

# Antibiofilm Activity of *Actinobacillus pleuropneumoniae* Serotype 5 Capsular Polysaccharide

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## Abstract

Cell-free extracts isolated from colony biofilms of *Actinobacillus pleuropneumoniae* serotype 5 were found to inhibit biofilm formation by *Staphylococcus aureus*, *S. epidermidis* and *Aggregatibacter actinomycetemcomitans*, but not by *A. pleuropneumoniae* serotype 5 itself, in a 96-well microtiter plate assay. Physical and chemical analyses indicated that the antibiofilm activity in the extract was due to high-molecular-weight polysaccharide. Extracts isolated from a mutant strain deficient in the production of serotype 5 capsular polysaccharide did not exhibit antibiofilm activity. A plasmid harboring the serotype 5 capsule genes restored the antibiofilm activity in the mutant extract. Purified serotype 5 capsular polysaccharide also exhibited antibiofilm activity against *S. aureus*. *A. pleuropneumoniae* wild-type extracts did not inhibit *S. aureus* growth, but did inhibit *S. aureus* intercellular adhesion and binding of *S. aureus* cells to stainless steel surfaces. Furthermore, polystyrene surfaces coated with *A. pleuropneumoniae* wild-type extracts, but not with capsule-mutant extracts, resisted *S. aureus* biofilm formation. Our findings suggest that the *A. pleuropneumoniae* serotype 5 capsule inhibits cell-to-cell and cell-to-surface interactions of other bacteria. *A. pleuropneumoniae* serotype 5 capsular polysaccharide is one of a growing number of bacterial polysaccharides that exhibit broad-spectrum, nonbiocidal antibiofilm activity. Future studies on these antibiofilm polysaccharides may uncover novel functions for bacterial polysaccharides in nature, and may lead to the development of new classes of antibiofilm agents for industrial and clinical applications.

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## Introduction

Biofilm is the predominant mode of growth for bacteria in most natural, industrial and clinical environments [1]. Biofilms typically contain millions of tightly-packed cells encased in a polymeric matrix attached to a tissue or surface. The biofilm mode of growth protects bacteria from cell stressors such as desiccation, predators and antibiotics. Biofilms cause corrosion and biofouling of industrial equipment and chronic infections in clinical settings [2]. New methods for treating and preventing biofilm formation are being sought.

Many biofilm bacteria secrete molecules such as quorum-sensing signals [3,4], surfactants [5], enzymes [6,7], polysaccharides [8], and D-amino acids [9] that function to regulate biofilm architecture or mediate the release of cells from biofilms during the dispersal stage of the biofilm life-cycle. These compounds often exhibit broad spectrum biofilm-inhibiting or biofilm-dispersing activity when tested against biofilms cultured in vitro. Such compounds may represent a novel source of antibiofilm compounds for technological development [10].

Previous studies showed that colony biofilms may be a useful source of novel antibiofilm compounds [11]. Colony biofilms are

lawns of bacterial cells cultured directly on an agar surface or on a semipermeable membrane that sits on an agar plate [12]. Colony biofilms have been shown to exhibit many properties characteristic of broth-cultured biofilms, including high cell density, extracellular matrix production, spatially dependent microbial growth, chemical gradients, and reduced susceptibility to antibiotics [13-18]. Cell-free extracts isolated from colony biofilms [11], or from biofilms cultured in a continuous-flow fermentor that produces a similarly high amount of biofilm biomass [19], have been shown to be enriched for soluble molecules produced within the biofilm matrix.

In the present study we screened a panel of 12 colony biofilm extracts isolated from various bacteria for their ability to inhibit *Staphylococcus aureus* biofilm formation in a 96-well microtiter plate assay. We found that colony biofilm extracts isolated from *Actinobacillus pleuropneumoniae* serotype 5 inhibited *S. aureus* biofilm formation without inhibiting *S. aureus* growth. Here we present results suggesting that the nonbiocidal antibiofilm activity in the *A. pleuropneumoniae* extract is due to high-molecular-weight serotype 5 capsular polysaccharide.

## Methods

### Bacterial Strains, Media and Growth Conditions

The bacterial strains used in this study are listed in Table 1. For solid media, Tryptic Soy agar was used for *A. pleuropneumoniae*, *A. actinomycetemcomitans* and *H. influenzae*, sheep blood agar (Catalog No. 221239; Becton, Dickinson and Co.) was used for staphylococci, and LB agar was used for all other bacteria. For broth cultures, Tryptic Soy broth supplemented with 6 g/L yeast extract and 8 g/L glucose was used for *A. pleuropneumoniae*, *A. actinomycetemcomitans*, *H. influenzae* and staphylococci, and LB broth was used for all others. *A. pleuropneumoniae* cultures were supplemented with 10 mg/L NAD. *H. influenzae* cultures were supplemented with 10 mg/L NAD and 10 mg/L hemin. Plasmid-harboring *A. pleuropneumoniae* strains were cultured in 80 mg/L spectinomycin. *A. pleuropneumoniae* and *A. actinomycetemcomitans* cultures were incubated in 10% CO<sub>2</sub>, whereas all other cultures were incubated in air. *P. carotovorum* cultures were incubated at 28°C, while all others were incubated at 37°C.

