Function of the bacteriophytochrome BphP in the RpoS/Las quorum-sensing network of *Pseudomonas aeruginosa*

Katalin Barkovits,^{1†} Britta Schubert,¹ Sabrina Heine,¹ Maurice Scheer² and Nicole Frankenberg-Dinkel¹

Correspondence

Nicole Frankenberg-Dinkel
nicole.frankenberg@rub.de

¹Physiology of Microorganisms, Ruhr-University Bochum, Universitaetsstr. 150, 44780 Bochum, Germany

²Institute for Microbiology, Technical University Braunschweig, 38106 Braunschweig, Germany

Received 11 February 2011
Revised 3 March 2011
Accepted 15 March 2011

The bacterial phytochrome of *Pseudomonas aeruginosa* (PaBphP) is an *in vitro*-active red/far-red light sensor histidine kinase of a two-component regulatory system. Despite solid biochemical data, its function in this heterotrophic, opportunistic pathogen is still unknown. Previous studies established that the genes encoding the two necessary phytochrome components BphO, a chromophore-producing haem oxygenase, and BphP, the apo-phytochrome, are co-transcribed in a bicistronic operon. Transcription has been shown to be induced in the stationary phase and to be dependent on the alternative sigma factor RpoS. Here we show an additional regulation of *bphP* expression through the quorum-sensing (QS) regulator LasR. This regulation is also reflected in a combination of expression profile experiments and proteome analyses of wild-type and phytochrome-deficient strains. While PaBphP has a pleiotropic effect on global gene expression, 66 % of the downregulated genes in the phytochrome mutant display a link to the Las QS system. Most of these genes seem to be indirectly regulated by LasR through BphP and the unknown response regulator BphR. A model of phytochrome function within the Las QS network is presented.

INTRODUCTION

Phytochromes are red/far-red light sensors in plants, where they are involved in the regulation of photomorphogenesis in processes such as seed germination, shade avoidance, chloroplast development and induction of flowering (Chory *et al.*, 1996; Rockwell *et al.*, 2006; Smith, 2000). For a long time phytochromes were thought to exist only in plants, but in recent years they have also been identified in fungi and prokaryotes (Blumenstein *et al.*, 2005; Davis *et al.*, 1999). By now, many prokaryotic and fungal phytochromes have been biochemically and photobiologically characterized (Blumenstein *et al.*, 2005; Froehlich *et al.*, 2005; Giraud *et al.*, 2002; Lamparter *et al.*, 2002; Tasler *et al.*, 2005). The response to red and far-red light

results in two spectrally distinct, photointerconvertible phytochrome forms: a red light-absorbing form (Pr form) and a far-red light-absorbing form (Pfr form) (Chen *et al.*, 2004). The photochemistry of phytochrome action is mediated by a covalently attached linear tetrapyrrole chromophore and is based on the isomerization of a double bond in the conjugated chromophore system (Li & Lagarias, 1992). While light sensing is an important characteristic for phototrophic prokaryotes such as *Rhodospseudomonas palustris* and the cyanobacterium *Synechocystis* sp. PCC 6803, the biological function of phytochromes in heterotrophic bacteria such as *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* is enigmatic (Giraud *et al.*, 2005; Hübschmann *et al.*, 2005; Lamparter, 2006; Oberpichler *et al.*, 2006). Despite the solid biochemical data which clearly point towards a light-sensing function of BphP in *P. aeruginosa* (Tasler *et al.*, 2005), another sensory function cannot be excluded. In general, the presence of a putative light sensor in *P. aeruginosa* would not be too surprising as this organism is very versatile in terms of adaptation and contains many sensor proteins to detect environmental signals (Rodrigue *et al.*, 2000). *P. aeruginosa* is ubiquitous in water and soil environments, and the extent of infections of plants, animals and humans triggered by *P. aeruginosa* is significant (Cao *et al.*, 2001; Prithiviraj *et al.*, 2005). In

[†]Present address: School of Pharmacy, University of Maryland, Baltimore, MD, USA.

Abbreviations: HHQ, 2-heptyl-4-quinolone; IPG, immobilized pH gradient; ppde, probability of differential expression; PQS, *P. aeruginosa* quinolone signal; qRT-PCR, quantitative real-time RT-PCR; QS, quorum sensing; WT, wild-type.

The microarray data discussed in this paper are available from ArrayExpress under accession number E-MEXP-2606.

A supplementary figure, supplementary methods and two supplementary tables are available with the online version of this paper.

particular, it can cause serious infections in immunocompromised individuals and in patients with cystic fibrosis (Wagner & Iglewski, 2008). During infection, virulence factors are produced in a cell density-dependent manner, via the quorum-sensing (QS) system as well as in a growth phase-dependent fashion, through the stationary phase alternative sigma factor RpoS (de Kievit & Iglewski, 2000; Schuster *et al.*, 2004; Suh *et al.*, 1999).

Biochemical analyses have established that the genes *bphO* (PA4116) and *bphP* (PA4117) encode the two necessary phytochrome components in *P. aeruginosa*: BphO, a haem oxygenase that produces the chromophore biliverdin IX α , and BphP, the apo-phytochrome. Both components autocatalytically assemble to a functional holo-protein (PaBphP) that exhibits the characteristic red/far-red light-induced photoconversion (Tasler *et al.*, 2005). *P. aeruginosa* harbours an additional haem oxygenase PigA (also known as HemO), which differs from BphO by its different regiospecificity of haem cleavage, resulting in the products BV IX β and BV IX δ (Ratliff *et al.*, 2001). Interestingly, the latter has been shown to be able to bind to apo-BphP and to constitute a functional phytochrome (Wegele *et al.*, 2004). Whether this is of any biological significance is unknown and is currently under investigation in our laboratory. However, it has been shown that PigA plays a role in the iron supply of the bacterium (Ratliff *et al.*, 2001).

PaBphP is an orphan cytoplasmic sensor histidine kinase of a two-component system whose autophosphorylation activity has been demonstrated (Tasler *et al.*, 2005). So far, the identity of the corresponding response regulator (which we named BphR) out of 72 putative response regulators in *P. aeruginosa* is still unknown (Rodrigue *et al.*, 2000).

The expression of the bicistronic phytochrome operon of *P. aeruginosa* has been shown to be induced in a growth phase-dependent manner through the action of the alternative sigma factor RpoS, suggesting a role of PaBphP in stationary phase. Phenotypic analyses of *bphO* and *bphP* chromosomal knockout mutants have revealed no obvious growth defect under various conditions, although increased levels of pyocyanin have been detected in the $\Delta bphO$ strain. This strain also shows decreased heat tolerance in the stationary phase and a potential protective role of the BphO reaction product biliverdin IX α has been postulated. Therefore, BphO has been postulated to have an additional function besides providing the chromophore for BphP, and both proteins are likely to have roles in the stationary phase (Barkovits *et al.*, 2008).

As the phenotypic analyses of the phytochrome and haem oxygenase knockout mutant have not answered the question about the function of PaBphP in *P. aeruginosa*, a global approach was undertaken to elucidate this puzzling question. With transcriptome analyses using $\Delta bphO$ and $\Delta bphP$ mutants in comparison with the wild-type (WT), this study identified several genes that are

interwoven in the RpoS/Las QS network of *P. aeruginosa*. Furthermore, proteome analyses of the WT and the $\Delta bphP$ mutant complemented this study at the protein level.

