

Role of PvdQ in *Pseudomonas aeruginosa* virulence under iron-limiting conditions

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PvdQ, an acylase from *Pseudomonas aeruginosa* PAO1, has been shown to have at least two functions. It can act as a quorum quencher due to its ability to degrade long-chain *N*-acylhomoserine lactones (AHLs), e.g. 3-oxo-C12-HSL, leading to a decrease in virulence factors. In addition, PvdQ is involved in iron homeostasis by playing a role in the biosynthesis of pyoverdine, the major siderophore of *P. aeruginosa*. In accordance with earlier studies on RNA level, we could show at the protein level that PvdQ is only expressed when iron is present at very low concentrations. We therefore set out to investigate the two functions of PvdQ under iron-limiting conditions. Gene deletion of *pvdQ* does not affect growth of *P. aeruginosa* but abrogates pyoverdine production, and results in an accumulation of 3-oxo-C12-HSL. Phenotypic analyses of our $\Delta pvdQ$ mutant at low iron concentrations revealed that this mutant is impaired in swarming motility and biofilm formation. Additionally, a plant and a *Caenorhabditis elegans* infection model demonstrated that the deletion of *pvdQ* resulted in reduced virulence. None of the phenotypes in the present study could be linked to the presence or absence of AHLs. These results clearly indicate that under iron-limiting conditions PvdQ plays a major role in swarming motility, in biofilm development and in infection that is more likely to be linked to the pyoverdine pathway rather than the LasI/LasR/3-oxo-C12-HSL quorum-sensing circuit.

Received 17 May 2009
Revised 10 September 2009
Accepted 18 September 2009

INTRODUCTION

Iron, which is essential for bacterial life, is not freely available and in many environments is present below the concentration required for bacterial growth (Braun & Hantke, 1997). Bacteria have established a system to sequester iron. They secrete iron-scavenging molecules, siderophores, that chelate iron from the environment, and transport it into the cells by binding to specific receptors on the cell surface (Neilands, 1993, 1995). Also multicellular organisms, e.g. mammals, have developed systems to strictly regulate iron homeostasis; as well as the need to scavenge iron ions present only at low concentrations, cells have to be protected from the damaging radicals that can be formed in the presence of excess iron (Carpenter *et al.*, 2009; Miethke & Marahiel, 2007). Molecules that regulate the exchangeable pool of iron (Freestone *et al.*,

2000, 2002, 2003), e.g. lactoferrin and transferrin, cause the concentration of free iron in serum to be as low as 10^{-24} M. Upon infection with pathogenic bacteria, the infected host and the pathogen will therefore start a fierce battle for iron. Studies of a pathogen such as *Pseudomonas aeruginosa* under iron-limiting conditions therefore seem appropriate to better understand its behaviour.

P. aeruginosa is an opportunistic pathogen infecting mainly immunocompromised individuals, such as HIV patients, as well as those suffering from burn wounds and cystic fibrosis (Holder, 1993). This bacterium produces two well-characterized siderophores. Pyoverdine has a high affinity for iron, whereas pyochelin, the second siderophore, has only a low iron affinity (Cox *et al.*, 1981; Cox & Adams, 1985; Poole *et al.*, 1996).

Pyoverdine is a complex molecule composed of a fluorescent chromophore linked to a peptide moiety (Meyer, 2000; Wendenbaum *et al.*, 1983). This siderophore is considered a virulence factor, capable of enhancing *P. aeruginosa* infection and virulence. Pyoverdine has been shown not only to increase its own expression, but also to

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Abbreviations: AHL, *N*-acylhomoserine lactone; EDDHA, ethylenediamine di(*o*-hydroxy)phenylacetic acid; HSL, homoserine lactone.

A supplementary figure is available with the online version of this paper.

influence the expression of at least two virulence genes: those encoding exotoxin A and PrpL protease (Beare *et al.*, 2003; Lamont *et al.*, 2002). Therefore, pyoverdine synthesis needs to be tightly regulated. Ferric uptake regulator (Fur) can be seen as the major suppressor of the expression of iron-regulated genes (Prince *et al.*, 1991, 1993; Vasil & Ochsner, 1999). Under low-iron conditions Fur is released from promoter regions, allowing transcription (Escolar *et al.*, 1999; Neilands, 1990). One of the Fur-regulated genes, the sigma factor gene *pvdS*, is the regulator of some genes involved in pyoverdine biosynthesis (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995; Ochsner *et al.*, 1995; Visca *et al.*, 2002).

Pyoverdine and subsequently iron acquisition are important for *P. aeruginosa* to develop different lifestyles. It has been shown that iron can serve as a signal for biofilm development (Banin *et al.*, 2005; Singh *et al.*, 2002; Yang *et al.*, 2007). Particularly iron limitation compromises biofilm formation (Banin *et al.*, 2005; Patriquin *et al.*, 2008). Mere iron diffusion is not enough to allow biofilm formation; to form biofilms, a functional iron-uptake system is required (Banin *et al.*, 2005). Low iron concentrations induce *P. aeruginosa* twitching motility, suggesting iron to be one of the links between biofilm formation and this type of motility (Singh *et al.*, 2002; Singh, 2004).

The *pvdQ* gene (PA2385 in strain PAO1 and PA14_33820 in strain PA14) is located within the pyoverdine (Pvd) locus, but its potential role in pyoverdine biosynthesis in *P. aeruginosa* is still unclear. PvdQ, which has homology to β -lactam acylases (Sio & Quax, 2004), belongs to the N-terminal nucleophile hydrolase (Ntn) superfamily and has been shown to degrade some *N*-acylhomoserine lactones (AHLs) (Sio *et al.*, 2006), the major communication molecules in Gram-negative bacteria. As for most Pvd genes, expression of this acylase occurs under iron

starvation (Lamont & Martin, 2003; Ochsner *et al.*, 2002), but little is known about the effects of the enzyme under those conditions. Therefore, we set out to investigate the role of *pvdQ* in *P. aeruginosa* PA14. A *pvdQ* deletion strain did not show any growth impairment compared to the wild-type. The deletion strain was analysed for phenotypes associated with iron, such as biofilm formation and motility. AHL levels were measured to see whether they correlated with the phenotypes observed. Our data suggest that PvdQ is a key enzyme regulating virulence in *P. aeruginosa*. At low iron concentration, PvdQ decreases the levels of 3-oxo-C12-HSL; it also controls pyoverdine production and swarming motility, increases virulence via the pyoverdine/iron pathway, and regulates biofilm formation via an as yet unidentified mechanism.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* S17-1 λ pir was used as the donor strain in bacterial conjugation (Simon *et al.*, 1983). *P. aeruginosa* competent cells were prepared as described by Choi *et al.* (2006). Bacteria were grown at 37 °C in Luria–Bertani (LB) medium or on LB agar plates (Sambrook *et al.*, 2001). For plasmid selection and maintenance, antibiotics were added to growth media at the following concentrations: *E. coli* – gentamicin, 10 mg l⁻¹; tetracycline, 10 mg l⁻¹; *P. aeruginosa* – gentamicin, 25 mg l⁻¹; tetracycline, 200 mg l⁻¹.