### Preparation of Colony Biofilm Extracts

A 100- $\mu$ l aliquot of an overnight broth culture ( $>10^8$  CFU) was spread onto the surface of a 100-mm-diam agar plate using a sterile glass spreader. The plate was incubated for at least 48 h until a robust lawn of microbial growth (colony biofilm) developed. Thereafter, the cell paste was scraped from the surface of the plate using a plastic inoculating loop or plastic cell scraper, and the cells were transferred to a microcentrifuge tube containing 750  $\mu$ l of saline (0.9% NaCl). The tubes were mixed by vortex agitation for 10 min, and the cells were pelleted by centrifugation. The supernatant was sterilized by passage through a 0.22- $\mu$ m pore-size filter, and the resulting colony biofilm extract was stored at 4°C until use.

### Screening Colony Biofilm Extracts for Antibiofilm Activity

*S. aureus* inocula were prepared from 18-h-old agar colonies as previously described [20]. A volume of 180  $\mu$ l of inoculum (ca.  $10^4$ – $10^5$  CFU/ml) was transferred to the well of a tissue-culture-treated polystyrene microtiter plate (Falcon no. 353047). A total of 20  $\mu$ l of colony biofilm extract, or 20  $\mu$ l of saline as a control, was mixed with the inoculum and the plate was incubated statically at 37°C. After 18 h, biofilms were rinsed with water and stained for 1 min with 200  $\mu$ l of Gram's crystal violet. Wells were then rinsed with water and dried. The amount of crystal violet binding was quantitated by destaining the wells for 10 min with 200  $\mu$ l of 33% acetic acid, and then measuring the absorbance of the crystal violet solution in a microplate spectrophotometer set at 595 nm.

### Physical and Chemical Analyses of *A. pleuropneumoniae* Colony Biofilm Extract

Size-exclusion filtration was carried out using a Microcon centrifugal concentrator (Millipore) with a 100-kDa molecular weight cut-off filter. For enzymatic treatments, extracts were incubated for 1 h at 37°C with 100 mg/L DNase I, RNase A, porcine pancreatic lipase or proteinase K (Sigma-Aldrich) or 10 mg/L dispersin B (Kane Biotech). Controls consisted of mock-treated extracts, or enzymes alone with no extract. For sodium metaperiodate treatment, 0.1 vol of 100 mM sodium metaperiodate was added to the extract, and the extract was incubated at 37°C for 1 h. Controls consisted of mock-treated extract and sodium metaperiodate alone with no extract. Following all treatments, extracts and controls were incubated at 100°C for 10 min prior to testing in the *S. aureus* biofilm assay described above.

**Table 1. Bacterial strains.**

Species	Strain	Source or reference*
<i>Acinetobacter lwoffii</i>	ATCC 15309	ATCC
<i>Acinetobacter haemolyticus</i>	ATCC 19002	ATCC
<i>Actinobacillus pleuropneumoniae</i>	J45 (wild-type; serotype 5a)	[21]
<i>Actinobacillus pleuropneumoniae</i>	J45-100 (J45 $\Delta$ cps5ABC; capsule-mutant)	[29]
<i>Actinobacillus pleuropneumoniae</i>	J45-100+ pJMMLCP55 (genetically-complemented capsule-mutant)	[29]
<i>Aggregatibacter actinomycetemcomitans</i>	CU1000	[36]
<i>Citrobacter freundii</i>	ATCC 43864	ATCC
<i>Enterobacter aerogenes</i>	ATCC 35029	ATCC
<i>Enterobacter amnigenus</i>	ATCC 51816	ATCC
<i>Haemophilus influenzae</i>	NJ9725	[37]
<i>Klebsiella pneumoniae</i>	ATCC BAA-1705	ATCC
<i>Lactococcus lactis</i>	525A	PIC
<i>Pectobacterium carotovorum</i>	ATCC 15713	ATCC
<i>Proteus vulgaris</i>	ATCC 8427	ATCC
<i>Pseudomonas aeruginosa</i>	PP	Environmental isolate
<i>Staphylococcus aureus</i>	SH1000	[38]
<i>Staphylococcus epidermidis</i>	NJ9709	[35]

\*ATCC, American Type Culture Collection, Manassas VA, USA; PIC, Presque Isle Cultures, Erie PA, USA.  
doi:10.1371/journal.pone.0063844.t001

## Purification of *A. pleuropneumoniae* Serotype 5 Capsular Polysaccharide

Capsular polysaccharide was purified from broth cultures of *A. pleuropneumoniae* strain J45 by Cetavlon precipitation of culture supernatant, extraction of the precipitate with NaCl and aqueous phenol, and Sepharose CL-4B gel filtration chromatography as previously described [21].

## Cell Binding Assay

A single-cell suspension of *S. aureus* (ca.  $10^6$ – $10^7$  CFU/ml) was prepared in fresh broth using a filtration protocol as previously described [22]. The cell suspension was supplemented with 10% *A. pleuropneumoniae* colony biofilm extract, or 10% saline as a control, and then aliquots of cells (0.5-ml each) were transferred to 1.5-ml polypropylene microcentrifuge tubes. Stainless steel rods (0.6-mm diam × 13-mm length) were placed in the tubes, and the tubes were incubated at 37°C. After 30 or 60 min, rods were removed from the tubes, rinsed three times with saline, and transferred to 15-ml conical centrifuge tubes containing 1 ml of saline. The rods were sonicated on ice (2 × 30 sec) using an IKA Labortechnik sonicator set to 50% power and 50% duty cycle. CFUs in the sonicate were quantitated by dilution plating.

