Localization of DING proteins on PstS-containing outer-surface appendages of Pseudomonas aeruginosa

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Abstract

Phosphate signaling and acquisition are critical for the bacterial response to phosphate limitation, and bacteria express multiple factors to scavenge phosphate. We previously found that multidrug-resistant strains of Pseudomonas aeruginosa from critically ill patients can form unusual outer-surface appendages harboring PstS proteins. Here, we have expanded our investigation to DING proteins that like PstS belong to the family of high-affinity phosphate-binding proteins but have strong similarity with eukaryotic DING proteins. We demonstrate the localization of DING on PstS-containing outer-surface appendages in both multidrug-resistant strain MDR25 and the PA14 strain of P. aeruginosa. However, the number of cells producing appendages and the amount of appendages on each cell in PA14 were found to be negligible, unless overexpression of either PstS or DING was achieved by transformation with constructed plasmids. We further noticed that DING expression under low phosphate conditions was significantly higher in MDR25 compared to PA14 which may explain the greater abundance of appendages in MDR25. Our finding that DING proteins are localized on extracellular appendages provides an opportunity to study the interaction of bacterial DING with host proteins by mimicking the action of host DINGs.

Introduction

Inorganic phosphate is the ultimate limiting nutrient for bacteria. As such bacteria have developed systems to cope with its extracellular content that, when deficient, shifts them to express a more virulent phenotype (Lamarche et al., 2008; Long et al., 2008; Zaborin et al., 2009, 2012; Zaborina et al., 2011; Bains et al., 2012). In host environments in which bacteria colonize, such as the intestinal tract, phosphate levels can become severely depleted in response to extreme physiologic stress (Long et al., 2008; Geerse et al., 2010).

Under phosphate-limiting conditions, Pseudomonas aeruginosa has been found to express PstS and DING both of which belong to the superfamily of high-affinity phosphate-binding proteins (Berna et al., 2002, 2008, 2009; Ahn et al., 2007). While PstS proteins are present in bacteria only, DING proteins are ubiquitous in animal and plants and microorganisms (Adams et al., 2002; Berna et al., 2009). In bacteria, they are mainly found in Pseudomonas spp. (Berna et al., 2008) and have 70–80% sequence identity when compared to eukaryotic DING proteins (Ahn et al., 2007; Zaborina et al., 2008).

We have previously shown that during conditions under which phosphate availability becomes limited, such as occurs in the human intestine following major surgery, multidrug-resistant (MDR) P. aeruginosa strains form unusual appendages incorporating the PstS protein (Zaborina et al., 2008). These appendages are visually distinct from flagella and pili, and promote adhesion to intestinal epithelial cells. In addition to PstS, we also found DING proteins in sheared fractions of these appendages when these strains grew on Pseudomonas isolation agar (PIA) (Zaborina et al., 2008). Whether DING proteins are also present in PstS-containing appendages is unknown. In the present work, we sought to determine whether DING, like PstS, can be localized to the extracellular appendages of MDR strain of P. aeruginosa and whether other strains
such as the well-studied *P. aeruginosa* PA14 also are capable of forming PstS/DING appendages.

**Materials and methods**

**Bacterial strains and plasmids used in the study**

*Pseudomonas aeruginosa* strains PA14 and derivative mutants were obtained from a PA14 mutant library (Liberati *et al.*, 2006), and *P. aeruginosa* MDR25 was previously isolated by our group (Zaborina *et al.*, 2006). *Escherichia coli* TOP10F' was used as a recipient strain for gene cloning and then as a donor for conjugative transfer into *Pseudomonas* cells. A summary of the other strains and plasmids used in the present study is provided in Table 1. *Pseudomonas aeruginosa* and *E. coli* strains were routinely grown in tryptic soy broth (TSB) at 37 °C. When required, antibiotics were added at the following concentrations (µg mL⁻¹): *E. coli*, ampicillin (Amp, 100), kanamycin (Km, 50), tetracycline (Tc, 10), or gentamicin (Gm, 10); For *P. aeruginosa* PA14: for routine maintenance of PA14 mutants, Gm, 15; for PA14 wild type overexpression strains, Gm, 100 or Tc, 50; for PA14 mutants with gene complementation and/or overexpression, Tc, 50.