General DNA manipulations. DNA manipulation was performed using standard techniques (Sambrook *et al.*, 2001). PCR fragments were purified using the QIAquick PCR Purification kit (Qiagen). DNA fragments were purified from agarose gels with the QIAquick Gel Extraction kit according to the manufacturer's instructions. Genomic DNA from *P. aeruginosa* strains was isolated using a genomic DNA isolation kit (GenElute bacterial genomic DNA kit, Sigma-Aldrich). Plasmid isolation was performed using Nucleospin Plasmid Isolation kit (Macherey-Nagel). DNA sequencing was carried out by Macrogen.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>E. coli</i>		
DH10B	pMCT- <i>pvdQ</i>	Sio <i>et al.</i> (2006)
S17-1 λ pir	<i>galU galK rpsL(Str^R) endA1 nupG thi pro hsdR hsdM⁺ recA</i> (RP4-2Tc::Mu Km::Tn7) λ pir	Simon <i>et al.</i> (1983)
OP50	A uracil auxotroph derived from <i>E. coli</i> B	Brenner (1974)
<i>P. aeruginosa</i>		
UCBPP-PA14	Clinical isolate; referred to as PA14	Lee <i>et al.</i> (2006)
PA14 Δ <i>pvdQ</i>	Δ <i>pvdQ</i> chromosomal deletion mutant of PA14	This study
PA14 Δ <i>pvdQ</i> ::pME6032- <i>pvdQ</i>		This study
PA14 <i>pvdQ</i> mutant ID27758	PA14 transposon insertion mutant	Liberati <i>et al.</i> (2006)
Plasmids		
pEX18Gm	Suicide plasmid carrying <i>sacBR</i> , Gm ^R	Hoang <i>et al.</i> (1998)
pME6032	<i>lacI^Q</i> -Ptac expression vector; pVS1-p15A shuttle vector Tet ^R	Heeb <i>et al.</i> (2002)
pME6032- <i>pvdQ</i>	<i>pvdQ</i> in pME6032	Sio <i>et al.</i> (2006)
pSB1075	<i>lasR lasI</i> (<i>P. aeruginosa</i> PAO1):: <i>luxCDABE</i> (<i>Photobacterium luminescens</i> ATCC 29999) fusion in pUC18 Ap ^R , acyl-HSL biosensor producing bioluminescence	Winson <i>et al.</i> (1998)

Construction of a *pvdQ* deletion mutant. An in-frame deletion of *pvdQ* was obtained via splicing by overlapping extension PCR (SOE-PCR: Horton *et al.*, 1989). Briefly, approximately 1 kb fragments located upstream and downstream of *pvdQ* with an additional short sequence of overlap (given in bold letters in their sequences below) were amplified from genomic DNA using primer pair ForA/RevA [ForA, 5'-GACAAGCTTGGTGTGCGAGAGCGAGTT-3', containing a *Hind*III restriction site (underlined); RevA, 5'-CATGAGACACGC-GTCCCCATCGATGTCGTTTC-3'] and primer pair ForB/RevB [ForB, 5'-GGGACGCGTGTCTCATGATAAGCAATGCCTATC-3'; RevB, 5'-CAGGAATTCGGCCATCGGTAGCA-3', containing an *Eco*RI restriction site (underlined)]. Next, the two DNA fragments were joined together, completed and the final product boosted by a third PCR using primers ForA and RevB. The resulting fragment was cloned into pEX18Gm carrying a *sacB* sucrose-sensitivity gene (Hoang *et al.*, 1998) using the *Eco*RI and *Hind*III restriction sites. This plasmid was transformed into *E. coli* S17-1 λ pir and conjugated into *P. aeruginosa* to generate an in-frame deletion of the *pvdQ* gene in the PA14 strain by allelic exchange. Gentamicin-resistant, sucrose-sensitive *P. aeruginosa* strains were selected, followed by selection of double recombinants on Vogel–Bonner minimal medium (Schweizer, 1991) containing 5% (w/v) sucrose. The deletion was confirmed by PCR of the *pvdQ* gene and Southern blot analysis of digested genomic DNA by using a DIG High Prime DNA labelling and detection starter kit I (Roche) according to the manufacturer's instructions.

Western blot assay. Purified protein was used to produce polyclonal PvdQ antibodies in rabbits (Eurogentec). *P. aeruginosa* PA14 and its Δ *pvdQ* mutant were grown in CAA medium (containing, per litre: 5 g low-iron Bacto Casamino Acids (Difco), 1.54 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$), LB medium and LB medium supplemented with the iron chelator 2,2'-dipyridyl (300 μ M). Samples were taken 3, 6, 9 and 24 h after inoculation. Cultures were spun down for 10 min at 13 000 g and the pellet resuspended in Bug Buster lysis buffer (Novagen). The lysate was boiled for 10 min, and 20 μ l was subsequently separated on a 4–12% polyacrylamide gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane, blocked with 5% milk, and probed with rabbit polyclonal PvdQ antibody (1:1000 dilution in TBS-T). Proteins were detected with goat anti-rabbit antibody (1:5000 dilution in TBS-T) conjugated to alkaline phosphatase.

Twitching motility assay. Twitching motility was assayed by the subsurface agar method (Alm & Mattick, 1995). A 2 μ l aliquot of a *P. aeruginosa* overnight culture was stab-inoculated through a 1% LB agar plate. The zone of twitching was visualized 24 and 48 h after incubation at 30 °C and 37 °C by staining with Coomassie brilliant blue R250 (Pierce). Twitching motility was also assayed after addition of 100 μ M 2,2'-dipyridyl.

