Institut Pasteur
Research in Microbiology 163 (2012) 685–691
www.elsevier.com/locate/resmic

Atomic force and super-resolution microscopy support a role for LapA as a cell-surface biofilm adhesin of *Pseudomonas fluorescens*

Ivan E. Ivanov [a], Chelsea D. Boyd [b], Peter D. Newell [b], Mary E. Schwartz [a], Lynne Turnbull [c], Michael S. Johnson [c], Cynthia B. Whitchurch [c], George A. O'Toole [b], Terri A. Camesano [a,*]

[a] *Department of Chemical Engineering, Worcester Polytechnic Institute, 100 Institute Rd., Worcester, MA 01609, USA*
[b] *Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Rm. 202 Remsen Building, N College St, Hanover, NH 03755, USA*
[c] *Microbial Imaging Facility, the ithree institute, University of Technology Sydney, P.O. Box 123, Broadway, NSW 2007, Australia*

Received 22 August 2012; accepted 27 September 2012
Available online 9 October 2012

Abstract

Pseudomonas fluorescence Pf0-1 requires the large repeat protein LapA for stable surface attachment. This study presents direct evidence that LapA is a cell-surface-localized adhesin. Atomic force microscopy (AFM) revealed a significant 2-fold reduction in adhesion force for mutants lacking the LapA protein on the cell surface compared to the wild-type strain. Deletion of *lapG*, a gene encoding a periplasmic cysteine protease that functions to release LapA from the cell surface, resulted in a 2-fold increase in the force of adhesion. Three-dimensional structured illumination microscopy (3D-SIM) revealed the presence of the LapA protein on the cell surface, consistent with its role as an adhesin. The protein is only visualized in the cytoplasm for a mutant of the ABC transporter responsible for translocating LapA to the cell surface. Together, these data highlight the power of combining the use of AFM and 3D-SIM with genetic studies to demonstrate that LapA, a member of a large group of RTX-like repeat proteins, is a cell-surface adhesin.

Keywords: AFM; Adhesin; Biofilm; Weibull analysis; 3D-SIM

1. Introduction

In natural settings, bacteria are commonly found in multicellular communities known as biofilms (Costerton et al., 1995). The transition between a planktonic state and a biofilm mode of growth is strictly regulated and is often required for pathogenesis or survival in hostile environments (Hall-Stoodley et al., 2004; Stanley and Lazazzera, 2004). The initiation of biofilm formation is dependent on environmental factors as well as expression of intact flagella, pili, lipopolysaccharides and production of extracellular DNA, among other molecules (Flemming et al., 1998; O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Whitchurch et al., 2002). Protein adhesins necessary for irreversible surface attachment have been identified largely via genetic studies, but there has been little direct experimental support for these surface proteins mediating cell–surface interactions (Cucarella et al., 2001; Hinsa et al., 2003; Latasa et al., 2005; Martínez-Gil et al., 2010; Pratt and Kolter, 1998; Syed et al., 2009; Theunissen et al., 2010).

Pseudomonas fluorescens Pf0-1 requires the large protein LapA for stable surface attachment (Hinsa et al., 2003). Biochemical studies have revealed that the protein is exported to the cell surface by an ABC transporter, encoded by the *lapEBC* genes. LapA is present in the cytoplasm, localizes to the cell surface and is released into the supernatant (Hinsa et al., 2003; Newell et al., 2011). Cells which do not encode

* Corresponding author.
E-mail addresses: ieivanov@stanford.edu (I.E. Ivanov), chelsea.d.boyd.gr@dartmouth.edu (C.D. Boyd), peter.d.newell@dartmouth.edu (P.D. Newell), mschwartz@wpi.edu (M.E. Schwartz), lynne.turnbull@uts.edu.au (L. Turnbull), michael.johnson@uts.edu.au (M.S. Johnson), cynthia.whitchurch@uts.edu.au (C.B. Whitchurch), georgeo@dartmouth.edu (G.A. O'Toole), terric@wpi.edu (T.A. Camesano).

0923-2508/$ - see front matter © 2012 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.resmic.2012.10.001

a fully functional LapA protein or those in which LapA transport to the cell surface is blocked are unable to initiate biofilm formation and achieve stable, "irreversible" binding to a large variety of surfaces, indicating a role for LapA as a biofilm adhesin (Hinsa et al., 2003).

In conditions unfavorable for biofilm formation (e.g. low P_i (inorganic phosphate) levels), the LapA protein is released from the cell surface by the periplasmic cysteine protease LapG, thus preventing attachment to surfaces (Newell et al., 2011). Under conditions favorable for biofilm formation, LapG no longer cleaves LapA and *P. fluorescens* is competent for stable surface attachment. Deletion of the *lapG* gene results in a hyper-adherent biofilm phenotype because LapA is not released from the cell surface, resulting in no detectable levels of LapA in the supernatant and a 2-fold increase in LapA on the bacterial cell surface (Newell et al., 2011).