METHODS

Bacterial strains and growth conditions. All *P. aeruginosa* strains were routinely grown and maintained on Luria–Bertani (LB) plates or LB broth at 37 °C (Barkovits *et al.*, 2008) and are listed in Table 1. The concentration of gentamicin used for selection of *P. aeruginosa* mutant strains was 200 $\mu\text{g ml}^{-1}$. Construction of mutant strains has been described previously (Barkovits *et al.*, 2008). The concentration of tetracycline used for selection of the strains containing chromosomally integrated promoter–*lacZ* fusions was 100 $\mu\text{g ml}^{-1}$.

RNA isolation. Total RNA from *P. aeruginosa* was isolated with the Total RNA Isolation NucleoSpin RNAII isolation kit (Macherey–Nagel) according to the manufacturer's instructions using 2 ml stationary phase culture (OD₅₇₈ 3.5) grown in LB broth. RNA was treated with RNase-free DNase (Macherey–Nagel) for 1 h at room temperature to remove contaminating chromosomal DNA. The quality and quantity of the prepared RNA were checked on an agarose gel and spectrophotometrically with a NanoDrop 100 spectrophotometer (PiqLab Biotechnologie).

Expression profiling experiments. Expression profiles of the *P. aeruginosa* WT, a $\Delta bphO$ and a $\Delta bphP$ mutant strain were compared. For this purpose, six independent cultures of each strain were grown in LB broth at 37 °C to stationary phase (OD₅₇₈ 3.5) and total RNA was isolated. Transcriptome experiments were performed with three independent samples, where each sample was composed of two individual RNA preparations. Labelled cDNA was generated according to the technical manual for the Affymetrix *Pseudomonas* microarray (<http://www.affymetrix.com>).

Synthesis of cDNA was done using random hexamers as primers for reverse transcription with SuperScript II (Invitrogen). Annealing of 10 μl primers (75 ng μl^{-1}) to 10 μg total RNA was performed for 10 min at 70 °C, followed by an incubation for 10 min at 25 °C. Elongation of cDNA using SuperScript II was performed under the following conditions: 10 min at 25 °C, 60 min at 37 °C, 60 min at 42 °C and 10 min at 70 °C [12 μl 5 $\times$ First Strand Buffer (Invitrogen), 6 μl 100 mM DTT, 3 μl 10 mM dNTPs, 1.5 μl 20 U SUPERase-In μl^{-1} (Ambion/Applied Biosystems) and 7.5 μl 200 U SuperScript II μl^{-1}]. Residual RNA was removed by addition of 1 M NaOH and incubation at 65 °C for 30 min, followed by neutralization with 1 M HCl. cDNA was purified with a MinElute PCR purification kit (Qiagen) and eluted in 45 μl buffer EB (10 mM Tris/HCl, pH 8.5), followed by quantification of cDNA using a NanoDrop 100 spectrophotometer.

Fragmentation of purified cDNA was carried out with DNase I (Invitrogen) in a ratio of 0.1 U DNase I:1 μg cDNA incubated for 5 min at room temperature. The reaction was subsequently inactivated by incubation at 98 °C for 10 min. Fragmented cDNA was end labelled with Biotin–ddUTP using GeneChip DNA Labelling Reagent (Affymetrix) and a terminal deoxynucleotidyltransferase (Promega). The labelling reaction (10 μl 15 $\times$ reaction buffer, 2 μl chip labelling reagent, 2 μl terminal deoxynucleotidyltransferase and 36 μl fragmented cDNA) was incubated for 60 min at 37 °C and stopped by adding 2 μl 0.5 M EDTA, pH 8. Verification of cDNA fragmentation between 50 and 200 bp and end-labelling with Biotin–ddUTP were confirmed by running samples on 15% polyacrylamide gels and visualizing via SYBR Gold staining (Invitrogen). Target hybridization, washing, staining and scanning

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description or sequence	Source or reference
<i>E. coli</i> strains		
DH5 α	F ⁻ Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>endA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44</i>	Hanahan (1983)
S17-1	<i>recA pro thi hsdR⁻ M⁺ Tp^r Sm^r; RP4::Tc::Mu-Km::Tn7/λpir</i>	de Lorenzo & Timmis (1994)
<i>P. aeruginosa</i> strains		
PAO1	WT	Dunn & Holloway (1971)
PA14	WT	He <i>et al.</i> (2004)
PAO-MW20	PAO1 <i>rpoS</i> ::Gm	Whiteley <i>et al.</i> (2000)
AHP01	PAO1 <i>bphO</i> ::Gm	Barkovits <i>et al.</i> (2008)
AHP02	PAO1 <i>bphP</i> ::Gm	Barkovits <i>et al.</i> (2008)
KBP08	PAO1 <i>att</i> ::(PA4739- <i>lacZ</i>)	This study
KBP10	AHP2 <i>att</i> ::(PA4739- <i>lacZ</i>)	This study
KBP12	PAO-MW20 <i>att</i> ::(PA4739- <i>lacZ</i>)	This study
SHP01	PA14 with Gm cartridge inserted into unique <i>Sal</i> I site of <i>lasR</i> (using pSB219.9A)	This study
JKP01	PA14 <i>att</i> :: <i>bphP-lacZ</i>	This study
SHP02	SHP01 <i>att</i> :: <i>bphP-lacZ</i>	
Plasmids		
mini-CTX- <i>lacZ</i>	Tc ^r ; promoterless <i>lacZ</i> gene	Becher & Schweizer (2000)
pEX18Ap	Ap ^r ; <i>oriT</i> ⁺ <i>sacB</i> ⁺ ; gene replacement vector with multiple cloning site from pUC18	Hoang <i>et al.</i> (1998)
pBBR1MCS3	Tc ^r , Mob, pBBR1 replicon	Kovach <i>et al.</i> (1995)
pBSP16	1263 bp fragment of the <i>bphO</i> promoter and coding region in pBBR1MCS3	This study
pKBP10	Ap ^r Tc ^r ; mini-CTX- <i>lacZ</i> with 706 bp of the PA4739 promoter region	This study
pJKP01	Ap ^r Tc ^r ; mini-CTX- <i>lacZ</i> with 818 bp of the putative <i>bphP</i> promoter region carrying the predicted <i>lasR</i> box	This study
pSB219.9A	pRIC380 carrying <i>lasR</i> ::Gm	Beatson <i>et al.</i> (2002)

were performed by the Affymetrix Core Facility at the Helmholtz Zentrum für Infektionsforschung, Braunschweig, Germany.

Data analysis of expression profiling experiments. Raw data obtained from the Affymetrix GeneArray Scanner were preprocessed with the Bioconductor software framework (Gentleman *et al.*, 2004). Expression values were calculated using the Robust Multichip Average (RMA) method (Irizarry *et al.*, 2003a, b) using quantile normalization (Bolstad *et al.*, 2003), background-corrected perfect match intensities and median polish as summarization method. Data were statistically analysed using the Cyber-T method. The *P* value threshold was 0.001 and the corresponding probability of differential expression (ppde) values were calculated (Baldi & Long, 2001; Hatfield *et al.*, 2003; Hung *et al.*, 2002) (Supplementary Table S1).