## Intercellular Adhesion Assay

*S. aureus* was cultured in 17-mm × 100-mm glass tubes in 2 ml of broth. The broth was supplemented with 7% (by vol) *A. pleuropneumoniae* colony biofilm extract isolated from strain J45 or J45-100, or with 7% saline as a control. Bacteria were incubated with shaking (200 rpm). After 7 h, tubes were incubated statically for 10 min and then photographed.

## Surface Coating Assay

A volume of 25 μl of *A. pleuropneumoniae* colony biofilm extract, or 25 μl of saline as a control, was transferred to the center of a well of a 24-well tissue-culture-treated polystyrene microtiter plate (Falcon no. 353047). The plate was incubated at 37°C for 30 min to allow complete evaporation of the liquid. The wells were then filled with 1 ml of broth containing  $10^4$  to  $10^5$  CFU/ml of *S. aureus*. After 18 h, wells were rinsed with water and stained with 1 ml of Gram's crystal violet. Stained biofilms were rinsed with water and dried, and the wells were photographed.

## Microscopy

*A. pleuropneumoniae* inocula were prepared from 24-h-old agar colonies as previously described [20]. Bacterial inocula were diluted in fresh broth to  $10^4$ – $10^5$  CFU/ml. Aliquots of diluted cells (250 μL each) were pipetted onto the surface of sterile glass slides, and the slides were placed inside a Petri dish. After incubation for 24 h, the slides were rinsed with water and stained with SYTO9 (Molecular Probes) for 20 min in the dark. Slides were then rinsed with water to remove excess stain. Biofilm bacteria were visualized at 10× magnification using a Nikon Eclipse 80i fluorescent microscope.

## Statistics and Reproducibility of Results

All microtiter plate assays were performed in duplicate wells, which exhibited an average variation of <10%. All assays were performed 2–3 times with similarly significant differences in absorbance values. The significance of differences between means was measured using the Student's *t*-test. A *P* value of ≤0.05 was considered significant.

## Results

### *A. pleuropneumoniae* Colony Biofilm Extract Exhibits Antibiofilm Activity

We isolated colony biofilm extracts from 12 different bacteria and tested the extracts for their ability to inhibit biofilm formation by *S. aureus* in a 96-well microtiter plate assay (Fig. 1). The bacteria that were tested comprised a convenience sample of 11 *Proteobacteria* and *Lactococcus lactis* (Table 1). Extracts were tested at a concentration of 10% by vol. Under these conditions, five extracts significantly inhibited *S. aureus* biofilm formation, while seven extracts had no significant effect on biofilm formation. Inhibition of *S. aureus* biofilm formation by *P. aeruginosa* extract was partially due to growth inhibition (data not shown). We selected *A. pleuropneumoniae* IA5 colony biofilm extract for further analysis because it exhibited a high level of biofilm inhibition, but it did not inhibit *S. aureus* growth or contain proteases or DNases that are known to inhibit *S. aureus* biofilm formation (data not shown). *A. pleuropneumoniae* IA5 colony biofilm extract inhibited biofilm formation by *S. aureus*, *S. epidermidis*, and the Gram-negative *Aggregatibacter actinomycetemcomitans* (Fig. 2). *A. pleuropneumoniae* colony biofilm extract did not inhibit the growth of *S. aureus*, *S. epidermidis* or *A. actinomycetemcomitans* (data not shown).

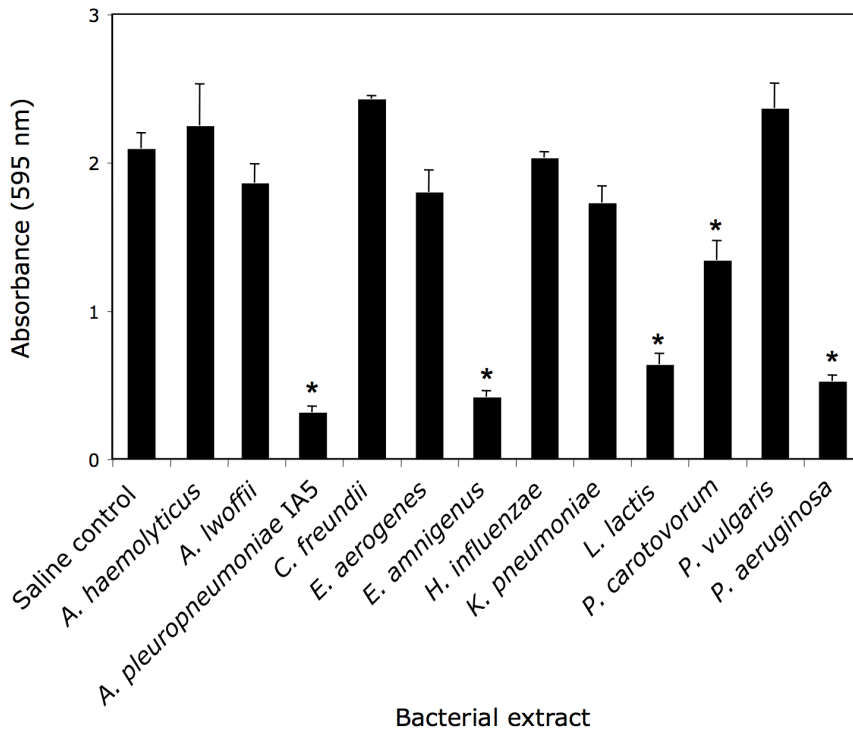
### The Antibiofilm Activity in *A. pleuropneumoniae* Colony Biofilm Extract is due to Capsular Polysaccharide

Physical analysis of the *A. pleuropneumoniae* IA5 colony biofilm extract indicated that the antibiofilm activity in the extract was >100 kDa in mass (Fig. 3A) and heat stable (Fig. 3B). Treatment of the extract with proteinase K, lipase, DNase or RNase had no effect on its antibiofilm activity (Fig. 3C). In contrast, treatment of the extract with the carbohydrate-active agent sodium metaperiodate significantly reduced its antibiofilm activity (Fig. 3C). These data suggest that the antibiofilm activity in the *A. pleuropneumoniae* colony biofilm extract was due to high-molecular-weight polysaccharide.

