Veterinary Microbiology 159 (2012) 187–194

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Immunoproteomic analyses of outer membrane antigens of *Actinobacillus pleuropneumoniae* grown under iron-restricted conditions

Jacqueline W. Chung [a,*], Elke Küster-Schöck [b], Bernard F. Gibbs [c], Mario Jacques [d], James W. Coulton [a]

[a] *Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, QC, Canada H3A 2B4*
[b] *Cell Imaging and Analysis Network, McGill University, 1205 Dr. Penfield Avenue, Montreal, QC, Canada H3A 1B1*
[c] *Sheldon Biotechnology Centre, McGill University, 3773 University Street, Montreal, QC, Canada H3A 3B4*
[d] *Groupe de recherche sur les maladies infectieuses du porc, Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada J2S 7C6*

ARTICLE INFO

Article history:
Received 22 September 2011
Received in revised form 30 March 2012
Accepted 31 March 2012

Keywords:
A. pleuropneumoniae
2D-DiGE
Immunoproteomics
Outer membrane proteins
Vaccine

ABSTRACT

Actinobacillus pleuropneumoniae, a bacterial pathogen of swine and agent of porcine pneumonia, causes a highly infectious disease of economic importance in the pig industry. Commercial vaccines for *A. pleuropneumoniae* include whole-cell bacterins and second generation subunit vaccines but they only confer partial protective immunity. Our search for new vaccine candidates identified antigens that are expressed during conditions that mimic infection; the outer membrane (OM) proteome of *A. pleuropneumoniae* serotype 5b was examined under iron restriction. Quantitative profiling by 2D-DiGE technology revealed that iron restriction induced expression of previously described transferrin binding proteins (TbpA, TbpB) plus four lipoproteins including spermidine/putrescine binding periplasmic protein 1 precursor (PotD2). Immunoproteomic analyses with antisera from naïve animals and from infected pigs were able to differentiate antigens within the OM proteome that were specifically recognized during *A. pleuropneumoniae* infection. Immunoblots of iron-restricted profiles detected PotD2, heme-binding protein A (HbpA), and capsule polysaccharide export protein (CpxD) as well as surface antigens TbpA, TbpB, and OmlA. These data identify OM proteins that demonstrate immunogenicity and upregulation under conditions mimicking infection, providing emphasis on lipoproteins as an important class of antigens to exploit for vaccine development for *A. pleuropneumoniae*.

1. Introduction

Actinobacillus pleuropneumoniae, etiological agent of porcine pneumonia, a highly contagious respiratory disease, contributes to major economic losses for the pig-rearing industry worldwide. All 15 serotypes of *A. pleuropneumoniae* are capable of causing disease, though prevalence of serotypes 1, 5 and 7 are specific to North America (Deslandes et al., 2007). While several virulence factors have been described, Apx toxins (Apx I–IV) contribute most to severity of disease and to the pathological effects of porcine pneumonia (Bossé et al., 2002; Frey, 1995). As potent antigens, these toxins elicit protection against bacterial challenge and are present in most commercially available subunit vaccines against *A. pleuropneumoniae* (Ramjeet et al., 2008). To date, a fully cross-protective, safe vaccine against all *A. pleuropneumoniae* serotypes does not exist (Haesebrouck et al., 2004). Animals that survive natural or experimental infection with *A. pleuropneumoniae* develop immunity that protects

* Corresponding author. Tel.: +1 514 398 5215; fax: +1 514 398 7052.
E-mail address: jacqueline.chung@mcgill.ca (J.W. Chung).

0378-1135/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2012.03.038

them from future infections with homologous and heterologous serotypes; however, the antigens that confer such protection are still unknown (Nielsen, 1984; Rycroft and Garside, 2000).

Current efforts in vaccine development apply genomic-based strategies and proteomic technologies to identify new antigens, with continued focus on surface proteins or secreted proteins as the most promising candidates. We previously described (Chung et al., 2007) *in silico* and proteomic data that defined the first OM proteome of *A. pleuropneumoniae* serotype 5b grown under nutrient-rich conditions. These studies revealed several OM proteins with functions associated with iron transport, a prominent virulence mechanism in the pathogenesis of *A. pleuropneumoniae*. Recent proteomic studies (Liao et al., 2009; Zhang et al., 2011) have reported immunoreactive proteins from total cell lysates of serotype 1 or from membrane and extracellular extracts of serotype 3. Likewise, several OM proteins were identified from immunoproteomic analyses of a DIVA subunit vaccine containing detergent-extracted proteins from serotypes 1, 2 and 5 (Buettner et al., 2011). These results underscore OM proteins as leading candidates for new vaccines against *A. pleuropneumoniae*.