Swimming assay. Swimming assays were performed using BM2 glucose minimal medium [62 mM potassium phosphate buffer (pH 7), 0.5 mM $MgSO_4$, 10 μ M Fe(II) sulfate, 0.5% Casamino acids and 0.4% glucose] (Overhage *et al.*, 2007) containing 0.3% (w/v) agar. Plates were spot-inoculated with 2 μ l of an overnight culture and the swimming zone was measured after incubation for 24 and 48 h at 30 °C or 37 °C.

Swarming assay. Swarming assays were done using BM2 glucose minimal medium containing 0.5% (w/v) agar. Approximately 8 h after they were poured, the plates were inoculated with 2.5 μ l of an overnight culture in triplicate. Comparisons were made only among plates poured from the same batch of agar. Swarming media were also supplemented with one of the following iron sources: Fe(II) sulfate (10–300 μ M), Fe(III) sulfate (10–300 μ M), Fe(III) chloride (18–300 μ M) and Fe(II) citrate (10–300 μ M). Swarming motility was also tested on media containing the iron chelator 2,2'-dipyridyl (50 μ M–1 mM), 3-oxo-C12-HSL, C4-HSL and partially purified pyoverdine

(as described by Koedam *et al.*, 1994). Compounds were added on a sterile disc at various concentrations.

Biofilm formation. Cultures of *P. aeruginosa* PA14 and the Δ *pvdQ* mutant were grown overnight in CAA medium and diluted in the same medium to OD₆₀₀ 0.1. To test the effects of iron on the Δ *pvdQ* mutant, the same iron sources that were tested in the swarming motility assay were added to the medium at 100 μ M, a concentration proven to restore swarming motility in the Δ *pvdQ* mutant. In order to minimize the intrinsic variability of the crystal violet assay (Peeters *et al.*, 2008) each test was performed in >20 wells of a round-bottomed polystyrene 96-well plate (Greiner Bio-One). A 100 μ l aliquot of the diluted cultures was added to each well and the plates were incubated at 30 °C (static biofilm). After 24 h of adhesion, the supernatant was removed and the wells were extensively rinsed with sterile physiological saline. Then 100 μ l of fresh medium was added to the wells and the plates were incubated for 24, 48 or 72 h. Biofilm biomass was quantified using the crystal violet method described by Christensen *et al.* (1985) with minor modifications (Peeters *et al.*, 2008). Briefly, after removal of the supernatant and extensive washing with 0.9% NaCl, 100 μ l of 99% methanol was added to each well. After 15 min of fixation, the methanol was removed and the plates were air-dried. Then 100 μ l crystal violet (10 mg ml⁻¹; Merck) was added to the wells and the plates were incubated at room temperature for 20 min. The excess of crystal violet was removed under tap water and the plates were subsequently air-dried. To release the crystal violet, 150 μ l of 33% acetic acid was added to the plates and absorbance was measured at 600 nm. For the iron complementation studies, the different iron salts tested in the motility assays were added to CAA medium to a final concentration of 100 μ M. After washing the biofilm, fresh CAA medium containing the same amount of iron salts was added to the wells. The following steps were identical to those described above.

Determination of autoinducer production. The amount of 3-oxo-C12-HSL produced was determined using the biosensor *E. coli*(pSB1075), which produces light in response to long-chain AHLs (Winson *et al.*, 1998). 3-Oxo-C12-HSL concentrations were determined at different stages of biofilm grown in round-bottomed polystyrene 96-well plates. Biofilm supernatants of wild-type PA14 and the *pvdQ* deletion mutant were collected after 24, 48 and 72 h. After 10 min centrifugation of the cultures, the supernatants were filtered using a 0.2 μ m pore filter (Whatman) and stored at –20 °C for later analysis. After collecting all the samples, a bioassay was started at 37 °C by adding 180 μ l of a 1/100 dilution of an overnight *E. coli*(pSB1075) culture and 20 μ l of each supernatant sample. The amount of light produced by the biosensor was read every hour during a 20 h time course in a multifunctional microplate reader (FLUOstar Omega, BMG Labtech). Data points obtained immediately prior to maximum light production were used for comparisons (about 10 h after initiation of the bioassay).

Testing *P. aeruginosa* virulence in a plant model. In order to develop a simple screening mechanism for infection by *P. aeruginosa*, we exploited the opportunistic plant infectious behaviour of this bacterium. Various plants were tested and potato (*Solanum tuberosum*) was selected due to easy handling and rapid visualization of infection. The potato tuber surface was sterilized with 70% ethanol to reduce microbial contamination. Slices about 3–5 mm thick were placed in sterile Petri dishes on paper dampened with sterile water. The slices were inoculated with 10 μ l of an overnight culture previously adjusted to OD₆₀₀ 0.3–0.4. Infection development was compared between slices inoculated with PA14 wild-type, PA14 Δ *pvdQ*, PA14 Δ *pvdQ*::pME6032-*pvdQ* and PA14 Δ *pvdQ*+partially purified pyoverdine. Non-inoculated slices were used as negative controls to discard unwanted spoilage and/or maceration. Global effects on infection development and tissue maceration were assessed visually 24, 48 and 72 h after incubation at 30 °C.

Caenorhabditis elegans killing assay. A *C. elegans* killing assay was performed to test the toxicity of PA14 and the *pvdQ* deletion mutant. *P. aeruginosa* strains were grown overnight at 37 °C in CAA medium (supplemented with Fe(II) sulfate, Fe(III) chloride or Fe(II) citrate where necessary) and then diluted 100-fold into fresh broth. Nematode growth medium (NGM; Brenner, 1974) plates (59 mm diameter) were then spread with 80 µl of the respective cultures. The plates were incubated at 37 °C for 24 h and allowed to equilibrate to room temperature for 30 min, then 40 L4 nematodes from stock plates were transferred onto the *P. aeruginosa* lawn. The plates were incubated at 24 °C and scored for living and dead worms every 3–4 h for 6 days. For statistical purposes a minimum of three replicates per trial were performed. The usual *C. elegans* food bacterium *E. coli* OP50 was used as a negative control to evaluate background levels of worm death. A worm was considered to be dead when it failed to respond to plate tapping or gentle touching with a platinum wire. Worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis. Results are presented as the percentage of living nematodes on the killing plates compared to their survival on the *E. coli* OP50 control strain.

