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Pseudomonas aeruginosa rhamnolipids disperse *Bordetella bronchiseptica* biofilms

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Abstract

We have previously reported that the respiratory pathogen *Bordetella bronchiseptica* can form biofilms in vitro. In this report, we demonstrate the disruption of *B. bronchiseptica* biofilms by rhamnolipids secreted from *Pseudomonas aeruginosa*. This suggests that biosurfactants such as rhamnolipids may be utilized as antimicrobial agents for removing *Bordetella* biofilms.

Keywords: *Bordetella bronchiseptica*; Biofilm; Rhamnolipids

1. Introduction

Bacterial biofilms are surface-adherent, multi-cellular communities that represent a fundamentally different physiological state compared to free-living planktonic bacteria [1–3]. Biofilms are thought to be representative of most bacterial growth on natural surfaces, and can be found in a wide array of natural and artificial environments ranging from rocks in a stream to shower curtains [4,5]. Biofilms are also important in pathogenesis of various bacterial infections. Nosocomial infections of pathogens such as *Staphylococcus* spp. occur via biofilm formation on catheters and other medical equipments. In addition, dental plaques, cystic fibrosis pneumonia, and infective endocarditis are thought to be caused by bacteria forming biofilms [5]. Various reports indicate that biofilms confer an enhanced resistance against antibiotics [6]. Hypotheses as to why biofilms show increased antibiotic resistance include decreased antibiotic penetration through biofilm structures [7], upregulation of expression of multi-drug efflux pumps [8], and expression of periplasmic glucans that directly bind and sequester antibiotics [9]. The phenotypes of antibiotic resistance and enhanced attachment to surfaces represent difficulties in combating bacterial biofilms in both medical and industrial settings.

Pseudomonas aeruginosa biofilms have been extensively studied due to its relative ease of biofilm formation under various conditions in vitro, its medical importance, and the genetic tractability of the organism [10]. During the later stages of *P. aeruginosa* biofilm development, the production of the biosurfactant rhamnolipid was shown to be important in modulating microcolony architecture by maintaining channels to allow fluids to flow through the biofilm [11]. Several surfactants produced by other bacteria have been shown

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to inhibit biofilm formation of heterospecific bacteria, and examples include surfactin produced by *Bacillus subtilis* [12] and surlactin produced by *Lactobacillus* spp. [13–15]. *P. aeruginosa* rhamnolipids may therefore be able to affect biofilms of other bacterial species in a similar fashion [11].

Bordetella bronchiseptica is a Gram-negative coccobacilli species closely related to the human pathogens *Bordetella pertussis* and *Bordetella parapertussis*, which cause whooping cough. *B. bronchiseptica* infects the respiratory tract of a wide range of mammals, often leading to life-long chronic colonization [16]. Once the respiratory tract of animals have been infected, it is extremely difficult to eliminate *B. bronchiseptica* from the host. Infection by *B. bronchiseptica* has been closely associated with various respiratory diseases including atrophic rhinitis in pigs, kennel cough in dogs, snuffles in rabbits and bronchopneumonia in cats. The most severe disease symptoms usually occur in co-infections with secondary agents including *Pasteurella multocida* and various respiratory viruses. *Bordetella* species utilize the BvgAS (*Bordetella* virulence gene) two-component signal transduction system to sense environmental stimuli and regulate the expression of various genes [17]. Many of these genes are virulence factors, including adhesins such as filamentous hemagglutinin (FHA), fimbriae, and pertactin; and toxins such as the bifunctional adenylate cyclase/haemolysin (ACY), pertussis toxin, and type III secretion system [16]. The differential control of the BvgAS system results in at least three distinct phases of growth under various environmental conditions: the virulent Bvg^+, intermediate Bvg^i, and the non-virulent Bvg^- phases. We have previously reported that *B. bronchiseptica* forms a maximal biofilm phenotype in Bvg^i phase [18]. This suggests a possible role of biofilm growth in infected hosts, as conditions in the nasal cavity of the upper respiratory tract is hypothesized to support a Bvg^i phase growth. The difficulty in removing this organism from infected hosts is also indirect evidence that biofilms may be involved in the colonization process.

In this report, we demonstrate that *B. bronchiseptica* biofilms can be effectively disrupted by treatment with the rhamnolipids secreted by *P. aeruginosa* PAO1 in vitro. Therefore, *P. aeruginosa* rhamnolipids may function to disrupt bacterial biofilms in nature, and this biosurfactant may be a potential candidate for use in treatment strategies to eliminate *B. bronchiseptica* infections.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. bronchiseptica strains RB50 (wild type) and RB53i (Bvg^i phase-locked mutant) were previously reported and characterized [19,20]. RB50 and RB53i were cultured in Stainer–Scholte (SS) liquid medium [21] or on BG agar (Becton Dickinson) supplemented with defibrinated sheep blood at 37 °C. For Bvg phase modulation, bacteria were grown in SS media with nicotinic acid (Sigma) added to appropriate final concentrations. *P. aeruginosa* PAO1 (wild type), 12985 (*rhlA′*::IS*lacZ*/hah Tn5 mutant strain), and 45577 (*rhlB′*::IS*phoA*/hah Tn5 mutant strain) were obtained from the University of Washington Genome Center *P. aeruginosa* PAO1 mutant collection. Unless otherwise stated, *P. aeruginosa* were propagated on either LB agar or in LB broth.

2.2. Preparation of conditioned medium

Pseudomonas spent medium was prepared from *P. aeruginosa* cultures grown in 100 ml SS for 36 h at 37 °C. Cultures were centrifuged at 10,000*g* at 4 °C, and the supernatant was filtered through 0.22 μm cellulose acetate filter unit (Corning). Conditioned medium was prepared by adding fresh 2× concentrated SS to the spent medium.

2.3. Purification of rhamnolipids

P. aeruginosa rhamnolipids were isolated using a modified protocol from previous reports [22,23]. Polystyrene resin Amberlite XAD-2 (Supelco) was incubated with methanol for 30 min prior to preparation. Resin was then washed with 0.1 M phosphate buffer, pH 6.1, and centrifuged to remove the supernatant. Twenty millilitres of *P. aeruginosa* spent medium was incubated with the equal volume of resin at 37 °C with agitation for 12 h, and washed with phosphate buffer three times. Rhamnolipids were extracted with methanol, evaporated to dryness under vacuum, and then re-suspended with 500 μl de-ionized H_2O.

2.4. Biofilm growth conditions

Quantitative assay of biofilm formation was performed in a 96-well plate format as previously described [18]. Overnight *B. bronchiseptica* cultures were inoculated 1:20 into 100 μl SS medium and incubated statically for 24 h at 37 °C. Biofilms were then washed vigorously with running water, and stained with 150 μl crystal violet for 30 min. The plates were dried, crystal violet stains were solubilised with 200 μl 33% acetic acid, and the biofilm formation was quantified by reading the absorbance at 595 nm using a plate reader. In experiments where biofilms were incubated with *Pseudomonas* conditioned media or rhamnolipids, media were first removed and replaced with 125 μl fresh medium containing antibiotics, conditioned medium, or rhamnolipid, and incubated at 37 °C. Biofilms for microscopy were grown in non-tissue culture-coated glass chamber slides