**Construction of pBBR1MCS-5-DING and pME6032-pstS**

The PA14_55410 (*DING*) gene was amplified using PA14 genomic DNA and the primers (forward PA14F1-EcoR1: 5'-GCATGAATTCA-TGAACTCAAGGTTTGAT-3' and reverse PA14R1-BamH1: 5'-GCATCTCGAGTTACAGGCCAGTT-CCTTGA-3'). The PCR products were first cloned into pGEMT vector (Promega), and sequence identity was confirmed by DNA sequencing. The cloned gene was retrieved and cloned into the *E. coli*-*P. aeruginosa* shuttle vector pBRR1MCS-5 (Kovach *et al.*, 1995) using EcoR1 and BamH1 restriction sites to create pBRR1MCS-5/PA14_55410 where PA14_55410 is expressed from the lac promoter, or *E. coli* – *P. aeruginosa* shuttle vector pME6032 (Zhang & Rainey, 2008) using EcoR1 and XhoI restriction sites to create pME6032-PA14_70860 where PA14_70860 is expressed from the Ptac promoter.

**Threshold of recognition of phosphate limitation**

In Pi limitation studies, *P. aeruginosa* strains were grown in TSB broth overnight at 37 °C with shaking at 200 r.p.m. The next day, overnight cultures were seeded in fresh TSB media at 1 : 100 dilution and the cultures

| Table 1. Bacterial strains and plasmids |
|----------------------------------------|
| **Strain, plasmid** | **Relevant properties and genotype** | **Source/reference** |
| *E. coli* TOP10F' | Recipient strain for cloning and conjugative transfer | Invitrogen |
| *P. aeruginosa* PA14 | Wild type isolated from burn patients | Liberati *et al.* (2006) |
| *P. aeruginosa* MDR 25 | Multidrug-resistant strain of *P. aeruginosa* isolated from tracheal aspirate of ICU patient | Zaborina *et al.* (2006) |
| PA14 ΔDING mutant ID 34094 | PA14 ΔDING, PA14 with a deletion of *DING*, Gm' | Liberati *et al.* (2006) |
| PA14 ΔpstS mutant ID 31354 | PA14 ΔpstS, PA14 with a deletion of *pstS*, Gm' | Liberati *et al.* (2006) |
| PA14/DING | PA14 complemented with pBBR1MCS-5 containing PA14_55410 gene, Gm' | This work |
| PA14/pstS | PA14 complemented with pME6032 containing PA14_70860 gene, Tc' | This work |
| PA14/DING/pstS | PA14 complemented with pBBR1MCS-5 containing PA14_55410 gene and pME6032 containing PA14_70860, Gm', Tc' | This work |
| **Plasmids** | | |
| pRK2013 | Helper plasmid, Tra', Km' | Gifted by Xue Xian Zhang |
| pBBR1MCS-5 | *E. coli* – *Pseudomonas* shuttle vector, inducible with IPTG | Gifted by Mark Ambrose |
| pME6032 | *E. coli* – *Pseudomonas* shuttle vector, inducible with IPTG | Gifted by Xue Xian Zhang |
| pGEMT | TA cloning vector | Promega |
were grown till the OD$_{600}$ nm reached 0.5. The bacterial cells were isolated by centrifuging at 4500 g, 6 min, following by washing and resuspension in defined citrate medium (DCM) that contained citric acid (4.0 g L$^{-1}$), (NH$_4$)$_2$SO$_4$ (1.0 g L$^{-1}$), and MgSO$_4$ $\cdot$7H$_2$O (0.2 g L$^{-1}$) supplemented with varying concentrations of phosphate: 0, 25, 100, and 300 µM using potassium phosphate buffer, pH 6.0. The DCM cultures were grown for 24 h at 37 °C, 200 r.p.m. The bacteria were centrifuged at 13 000 g, and the supernatants were lyophilized and reconstituted using 200 µL of phosphate-buffered saline (PBS). The pellet was lysed using Bug Buster Master Mix (Novagen). The samples were then used for immunoblot analysis. Three biologic replicates in each group were used for the analysis.

**Immunoblot analysis**

For immunoblot analysis, 20 µg proteins per well was separated on a 12% SDS-PAGE gel and electro-transferred to PVDF membrane (Immobilon-P, Millipore). The protein was primed with either anti-DING antibodies at 1 : 1000 dilution or anti-PstS antibodies at 1 : 500 dilution. Anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) at 1 : 5000 dilution or anti-PstS antibodies at 1 : 500 dilution was used as secondary antibody. Detection was performed using 3,3' diaminobenzidine hydrochloride (Sigma; DAB) with metal enhancer tablets (Sigma). The antibodies used for TEM and IEM were performed as previously described (Zaborina et al., 2008). For IEM 1 : 10 dilution of anti-
PstS antibodies was used. Goat anti-rabbit IgG conjugated with 15 nm gold particles (TED PELLA) at 1 : 10 dilution was used as secondary antibody. Independent experiments were performed at least in triplicate visualizing at least 500 cells on each grid.