To determine whether the *A. pleuropneumoniae* serotype 5 capsule contributes to the antibiofilm activity of the colony biofilm extract, we isolated extracts from wild-type serotype 5 strain J45, and from the isogenic serotype 5 capsule-mutant strain J45-100. Like extracts isolated from strain IA5, extracts isolated from strain J45 inhibited *S. aureus* biofilm formation (Fig. 4A). In contrast, extracts isolated from capsule-mutant strain J45-100 did not inhibit *S. aureus* biofilm formation (Fig. 4A). Extracts isolated from capsule-mutant strain J45-100 transformed with plasmid pJMLCPS5, which restores capsule production, exhibited significantly greater antibiofilm activity than extracts isolated from uncomplemented J45-100 (Fig. 4B). This biofilm inhibition was not due to growth inhibition caused by antibiotic carryover from the genetically-complemented *A. pleuropneumoniae* culture (data not shown). In addition, purified serotype 5 capsular polysaccharide inhibited *S. aureus* biofilm formation in a dose-dependent manner (Fig. 4C). These findings confirm that the *A. pleuropneumoniae* serotype 5 capsule exhibits antibiofilm activity against *S. aureus*.

### *A. pleuropneumoniae* Colony Biofilm Extract Inhibits *S. aureus* Cell-to-cell and Cell-to-Surface Interactions

*S. aureus* cells cultured in broth with shaking aggregated and settled to the bottom of the tube, resulting in a visible clearing of the broth (Fig. 5A, left panel). When *A. pleuropneumoniae* J45 extract was present in the culture, the *S. aureus* cells exhibited less settling (Fig. 5A, middle panel). *S. aureus* cells cultured in the presence of extract isolated from serotype 5 capsule-mutant strain J45-100



**Figure 1. *S. aureus* biofilm formation in the presence of 12 different cell-free colony biofilm extracts.** *S. aureus* was cultured in the presence 10% extract isolated from the bacteria indicated along the bottom. Control cultures were incubated with 10% saline. After 18 h, biofilms were rinsed and stained with crystal violet. Values show the average amount of crystal violet binding for duplicate wells and error bars indicate range. Asterisks indicate absorbance values significantly different from saline control ( $P < 0.05$ ). doi:10.1371/journal.pone.0063844.g001

exhibited the same amount of settling as control cultures (Fig. 5A, right panel). These data suggest that *A. pleuropneumoniae* serotype 5 capsule inhibits *S. aureus* intercellular adhesion.

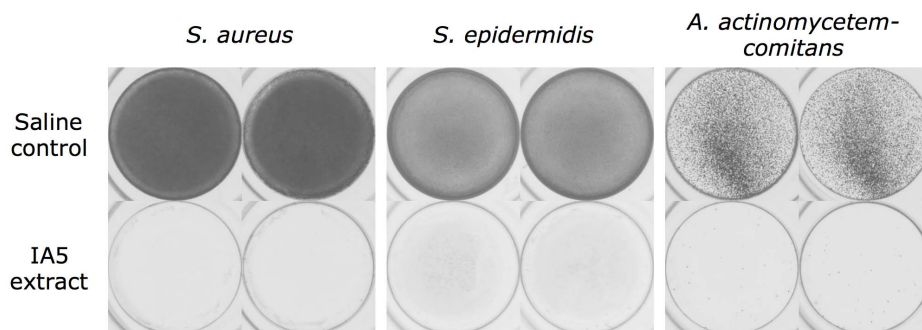
We also measured binding of *S. aureus* planktonic cells to stainless steel rods in the presence and absence of *A. pleuropneumoniae* J45 colony biofilm extract. As shown in Fig. 5B, the *A. pleuropneumoniae* extract significantly inhibited *S. aureus* cell binding after both 30 and 60 min.

We also tested whether *A. pleuropneumoniae* J45 colony biofilm extract could modify the surface properties of an abiotic substrate. To do this, we used evaporation coating to deposit the extract onto the surface of polystyrene wells, and then tested the ability of the coated surfaces to resist biofilm formation by *S. aureus*. When *A. pleuropneumoniae* extract was applied to the polystyrene surfaces, the

