The *Pseudomonas aeruginosa* Exopolysaccharide Psl Facilitates Surface Adherence and NF-κB Activation in A549 Cells

Matthew S. Byrd,a Bing Pang,a Meenu Mishra,b W. Edward Swords,a and Daniel J. Wozniakb

Department of Microbiology and Immunology, Wake Forest University Health Sciences, Winston-Salem, North Carolina, USA,a and Center for Microbial Interface Biology, The Ohio State University, Columbus, Ohio, USAb

M.S.B. and B.P. contributed equally to this work.

**ABSTRACT** In order for the opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* to cause an airway infection, the pathogen interacts with epithelial cells and the overlying mucous layer. We examined the contribution of the biofilm exopolysaccharide Psl to epithelial cell adherence and the impact of Psl on proinflammatory signaling by flagellin. Psl has been implicated in the initial attachment of *P. aeruginosa* to biotic and abiotic surfaces, but its direct role in pathogenesis has not been evaluated (L. Ma, K. D. Jackson, R. M. Landry, M. R. Parsek, and D. J. Wozniak, J. Bacteriol. 188:8213–8221, 2006). Using an NF-κB luciferase reporter system in the human epithelial cell line A549, we show that both Psl and flagellin are necessary for full activation of NF-κB and production of the interleukin 8 (IL-8) chemokine. We demonstrate that Psl does not directly stimulate NF-κB activity, but indirectly as a result of increasing contact between bacterial cells and epithelial cells, it facilitates flagellin-mediated proinflammatory signaling. We confirm differential adherence of Psl and/or flagellin mutants by scanning electron microscopy and identify Psl-dependent membrane structures that may participate in adherence. Although we hypothesized that Psl would protect *P. aeruginosa* from recognition by the epithelial cell line A549, we instead observed a positive role for Psl in flagellin-mediated NF-κB activation, likely as a result of increasing contact between bacterial cells and epithelial cells.

**IMPORTANCE** *Pseudomonas aeruginosa* is the predominant airway pathogen causing morbidity and mortality in individuals affected by the genetic disease cystic fibrosis. *P. aeruginosa* can also cause severe pneumonia, burn wound infections, and sepsis, making its overall impact on human health significant. The attachment of *P. aeruginosa* to host tissues, often leading to recalcitrant biofilm infections, and inflammation induced by flagellin are both important mechanisms of virulence. We explored the role of the biofilm exopolysaccharide Psl in the pathogenesis of *P. aeruginosa* and found that Psl is required for surface adherence to A549 epithelial cells, and as an adhesin, it facilitates flagellin-mediated NF-κB activation. This work was done to better understand the initial events of infection and revealed that a biofilm polysaccharide contributes to inflammation in a novel manner.

Received 13 May 2010 Accepted 1 June 2010 Published 29 June 2010

Citation Byrd, M. S., B. Pang, M. Mishra, W. E. Swords, and D. J. Wozniak. 2010. The *Pseudomonas aeruginosa* exopolysaccharide Psl facilitates surface adherence and NF-κB activation in A549 cells, mBio 1(3):e00140-10. doi:10.1128/mBio.00140-10.

Editor Gerald Pier, Harvard Medical School

Copyright © 2010 Byrd et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Daniel J. Wozniak, Daniel.wozniak@osumc.edu.

In lung infections, the Gram-negative opportunist *Pseudomonas aeruginosa* initially encounters airway epithelial cells and their associated covering of mucus, which is characteristically thick and dehydrated in cystic fibrosis (CF) patients (1). Psl is a mannosereich polysaccharide adhesin involved in biotic and abiotic surface attachment and biofilm formation, and it is hypothesized to be important in adherence to epithelial cells early in infection, likely by facilitating interactions between bacteria (2–7). Although its role in *P. aeruginosa* pathogenesis has not been explored, Psl may have direct stimulatory or inhibitory effects on the host immune response, or it may function primarily to facilitate interactions between *P. aeruginosa* and host cells. An intact flagellum is also required for attachment to a variety of surfaces, including respiratory mucins, and for surface motility associated with biofilm formation (8–12). In addition to the role of flagella in attachment, the flagellar structural subunit FlIC elicits proinflammatory signaling through Toll-like receptor 5 (TLR5), resulting in activation of the NF-κB transcription factor among other factors (13–15).