RESULTS

Expression of PvdQ under iron-limiting conditions

pvdQ expression under iron-limiting conditions was verified by growing strain PA14 in CAA medium with or without additional Fe(III) chloride (100 µM) as well as in LB medium and LB medium supplemented with the iron chelator 2,2'-dipyridyl (300 µM) (Fig. 1). As a control, we grew the $\Delta pvdQ$ mutant in the same media (only shown for CAA medium). Our results demonstrate that PvdQ can only be detected on Western blots when the bacteria are grown in media with low amounts of iron, indicating iron-dependent regulation of PvdQ production.

Influence of *pvdQ* deletion on growth in iron-depleted conditions

Pyoverdine-negative strains normally show growth impairment under low-iron conditions. Hence, we compared PA14 to the $\Delta pvdQ$ mutant. Both were grown in CAA medium and samples were taken over 24 h. OD₆₀₀ was

measured to compare growth, whereas absorbance of the supernatant at 405 nm (A_{405}) was measured to examine pyoverdine production. Fig. 2 shows no difference in growth during low-iron conditions, but clearly demonstrates the absence of pyoverdine in the deletion strain. It should be noted here that the addition of 0.5 g l⁻¹ of the strong chelator ethylenediamine di(*o*-hydroxy)phenylacetic acid (EDDHA) impaired growth severely in the *pvdQ* deletion strain but only slightly in the wild-type strain (see Supplementary Fig. S1, available with the online version of this paper).

Influence of PvdQ on motility

Motility has been associated with nutrient availability (Deziel *et al.*, 2003; Kohler *et al.*, 2000; Rashid & Kornberg, 2000; Singh *et al.*, 2002). We examined the effects of *pvdQ* deletion in *P. aeruginosa* on three different types of motility: flagellar-mediated swimming motility, swarming, and type IV pili-mediated twitching motility. Swimming and swarming motility assays were performed in the standard BM2 medium or BM2 medium without Fe(II) sulfate. No difference was observed in twitching and swimming motility between wild-type and deletion mutant (data not shown). In contrast to the wild-type strain, no swarming motility could be observed for the *pvdQ* deletion strain (Fig. 3a). Plasmid-borne expression of *pvdQ* or addition of partially purified pyoverdine allowed complementation of the mutant strain, restoring the swarming level to that of the parent strain (Fig. 3b, c). Addition of 3-oxo-C12-HSL and C4-HSL did not restore swarming motility in the mutant (not shown).

In a recent study, a *pvdQ* transposon mutant from the *P. aeruginosa* PA14 mutant library (Liberati *et al.*, 2006) showed a decrease in swarming motility (~75% diminished compared to wild-type) (Overhage *et al.*, 2008). This *P. aeruginosa* PA14 *pvdQ* transposon mutant (ID27758) was compared to our *pvdQ* deletion strain on the same type of swarming plates. Interestingly, the transposon mutant also showed total absence of swarming motility (Fig. 3d). The differences seen in the earlier study (Overhage *et al.*,

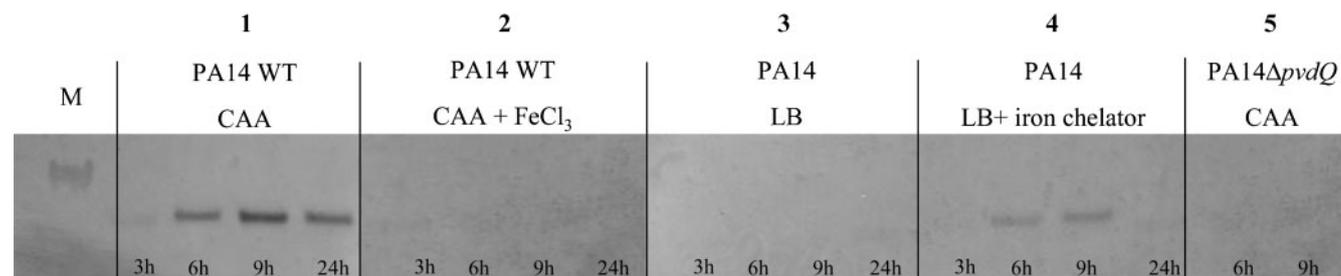


Fig. 1. PvdQ expression in *P. aeruginosa* PA14. PvdQ expression was monitored over 24 h. Samples were taken from cultures at various times after inoculation as indicated. Proteins were separated on a 4–12% polyacrylamide gel and then transferred to a nitrocellulose membrane. PvdQ was detected with polyclonal PvdQ antibodies. Sections 1 and 2 show expression in CAA medium, whereas sections 3 and 4 were conducted in LB with and without additional 2,2-dipyridyl. Monitoring expression in the deletion strain 5 (section 6) served as a control. The marker (M) indicates 72 kDa; the β -subunit of PvdQ shown has a size of 60 kDa.

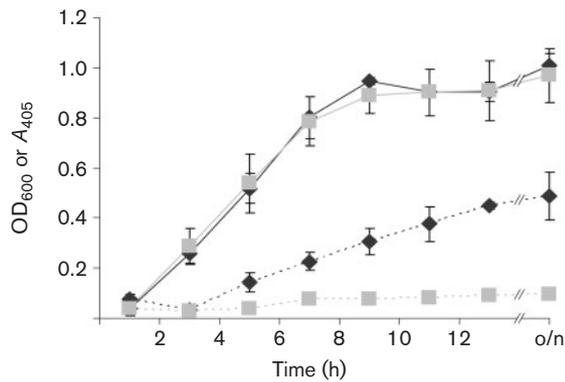


Fig. 2. Growth of wild-type PA14 (black diamonds) and the $\Delta pvdQ$ mutant (grey squares). Growth was monitored in iron-limited CAA medium. Wild-type and deletion strain show no differences in growth (full lines). However, when measuring the A_{405} of the cell-free supernatant (dashed lines) a clear difference is visible: PA14 $\Delta pvdQ$ lacks the production of pyoverdine. o/n, overnight.

2008) can thus be attributed to laboratory conditions and not to any polar effects from the transposon.