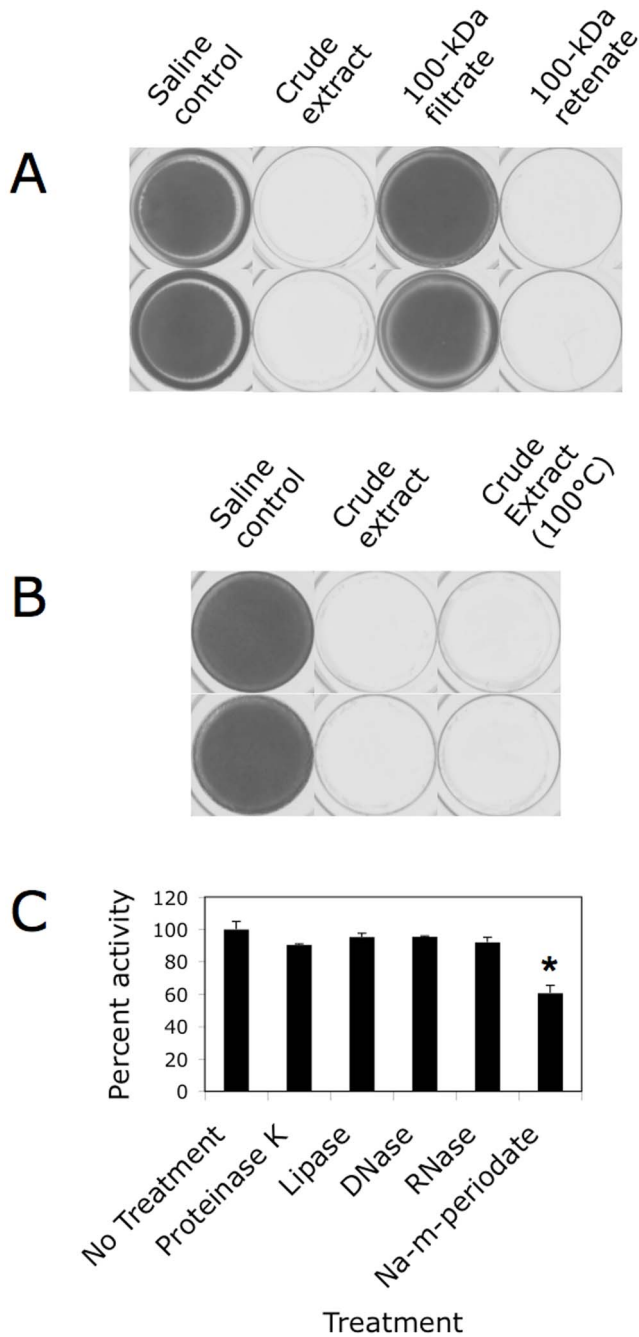
coated surfaces efficiently repelled *S. aureus* biofilm formation in the area where the extract was deposited (Fig. 5C). Surfaces coated with extract isolated from the serotype 5 capsule-mutant strain did not resist *S. aureus* biofilm formation (Fig. 5C).

#### *A. pleuropneumoniae* Colony Biofilm Extract does not Inhibit *A. pleuropneumoniae* Biofilm Formation

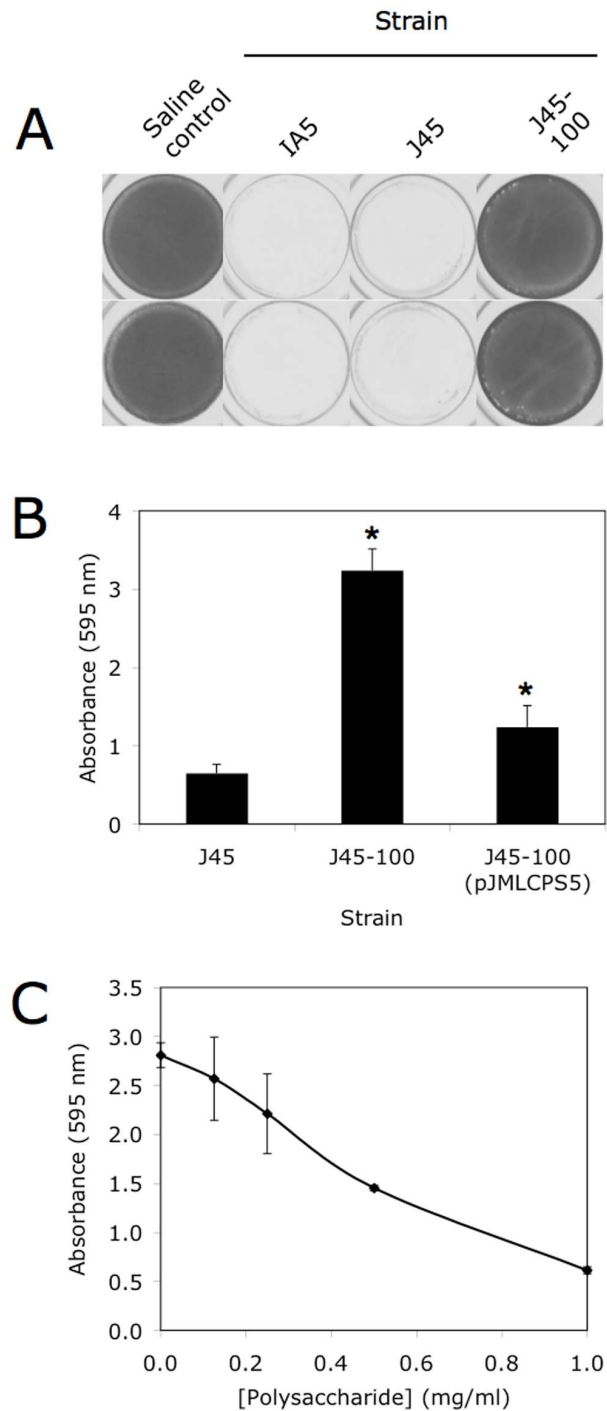
Extracts isolated from *A. pleuropneumoniae* wild-type strain J45 and capsule-mutant strain J45-100 were tested for their ability to inhibit biofilm formation by *A. pleuropneumoniae* J45 and J45-100. There was no significant difference between the amount of biofilm formed by strains J45 and J45-100 when quantitated by spectrophotometry (Fig. 6A). Neither the J45 nor the J45-100