The LapA protein has an estimated molecular weight of ~520 kDa and contains an extensive repetitive region consisting of 37 repeats of ~100 amino acids. Bioinformatics tools have predicted an N-terminal transmembrane region and several conserved motifs and domains at the C-terminus of the protein, namely Calx-β, von Willebrand factor type A (vWA), seven repeats-in-toxins (RTX) sequences and a type I secretion system (T1SS) signal. With the exception of the Calx-β domain, the function of those motifs and domains in LapA has not been experimentally investigated. While in other proteins, the Calx-β domain has demonstrated involvement in calcium binding and regulation (Schwarz and Benzer, 1997), deletion of the Calx-β domain in LapA does not impact biofilm formation or LapA localization (Boyd et al., 2012).

The *lapABCE* genes appear to be conserved among many environmental pseudomonads (*P. fluorescens*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas entomophila*), but are absent from pathogenic pseudomonads such as *Pseudomonas aeruginosa* and *Pseudomonas syringae* (Hinsa et al., 2003). The vast majority of these LapA proteins contain conserved domains, like Calx-β, as well as domains with a variable number of amino acid repeat sequences. Interestingly, large repeat surface proteins are not only constrained to the pseudomonads, but are relatively widespread in the microbial domain. Yousef and Espinosa-Urgel (2007) have classified these proteins into seven families based on phylogenic similarities with the most prominent member of the group, namely AidA in *Magnetospirillum magneticum*, Bap in *Staphylococcus aureus* and *Staphylococcus epidermidis*, Bsp in *Bacillus* spp., Ebh in *S. aureus*, FhaL in *Bordetella pertussis*, FhaB in *Xanthomonas axonopodis pv. citri* and LapA in *P. fluorescens*. Common characteristics among most of these proteins are the presence of vWA, calcium binding, cadherin, RGD, hemagglutinin or leucine zipper domains and involvement in cellular adhesion and tissue colonization. Other widely studied large adhesins with characteristic amino acid repeat stretches include LapF, a protein expressed by *P. putida* that is required for late stages of biofilm formation (Martínez-Gil et al., 2010), SiiE in *Salmonella enterica*, which functions as an adhesin for epithelial cells (Gerlach et al., 2007), FrhA in *Vibrio cholerae*, which is associated with hemagglutination, adherence to epithelial cells, biofilm formation and chitin binding (Syed et al., 2009), the biofilm-promoting factor BpfA in *Shewanella oneidensis* (Theunissen et al., 2010) and RtxA in *Legionella pneumophila*, which has demonstrated involvement in adhesion and entry into macrophages and amoebae (Cirillo et al., 2002).

The function of specific domains and amino acid repeats in adhesion proteins, however, has often not been experimentally studied and their role as adhesins has only been inferred indirectly from genetic studies and biofilm assays (O'Toole et al., 1999). Atomic force microscopy (AFM) presents a sensitive tool to study bacterial adhesion molecules and the process of bacteria adhesion at the single cell level (Dufrêne, 2002). AFM is increasingly used in biology with recent applications in differentiating normal from cancerous cells, studying pathogen–host interactions or correlating force of adhesion to bacterial virulence (Ivanov et al., 2011; Iyer et al., 2009; Ovchinnikova et al., 2012; Park et al., 2009). In this study, we utilized AFM, together with super-resolution 3D structured illumination microscopy (3D-SIM), to support the role of the LapA protein as a cell-surface adhesin.

2. Materials and methods

2.1. Bacterial culture and harvesting

Bacterial strains were cultured overnight in 50 mL of lysogeny broth (LB) broth at 30 °C and with shaking (250 rpm). Overnight cultures were diluted 1:100 in K10T-1 growth medium (50 mM Tris–HCl pH 7.4, 0.2% (w/v) tryptone, 0.15% (v/v) glycerol, 0.6 mM $MgSO_4$, and 1 mM K_2HPO_4) and incubated for an additional 6 h until reaching the exponential growth phase. Bacterial cells were harvested by centrifugation at 7000 rpm for 10 min and washed once with saline (0.85% (w/v) NaCl in water). The wild-type strain analyzed here, designated SMC4798, has three HA epitopes inserted after residue 4093 of LapA, and as reported, the strain carrying this variant behaves like the parent, non-HA-tagged strain (Monds et al., 2007). All other strains studied here are a derivative of SMC4798. The other strains analyzed here have been reported: SMC5145 (*lapA*::pKO, Monds et al., 2007), SMC5164 (*lapB*::pMQ89, Monds et al., 2007), SMC5207 (Δ*lapG*, Newell et al., 2011) and SMC5678 (ΔCalX-β, Boyd et al., 2012).

2.2. Bacterial immobilization on glass slides

Glass slides were cleaned by sonication in 2% (v/v) RBS-35 (Thermo Fisher Scientific, Rockford, IL) for 10 min followed by rinsing with copious amounts of ultrapure water. Cleaned slides were rinsed with 100% methanol and immersed in 30% (v/v) 3-aminopropyltrimethoxysilane (Sigma–Aldrich, St. Louis, MO) in methanol for 20 min. Functionalized slides were rinsed with copious amounts of methanol and ultrapure water. Washed bacterial cells were resuspended in saline, supplemented with 3 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Thermo