To investigate whether the reduced swarming motility was influenced by differences in production of rhamnolipids (Caiazza *et al.*, 2005; Deziel *et al.*, 2003; Kohler *et al.*, 2000; Overhage *et al.*, 2007), an orcinol test and a TLC analysis (as described by Wilhelm *et al.*, 2007) were conducted with our parent and mutant strain. However, no differences were observed (data not shown).

Role of iron in swarming motility

Based on the lack of pyoverdine production in our deletion strain and the evidence that low levels of iron stimulate

surface motility (Singh *et al.*, 2002; Singh, 2004), we tested the influence of different iron sources on the swarming behaviour of the $\Delta pvdQ$ mutant. Fe(II) sulfate, Fe(III) sulfate, Fe(III) chloride and Fe(II) citrate were used as individual iron sources. Additionally, swarming behaviour was studied on iron-depleted swarming agar (containing 2,2'-dipyridyl). Individual addition of each iron compound (shown for Fe(III) chloride in Fig. 4b) resulted in increasing swarming motility in the deletion mutant (up to wild-type level; Fig. 4a). Addition of 2,2'-dipyridyl resulted in inhibition of swarming of the wild-type PA14 (Fig. 4c).

We also tested whether addition of a partially purified batch of pyoverdine had any effect on swarming motility (Fig. 4d). Indeed swarming motility was restored, indicating a direct relation between PvdQ and pyoverdine. These experiments show that sufficient iron is needed for swarming motility.

PvdQ plays a role in iron uptake and biofilm formation

Staining with crystal violet revealed a significant 32-fold reduction in biofilm production in CAA medium in the $\Delta pvdQ$ mutant in comparison to the parental strain (Fig. 5). To test the hypothesis that iron was directly responsible for these differences, we added the four abovementioned iron sources. At the concentrations tested (10–300 μ M) these iron salts did not restore biofilm formation (Fig. 5: data shown only for Fe(III) chloride). These results indicate that iron alone does not account for the differences in the amount of biofilm produced by the $\Delta pvdQ$ mutant, suggesting that PvdQ plays a crucial role in biofilm formation, which seems to be independent of its role in the iron/pyoverdine pathway.

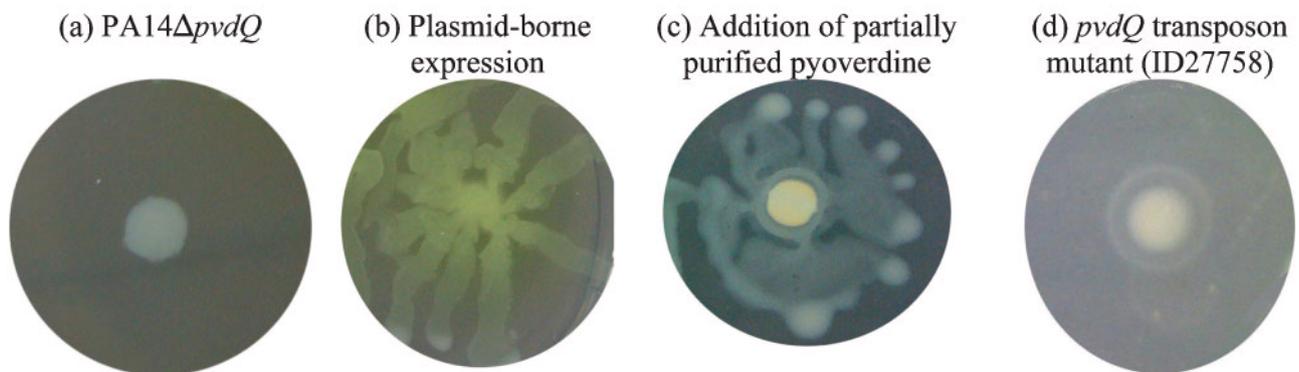


Fig. 3. Swarming motility of PA14 $\Delta pvdQ$. Swarming motility was assayed on BM2 medium solidified with 0.5% agar. (a) Swarming impairment in PA14 $\Delta pvdQ$. (b, c) Swarming motility can be fully restored by plasmid-borne gene expression (b) or by addition of partially purified pyoverdine (c). The *pvdQ* transposon mutant (ID27758) shows the same impairment as the clean deletion used in our studies (d).

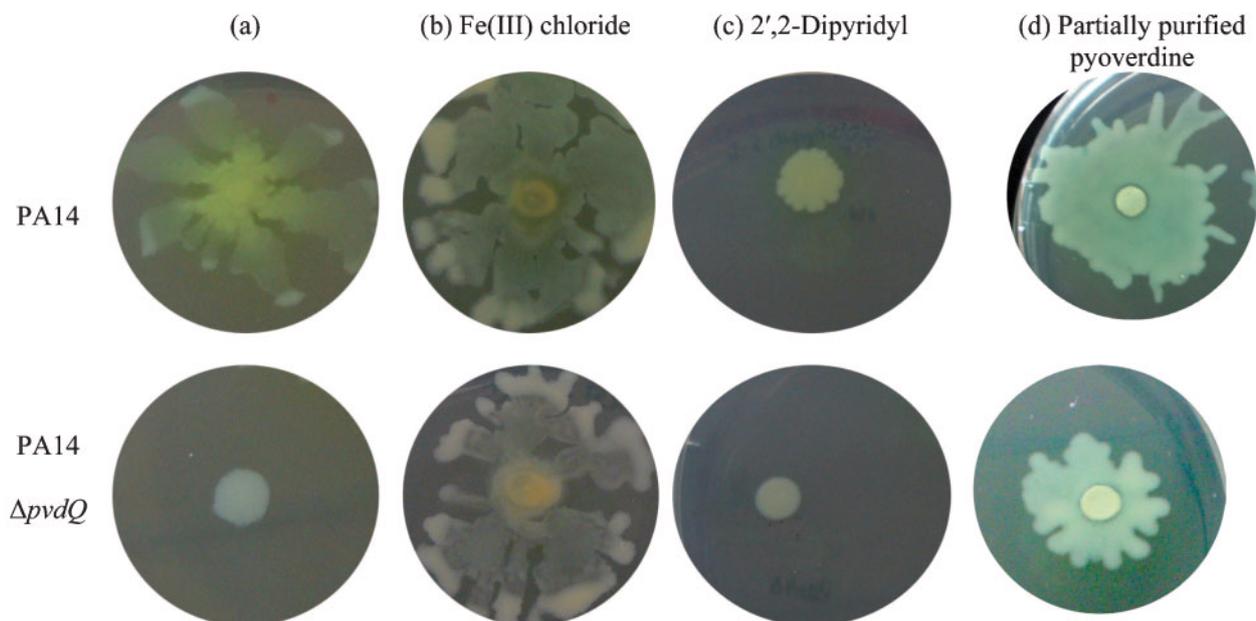


Fig. 4. Influence of iron on swarming motility. PA14 $\Delta pvdQ$ is impaired in swarming motility compared to wild-type (a). However, this swarming motility can be restored by the addition of iron (Fe(III) chloride) (b). Enough iron needs to be present in order for the cells to perform swarming motility; addition of iron chelators also inhibits swarming in the wild-type (c). Partially purified pyoverdine can restore swarming motility in the deletion strain (d).