We hypothesized that if Psl protects against immune recognition, then a *psl* mutant would generate a greater NF-κB response in A549 epithelial cells than the parental strain would. However, we observed that Psl is required for full activation of NF-κB and that this phenotype is likely a result of Psl-mediated NF-κB activation. This work was done to better understand the initial events of infection and revealed that a biofilm polysaccharide contributes to inflammation in a novel manner.

Psldoes not directly stimulate NF-κB activation in A549/NF-κB-luc cells. Initial experiments using A549 cells stably transfected with an NF-κB-responsive luciferase reporter (*A549/NF-κB-luc* cells), cultured in 24-well plates as described previously (16), showed a decrease in NF-κB activation in A549 cells infected with *P. aeruginosa* lacking Psl compared to the parental strain expressing Psl (M. S. Byrd and B. Pang, unpublished data). To determine whether the effect of Psl on NF-κB activation is direct, *A549/NF-κB-luc* cells (~4 × 10⁵ cells/well) were infected in triplicate with mid-log-phase *P. aeruginosa* at a multiplicity of infection...
and then incubated for 1 h at 37°C with 5% CO2 before fresh FliC (final concentration of 10^-9 M) (Fig. 1A). An isogenic bars) for three experiments. (B) Western blots of whole-cell lysates against NF-B activity were in Text S1 in the supplemental material. In the leftmost lane with an asterisk, purified Salmonella FliC (final concentration of 10^-9 M) was used as a positive control. Values are means plus standard errors of the means (error bars) for three experiments.

(A) Fold change in luminescence over DMEM, normalized to an MOI of 10 for P. aeruginosa PAO1, Δpsl and Δflic single and double mutants, and the arabinose-inducible Δflic araC-pBAD-psl mutant grown in 0, 0.05, or 1.0% L-Ara. Purified Salmonella FliC (final concentration of 10^-9 M) was used as a positive control. Values are means plus standard errors of the means (error bars) for three experiments.

(B) Western blots of whole-cell lysates against P. aeruginosa flagellin type B (using an antibody against FlaB (α-FlaB)) and Psl immunoblot of 1.5-µl EDTA extracts (α-Psl), corresponding with strains from panel A. Conditions for flagellin Western blotting and Psl immunoblotting are in Text S1 in the supplemental material. In the leftmost lane with an asterisk, purified P. aeruginosa type B flagellin (1 µg) was used as a positive control. N/D, not done. (C) Percent adherence of strains from panels A and B compared to inocula by plate counts following washing and lysis with 1% saponin. Values are means plus standard errors of the means (error bars) for three experiments.

FIG 1 NF-κB activation in A549/NF-κB-luc cells by P. aeruginosa. (A) Fold change in luminescence over DMEM, normalized to an MOI of 10 for P. aeruginosa PAO1, Δpsl and Δflic single and double mutants, and the arabinose-inducible Δflic araC-pBAD-psl mutant grown in 0, 0.05, or 1.0% L-Ara. Purified Salmonella FliC (final concentration of 10^-9 M) was used as a positive control. Values are means plus standard errors of the means (error bars) for three experiments.

(B) Western blots of whole-cell lysates against P. aeruginosa flagellin type B (using an antibody against FlaB (α-FlaB)) and Psl immunoblot of 1.5-µl EDTA extracts (α-Psl), corresponding with strains from panel A. Conditions for flagellin Western blotting and Psl immunoblotting are in Text S1 in the supplemental material. In the leftmost lane with an asterisk, purified P. aeruginosa type B flagellin (1 µg) was used as a positive control. N/D, not done. (C) Percent adherence of strains from panels A and B compared to inocula by plate counts following washing and lysis with 1% saponin. Values are means plus standard errors of the means (error bars) for three experiments.

To visualize the contribution of Psl and flagellin to P. aeruginosa adherence to A549/NF-κB-luc cells, the adherence experi-
Psl production was induced by the addition of L-Ara at either 0.05% or 1.0%, and percent adherence was calculated. Aggregates of adherent bacteria were counted in 20 predetermined fields for electron microscopy (SEM) analysis as described previously (2). Following washing, the third coverslip was processed for scanning electron microscopy (SEM) as described previously (2).

Each sample, and percent adherence was calculated. Aggregates of adherent bacteria were counted in 20 predetermined fields for electron microscopy (SEM) analysis as described previously (2). Following washing, the third coverslip was processed for scanning electron microscopy (SEM) as described previously (2).