In this study we exploited 2D-DiGE technology and immunoproteomics to compare OM protein profiles between *A. pleuropneumoniae* grown under nutrient-rich and under iron-restricted conditions. Our objective was to define a selection of potential vaccine candidates for *A. pleuropneumoniae* that are both immunogenic and expressed under conditions resembling infection in the porcine host.

2. Materials and methods

2.1. Bacterial strain, growth conditions and OM protein preparations

A. pleuropneumoniae serotype 5b isolate L20 was routinely cultured in brain heart infusion (BHI) media containing 10 μg/ml nicotinamide adenine dinucleotide (NAD). As previously described (Mikael et al., 2003; Srikumar et al., 2004), iron restriction was achieved by adding 50 μM EDDHA (Sigma) to broth cultures that reached early log phase (OD_{600} 0.1). Growth rates for all conditions were similar. OM protein profiles from each growth condition were compared to those from *A. pleuropneumoniae* grown under nutrient-rich conditions. After harvesting cultures in late log phase and lysing bacteria in a French pressure cell, OM vesicles were isolated by sucrose density gradient and subjected to membrane washes of 2.5 M sodium bromide combined with a single wash of 0.1 M sodium carbonate as previously described (Chung et al., 2007). Washed OM were suspended in a final volume (500 μl) of 10 mM Tris–HCl pH 7.5. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay. All SDS–PAGE was performed according to Laemmli. OM protein preparations (10 μg) were boiled with SDS sample buffer for 1D-PAGE, then applied to 10% polyacrylamide gels and visualized by Coomassie blue.

2.2. Pig sera, immunoglobulin purification and immunoblot analysis

Pig sera that had previously been used to detect antigens and LPS of serotype 5, were kindly provided by M. Gottschalk (Département de pathologie et microbiologie, Université de Montréal, St-Hyacinthe, QC). Pre-immune sera were collected from healthy pigs; pooled immune sera were obtained from 15 convalescent pigs that had been experimentally infected with *A. pleuropneumoniae* serotype 5 (Stenbaek et al., 1997). Immune sera that were not purified showed nonspecific background on Western blots; immunoglobulin purification significantly reduced high background reactivity. Hence immunoglobulins from sera were isolated by a 1-ml gravity column packed with Protein A-Sepharose (Sigma). Resin was equilibrated with buffer A (0.2 M NaH_2PO_4, 0.15 M NaCl, pH 8) and pig serum (1 ml) was passed through the column. Following washes with buffer A, immunoglobulins were eluted in buffer B (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 3). Fractions were collected, pooled, and brought to pH 7 with 0.1 M NaOH. The solution was concentrated by Amicon filter units (Millipore) with a molecular weight cut-off of 10 kDa and purified immunoglobulins were resuspended in 1 ml of phosphate-buffered saline, pH 7 prior to freezing. For immunoblot analysis, OM proteins, separated by minigels or slab-sized 2D gels, were electrotransferred onto PVDF membrane (Bio-Rad) for 1 h at 100 V or by semi-dry transfer for 40 min at 15 V. After transfer, 2D immunoblots were stained with Ponceau S to visualize and to landmark selected spots for orientation, then rinsed with distilled water. Immunoblots were probed with sera at a 1:200 dilution and secondary antibody [alkaline phosphatase-conjugated AffiniPure goat anti-swine IgG (H+L), Jackson ImmunoResearch Laboratories, Inc.] at a 1:5000 dilution. Proteins were detected by adding NBT/BCIP substrate (Roche) in developing buffer.

2.3. DiGE and 2D-PAGE

For DiGE, three independent OM protein preparations of each growth condition (nutrient-rich and iron restriction) were minimally labeled with fluorescent CyDyes (GE Healthcare) following the manufacturer's instructions. Labeling of samples was randomized with Cy3 and Cy5 DiGE dyes to prevent preferential binding and dye bias. A pooled internal standard, composed of equal amounts of each sample replicate, was labeled with Cy2 DiGE dye. All incubations were performed in the dark and on ice. Each labeling reaction contained 50 μg of protein with 400 pmol CyDye; incubation was 30 min. Labeling reactions were quenched with 1 μl of 10 mM lysine (per 400 pmol of dye) and further incubated for 10 min. Iron-restricted samples were paired with nutrient-rich samples, combined with a Cy2-labeled internal standard (150 μg total protein) and mixed 1:1 with rehydration buffer (7 M urea, 2 M thiourea, 1% (w/v) dithiothreitol [DTT; Sigma], 1% (w/v) ASB-14, 4% (w/v) CHAPS) as described (Twine et al., 2005); incubation was 15 min. Samples were treated with additional rehydration buffer (to 300 μl) supplemented with 0.5%