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Poly-*N*-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*

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Abstract

Most field isolates of the swine pathogen *Actinobacillus pleuropneumoniae* form tenacious biofilms on abiotic surfaces in vitro. We purified matrix polysaccharides from biofilms produced by *A. pleuropneumoniae* field isolates IA1 and IA5 (serotypes 1 and 5, respectively), and determined their chemical structures by using NMR spectroscopy. Both strains produced matrix polysaccharides consisting of linear chains of *N*-acetyl-D-glucosamine (GlcNAc) residues in β(1,6) linkage (poly-β-1,6-GlcNAc or PGA). A small percentage of the GlcNAc residues in each polysaccharide were *N*-deacetylated. These structures were nearly identical to those of biofilm matrix polysaccharides produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. PCR analyses indicated that a gene encoding the PGA-specific glycoside transferase enzyme PgaC was present on the chromosome of 15 out of 15 *A. pleuropneumoniae* reference strains (serotypes 1–12) and 76 out of 77 *A. pleuropneumoniae* field isolates (serotypes 1, 5 and 7). A *pgaC* mutant of strain IA5 failed to form biofilms in vitro, as did wild-type strains IA1 and IA5 when grown in broth supplemented with the PGA-hydrolyzing enzyme dispersin B. Treatment of IA5 biofilms with dispersin B rendered them more sensitive to killing by ampicillin. Our findings suggest that PGA functions as a major biofilm adhesin in *A. pleuropneumoniae*. Biofilm formation may have relevance to the colonization and pathogenesis of *A. pleuropneumoniae* in pigs.

Keywords: Crystal violet; Dispersin B; *dspB*; *pgaABCD*

1. Introduction

Surface-associated colonies of bacteria known as biofilms play a role in the pathogenesis of many chronic infections [1]. Bacterial cells in a biofilm are encased in a self-synthesized, extracellular hydrogel matrix that holds the cells together in a mass and firmly attaches the bacterial mass to the underlying surface [2]. This matrix, also referred to as the slime layer, glycocalyx, or extracellular polymeric substance (EPS) matrix, can comprise up to 90% of the biofilm biomass [3]. In addition to its structural role, the EPS matrix provides biofilm cells with a protected microenvironment containing dissolved nutrients, secreted enzymes, DNA, and bacteriophages. The EPS matrix also contributes to the increased resistance to antibiotics and host defenses exhibited by biofilm cells [4]. Polysaccharide is a major component of the EPS matrix in most bacterial biofilms [2].

Abbreviations: BHI, brain heart infusion; EPS, extracellular polymeric substance; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNH_2$, glucosamine; MHB, Mueller–Hinton broth; PGA, poly-β-1, 6-*N*-acetyl-D-glucosamine; PBS, phosphate buffered saline; TSA, Tryptic Soy agar

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Actinobacillus pleuropneumoniae is a member of the *Pasteurellaceae*, a family of Gram-negative bacteria that includes many important human and animal pathogens. *A. pleuropneumoniae* colonizes the lungs of pigs and causes the severe and contagious respiratory disease swine pleuropneumonia [5]. Most field isolates of *A. pleuropneumoniae* form tenacious biofilms on abiotic surfaces in vitro [6]. *A. pleuropneumoniae* biofilms contain a hexosamine-rich polysaccharide that is functionally and genetically related to extracellular polysaccharide adhesins produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* [7]. These polysaccharides, usually referred to as PGA, PNAG or polysaccharide intercellular adhesin (PIA), consist of linear chains of *N*-acetyl-D-glucosamine (GlcNAc) residues in β(1,6) linkage (hereafter referred to as PGA). Various forms of PGA appear to differ in their molecular weight, in the degree of *N*-deacetylation of the GlcNAc residues, and in the presence of *O*-succinate substituents [8–11]. PGA has been shown to play a role in abiotic surface attachment and intercellular adhesion [12–15], protection from host innate defenses including phagocytosis and antimicrobial peptides [16], and virulence [17]. PGA appears to be essential for *A. pleuropneumoniae* biofilm formation in vitro because biofilms treated with a PGA-hydrolyzing enzyme were efficiently detached from surfaces [7].