**Transmission- and immuno-electron microscopy (TEM and IEM)**

TEM and IEM were performed as previously described (Zaborina et al., 2008). For IEM 1 : 10 dilution of anti-PA5369 (anti-PstS) and 1 : 10 dilution of anti-DING, antibodies were used. Goat anti-rabbit IgG conjugated with 15 nm gold particles (TED PELLA) at 1 : 10 dilution was used as secondary antibody. Independent experiments were performed at least in triplicate visualizing at least 500 cells on each grid.

**QRT-PCR**

Strains PA14 and MDR25 were grown overnight on PIA plates. RNA was isolated from overnight-grown plates with high colony density (90–95% confluence). Three individual plates per group were included in the experiment. The colonies were collected directly in the RNA protect buffer (Qiagen), and RNA isolation, DNA degradation, and cDNA synthesis were performed as previously described (Zaborina et al., 2012). Real-time PCR was performed on the ABI 7900HT System using SYBR Green qPCR SuperMix-UDG (Invitrogen), cDNA, and respective primers: for PA4748 gene, forward primer PA4748-F 5′ AACAAGCAAGGGCCGATACA 3′ and reverse primer PA4748-R 5′ TGACCGTACGCATTCCAGTG 3′; for PstS PA5369 and PstS MDR25, forward primer PstS-F 5′ CTGCCGAATATCAGAAACG 3′ and reverse primer PstS-R 5′ GTCATCAAGGTGCCAGAGT 3′; for MDR25 DING gene, forward primer MDR25-DING-F 5′ CCGGCA CTACCAACAGAAGC 3′ and reverse primer MDR25-DING-R 5′ TGAAGATAGGGTTTTCAG 3′, and for PA14 DING gene, forward primer PA14-DING-F 5′ CCGGCACTACCAACAGAAGC 3′ and reverse primer PA14-DING-R 5′ TGAAGATAGGGTTTTCAG 3′. RNA concentration was measured with the Qubit 2.0 Fluorimeter (Invitrogen) using Qubit RNA assay kit Q32855, and 10 ng of RNA was used for cDNA synthesis with high-capacity RNA-to-cDNA kit (Applied Biosynthesis). Control samples (−RT) confirming the absence of DNA were obtained from 10 ng of RNA with the same RNA-to-cDNA kit without including RT Enzyme mix. One microliter of cDNA (or control −RT) was used in qRT-PCR assay in total 10 µL reaction mixture containing 5 µL SYBR green (Platinum SYBR Green qPCR Super Mix-UDG with ROX, Invitrogen, Cat# 11744-100) and 0.2 µM of each primer. The quantitative PCR (qPCR) was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems). The program for amplification had an initial heat step at 50 °C for 2 min, followed by the denaturation step at 95 °C for 15 s, and then followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the reaction was monitored by melt-curve analysis following the real-time program. Each sample was run in triplicate, and mean values were used in calculations. Expression levels were calculated based on differences in C$\_i$ levels. Statistical analysis was performed by Student’s t-test, with P < 0.05 accepted as the criterion for a statistically significant value.

**Surface shearing procedure**

The shearing of bacterial surface for MDR strains as well as overexpression and gene-complemented strains of PA14 was adapted from a previously described procedure (Zaborina et al., 2008) with slight modification. Briefly, overnight-grown bacteria were harvested from PIA plates into PBS, washed once, and then suspended in protease inhibitor cocktail (Sigma) to an optical density at 600 nm
(OD$_{600\text{ nm}}$) of 5.0. The suspension was vortexed at 2500 g for 2 min and then centrifuged at 13 000 g for 10 min at 4 °C to separate the extracellular-appendage-enriched supernatant. The supernatant was centrifuged again to eliminate residual bacterial cells and then was used for immunoblot analysis as described previously (Zaborina et al., 2008).