Detection of 3-oxo-C12-HSL under iron-limiting conditions

Analysis of the cell-free supernatants of biofilms formed in CAA minimal medium by the wild-type strain and the $\Delta pvdQ$ mutant in a bioassay indicated a significant decrease in 3-oxo-C12-HSL concentration in the wild-type strain (Fig. 6), which can be attributed to the degradation of this autoinducer following PvdQ production. Reduced levels of 3-oxo-C12-HSL were observed by other authors

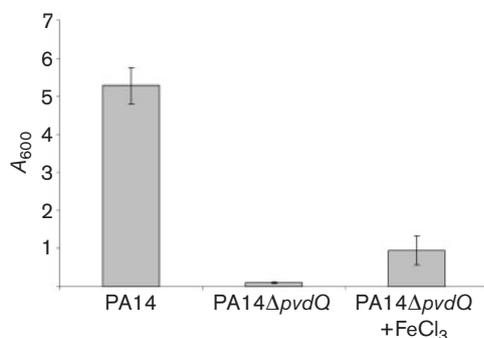


Fig. 5. Effects of PvdQ on biofilm formation: comparison of biofilm formation between PA14 (wild-type) and PA14 $\Delta pvdQ$. Biofilms were grown in CAA medium at 30 °C. Supplementation of the medium with iron does not restore biofilm formation in PA14 $\Delta pvdQ$.

while studying the amounts of AHLs in the cystic fibrosis lung (Singh *et al.*, 2000). A link between PvdQ and low 3-oxo-C12-HSL levels has been suggested as a possible explanation for the observed autoinducer decrease (Hentzer *et al.*, 2005).

In vivo effect of PvdQ in a plant infection model

The effects of PvdQ on virulence were studied in a plant model system. Potato slices inoculated with the $\Delta pvdQ$ strain exhibited a pronounced decrease in infection compared to the parental strain, for which clear infection was observed 48 h after incubation at 30 °C (Fig. 7). Complementation of the *pvdQ* mutant with plasmid pME6032-*pvdQ* or addition of partially purified pyoverdine restored infection to the wild-type level (Fig. 7). Restoration with iron sources could not be performed, as iron alone already caused fouling of the potato surface.

In vivo effect of *pvdQ* expression in a *C. elegans* infection model under iron-limiting conditions

C. elegans nematodes were exposed to PA14 and the $\Delta pvdQ$ mutant in order to study the effect of PvdQ under iron-limiting conditions *in vivo*. The nematodes were transferred to CAA plates with lawns of the respective bacterial strains and monitored over a 6 day period. It should be noted that CAA medium is different from the normally used *C. elegans* infection medium (Papaioannou *et al.*, 2009).

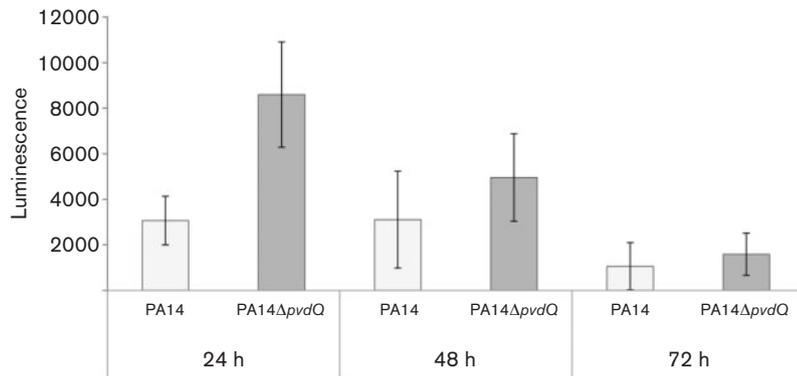


Fig. 6. Quantification of 3-oxo-C12-HSL. Strains PA14 and PA14ΔpvdQ were grown in a biofilm, and cell-free extracts prepared on three consecutive days were analysed using the biosensor strain *E. coli*(pSB1075). Light produced in response to 3-oxo-C12-HSL was quantified. For comparison, light values prior to the exponential phase were selected. All values are the means ± SE from at least three independent experiments.

During the first 2 days the nematodes did not show any alterations in their behaviour in any of the plates. However, on the third day post-infection the animals exposed to the wild-type strain showed locomotion problems and their pharyngeal pumping rate started to decrease (Fig. 8a). The number of eggs present on the plates after 4 days was very limited. The few offspring that could be seen on the plates showed a reduced growth rate and by the fifth day, the LT_{50} was reached. In contrast to this, the ΔpvdQ mutant was avirulent to the nematodes, which showed no disease-like symptoms throughout the period of the assay. Hundreds of eggs were present on the plates by the third day and the offspring went through their life cycle without any complications. No decrease in movement or pharyngeal pumping rates was observed. After the completion of the assay, bacterial lawns on the ΔpvdQ mutant plates were almost completely consumed.

To test if the different iron sources restore the toxicity of the ΔpvdQ mutant, we added one of the following iron sources to the CAA plates: Fe(II) sulfate, Fe(III) chloride or Fe(II) citrate. Each of these iron compounds restored the toxicity of the deletion strain to at least wild-type level (shown for Fe(III) chloride in Fig. 8b). In many cases the animals showed egg-laying defects and the eggs were hatching inside

the adult. Medium supplemented with Fe(III) chloride appeared to result in the highest overall toxicity levels, compared to all other media used: after 1 day of exposure to this medium only 30 % of the animals were alive (Fig. 8b). It should be noted here that PA14 was more virulent under all the iron-supplemented conditions tested compared to the iron-limiting conditions.

