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The Lipase LipA (PA2862) but Not LipC (PA4813) from *Pseudomonas aeruginosa* Influences Regulation of Pyoverdine Production and Expression of the Sigma Factor PvdS▽

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A key element in iron-dependent regulation of iron metabolism and virulence-related functions for *Pseudomonas aeruginosa* is the sigma factor PvdS. PvdS expression itself is also influenced by iron-independent stimuli. We show that pyoverdine production and *pvdS* expression depend on one of the two lipases of *P. aeruginosa*.

Due to its fundamental functions in cell metabolism, iron is an essential element for most living cells. However, although this element is present in high abundance, but with very low bioavailability in aerobic environments at neutral pH, both prokaryotes and eukaryotes must mitigate this paradox in order to acquire this metal for key metabolic processes.

The Gram-negative bacterial pathogen *Pseudomonas aeruginosa* has become one of the best-understood bacterial models to study regulatory processes related to iron acquisition and metabolism (26). *P. aeruginosa* is a ubiquitously distributed bacterium that has an enormous metabolic versatility which enables it to survive in a range of diverse environmental niches. It has evolved a variety of strategies for both iron acquisition and for the regulation of its metabolism (3, 4, 11, 18).

In Gram-negative bacteria, the repressor Fur (*f*erric *u*ptake *r*egulator) plays the master role in regulation of iron metabolism (7, 27). Fur and its cofactor Fe(II) bind to so-called Fur boxes within promoters of iron-regulated genes (encoding, e.g., proteins for siderophore biosynthesis and uptake), thereby repressing their expression. In *P. aeruginosa* more than 200 genes are regulated in response to changes in iron availability (17). However, only a subpopulation of these genes are directly repressed by Fur (17), and several Fur-dependent mechanisms have been described to act downstream of Fur to regulate a subset of iron-regulated genes (reviewed in references 26 and 30). Among them is the extracytoplasmic function sigma factor PvdS, which plays a central role in regulation of at least 26 iron-repressible genes or operons in *P. aeruginosa* involved in iron uptake and metabolism (17, 24). PvdS controls the expression of major *P. aeruginosa* virulence factors, including pyoverdine, the extracellular protease V (PrpL), and exotoxin A, suggesting a prominent role of PvdS in regulation of virulence (5, 21, 29, 31). PvdS is known to be directly regulated by Fur and is expressed under iron limitation (13, 16). However, additional mechanisms control PvdS sigma factor activity at posttranslational levels (12, 28), and there are indications that expression of PvdS itself and PvdS-regulated genes are directly or indirectly affected by additional environmental conditions, like carbon sources, oxygen tension, copper starvation, and population density (8, 15, 22), suggesting a functional integration of different signals into an appropriate physiological and regulatory answer to a given sum of different stimuli. Recently, CysB, the first non-iron-stimulated regulator with an effect on PvdS expression, was described (10).

Lipolytic enzymes have been suggested to be involved in lipid signaling pathways, since they influence virulence-relevant phenotypes of *P. aeruginosa* (1, 14, 25, 33). Among those enzymes is the lipase LipC (PA4813), which was found to affect motility, biofilm formation, and rhamnolipid production (19), whereas the second lipase, LipA (PA2862), was unobtrusive with respect to virulence-related phenotypes. However, mutant strains (Δ*lipA* [32] and Δ*lipA* Δ*lipC* [Δ*lipA*] with an additional GM cassette in *lipC* [19]) with deletions of lipase genes lack the characteristic green color of *P. aeruginosa*, thereby suggesting reduced pyoverdine production. Comparison of the pyoverdine concentration in cultures of the wild type, a *pvdS* mutant (16), and a *lipC lipA* double mutant grown under iron-limiting conditions in M9 medium (20) with 3 g/liter succinate as the carbon source (20 ml in 250-ml flasks; 8 h, 37°C, 150 rpm) proved this assumption (Fig. 1A). Pyoverdine production in this mutant was reduced to levels comparable to those observed with the *pvdS* mutant and could be restored by complementation with LipA, but not with LipC (Fig. 1A). Complementation was performed using plasmids pVLT33-*lipAH* and pVLT33-*lipCH*, with the target genes transcriptionally regulated from the plasmid-carried *tac* promoter (6), and lipase expression was verified by spectrophotometric activity measurement with *p*-nitrophenyl-palmitate as the substrate (23, 34)

