH-amino acid separation system.

2.5. Transmission electron microscopy (TEM)

Fimbriae were visualized on intact HS25 cells grown in CDM and in a purified fimbriae preparation (MgCl₂ precipitation). Carbon coated copper grids were touched onto a 10–20 μl drop of cells or purified fimbriae and the sample was left to settle onto the grids for 1 min before blotting. Cells or purified fimbriae were stained for 45 or 30 s, respectively, with 1% ammonium molybdate solution. The grids were air dried prior to visualization in a Phillips CM12 electron microscope at a voltage of 60 kV.

3. Results and discussion

3.1. Visualization of fimbriae on A. pleuropneumoniae

TEM detected fimbriae extending from the cell surface of HS25 when the bacteria were grown on CDM under a microaerophilic atmosphere (Fig. 1). Interestingly, fimbriae could not be detected following growth of cells on BHIA containing horse blood or BHI broth. Different growth conditions, such as degree of aeration, temperature or the inclusion of glucose, cyclic adenosine monophosphate and alanine have been found to variously enhance or inhibit fimbriae expression in Escherichia coli [18,19]. Similarly, our results suggested that the expression of fimbriae by A. pleuropneumoniae was influenced by defined growth conditions and may involve specialized regulatory elements.

3.2. Identification of type 4 fimbrial protein

Whole cell lysates of HS25, HS134, HS93 and HS56 were analyzed by SDS–PAGE and Western blotting (Fig. 2). A 17 kDa protein present in all four strains showed strong reactivity with antibodies specific to type 4 fimbriae (Fig. 2B) but was not distinctly visible in Coomassie blue stained gels, indicating that the protein was present in a very small amount (Fig. 2A). Type 4 fimbriae display many conserved antigenic and immunogenic determinants located at the N-terminal domain and often the
antiserum raised against type 4 fimbriae from one bacterial species can cross-react with that of other bacteria [20]. The results presented in Fig. 2 suggest that the 17 kDa band identified by Western blot is the type 4 fimbrial subunit of *A. pleuropneumoniae*. The antiserum raised against type 4 fimbriae also detected an approximately 30 kDa protein in the whole cell lysates (Fig. 2B). This reaction was not observed when purified subunit or fimbriae preparations were separated by SDS–PAGE and reacted with the *M. bovis* type 4 fimbrial antiserum (Fig. 2C and D), suggesting that it was due to non-specific reaction between the sera and an unrelated soluble protein.

### 3.3. Sequencing

In order to confirm that the 17 kDa protein of *A. pleuropneumoniae* was indeed the type 4 fimbrial subunit protein, N-terminal sequencing was undertaken on two purified protein preparations, viz. total membrane preparations obtained by reverse-phase HPLC and fimbriae purified by MgCl₂ precipitation. These proteins were subjected to direct amino acid sequencing and revealed the following N-terminal sequence: (X)TLIELMIVAL. Sequence alignment analysis showed that the obtained sequence was identical to those of type 4 fimbrial subunit proteins previously identified in *H. influenzae* (pilin), *M. bovis* (β pilin), *M. nonliquefaciens* (TfpA), *Eikenella corrodens* (Ecp), *N. gonorrhoeae* (piliE), *N. meningitidis* (pilE), *Aeromonas hydrophila* (TapA), *P. aeruginosa* (pilA), and *D. nodosus* (FimA) [20–23]. The first amino acid of the *A. pleuropneumoniae* fimbrial subunit could not be identified probably due to the likelihood that it was a methylated phenylalanine residue, as is the case for each of other identified type 4 fimbrial proteins. Taken together, the results of TEM, Western blots and N-terminal sequence analysis indicated that the 17 kDa protein, which we have named ApfA, is the fimbrial subunit of *A. pleuropneumoniae* type 4 fimbriae.

Expression of fimbriae is believed to be important for the initial stages of bacterial infection [4]. Type 4 fimbriae have been shown to increase bacteria–bacteria interactions and to promote bacterial adherence and colonization, thereby facilitating the progress of bacterial infection [4]. It has been well established that *A. pleuropneumoniae* attaches to the epithelial cells of the host respiratory tract [7]. With the identification of type 4 fimbriae it will now be possible to study the role of these structures in the development of pleuropneumonia and in the adherence and colonization of *A. pleuropneumoniae* to host tissue.

### Acknowledgements

We express appreciation to Dr. Robert Moore for critical reading of the manuscript. The contributions made by Vicki Bennett-Wood and Roy Robins-Browne in electron microscopy and Brian Shiell in amino acid sequencing are gratefully acknowledged.

### References