Construction of reporter gene fusions. Chromosomally integrated transcriptional *lacZ* reporter gene fusions were generated using the mini-CTX-*lacZ* vector system (Becher & Schweizer, 2000). The putative promoter region of PA4739 was amplified using *Pfu* DNA polymerase (Fermentas) with the primers PA4739profwd (containing an *Xho*I site) and PA4739prorev (containing an *Eco*RI site) (Supplementary Table S2). The 706 bp *Xho*I/*Eco*RI-digested PCR amplicon was cloned into the respective sites of mini-CTX-*lacZ* to construct pKBP10. This plasmid was transferred to *P. aeruginosa* WT, Δ *bphP* and Δ *rpoS* via diparental mating using *Escherichia coli* S17-1 as a donor to generate the strains KBP08, KBP10 and KBP12, respectively. The putative promoter region within the intergenic region of *bphO* and *bphP* was amplified using primers PA4117PHindIII fwd and PA4117PXmalrev (Supplementary Table S2). The 818 bp amplicon was digested with *Hind*III and *Xma*I and subsequently cloned into similarly digested miniCTX-*lacZ* to construct pJKP01. This plasmid was transferred to *P. aeruginosa* PA14 WT and an isogenic Δ *lasR* strain generated with plasmid

pSB219.9A (Beatson *et al.*, 2002) as described above. The use of strain PA14 became necessary as we were unable to obtain the respective mutants in the PAO1 WT. The integration of the plasmid into the *attB* site of the *P. aeruginosa* genome was obtained by selecting for tetracycline resistance. For determination of β -galactosidase activity from the chromosomally integrated *lacZ* fusion the *P. aeruginosa lacZ* reporter strains were grown overnight at 37 °C, diluted to OD₅₇₈ 0.05 and grown at 37 °C. β -Galactosidase activity was measured (Miller, 1972) at different time intervals.

Construction of the *bphO* complementation vector. A 980 bp PCR fragment containing the *bphO* coding region and 283 bp of the upstream region was generated with *Pfu* DNA polymerase using primers PA4115-16fwd and PA4115P2rev (Supplementary Table S2). The amplicon was blunt end-cloned into the *Sma*I restriction site of pBBR1MCS3 (Kovach *et al.*, 1995), resulting in pBSP16. The integrity of pBSP16 was confirmed by DNA sequencing (MWG). For complementation studies, pBSP16 was conjugated via diparental mating into the PAO1 WT and the Δ *bphO* mutant strain.

Quantitative real-time RT-PCR (qRT-PCR). Transcript abundance was measured by qRT-PCR using the MESA Green qPCR MasterMix Plus for SYBR assay (Eurogentec). RNA was isolated from *P. aeruginosa* WT and mutant strains grown in LB to stationary phase (OD₅₇₈ 3.5). Synthesis of cDNA was carried out as described above for the expression profile experiments. Oligonucleotide primers were designed using the *Pseudomonas* genome database (<http://www.pseudomonas.com>) and the Primer3 software on <http://frodo.wi.mit.edu>. Selected primers had melting temperatures of 61–62 °C and yielded amplicons of 100–200 bp. Moreover, the functionality of each primer pair was verified by PCR with genomic DNA and subsequent gel electrophoresis before use for further quantification analysis by real-time RT-PCR. The primers used are listed in Supplementary

Table S2. All real-time RT-PCRs were performed in a volume of 20 µl in a DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories) with the following PCR conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Obtained melting curves were analysed for optimal PCR conditions, whereas reaction with primer pairs that produced dimer were excluded for quantification of transcript levels. Amplicon size for each primer pair was verified by gel electrophoresis. Control PCRs were performed with samples that lacked reverse transcriptase to exclude contamination with chromosomal DNA. Only RNA samples that did not yield a PCR product in the absence of reverse transcriptase were used for further studies. The efficiency of each primer pair was calculated from standard curves (Pfaffl, 2001) or using LinRegPCR (Ramakers *et al.*, 2003). Mean threshold cycle (C_t) values were calculated from the triplicates and used for calculations of expression ratios according to Pfaffl (2001) with primer-specific efficiencies. The C_t values for an amplicon derived from the *recA* gene (Waite *et al.*, 2006) were used as a reference for normalization. Real-time PCR experiments with each strain and primer pair were carried out at least twice with biologically independent samples, and all reactions were performed in triplicate. Complementation strains were treated similarly and compared with the WT strain carrying the complementation plasmid.

Proteome analysis. For separation of whole-cell proteins, *P. aeruginosa* cultures (WT and $\Delta bphP$) were grown to stationary phase (OD₅₇₈ 3.5) in LB broth, and 10 ml of bacterial suspension was harvested for protein extraction by centrifugation (10 000 g for 3 min). All preparative steps were carried out at 4 °C unless otherwise noted. Bacterial cells were washed twice with PBS (pH 7), and the obtained pellet was suspended in 1.6 ml SDS buffer [100 mM Tris/HCl, pH 9.5, 1% (w/v) SDS]. Cell disruption was achieved with an ultrasonic homogenizer (2 × 30 pulses; 4 mm probe tip; interval: 1 Hz; pulse duration: 0.3 s; 20 kHz homogeneous sound; power output: 50 W) on ice. The cell lysate was centrifuged at 10 000 g for 30 min and aliquots of the supernatant were frozen at −80 °C.

Prior to the IEF with immobilized pH gradient (IPG) strips (GE Healthcare), the protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Approximately 250 µg whole-cell protein was used for IPG strip rehydration in IPG strip rehydration buffer [8 M urea, 1% CHAPS (w/v), 0.4% DTT (w/v) and 0.5% Pharmalyte 3–10 (v/v)] for 16 h. IEF was performed across a nonlinear pH range between 3 and 7 on 24 cm IPG strips on an IPGphor 3 isoelectric focusing system (GE Healthcare) at 20 °C with the following focusing settings: 200 V constant for 2 h, 500 V constant for 2 h, 1000 V gradient for 2 h, 8000 V gradient for 2.4 h, and final 8000 V for 4.5 h, giving a total of 48 000 Vh. IPG strips were stored at −80 °C or used immediately for second-dimension electrophoresis by SDS-PAGE.

Separation of the whole-cell protein in the second dimension was accomplished in 15% and 1 mm thick vertical SDS-polyacrylamide gels using the Ettan DALT six system (GE Healthcare) at 20 °C. Before application of IPG strips to the SDS-PAGE, the separated proteins in the strips were first reduced with equilibration buffer I [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT in 0.05 M Tris/HCl buffer, pH 8.8] for 20 min. Next, strips were incubated in equilibration buffer II [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 4.8% (w/v) iodoacetamide in 0.05 M Tris/HCl buffer, pH 8.8] for 20 min to alkylate free thiol groups on separated proteins with iodoacetamide. The IPG strips were then placed on top of the SDS-polyacrylamide gels and fixed with 0.5% agarose sealing buffer (0.5% agarose in running buffer dyed with bromophenol blue). Gels were run using an Ettan DALT six system with 1 × running buffer (24 mM Tris, 0.2 M glycine, 0.1% SDS) in the lower chamber and 2.5 × running buffer in the upper chamber. SDS-PAGE was

performed at 2 W per gel in a first step for 1 h, and in a second final step at 80 W per gel at 20 °C until the dye front reached the end of the gel. To estimate the molecular mass of proteins the Unstained Protein Molecular Weight Marker from Fermentas was used.