**Results**

**DING proteins localize on PstS appendages of P. aeruginosa MDR25**

To confirm the presence of DING proteins on the PstS-containing appendages, we performed immuno-gold electron microscopy (IEM) of *P. aeruginosa* MDR25 isolated from a tracheal aspirate of a critically ill patient (Zaborina et al., 2006) that was previously demonstrated to form these appendages (Zaborina et al., 2008). Whole cells of MDR25 were directly harvested from PIA plates and subjected to IEM using the rabbit anti-DING antibodies followed by gold-labeled goat anti-rabbit antibody. We also performed IEM using anti-PstS antibodies to confirm the presence of PstS proteins. As seen by the dense spots localized on the cell surface structures (Fig. 1), both DING and PstS proteins localize predominantly to these outer-surface structures in MDR25. Some gold spots were found outside of the appendages that may label secreted DING and PstS proteins, or disintegrated parts of the appendages, as we
previously noticed the fragility of their structures. Gold spots were not found in negative controls performed in the absence of primary antibody (Fig. 1).

**Formation of PstS/DING-containing appendages by *P. aeruginosa* PA14**

We next used the well-characterized virulent strain PA14 that harbors *pstS* and *DING* genes to determine whether appendages were formed. Using IEM, we were able to detect outer-surface appendages containing PstS and DING (Fig. 2); however, fewer than 1% of PA14 cells produced these appendages compared with 50% of MDR25 cells.

**Overexpression of DING and PstS from transformed plasmids enhances the production of DING/PstS appendages by *P. aeruginosa* PA14**

We then constructed PA14-derivative strains with overexpression of either DING (PA14/pBBR1MCS-5-*DING*), or PstS (PA14/pME6032-*pstS*), or PstS and DING simultaneously (PA14/pBBR1MCS-5-*DING*/pME6032-*pstS*). Overexpression of either DING or PstS or both resulted in less than fivefold higher number of cells displaying appendages as counted by TEM. The number of appendages per cell was also increased (Fig. 3a–c). Immunoblot analysis to quantify the protein in sheared appendage fractions confirmed the higher amount of PstS and DING in constructed strains (Fig. 3d and e). The band density corresponding to PstS protein was increased in PA14/pME6032-*pstS* and further increased in PA14/pBBR1MCS-5-*DING*/pME6032-*pstS*. The density of the band corresponding to DING protein was increased in PA14/pBBR1MCS-5-*DING* but was not however further increased during double overexpression, suggesting that PstS and DING use the same mechanism of secretion or extrusion, resulting in competitive inhibition when both are overexpressed.

**Either PstS or DING alone can contribute to appendage formation**

In order to determine whether PstS-DING cointeraction is necessary for appendage formation, we first performed...
TEM analysis of the PA14 mutants, ΔDING, and ΔPstS. We were able to detect ΔDING and ΔPstS cells producing appendages, suggesting that either PstS or DING alone can lead to appendage formation (Fig. 4a and b). Plasmid transformation was used to overproduce DING and PstS in ΔDING and ΔPstS mutants of PA14. Using immunoblot analysis, we found DING proteins in ΔPstS and PstS proteins in ΔDING sheared fractions (Fig. 4c and d). The amounts of PstS and DING proteins were greatly increased in plasmid-bearing mutant strains (Fig. 4c and d) suggesting that PstS or DING alone can contribute to appendage formation. These data were confirmed using IEM analysis with DING and PstS antibodies (Fig. 4e and f). To further confirm that PstS and DING are the main proteins contributing to the appendage formation, we performed TEM analysis of the PA14 mutants, ΔPstS, ΔDING, and ΔPstS/ΔDING stained with anti-PstS (c) and anti-PstS (d) antibodies. (e and f) IEM images of ΔPstS/ΔDING stained with anti-DING antibodies and (f) ΔDING/ΔPstS stained with anti-PstS antibodies. Arrows indicate localization of gold spots.

**Abundant DING expression in MDR25**

We hypothesized that the abundance of appendages in MDR25 compared with PA14 may be related to differential expression of PstS and/or DING. To test this hypothesis, we first sequenced the MDR25 DING gene (deposited in GenBank: JX500097.1). Amino acid sequence alignment revealed 95–99% of identity with hypothetical proteins NCGM2_1352 (P. aeruginosa NCGM2.S1), PA39016_000050001 (P. aeruginosa 39016), and the DING proteins PA14_55410 (P. aeruginosa PA14), PSPA7_4822 (P. aeruginosa PA7), and P. aeruginosa MDR1 (Zaborina et al., 2008). Comparative transcriptional analysis of pstS and DING expression in PA14 and MDR25 under equivalent growth conditions suitable for appendage formation was performed. RNA was isolated from cells grown overnight on PIA plates with a colony density of 90–95% confluence. Gene expression was estimated relative to the expression of housekeeping gene PA4748, encoding triose phosphate isomerase (Zaborin et al., 2009). There was no significant difference in pstS expression between PA14 and MDR25 strains; however, the expression of DING in MDR25 was close to 25-fold higher compared with PA14 (Fig. 5a). We tested for a difference in copy numbers between PA14 and MDR25. The quantitative PCR analysis was performed using 1 ng of DNA as a template, and C<sub>t</sub> levels of PA4748, pss<sub>t</sub>, and DING gene amplification were compared. As Fig. 5b shows, there was no apparent difference in DING copy number, suggesting that changes in the mechanism of regulation of DING expression rather than gene copy number are responsible for the highly enhanced DING expression in MDR25.