The purpose of the present study was to gain better insight into the structural and functional role of PGA in *A. pleuropneumoniae* biofilm formation. We purified PGA polysaccharides from two biofilm-producing field isolates of *A. pleuropneumoniae* and determined their chemical structures by using NMR spectroscopy. We also investigated the phylogenetic distribution of PGA biosynthetic genes among 92 *A. pleuropneumoniae* reference strains and field isolates by using a PCR assay. Finally, we investigated the role of PGA in *A. pleuropneumoniae* biofilm formation in vitro by using both a PGA mutant strain and a PGA-degrading enzyme. In this report, we present the structure of *A. pleuropneumoniae* PGA along with evidence that PGA mediates intercellular adhesion, biofilm formation and antibiotic resistance in phylogenetically diverse *A. pleuropneumoniae* strains.

2. Results

2.1. Purification of A. pleuropneumoniae PGA

PGA was purified from two biofilm-positive *A. pleuropneumoniae* field isolates, IA1 and IA5 (serotypes 1 and 5, respectively). Extraction of IA1 and IA5 biofilms with saline was not sufficient to release PGA from the cells. Sonication, however, liberated large amounts of PGA, which eluted as a single peak on a gel filtration column (Fig. 1). Based on the elution profile, the molecular weight of *A. pleuropneumoniae* PGA was similar to that of staphylococcal PGA (>20 kDa; [10]). *A. pleuropneumoniae* PGA was partially soluble in water. Complete solubility of PGA was achieved by solubilizing the samples in a small volume of 5 M HCl. GLC analysis revealed that glucosamine was the only monosaccharide component of *A. pleuropneumoniae* PGA.

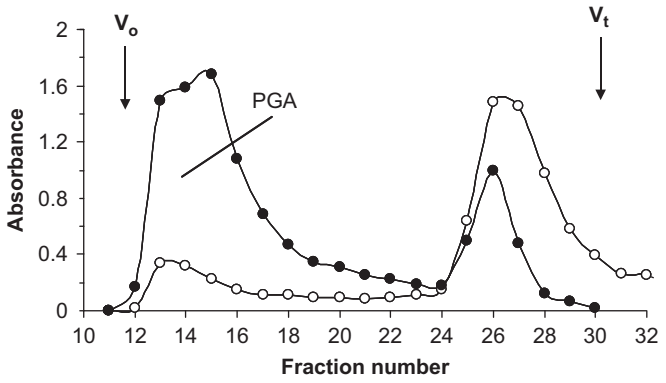

Fig. 1. Elution profile of a crude extracellular extract of *A. pleuropneumoniae* IA1 biofilm cells on a Sephacryl S-300 column irrigated with water. Aliquots (200 µL) of each 5-mL fraction were assayed for neutral sugars (○, A_{485}) and aminosugars (●, A_{530}). Void (V_o) and total (V_t) volumes of the column are indicated with arrows.

2.2. A. pleuropneumoniae PGA is a β(1,6)-linked GlcNAc polymer

Purified PGA polysaccharides were analyzed by ^{1}H-NMR spectroscopy (Fig. 2). The ^{1}H-NMR spectra of PGA from *A. pleuropneumoniae* IA1 and IA5 (Fig. 2, spectra b and a, respectively) were nearly identical to each other and to that of PGA purified from *S. epidermidis* strain RP62A (Fig. 2, spectrum c). In addition, all of the major signals of the β(1,6)-linked GlcNAc residues had shift assignments that were nearly identical to those reported for ^{1}H-NMR spectra of PGA isolated from various other staphylococcal strains and from *E. coli* [8–11,15,18]. The presence of minor peaks, including one at 2.7 ppm (H2 of $GlcNH_2$), may be due to partial *N*-deacetylation of GlcNAc residues [8,9]. These results indicate that *A. pleuropneumoniae* PGA is a linear polymer with the structure →6)-β-GlcNAc-(1→.

The chemical structure of *A. pleuropneumoniae* PGA was confirmed by 2D-NMR spectroscopy (Fig. 3). ^{1}H and ^{13}C chemical shifts of both $GlcNH_2$ and GlcNAc residues (Table 1) closely corresponded to those reported for PGA purified from *S. aureus* strain MN8m [8]. PGA from strain IA1 showed more heterogeneity than PGA from IA5 due to a higher degree of *N*-deacetylation, which was evident by the relative intensity of the peak at 2.7 ppm. According to the NMR data, ~25% of the total glucosamine residues in this IA1 PGA preparation were *N*-deacetylated. This result was in good agreement with the value obtained colorimetrically using the Smith and Gilkerson method (22±2%). The degree of *N*-deacetylation in PGA from strain IA5 varied from 1% to 16%, depending on the PGA preparation. No evidence for partial *O*-succinate substitution of *A. pleuropneumoniae* PGA was found.