In order to analyse the presence and absence of separated proteins, after 2D electrophoresis the proteins were stained with SYPRO Ruby as described in Lamanda *et al.* (2004). Stained gels were scanned with a flatbed scanner (Typhoon imager). After image scanning, the 2D gels were evaluated with Delta2D software, version 4.0 (DECODON). Selected protein spots were cut out, digested with trypsin and identified by Peptide Mass Fingerprint (PMF) using a 4800 MALDI TOF/TOF analyser (Applied Biosystems) (Eymann *et al.*, 2004).

RESULTS

Expression profiles of the $\Delta bphO$ and $\Delta bphP$ mutants of *P. aeruginosa*

In order to learn more about the function of *PaBphP*, the effects of both a *bphO* and a *bphP* deletion on global gene expression were analysed using *Pseudomonas* Affymetrix GeneChips. For this purpose expression profiles of a $\Delta bphO$ mutant, a $\Delta bphP$ mutant and the *P. aeruginosa* WT grown to stationary phase (OD₅₇₈ 3.5) were compared. This revealed 89 genes whose expression was influenced by the loss of *bphO* and 124 genes influenced by the loss of *bphP*. In the $\Delta bphO$ mutant, 67 genes were significantly downregulated and 22 genes were significantly upregulated, while 105 genes were downregulated and 19 genes were upregulated in the $\Delta bphP$ mutant. Comparison of the two expression profiles indicated that 74 genes were regulated in both deletion mutants (Tables 2 and 3). These 74 genes are considered true phytochrome-regulated genes as we propose that both *BphO* and *BphP* are required for establishing a functional holo-phytochrome sensor histidine kinase. Among those 74 regulated genes, 60 were significantly downregulated and 14 were significantly upregulated in both mutants. For further analyses, these genes were divided into functional classes and are listed in Tables 2 and 3. The majority of the downregulated genes encode hypothetical proteins, but other identified genes can be attributed to specific functional classes. The upregulated genes can be placed within the same functional classes; however, no genes involved in adaptation were significantly upregulated in either mutant strain.

Validation of expression profile data with a *lacZ* reporter gene fusion and qRT-PCR

Expression profile data were independently confirmed via a promoter-*lacZ* reporter gene fusion of PA4739 and qRT-PCR of several regulated genes. PA4739 was chosen for the *lacZ* analyses as it was the gene downregulated to the greatest extent in our expression analyses. Transcription of PA4739 is growth phase-dependent and was approximately threefold reduced in the $\Delta bphP$ strain after 6 h of growth (i.e. at OD₅₇₈ ~3.5) (Fig. 1). qRT-PCR of selected genes confirmed the microarray data and results are shown in Table 4.

Table 2. Genes downregulated in the $\Delta bphO$ and $\Delta bphP$ mutants compared with the WT classified by functional group

Functional group*, PA no. and gene	Product name*	Fold change†		Known regulation‡
		<i>ΔbphO</i>	<i>ΔbphP</i>	
Hypothetical				
PA0567	Conserved hypothetical protein	−3.0	−4.8	QS (↑); [LasR]
PA2116	Conserved hypothetical protein	−4.1	−2.2	
PA2134	Hypothetical protein	−2.3	−3.3	QS (↑)
PA2146 (<i>yciG</i>)	Conserved hypothetical protein	−2.0	−2.7	QS (↑); [LasR]
PA2159	Conserved hypothetical protein	−3.8	−5.1	QS (↑)
PA2161	Hypothetical protein	−2.2	−2.4	QS (↑)
PA2166	Hypothetical protein	−3.3	−6.0	QS (↑); [LasR, RpoS]
PA2169	Hypothetical protein	−2.2	−3.0	QS (↑)
PA2170	Hypothetical protein	−2.5	−3.1	QS (↑); [LasR]
PA2171	Hypothetical protein	−3.4	−6.7	QS (↑); [LasR]
PA2172	Hypothetical protein	−3.1	−4.0	QS (↑)
PA2173	Hypothetical protein	−3.9	−6.2	QS (↑)
PA2184 (<i>yciE</i>)	Conserved hypothetical protein	−2.0	−2.2	
PA2190	Conserved hypothetical protein	−2.2	−4.9	QS (↑); [LasR]
PA2433	Hypothetical protein	−2.6	−5.3	QS (↑)
PA3369	Hypothetical protein	−4.0	−4.9	QS (↑); [LasR]
PA3370	Hypothetical protein	−3.0	−3.9	QS (↑); [LasR]
PA3371	Hypothetical protein	−3.3	−3.5	QS (↑); [LasR]
PA4345	Hypothetical protein	−2.0	−2.8	
Transcriptional regulators				
PA0610 <i>prtN</i>	Transcriptional regulator PrtN	−2.3	−2.5	
PA0612 <i>ptrB</i>	Repressor PtrB	−3.5	−3.3	
Translation, modification, degradation				
PA0355 <i>pfpI</i>	Protease PfpI	−2.1	−3.2	QS (↑); [LasR]
PA3162 <i>rpsA</i>	30S ribosomal protein S1	−3.2	−2.4	
PA3834 <i>valS</i>	Valyl-tRNA synthetase	−2.0	−2.2	
PA4247 <i>rplR</i>	50S ribosomal protein L18	−2.1	−2.1	
PA4248 <i>rplF</i>	50S ribosomal protein L6	−2.0	−2.2	
PA4262 <i>rplD</i>	50S ribosomal protein L4	−2.0	−2.0	
PA4266 <i>fusA1</i>	Elongation factor G	−2.2	−2.2	
Putative enzymes				
PA2158	Probable alcohol dehydrogenase	−2.1	−2.5	QS (↑)
PA2160 (<i>glgX</i>)	Probable glycosyl hydrolase	−2.6	−3.6	QS (↑)
PA2164	Probable glycosyl hydrolase	−2.3	−2.3	QS (↑); [LasR]
PA2815 (<i>yafH</i>)	Probable acyl-CoA dehydrogenase	−2.5	−4.1	[RpoS] (↑)
PA3795	Probable oxidoreductase	−2.2	−2.7	
Energy metabolism				
PA1552	Probable cytochrome <i>c</i>	−2.2	−2.6	
PA1553	Probable cytochrome <i>c</i> oxidase subunit	−2.3	−2.1	
PA1581 <i>sdhC</i>	Succinate dehydrogenase (C subunit)	−2.0	−2.5	
PA2165 (<i>glgA</i>)	Probable glycogen synthase	−2.2	−2.3	QS (↑); [LasR]
PA4430	Probable cytochrome <i>b</i>	−2.3	−2.3	
Biosynthesis of cofactors				
PA0996 <i>pqsA</i>	Probable coenzyme A ligase	−2.0	−2.5	QS (↑)
PA0998 <i>pqsC</i>	Beta-keto-acyl-acyl-carrier protein synthase	−2.1	−2.7	QS (↑)
Other				
PA1288 (<i>ompP1</i>)	Probable outer-membrane protein precursor	−2.2	−2.3	
PA1800 <i>tig</i>	Trigger factor	−2.0	−2.1	
PA2144 <i>glgP</i>	Glycogen phosphorylase	−2.2	−2.5	QS (↑)
PA2414	L-Sorbose dehydrogenase	−2.1	−2.3	QS (↑)

Table 2. cont.