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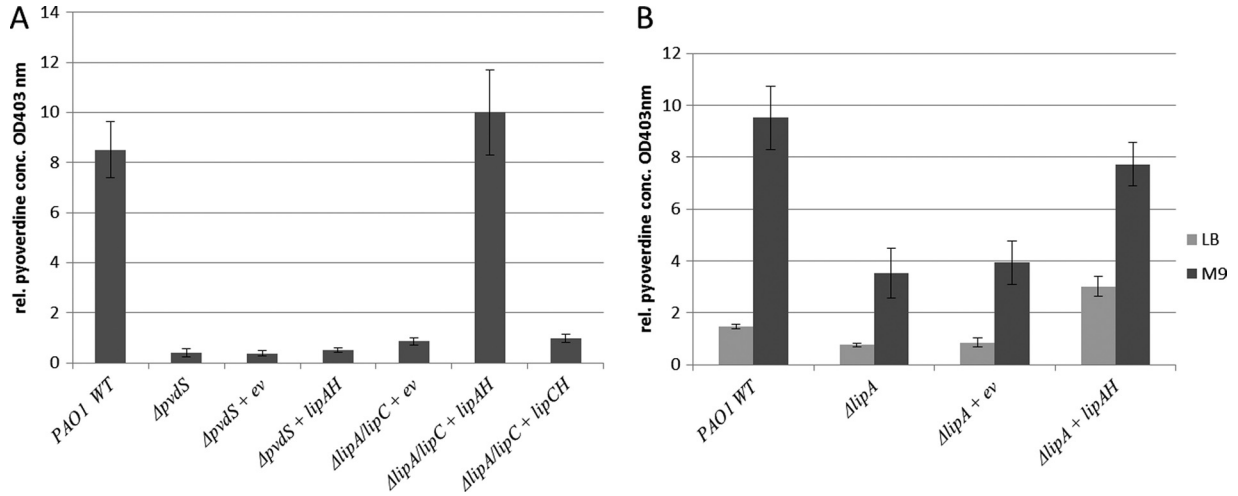

FIG. 1. Pyoverdine concentrations in *P. aeruginosa* culture supernatants. (A) Wild-type (WT), Δ*pvdS* mutant, Δ*lipA* Δ*lipC* double mutant, and the double mutant with a *lipA* or *lipC* expression plasmid, which also harbored the gene for the lipase-specific chaperone *lipH* for proper folding of the lipases, were grown for 8 h at 37°C in M9 minimal medium without iron supplementation, and pyoverdine concentrations were determined in cell-free supernatants (9). (B) The wild type and a Δ*lipA* mutant harboring the empty vector (ev) or a *lipA* expression plasmid were grown in rich LB or minimal M9 medium, and the relative pyoverdine concentrations were determined after 8 h of growth.

(data not shown). As expected, in LB as a rich medium, pyoverdine production was decreased compared to cultivation in M9 medium without extra iron (Fig. 1B). Interestingly, expression of *lipA* in the LipA-deficient mutant led to increased pyoverdine levels, even in the non-iron-restricted rich LB medium (Fig. 1B). As the wild type and the empty vector control appeared not to produce pyoverdine under these non-iron limitation conditions, this finding suggests a decoupling of pyoverdine production from iron limitation. Moreover, the results show that reaching full pyoverdine production capacity under iron limitation conditions requires the presence of LipA. To distinguish whether LipA was needed up or downstream of PvdS, this lipase was also expressed in the *pvdS* mutant strain. In fact, LipA expression in this iron regulation mutant could not restore pyoverdine production, thus strongly suggesting that LipA acts upstream of PvdS. Expression of the *pvdS* gene was measured in the *lipA* mutant by real-time PCR with SYBR green assays. Total RNA was isolated using the RNA minikit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA with the High Cap RNA-cDNA kit (Life Technology, Darmstadt, Germany), which was then used as template for quantitative PCR on an HT7900 cycler (Applied Biosystems, Darmstadt, Germany). Changes were determined using the comparative threshold cycle (C_T) method by normalizing to the levels of mRNA transcribed from the reference gene *rpoD* (2). The transcript level of *pvdS* was found to be 4-fold reduced in the *lipA* mutant compared to the wild type. Consequently, complementation with functional LipA (as determined by the