All of the above assays were performed using *E. coli* OP50 as a negative control to evaluate the background death levels of the worms. This strain was avirulent in all the assays performed under these conditions, and the nematodes fed on this strain went through a normal life cycle without any complications.

DISCUSSION

Studies on quorum-quenching acylases have focused on the potential of these enzymes to target infections by a broad range of Gram-negative pathogens. As a recent example *P. aeruginosa* has been shown to produce PvdQ, an acylase capable of degrading its own quorum-sensing molecule (3-oxo-C12-HSL) (Huang *et al.*, 2003; Sio *et al.*, 2006).

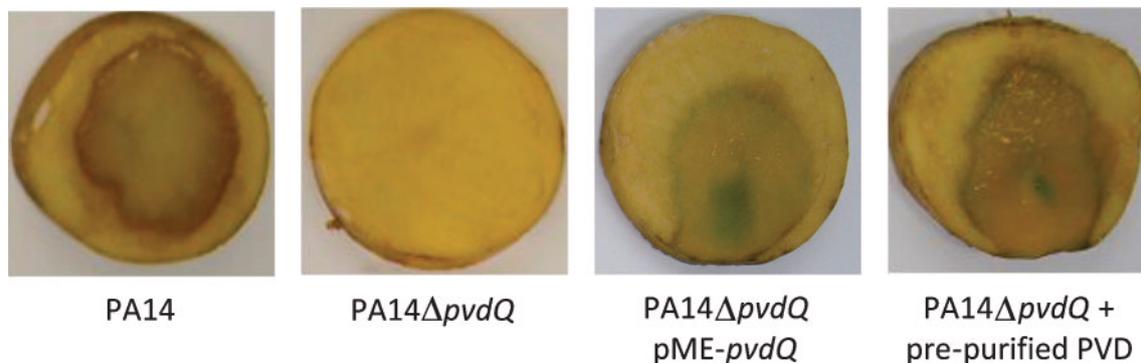


Fig. 7. PvdQ stimulates *P. aeruginosa* virulence in a plant model system. Infection of *P. aeruginosa* PA14 and the ΔpvdQ mutant was visualized on potato slices. Infection is clearly visible for the WT strain and absent for the ΔpvdQ mutant. Complementation with plasmid pME6032-pvdQ and addition of partially purified pyoverdine (PVD) restore infective behaviour in the ΔpvdQ mutant.

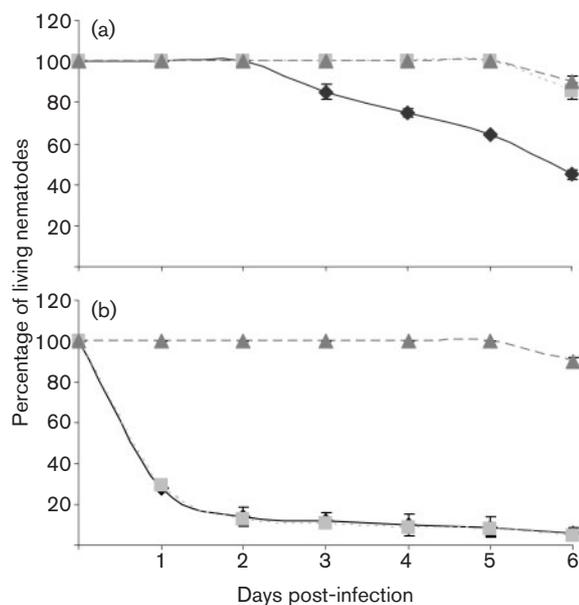


Fig. 8. Effects of deletion of *pvdQ* on *P. aeruginosa* toxicity under iron-limiting conditions in the *C. elegans* infection model and complementation by Fe(III) chloride. *P. aeruginosa* PA14 and its $\Delta pvdQ$ mutant were screened for virulence in the *C. elegans* model. (a) Under iron-limiting conditions (CAA medium) the $\Delta pvdQ$ mutant (grey squares) is clearly attenuated in pathogenicity compared to the PA14 wild-type (black diamonds). (b) Addition of 100 μ M Fe(III) chloride restores the toxicity of the $\Delta pvdQ$ mutant (grey squares) to the wild-type levels (black diamonds) (symbols superimposed). The negative control strain *E. coli* OP50 (black triangles) did not show any significant virulence against the nematodes.

However, little is known about the physiological role of this enzyme in *P. aeruginosa*. Interestingly, *pvdQ* is part of the Pvd locus and as such is involved in biosynthesis of pyoverdine, the major siderophore of this bacterium. As with most Pvd genes, *pvdQ* is only expressed under iron-limiting conditions as shown by microarray studies (Ochsner *et al.*, 2002). The consequence of this is that, apart from the involvement in pyoverdine biosynthesis, the quorum-quenching capabilities of PvdQ are likely to become apparent only when low concentrations of available iron are present in the environment.

To study the control of quorum quenching and siderophore production by one enzyme we set out to investigate the phenotypes affected by PvdQ. We confirmed that *pvdQ* is only expressed under low-iron conditions (Fig. 1). This result strongly suggests that PvdQ is only produced, and thus fulfils a physiological role, when the iron concentration is low. The deletion of *pvdQ* leads to the lack of pyoverdine synthesis as judged by the absence of the typical green colour that is present in the parent strain grown in CAA medium (Fig. 2) and as analysed by HPLC (unpublished results).

In CAA medium the deletion strain grew at a rate similar to the parent strain (Fig. 2). The absence of a growth defect in our pyoverdine mutant suggests that in the absence of a strong iron chelator, the other siderophore pyochelin is sufficient for unaltered growth of the *pvdQ* mutant under the conditions tested. This hypothesis was confirmed by addition of the strong iron chelator EDDHA to the growth medium, which resulted in a growth defect for the $\Delta pvdQ$ mutant (Supplementary Fig. S1), supporting the observation made by others that pyoverdine is necessary for growth in the presence of strong iron chelators (Lamont & Martin, 2003; Ochsner *et al.*, 2002).