Functional group*, PA no. and gene	Product name*	Fold change†		Known regulation‡
		<i>ΔbphO</i>	<i>ΔbphP</i>	
PA3692 (<i>ompA</i>)	Probable outer-membrane protein precursor	−2.3	−6.8	QS (↑); [LasR]
Adaptation (osmotic stress)§				
PA0059 <i>osmC</i>	Osmotically inducible protein OsmC	−2.4	−5.4	QS (↑); [LasR]
PA1323	Hypothetical protein	−2.4	−7.1	QS (↑); [LasR]
PA1324	Hypothetical protein	−2.5	−7.1	QS (↑); [LasR]
PA2143	Hypothetical protein	−2.7	−4.0	QS (↑); [LasR]
PA2152	Probable trehalose synthase	−1.9	−2.3	QS (↑)
PA2747	Hypothetical protein	−4.4	−10.1	QS (↑); [LasR]
PA3460	Probable acetyltransferase	−2.6	−4.5	
PA3691	Hypothetical protein	−2.0	−3.8	QS (↑); [LasR]
PA4171	Probable protease	−2.6	−2.9	QS (↑); [LasR]
PA4738 (<i>yjbI</i>)	Conserved hypothetical protein	−3.1	−8.9	QS (↑); [LasR, RpoS]
PA4739 (<i>osmY</i>)	Conserved hypothetical protein	−3.4	−19.1	QS (↑); [LasR, RpoS]
PA4876 <i>osmE</i>	Osmotically inducible lipoprotein OsmE	−2.6	−5.2	QS (↑); [LasR, RpoS]
PA4880	Probable bacterioferritin	−2.4	−5.9	QS (↑); [LasR, RpoS]
PA5481	Hypothetical protein	−3.2	−3.4	QS (↑); [LasR, RpoS]
PA5482	Hypothetical protein	−5.0	−6.2	QS (↑); [LasR, RpoS]

*Functional groups, PA numbers, genes and product names are annotated according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>). Genes in parentheses are predicted or alternative gene names. Genes in bold type represent putative operons.

†Fold changes (>2) are indicated for comparisons of gene expression in the mutant strain with that in the *P. aeruginosa* WT strain. Fold changes of QS- or RpoS-regulated genes are shown in bold type.

‡Genes which were shown to be QS- or RpoS-dependently regulated via the ProdoNet tool of the PRODORIC database or in other transcriptome studies of *P. aeruginosa* (Hentzer *et al.*, 2003; Münch *et al.*, 2003; Schuster & Greenberg, 2007; Schuster *et al.*, 2003, 2004; Wagner *et al.*, 2003). (↑) Indicates activation by QS or RpoS. QS includes both the Las and/or the Rhl system (Schuster *et al.*, 2003; Wagner *et al.*, 2003). Regulators denoted in brackets have been shown to activate gene expression (Schuster & Greenberg, 2007).

§Aspedon *et al.* (2006).

Complementation studies

In order to confirm that the changes in the expression of certain genes were indeed due to the loss of *bphO*, complementation experiments employing *bphO* under its own promoter were performed. A replicative vector harbouring a PCR fragment containing the *bphO* promoter and the *bphO* gene was introduced into the WT and the *ΔbphO* strain and subsequently expression levels of several selected genes were determined by qRT-PCR (Table 4). Our data indicated that the mutant was complemented and that expression levels in the complemented strains were closer to those observed in the WT.

Many genes affected by *PaBphP* are linked to QS

Closer inspection of the 60 downregulated genes revealed that 40 of them had been shown to be regulated by the two homoserine lactone QS systems LasR–LasI and/or RhlR–RhlI (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). Specifically, 26 of these QS-regulated genes had been shown to be induced in a LasR-overexpression strain of *P. aeruginosa* (Schuster & Greenberg, 2007) (Table 2). In addition, seven of the LasR-dependent genes had been shown to be regulated by the alternative sigma factor RpoS (Schuster & Greenberg, 2007; Schuster *et al.*, 2004).

PaBphP itself is LasR-regulated

In order to understand the role of *PaBphP* in regulating the identified genes we again had a closer look at *bphOP* expression itself. We and others have previously identified the RpoS-dependent expression of the bicistronic *bphOP* operon (Barkovits *et al.*, 2008; Schuster *et al.*, 2004). In addition, several microarray studies and a recent chromatin immunoprecipitation (ChIP)-Chip experiment have suggested additional regulation via LasR (Gilbert *et al.*, 2009; Schuster *et al.*, 2003, 2004; Wagner *et al.*, 2003). Specifically, a LasR-dependent promoter for *bphP* has been postulated in the intergenic region of *bphO* and *bphP* (Gilbert *et al.*, 2009). Since no direct binding of LasR to the intergenic region could be shown by gel-shift analysis (Gilbert *et al.*, 2009) we constructed a *bphP*–*lacZ* reporter gene fusion harbouring the intergenic region between *bphOP* and introduced it into the WT and an isogenic *lasR* mutant strain. The expression in the *lasR* background was approximately 3.5-fold reduced compared with the WT (Fig. 2), suggesting a second level of regulation of *bphP* through the QS regulator LasR. However, transcription from this promoter would require a rather unconventional binding of the RNA polymerase, as the predicted LasR box is only 8 bp upstream of the annotated AUG start codon. These data indicate that *bphO* and *bphP*

Table 3. Genes upregulated in the $\Delta bphO$ and $\Delta bphP$ mutants compared with the WT strain classified by functional group

Functional group*, PA no. and gene	Product name*	Fold change†		Known regulation‡§
		$\Delta bphO$	$\Delta bphP$	
Hypothetical				
PA0200	Hypothetical protein	2.4	2.3	BF
PA1673	Hypothetical protein	2.3	2.0	RpoS (↓), BF
PA4352	Conserved hypothetical protein	3.0	2.7	BF
PA4577	Hypothetical protein	3.2	2.8	
PA5027	Hypothetical protein	3.1	2.8	QS (↑) [RhlR, RpoS], BF
PA5460	Hypothetical protein	2.1	2.0	BF
PA5475	Hypothetical protein	2.6	2.5	BF
Transcriptional regulators				
PA1196	Probable transcriptional regulator	3.1	2.6	
Putative enzymes				
PA0836 <i>ackA</i>	Acetate kinase	2.1	2.2	Anr
Energy metabolism				
PA4587 <i>ccpR</i>	Cytochrome <i>c</i> ₅₅₁ peroxidase precursor	2.8	2.5	QS (↓), RpoS (↓), Anr
PA5427 <i>adhA</i>	Alcohol dehydrogenase	3.8	3.5	BF, Anr
Biosynthesis of cofactors				
PA1546 <i>hemN</i>	O ₂ -independent coproporphyrinogen III oxidase	2.5	2.2	BF, Anr
Other				
PA1429	Probable cation-transporting P-type ATPase	2.0	2.0	
PA3337 <i>rfaD</i>	ADP-L-glycero-D-mannoheptose 6-epimerase	2.4	2.0	QS (↓), BF

*Functional groups, PA numbers, genes and product names are annotated according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>).