**Figure 2. Biofilm formation by *S. aureus*, *S. epidermidis* and *A. actinomycetemcomitans* in the presence of *A. pleuropneumoniae* colony biofilm extract.** Inocula were supplemented with 10% *A. pleuropneumoniae* IA5 extract, or 10% saline as a control. Bacteria were cultured in 96-well microtiter plates. After 18 h, biofilms were rinsed, stained with crystal violet, and photographed. Duplicate wells are shown. doi:10.1371/journal.pone.0063844.g002

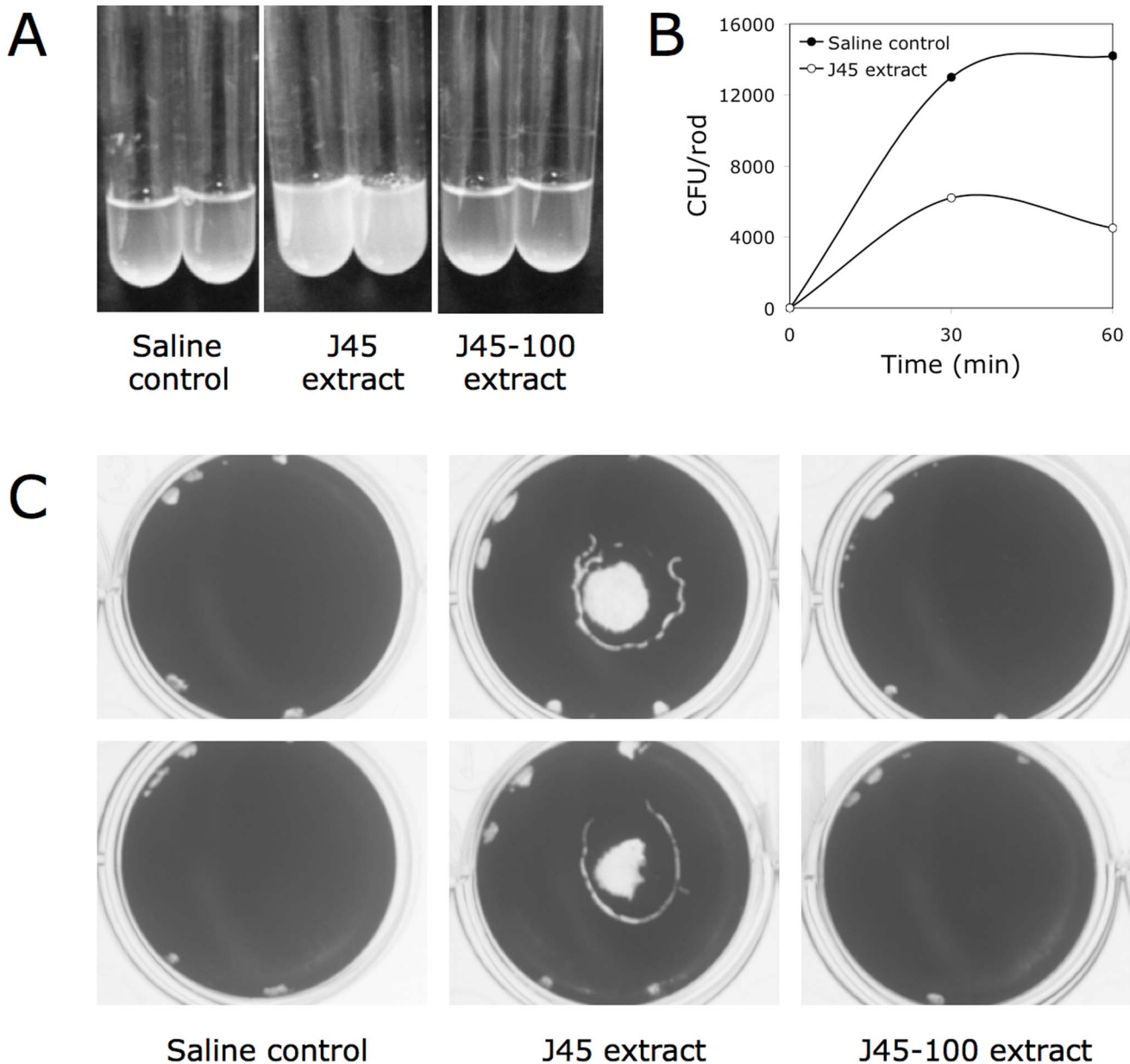


**Figure 3. Physical and chemical analyses of the antibiofilm activity in *A. pleuropneumoniae* IA5 colony biofilm extract.** (A) Biofilm formation by *S. aureus* in the presence of 10% saline (control), crude extract, or the filtrate and retentate of crude extract passed through a 100-kDa pore-size filter. Duplicate wells are shown. (B) Biofilm formation by *S. aureus* in the presence of 10% saline, crude extract, or crude extract that was incubated at 100°C for 15 min. Duplicate wells are shown. (C) Biofilm-inhibiting activity of IA5 extract treated with proteinase K, lipase, DNase, RNase or sodium metaperiodate. Biofilm inhibition was measured against *S. aureus* as in panels A and B. Percent activity was calculated as the ratio of the reduction in crystal violet absorbance exhibited by the treated extract to the reduction in absorbance exhibited by the mock-treated extract ( $\Delta A_{595}(\text{treated extract})/\Delta A_{595}(\text{mock-treated extract}) \times 100$ ). The graph shows mean and range percent activity values from 2-3 experiments. Asterisk indicates a significant reduction in activity ( $P < 0.05$ ). doi:10.1371/journal.pone.0063844.g003