Interestingly, we could not observe any change in twitching or swimming motility, although low iron concentrations have been shown to enhance twitching motility (Patriquin *et al.*, 2008; Singh *et al.*, 2002; Singh, 2004). Similar results were obtained by others (Banin *et al.*, 2005), where biofilm formation and twitching motility showed no correlation.

PvdQ plays a role in swarming motility of *P. aeruginosa*, as shown by the observation that the *pvdQ* deletion strain was impaired in swarming motility (Fig. 3a), and that expression of plasmid-borne *pvdQ* was able to restore swarming to the wild-type level (Fig. 3b). Addition of 3-oxo-C12-HSL and C4-HSL had no effect on restoration of swarming motility, neither could any difference in rhamnolipid production be observed, strongly suggesting that this phenotype is quorum quenching independent. Addition of different iron sources (Fig. 4b) or partially purified pyoverdine (Fig. 4d) restored swarming motility, indicating that this phenotype is under control of iron. These results are consistent with the observations made in *Pseudomonas putida*, where swarming could be restored in a pyoverdine mutant of *P. putida* KT2440 by addition of iron or pyoverdine (Matilla *et al.*, 2007).

Biofilm formation has been shown in previous studies to be disrupted in pyoverdine-negative strains; these biofilm defects could be restored by addition of Fe(II) citrate or Fe(III) chloride (Banin *et al.*, 2005; Patriquin *et al.*, 2008). Interestingly, most biofilm-deficient mutants were demonstrated to have enhanced swarming motility, suggesting that these two phenotypes are inversely regulated (Caiazza *et al.*, 2007). In our case, the $\Delta pvdQ$ deletion strain is not able to form biofilms in low-iron medium (CAA). Addition of different iron sources to the medium could not rescue this phenotype in our mutant strain (Fig. 5). These observations give us an indication that in *P. aeruginosa*, PvdQ plays a role in biofilm formation that goes beyond the acquisition of iron.

To rule out the possibility of the quorum-quenching ability of PvdQ having an influence on the results observed, 3-oxo-C12-HSL levels were measured. These levels were higher in the $\Delta pvdQ$ mutant than in the wild-type, corroborating the enzyme's capability to degrade *in vivo* long-chain AHLs (Fig. 6). Two other studies indicate a low level of 3-oxo-C12-HSL in biofilm-forming *P. aeruginosa*

cells: low levels of 3-oxo-C12-HSL were found in the sputum of cystic fibrosis patients (Singh *et al.*, 2000), and in a more recent study microarray data linked the reduction in 3-oxo-C12-HSL to an increase in *pvdQ* expression in *P. aeruginosa* biofilms (Hentzer *et al.*, 2005). However, it seems more likely that the 3-oxo-C12-HSL levels are simply a consequence of the presence of PvdQ, but not instrumental in biofilm formation. Overall, a relationship between PvdQ and biofilm formation seems clear from all the reported evidence.

Studies in a burned mouse infection model revealed that pyoverdine/iron acquisition is important for virulence (Meyer *et al.*, 1996). But what role does PvdQ play? If deletion of *pvdQ* has an opposite effect on biofilm formation under low-iron conditions compared to rich medium, then what about its effect on virulence? Our findings show that the $\Delta pvdQ$ mutant is strictly avirulent in two different models, a plant model and a *C. elegans* model. When applying wild-type *P. aeruginosa* strain on potato slices, fouling lesions were evident, while infection was suppressed in the $\Delta pvdQ$ mutant (Fig. 7). These results are in line with the biofilm results, especially because infection could be restored by *pvdQ* complementation with plasmid pME6032-*pvdQ* (Fig. 7). Under iron-limiting conditions, PvdQ positively influences a number of virulence phenotypes. In a *C. elegans* slow-killing assay, lack of iron in the medium resulted in the PA14 $\Delta pvdQ$ mutant being avirulent to the nematodes whereas the PA14 wild-type, under the same conditions, had toxic effects. This observation confirms the important role that PvdQ has in virulence. However, the virulence enhancement can only be observed under iron-limiting conditions. Once the media are supplemented with iron sources, the results obtained from the slow-killing assays dramatically change. Addition of any one of the three iron compounds, Fe(II) sulfate, Fe(III) chloride and Fe(II) citrate, to CAA medium resulted in the PA14 $\Delta pvdQ$ mutant reaching toxicity levels comparable to the PA14 wild-type (shown for Fe(III) chloride in Fig. 8b). Taking the results together, we can conclude that the use of the PvdQ protein as a quorum-quenching agent for therapeutic purposes (Papaioannou *et al.*, 2009) should only be considered under conditions where enough iron is present.

The recent postulation that quorum sensing and iron uptake are related in a complicated and nutritionally conditioned manner (Shrout *et al.*, 2006) is in line with the results from our experiments demonstrating that the effect of PvdQ under iron-limiting conditions is different from that in rich medium. Deletion of the gene does not lead to improved biofilm formation or virulence as would be expected by the resulting higher levels of AHLs. Complementation of *pvdQ*-related phenotypes in swarming and virulence by addition of iron compounds leads to the conclusion that the role of PvdQ in the iron-uptake pathway overrules its deacylase activity under iron-limiting conditions. However, the inability of iron compounds to restore biofilm formation in the $\Delta pvdQ$ mutant and the parallel swarming complementation (by addition of iron

sources) suggest that PvdQ is a key enzyme where these – and possibly more – pathways are involved in a complicated interplay that needs further elucidation.

ACKNOWLEDGEMENTS

We gratefully acknowledge Miguel Cámara and Paul Williams for providing the biosensor strain pSB1075 and 3-oxo-C12-HSL, and Fred Ausubel and Elina Drenkard for providing the PA14 transposon strain ID27758. We also thank Sandra Matthijs and Pierre Cornelis for helpful discussion, Nele Matthijs for excellent technical assistance, and Diane Black for critically reading the manuscript. This research was partly funded by EU grant Antibiotarget MEST-CT-2005-020278 to P. N. J., G. K. and E. P.

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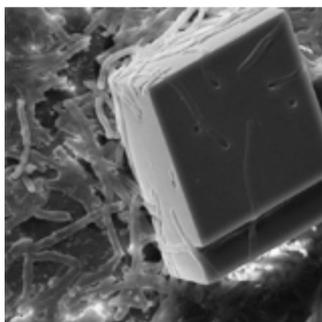
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