†Fold changes (>2) are indicated for comparisons of gene expression of the $\Delta bphP$ mutant strain with that of the *P. aeruginosa* WT. Fold changes of QS- or RpoS-regulated genes are shown in bold type.

‡Genes which were shown to be QS- or RpoS-dependently regulated via the ProdoNet tool of the PRODORIC database or in other transcriptome studies of *P. aeruginosa* (Hentzer *et al.*, 2003; Münch *et al.*, 2003; Schuster & Greenberg, 2007; Schuster *et al.*, 2003, 2004; Wagner *et al.*, 2003). (↑) Indicates activation and (↓) repression by QS or RpoS. QS includes both the Las and/or the Rhl system (Schuster *et al.*, 2003; Wagner *et al.*, 2003). Regulators in brackets have been shown to activate gene expression (Schuster & Greenberg, 2007).

§Genes which are known to be induced in biofilm (BF) cultures compared with planktonic cells (Hentzer *et al.*, 2005).

are directly regulated by RpoS (bicistronic transcript), with *bphP* additionally regulated through LasR (monocistronic transcript).

Osmoresponsive genes are influenced by *PaBphP*

In general, it appears that the loss of a functional phytochrome has a pleiotropic effect on global gene expression. Careful analysis of our data revealed a whole set of genes that had previously been described as osmoresponsive genes in *P. aeruginosa* but also in other Gram-negative bacteria such as *E. coli* (Aspedon *et al.*, 2006; Wood, 1999). In total, 15 genes related to osmotic stress were downregulated in both mutants and are listed in Table 2. All of them, except PA3460, have been identified as QS-regulated genes in other microarray analyses (Hentzer *et al.*, 2003; Schuster & Greenberg, 2007; Schuster *et al.*, 2003; Wagner *et al.*, 2003). Among them is *osmY* (PA4739), the most downregulated gene in the $\Delta bphP$ mutant. The gene product of *osmY* is a hypothetical periplasmic protein and

possesses a predicted BON domain that is typical of OsmY-type proteins involved in protection against osmotic shock (Yeats & Bateman, 2003). In *E. coli* and *P. aeruginosa*, *osmY* is induced by hyperosmotic shock (Aspedon *et al.*, 2006; Yim & Villarejo, 1992). It is hypothesized that OsmY contacts the phospholipid interface within the periplasmic space, thereby preventing shrinkage of the cytoplasmic compartment. In this way, the inner membrane would be attached to the more rigid outer membrane and shrinking would be reduced (Liechty *et al.*, 2000; Oh *et al.*, 2000). *osmY* is most likely the first gene of a bicistronic operon (PA4739–PA4738). Therefore, it is not unexpected that PA4738 is also downregulated in both mutant strains and that PA4738 is also induced under osmotic stress (Aspedon *et al.*, 2006).

Furthermore, the genes *osmC* and *osmE* were significantly downregulated in both mutants (Table 2). Transcription of *osmC* and *osmE* is osmotic stress-inducible, and the corresponding products are also proposed to be involved directly or indirectly in defence against oxidative compounds (Atichartpongkul *et al.*, 2001; Conter *et al.*, 2001;

Fig. 1. Expression of a chromosomally integrated transcriptional PA4739 (*osmY*)-*lacZ* reporter gene fusion during growth in the *P. aeruginosa* WT, and the $\Delta bphP$ and $\Delta rpoS$ mutants. As a control, a promoterless *lacZ* was used. β -Galactosidase activity is shown in Miller units and each value is the mean $\pm$ SD of two independent triplicate measurements.

Gutierrez & Devedjian, 1991; Gutierrez *et al.*, 1995). Transcription of *osmE* in *P. aeruginosa* is RpoS-dependent (Lacour & Landini, 2004; Schuster & Greenberg, 2007). In addition, expression of both genes is activated by the Las system and has also been shown to be affected by PsrA, a transcriptional regulator involved in stationary phase-induced transcriptional regulation of *rpoS* (Kang *et al.*, 2008; Kojic *et al.*, 2005; Schuster *et al.*, 2003). *osmC* and *osmE* transcription has furthermore been shown to be

activated by AlgU, an alternative sigma factor, in a mucoid strain of *P. aeruginosa* (Firoved & Deretic, 2003). Thus, several regulatory players are involved in *osmC* and *osmE* expression, supporting the physiological significance of their regulation in stationary phase.

In order to test the link between *PaBphP* and osmoprotection, hyperosmotic conditions (0.8 M NaCl) were applied to the PAO1 WT and the $\Delta bphO$ and $\Delta bphP$ mutant strains. However, no survival rate reduction or growth deficiencies in the mutant strains were observed compared with the WT (data not shown).

Impact of *PaBphP* on PQS synthesis genes

Our microarray experiment furthermore revealed an influence on the expression of *pqsA* and -C in both the $\Delta bphO$ and the $\Delta bphP$ strain, and additionally *pqsB* and -D in the $\Delta bphP$ strain alone (data not shown). These genes were downregulated approximately 2.5-fold in response to the absence of *PaBphP*. The *pqs* genes form an operon, which encodes enzymes involved in synthesis of the *P. aeruginosa* quinolone signal (PQS) precursor 2-heptyl-4-quinolone (HHQ). PQS and HHQ belong to a family of compounds called 2-alkyl-4-quinolones and are involved in the QS regulatory system, where they are important for virulence factor expression and biofilm formation (Camilli & Bassler, 2006; Dubern & Diggle, 2008; Pesci *et al.*, 1999). Recently, PQS was identified as having pro- as well as antioxidant activities when cells enter the stationary phase. PQS sensitizes bacteria to exogenous stresses but also promotes an efficient bacterial anti-stress response (Häussler & Becker, 2008).

To analyse the effect of *PaBphP* on *pqsABCD* expression, PQS and HHQ production of the WT was compared with

Table 4. Comparison of expression data obtained from microarray analysis and from qRT-PCR experiments

PA no.	Gene or product	Microarray: fold change WT vs $\Delta bphO$ WT vs $\Delta bphP$	qRT-PCR: fold change WT vs $\Delta bphO$ WT vs $\Delta bphP$	qRT-PCR: fold change WT/pBSP16 vs $\Delta bphO$ / pBSP16
PA3617	<i>recA</i>	0	0	0
PA0059	<i>osmC</i>	-2.4	-3.8	ND*
		-5.4	-5.3	ND
PA2747	Hypothetical protein	-4.4	-4.8	-2.3
		-10.1	-7.7	ND
PA4577	Hypothetical protein	+3.2	ND	ND
		+2.8	+1.32	ND
PA4876	<i>osmE</i>	-2.6	-5.75	ND
		-5.2	-7.6	ND
PA5027	Hypothetical protein	+3.1	+2.3	+1.3
		+2.8	+1.4	ND
PA5482	Hypothetical protein	-5.0	-4.4	-1.9
		-6.2	-5.4	ND

*ND, Not determined or no reliable data obtained.