FIG 2  Representative SEM images of adherent P. aeruginosa on A549/NF-κB-luc cells infected with P. aeruginosa PAO1 (A), Δpss mutant (B), ΔflIC mutant (C), Δpsl ΔflIC mutant (D), and ΔflIC pBAD Δpsl mutant (E to G) at three different concentrations of L-Ara, 0% (E), 0.05% (F), and 1.0% (G). The white arrows indicate Psl-dependent membrane structures. Bar, 1 μm.

ACKNOWLEDGMENTS

We thank Gayle Foster and Jim Turner for technical assistance and Ken Grant for SEM assistance. Steven Mize generously provided Salmonella and P. aeruginosa type B purified flagellins.

This work was supported by Public Health Service grants AI061396 and HL058334 (D.J.W.) and NRSA fellowship AI0787002 (M.S.B.).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00140-10/-/DCSupplemental.

Text S1, DOC file, 0.06 MB.

Fig. S1, TIF file, 0.14 MB.

Fig. S2, TIF file, 0.16 MB.

Fig. S3, TIF file, 0.16 MB.

REFERENCES

1. Govan, J. R., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol. Rev. 60:539–574.

2. Byrd, M. S., I. Sadovskaya, E. Vinogradov, H. Lu, A. B. Sprinkle, S. H. Richardson, L. Ma, B. Ralston, M. R. Parsek, E. M. Anderson, J. S. Lam, and D. J. Wozniak. 2009. Genetic and biochemical analyses of the Pseudomonas aeruginosa Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. Mol. Microbiol. 73:622–638.

3. Friedman, L., and R. Kolter. 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. J. Bacteriol. 186:4457–4465.

4. Jackson, K. D., M. Starkey, S. Kromer, M. R. Parsek, and D. J. Wozniak. 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for Pseudomonas aeruginosa PA01 biofilm formation. J. Bacteriol. 186:4466–4475.

5. Ma, L., M. Conover, H. Lu, M. R. Parsek, K. Bayles, and D. J. Wozniak. 2009. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. PLoS Pathog. 5(3):e1000354.
Ma, L., K. D. Jackson, M. R. Parsek, and D. J. Wozniak. 2006. Analysis of Pseudomonas aeruginosa conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. J. Bacteriol. 188:8213–8221.

Matsukawa, M., and E. P. Greenberg. 2004. Putative exopolysaccharide synthesis genes influence Pseudomonas aeruginosa biofilm development. J. Bacteriol. 186:4449–4456.

Lillehoj, E. P., B. T. Kim, and K. C. Kim. 2002. Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin. Am. J. Physiol. Lung Cell. Mol. Physiol. 282:L751–L756.

O’Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol. Microbiol. 30:295–304.

Ramphal, R., and S. K. Arora. 2001. Recognition of mucin components by Pseudomonas aeruginosa. Glycoconj. J. 18:709–713.

Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J. Bacteriol. 184:1140–1154.

Scharfman, A., S. K. Arora, P. Delmotte, E. Van Brussel, J. Mazurier, R. Ramphal, and P. Roussel. 2001. Recognition of Lewis x derivatives present on mucins by flagellar components of Pseudomonas aeruginosa. Infect. Immun. 69:5243–5248.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410:1099–1103.

Honko, A. N., and S. B. Mizel. 2005. Effects of flagellin on innate and adaptive immunity. Immunol. Res. 33:83–101.

Zhang, Z., J. P. Louboutin, D. J. Weiner, J. B. Goldberg, and J. M. Wilson. 2005. Human airway epithelial cells sense Pseudomonas aeruginosa infection via recognition of flagellin by Toll-like receptor 5. Infect. Immun. 73:7151–7160.

Yoon, S. S., and J. J. Mekalanos. 2008. Decreased potency of the Vibrio cholerae sheathed flagellum to trigger host innate immunity. Infect. Immun. 76:1282–1288.

Weimer, E. T., H. Lu, N. D. Kock, D. J. Wozniak, and S. B. Mizel. 2009. A fusion protein vaccine containing OprF epitope 8, OprI, and type A and B flagellins promotes enhanced clearance of nonmucoid Pseudomonas aeruginosa. Infect. Immun. 77:2356–2366.

Ryder, C., M. Byrd, and D. J. Wozniak. 2007. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr. Opin. Microbiol. 10:644–648.

Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O’Toole. 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J. Bacteriol. 189:8154–8164.

Jensen, S. E., I. T. Fecycz, and J. N. Campbell. 1980. Nutritional factors controlling exocellular protease production by Pseudomonas aeruginosa. J. Bacteriol. 144:844–847.

McBroom, A. J., and M. J. Kuehn. 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol. Microbiol. 63:543–558.