**Figure 4. Biofilm formation by *S. aureus* in the presence of *A. pleuropneumoniae* serotype 5 wild-type and capsule-mutant colony biofilm extracts and purified serotype 5 capsular polysaccharide.** (A) *S. aureus* biofilm formation in the presence of colony biofilm extracts isolated from wild-type strains IA5 and J45, and isogenic J45 capsule-mutant J45-100. Duplicate wells are shown. \*, significantly different from J45 control extract ( $P < 0.05$ ). (B) Quantitation of *S. aureus* biofilm formation in the presence of extracts isolated from wild-type J45, capsule mutant J45-100, and genetically-complemented J45-100 capsule-mutant. (C) Quantitation of *S. aureus* biofilm formation in the presence of purified serotype 5 capsular polysaccharide. Values in panels B and C show averages for duplicate wells and error bars indicate range. doi:10.1371/journal.pone.0063844.g004





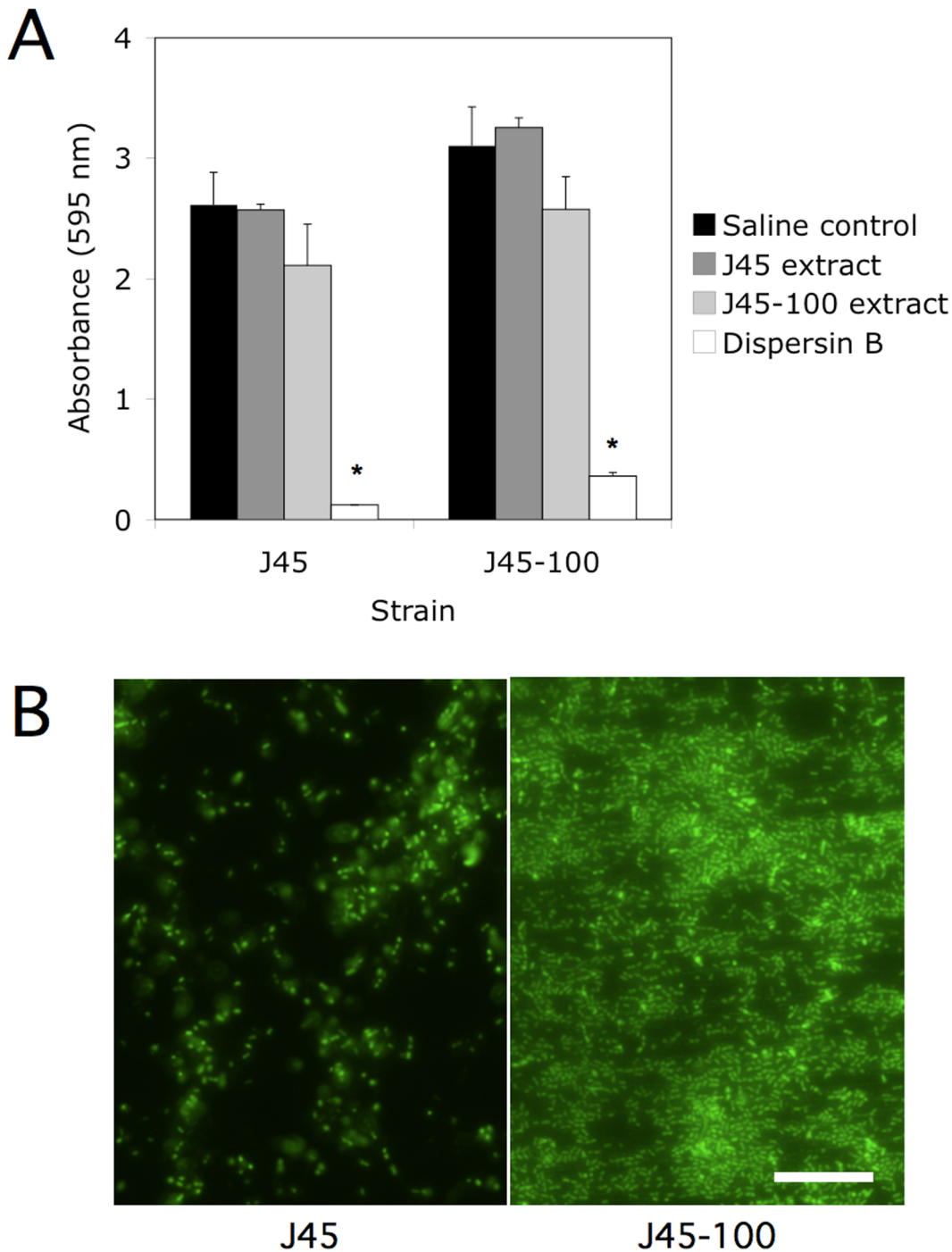
**Figure 5. *A. pleuropneumoniae* serotype 5 colony biofilm extract exhibits surfactant-like properties.** (A) Interference microscopy images of *S. aureus* planktonic cells cultured in 10% saline (control), wild-type J45 extract, or capsule-mutant J45-100 extract. Duplicate tubes are shown. (B) Attachment of *S. aureus* planktonic cells to stainless steel rods in the presence of 10% *A. pleuropneumoniae* J45 colony biofilm extract. Values show average for duplicate rods. (C) Biofilm formation by *S. aureus* in polystyrene microtiter plate wells coated with saline, J45 extract, or J45-100 extract. Duplicate wells are shown. doi:10.1371/journal.pone.0063844.g005