**Fig. 4.** Either PstS or DING alone can contribute to the appendages formation by PA14 that is further increased due to overproduction. (a and b) TEM images of (a) ΔDING, (b) ΔPstS isolated from PA plates at 95% confluence. (c and d) Immunoblot analysis of sheared fractions using anti-DING (c) and anti-PstS (d) antibodies. (e and f) IEM images of (e) ΔPstS/ΔDING stained with anti-DING antibodies and (f) ΔDING/ΔPstS stained with anti-PstS antibodies. Arrows indicate localization of gold spots.
Higher concentrations of inorganic phosphate are required to suppress the expression of PstS and DING in MDR25 compared with PA14

We next decided to check whether \( \text{pstS} \) and DING expression differentially respond to varying phosphate concentrations in PA14 and MDR25. We performed the experiment in liquid DCM, complemented with different concentrations of potassium phosphate to determine the threshold concentration of phosphate at which the expression of PstS and DING is suppressed. Results demonstrated that 100 \( \mu \text{M} \) of inorganic phosphate significantly suppressed the DING expression and 300 \( \mu \text{M} \) suppressed the PstS expression in PA14, but not in MDR25 (Fig. 5c and d). These data demonstrate that MDR25 expresses the two proteins at phosphate levels which repress expression in PA14.

Discussion

The effect of stressful environmental conditions on microbial behaviors, such as those that occur during nutrient limitation, is an important aspect of infection pathogenesis. Prolonged exposure to such conditions, as often occurs when MDR strains colonize epithelial surfaces of critically ill humans, can result in the selection of strains that express novel virulence properties. Studying clinical strains exposed to these complex conditions offers an opportunity to discover emergent properties in pathogens that may lead to novel treatment strategies.

Results from the current experiments have extended our knowledge regarding the role of the identified novel adhesive appendages discovered on some MDR strains of \( \text{Pseudomonas aeruginosa} \) that appear during low phosphate conditions. Two phosphate-binding proteins, PstS and DING, coded by genes of the \text{pho} \_ \text{regulon} and that are known to be induced at low phosphate concentrations, were localized to these nonflagellar appendages. PstS/DING appendages are much rarer on PA14 cells, but can be increased by recombinant overexpression of either DING or PstS. The comparative expression of these proteins in PA14 and MDR25 revealed DING protein expression is c. 25-fold higher in MDR25 and that a higher concentration of phosphate is required to suppress its expression. Taken together, it is plausible that PstS/DING appendages can be evolved among clinical MDR strains as a result of the unprecedented conditions to which they are exposed during extreme medical interventions, most notably phosphate limitation. They may have developed to ensure their ability to acquire phosphate under such environmentally harsh conditions.

The function of bacterial DING proteins is still not known. Their proposed role in phosphate scavenging has not yet been supported by direct experimental evidence. Given that bacterial DING proteins share very high amino acid sequence similarity with mammalian DING proteins, it is possible that they may be involved in the modulation of host cell signaling. Several examples exist indicating that eukaryotic DING proteins act in this way (Berna et al., 2009), and there is also evidence that bacterial DING proteins influence gene expression and proliferation of human cells (Ahn et al., 2007; Suh et al., 2013). Work by Suh et al. (2013) is particularly interesting in this regard, in that it demonstrates transcriptional...
regulation when _P. aeruginosa_ DING proteins are transiently expressed in human cells. However, transient expression of PstS displays no such activity indicating a clear functional difference between these two proteins, which may also be relevant to bacterial pathogenesis.

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**Authors’ contribution**

J.C.A, K.S., and O.Z. are senior co-authors.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Immunoblot analysis of sheared appendages of MDR25 grown on PIA demonstrating no cross-reaction between anti-PstS and anti-DING antibodies.

**Fig. S2.** PA14/pBBR1MCS-5–DING and PA14/pME6032–pstS in which expression of DING and pstS is induced by IPTG, produce appendages in phosphate-rich media.