Fig. 2. Expression of a chromosomally integrated transcriptional *bphP-lacZ* reporter gene fusion in *P. aeruginosa* PA14 WT and an isogenic $\Delta lasR$ mutant strain. As a control, a promoterless *lacZ* was used. β -Galactosidase activity is shown in Miller units and each value is the mean $\pm$ SD of two independent duplicate measurements.

that of the $\Delta bphP$ mutant. The synthesis of both PQS and HHQ was only marginally reduced in the $\Delta bphP$ mutant at a cell density of OD₅₇₈ 3.5, at which gene expression was also analysed by microarray (Supplementary Fig. S1).

Many of the upregulated genes are also upregulated in biofilm cultures

Closer inspection of the 14 significantly upregulated genes revealed that eight of them had been shown to be induced in biofilm cultures compared with planktonic cells (Hentzer *et al.*, 2005). Among the upregulated genes are several related to oxygen limitation and anaerobic growth: PA4587, a dihaem-containing cytochrome *c*₅₅₁ peroxidase involved in the reduction of H₂O₂, PA5427, alcohol dehydrogenase, and PA0836, acetate kinase, both fermentation pathway enzymes, and PA1546, the O₂-independent coproporphyrinogen III oxidase, a key regulatory enzyme of haem biosynthesis. All these genes have recently also been shown to be regulated through the oxygen sensor Anr (Trunk *et al.*, 2010). As previously reported, we did not see any growth difference between the WT and the phytochrome mutants under anaerobic denitrifying growth conditions (Barkovits *et al.*, 2008).

Proteome analyses support the transcription profile of the $\Delta bphP$ mutant

In a complementary approach to the presented expression profiles of the $\Delta bphO$ and $\Delta bphP$ mutants, the proteome profiles of a $\Delta bphP$ mutant and the PAO1 WT grown

under identical conditions were analysed by means of 2D gel electrophoresis. Among the obtained protein patterns, 10 proteins with significantly decreased or increased levels in the $\Delta bphP$ mutant were identified and selected for protein identification by peptide mass fingerprinting using MALDI-TOF MS analysis (Fig. 3, Table 5). Interestingly, three proteins [PA4739 (OsmY), PA0059 (OsmC) and PA4880] were identified whose coding genes were also detected in our expression profiling experiments (Table 5, highlighted in bold type). Among them also was OsmY, the most downregulated gene in our expression profile data. Both OsmC and OsmY have recently also been identified in a proteome study and are clearly downregulated in a *P. aeruginosa oxyR* mutant (Vinckx *et al.*, 2011). Other identified proteins included PpiA (PA3227), a peptidyl-prolyl *cis-trans* isomerase A, which has been reported in a proteomic study of the QS regulon (Arevalo-Ferro *et al.*, 2003). PpiA is a QS-regulated protein with chaperone activity that catalyses the *cis-trans* isomerization of proline peptide bonds and thereby helps proteins to fold into their active conformation. This process has been reported to play a role in pathogenesis (Kromina *et al.*, 2008).

Overall, the observation that genes and their corresponding proteins were affected at both the transcriptional and the protein level supports the role of *PaBphP* in stationary phase and furthermore the coherence of the presented data. On the other hand, some of the identified proteins appear to be translated from an mRNA with a short half-life, which might explain why we did not observe them in our microarray studies.

DISCUSSION

Bacterial adaptation to environmental conditions is maintained by complex regulatory systems, which are adjusted to the current stimuli. Since the discovery of bacterial phytochromes in heterotrophic organisms, many biological functions of these putative photoreceptors have been discussed, including roles as bilin, light and/or oxygen sensors (Montgomery & Lagarias, 2002). Up to now, only a function for the phytochrome from *Deinococcus radiodurans*, *DrBphP*, in the light-dependent regulation of carotenoid biosynthesis has been postulated. In this case, the pigment protects the bacterium from UV radiation (Davis *et al.*, 1999). In contrast, the opportunistic pathogen *P. aeruginosa* does not produce any carotenoids, and therefore *PaBphP* must have another biological function.

Bacterial phytochromes are typical sensor histidine kinases of a two-component signalling system. The phytochrome operons of *D. radiodurans*, *Rhizobium leguminosarium* and *Synechocystis* sp. PCC6803 also encode a response regulator involved in the sensory cascade as the direct phospho-acceptor of the activated kinase (Bhoo *et al.*, 2001). This is in contrast to *P. aeruginosa*, in which the corresponding response regulator is unknown and not encoded within the phytochrome operon. Although *PaBphP* is still postulated

Fig. 3. 2D PAGE of whole-cell proteins from the *P. aeruginosa* WT strain (a) and the $\Delta bphP$ mutant (b) were grown to stationary phase in LB broth and whole-cell proteins were used for 2D separation. Spots in the gel images marked with arrows show significant changes in proteins synthesized at higher or lower amounts in the $\Delta bphP$ mutant strain and were further used for protein identification. The numbers correspond to the spot ID numbers given in Table 5. Two representative gel images from three replicate gels are shown. Proteins were stained with SYPRO Ruby.

to be part of a two-component regulatory system, the constituents of the phytochrome regulon are still unknown (Ventre *et al.*, 2004). This includes the nature of the signal and the response regulator as well as the overall function of

PaBphP. A first hint indicating the function of *PaBphP* was the RpoS-dependent stationary phase-induced expression of the phytochrome operon (Barkovits *et al.*, 2008). In the current study we show that the expression of *bphP* is

Table 5. Identified proteins with a decreased protein level in the $\Delta bphP$ mutant proteome

Spot ID*	PA no. and protein†	Product name†	Transcriptome‡
1	PA4739 OsmY	Conserved hypothetical protein	+
2	PA0059 OsmC	Osmotically inducible protein OsmC	+
3	PA3838	Probable ATP-binding component of ABC transporter	
4	PA2007 MaiA	Maleylacetoacetate isomerase	
5	PA4880	Probable bacterioferritin	+
6	PA3227 PpiA	Peptidyl-prolyl <i>cis-trans</i> isomerase A	
7	PA3139	Probable amino acid aminotransferase	
8	PA4745 NusA	N utilization substance protein A	
9	PA3001	Probable glyceraldehyde-3-phosphate dehydrogenase	
10	PA0447 GcdH	Glutaryl-CoA dehydrogenase	

*Corresponding spot ID for the selected and identified proteins. The same ID numbers on the 2D PAGE indicate the identified protein spot.

†PA number, protein and product name are annotated according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>).

‡‘+’ Indicates agreement with the transcriptome profile of the $\Delta bphP$ mutant.

additionally modulated by the QS regulator LasR, and that more than 50 % of *PaBphP*-promoted genes have been shown elsewhere to be activated by the QS system. The QS system of *P. aeruginosa* is involved in the regulation of over 300 genes and consists of three associated systems: the Las, the Rhl and the PQS systems (Williams & Cámara, 2009). The first two are well characterized and are composed of a respective transcriptional regulator (LasR/RhlR) and the cognate autoinducer *N*-(3-oxohexanoyl)-L-homoserine lactone/*N*-butanoyl-L-homoserine lactone, which are synthesized by LasI and RhlI, respectively. In addition it is well established that both systems are hierarchically organized, with LasR controlling the expression of *rhlI* and *rhlR* (Latifi *et al.*, 1996; Medina *et al.*, 2003; Pesci *et al.*, 1997). Therefore, it is not unexpected that many of the identified downregulated genes in Table 2 are regulated via both LasR and RhlR (for simplicity only designated QS in Table 2) (Schuster *et al.*, 2003).