extract significantly inhibited biofilm formation by either strain. Dispersin B significantly inhibited biofilm formation by both strains, indicating that strain J45, like *A. pleuropneumoniae* serotype 5 strain IA5 [23], produces PNAG-dependent biofilms. Microscopic analysis revealed that biofilms produced by the capsule mutant strain J45-100 appeared much denser than biofilms produced by wild-type strain J45 (Fig. 6B).

**Discussion**

The Gram-negative bacterium *A. pleuropneumoniae* is the causative agent of swine pleuropneumonia, a severe and contagious

respiratory disease that affects pigs worldwide [24]. *A. pleuropneumoniae* strains are divided into 15 serotypes based on the structures of their capsular polysaccharide (CPS) [25]. Serotype 5 is a prevalent *A. pleuropneumoniae* serotype in the U.S., Canada, Brazil, Chile, Korea and Taiwan [26]. *A. pleuropneumoniae* serotype 5 CPS consists of a linear polymer with the structure  $\rightarrow 6)-\alpha\text{-D-Glc}p\text{NAc}-(1\rightarrow 5)-\beta\text{-D-dOclAp}-(2\rightarrow$  [27]. A subset of serotype 5 strains (designated serotype 5b) contain an additional  $\beta\text{-D-Glc}p$  residue covalently joined to the  $\beta\text{-D-dOclAp}$  residue in (1 $\rightarrow$ 4) linkage [26]. Mutant strains lacking serotype 5 CPS have been shown to exhibit decreased serum resistance *in vitro* and decreased virulence in pigs [28,29].



**Figure 6. Biofilm formation by *A. pleuropneumoniae* wild-type J45 and capsule-mutant J45-100.** (A) Quantitation of biofilm formation in 96-well polystyrene microtiter plates. Biofilms were grown in the presence of 10% saline (control), J45 colony biofilm extract, J45-100 colony biofilm extract, or 10 mg/L dispersin B. Biofilms were stained with crystal violet. Values show mean absorbance values and standard deviation from four independent experiment. \*, significantly different from saline control ( $P < 0.05$ ). (B) Micrographs of 24-h-old biofilms cultured on glass slides. Cells were stained with SYTO9. Measure bar = 20  $\mu$ m. doi:10.1371/journal.pone.0063844.g006

Our findings demonstrate that *A. pleuropneumoniae* serotype 5 CPS exhibits nonbiocidal antibiofilm activity against other Gram-negative and Gram-positive bacteria. Several other bacteria produce nonbiocidal antibiofilm polysaccharides including *Kingella kingae* [11], *Escherichia coli* [8,19], *Bacillus licheniformis* [30], *Lactobacillus acidophilus* [31], *Streptococcus phocae* [32] and *Vibrio* sp.

[33]. The biological functions of these antibiofilm polysaccharides are not known, but they may include water channel formation or biofilm dispersal [34]. The fact that the *A. pleuropneumoniae* CPS mutant strain exhibited increased biofilm formation compared to the wild-type strain (Fig. 6B) suggests that the serotype 5 capsule may function in water channel formation or biofilm dispersal.

Previous studies showed that *A. pleuropneumoniae* produces dispersin B, which may also function in maintaining biofilm architecture or in biofilm dispersal [23].

We found that *A. pleuropneumoniae* serotype 5 CPS inhibited biofilm formation by *S. aureus*, *S. epidermidis* and *A. actinomycetemcomitans*, but not by *A. pleuropneumoniae* serotype 5 itself. Biofilm formation by all four of these species is dependent on the production of poly-*N*-acetylglucosamine (PNAG) matrix polysaccharide [20,35]. Although PNAG is the major biofilm matrix adhesin in both *A. pleuropneumoniae* and *S. epidermidis* biofilms, *A. pleuropneumoniae* serotype 5 CPS inhibited only *S. epidermidis* biofilm formation and not *A. pleuropneumoniae* serotype 5 biofilm formation. In addition, colony biofilm extracts isolated from a PNAG-deficient strain of *A. pleuropneumoniae* serotype 5 exhibited the same antibiofilm activity as extracts isolated from a wild-type strain (unpublished results). These observations suggest that the anti-

biofilm activity of serotype 5 CPS is independent of the intercellular adhesion activity of PNAG.

Our screen of colony biofilm extracts isolated from 12 phylogenetically diverse bacteria identified five extracts that exhibited antibiofilm activity against *S. aureus*. In a similar screen, Rendueles *et al.* [19] found that 20% of cell-free biofilm extracts isolated from 122 natural *E. coli* isolates exhibited antibiofilm activity against a panel of seven biofilm-forming Gram-positive and Gram-negative bacteria. These findings suggest that bacterial biofilms constitute untapped sources of natural bioactive molecules antagonizing adhesion or biofilm formation of other bacteria.

## Author Contributions

Conceived and designed the experiments: DEK TJI JBK. Performed the experiments: MTK MB EAI VS TJI. Analyzed the data: MTK DEK TJI JBK. Wrote the paper: MTK DEK TJI JBK.

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