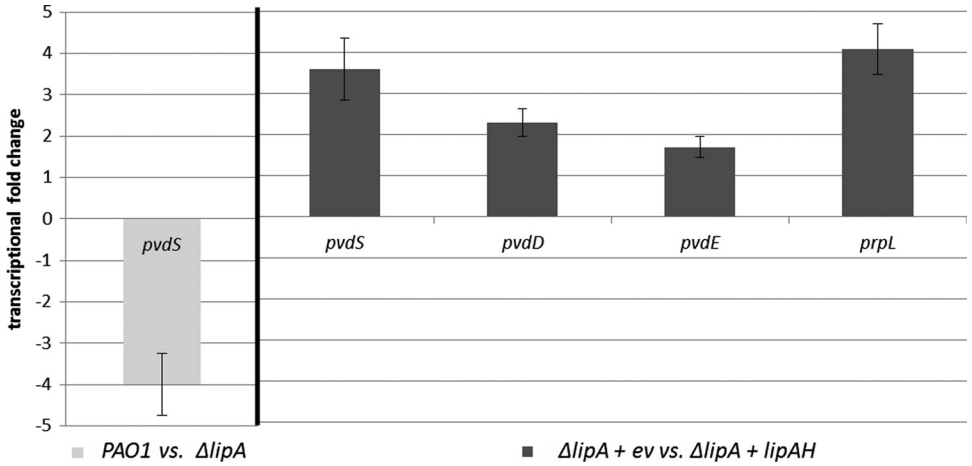

FIG. 2. Change in gene expression of *pvdS* in a *P. aeruginosa* Δ*lipA* mutant relative to the wild type (light gray). The right side of the graph compares (based on the fold change) the regulation of *pvdS* and PvdS-regulated genes (*pvdD*, *pvdE*, and *prpL*) in a Δ*lipA* mutant with empty vector (ev) versus a Δ*lipA* mutant which harbored the *lipA* gene and the gene for the lipase-specific chaperone *lipH* on plasmid pVLT33-LipAH (*lipAH*; dark gray). In all cases transcription was normalized to *rpoD*. Cultures were grown for 8 h in LB medium at 37°C before RNA was isolated. Error bars represent standard deviations from three independent experiments.

lipase activity assay [data not shown]) led to an increase not only of the transcript levels for *pvdS* but also of the *pvdD* (pyoverdine synthetase D), *pvdE* (pyoverdine biosynthesis protein PvdE), and *prpL* genes (Fig. 2), which are known as PvdS-regulated target genes and play important roles in pyoverdine biosynthesis (10, 17) or, in the case of *prpL*, function in virulence (24). These results demonstrate that the lipase LipA, or a yet-uncharacterized lipase-dependent signaling pathway, is involved in regulation of *pvdS* gene expression at the transcriptional level, with all consequences for PvdS-dependent regulation of downstream genes. Consequently, with an influence on iron acquisition, LipA affects one of the key processes required for full virulence of *P. aeruginosa*. This effect appears to be specific for the lipase LipA, and not for the second lipase, LipC, which has been shown to influence other virulence-related functions. Accordingly, these data show that both lipases are likely important for *P. aeruginosa* pathogenicity while they appear to have very distinct functions.

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