Based on our results we propose that *bphP* is likely one of the few direct LasR targets in *P. aeruginosa*. We assume that regulation through LasR will increase the number of phytochrome sensor molecules in the stationary phase to integrate an unknown signal. It is conceivable that only expression of the sensor is modulated and not that of the chromophore-producing haem oxygenase. The haem oxygenase BphO is able to supply the apo-phytochrome with enough chromophore even when the demand becomes higher, since the rate-limiting step of this enzymic conversion is the release of the product (Wegele *et al.*, 2004). From a physiological point of view there is not the necessity to further upregulate *bphO* expression.

The present study suggests that the signal sensed by BphP is integrated into the QS system of *P. aeruginosa*. Loss of *PaBphP* initiated changes in the transcription levels of various genes, many of which have been reported to be under the control of LasR. However, many of them appear to be indirect LasR targets and might instead be directly regulated by a *PaBphP* downstream partner, such as BphR (Fig. 4). Others, such as *osmY*, contain a predicted LasR box in their promoter region and therefore might be directly and indirectly regulated by LasR. An additional level of regulation is introduced through the alternative sigma factor RpoS. RpoS directly regulates the expression of *bphOP* (Barkovits *et al.*, 2008) and itself is regulated by LasR (Latifi *et al.*, 1996). We propose that many of the genes identified in this study (such as *osmY*) which are also regulated by LasR and/or RpoS might be direct downstream targets of *PaBphP* and only indirectly regulated by the QS system and the alternative sigma factor. However, a more complex mode of regulation involving several transcriptional activators and an alternative sigma factor is also feasible (Ishihama, 2010).

Under the assumption that both components, BphO and BphP, are required to establish a functional *PaBphP*, as biochemically observed *in vitro* (Tasler *et al.*, 2005), only those genes significantly regulated in both mutant strains

Fig. 4. Working model of *PaBphP* function within the RpoS/LasR QS network of *P. aeruginosa*. RpoS and LasR directly regulate the expression of *bphP*. OsmY is a proposed direct target of the unknown response regulator BphR, with putative additional regulation through LasR. The observed RpoS dependence of *osmY* expression could also be indirectly through BphP. Solid lines indicate regulation for which experimental evidence exists.

were included in our further analyses. Although the loss of a functional phytochrome has a pleiotropic effect, it is evident that a number of genes encoding proteins involved in the synthesis of stress protectants are downregulated. We therefore propose that *PaBphP* senses an environmental signal and subsequently induces the transcription of these genes to protect the cell. Most of the identified genes have been described in the context of osmotic stress and QS; however, it is known from other bacteria such as *E. coli* that certain stressors induce a general stress response which enables the cell to cross-protect itself from other stresses (Hengge-Aronis, 2000). On the other hand it is also obvious that in contrast to a hyperosmotic stress response we did not observe the induction of transporters in any of our studies. Transporters are among the main target genes to be induced under osmotic stress conditions, enabling influx of K⁺ and glutamate in response to hyperosmotic conditions (Aspedon *et al.*, 2006). Our study suggests rather that the biosynthesis of cell-stabilizing substances or proteins is induced by *PaBphP*. Unfortunately, phenotypic analysis to prove this assumption did not result in any significant changes in stress tolerance.

Based on the light-responsive activity of recombinant *PaBphP* *in vitro*, one would still postulate light as the primary signal. However, so far we have no experimental evidence that this is the case *in vivo*, and other signals have been proposed to be sensed by bacterial phytochromes. One would be the presence or absence of the bilin chromophore, which could indirectly represent a certain environmental condition [i.e. the presence or absence of oxygen or iron due to the activity of haem oxygenases (Wegele *et al.*, 2004)]. In summary, *PaBphP* integrates not only a growth-phase effect (through RpoS) into the LasR QS network but also a so-far-unknown signal, which might

be light, oxygen or some other environmental condition. The identification of the signal and the response regulator is the subject of current research in our laboratory.

ACKNOWLEDGEMENTS

We thank Julia Konieczny for technical assistance. Thanks are also due to Dr Max Schobert (Braunschweig) for the helpful discussion and technical support, Dr Dirk Albrecht and Knut Büttner (Greifswald) for MALDI-TOF MS analysis, and Dr Julia Bandow and DECODON GmbH for the use of a prototype software package. We thank Dr Minou Nowrousian and Alexandra Müller for the help with qRT-PCR, and Dr Jessica Wiethaus for critical reading of the manuscript. This work was financially supported by a grant from the Deutsche Forschungsgemeinschaft to N.F.-D.

REFERENCES

- Arevalo-Ferro, C., Hentzer, M., Reil, G., Görg, A., Kjelleberg, S., Givskov, M., Riedel, K. & Eberl, L. (2003). Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol* 5, 1350–1369.
- Aspedon, A., Palmer, K. & Whiteley, M. (2006). Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. *J Bacteriol* 188, 2721–2725.
- Atichartpongkul, S., Loprasert, S., Vattanaviboon, P., Whangsuk, W., Helmann, J. D. & Mongkolsuk, S. (2001). Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. *Microbiology* 147, 1775–1782.
- Baldi, P. & Long, A. D. (2001). A Bayesian framework for the analysis of microarray expression data: regularized *t*-test and statistical inferences of gene changes. *Bioinformatics* 17, 509–519.
- Barkovits, K., Harms, A., Benkartek, C., Smart, J. L. & Frankenberg-Dinkel, N. (2008). Expression of the phytochrome operon in *Pseudomonas aeruginosa* is dependent on the alternative sigma factor RpoS. *FEMS Microbiol Lett* 280, 160–168.
- Beatson, S. A., Whitchurch, C. B., Semmler, A. B. & Mattick, J. S. (2002). Quorum sensing is not required for twitching motility in *Pseudomonas aeruginosa*. *J Bacteriol* 184, 3598–3604.
- Becher, A. & Schweizer, H. P. (2000). Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques* 29, 948–950, 952.
- Bhoo, S. H., Davis, S. J., Walker, J., Karniol, B. & Vierstra, R. D. (2001). Bacteriophytochromes are photochromic histidine kinases using a biliverdin chromophore. *Nature* 414, 776–779.
- Blumenstein, A., Vienken, K., Tasler, R., Purschwitz, J., Veith, D., Frankenberg-Dinkel, N. & Fischer, R. (2005). The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. *Curr Biol* 15, 1833–1838.
- Bolstad, B. M., Irizarry, R. A., Astrand, M. & Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19, 185–193.
- Camilli, A. & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science* 311, 1113–1116.
- Cao, H., Baldini, R. L. & Rahme, L. G. (2001). Common mechanisms for pathogens of plants and animals. *Annu Rev Phytopathol* 39, 259–284.
- Chen, M., Chory, J. & Fankhauser, C. (2004). Light signal transduction in higher plants. *Annu Rev Genet* 38, 87–117.
- Chory, J., Chatterjee, M., Cook, R. K., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A. & other authors (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proc Natl Acad Sci U S A* 93, 12066–12071.
- Conter, A., Gangneux, C., Suzanne, M. & Gutierrez, C. (2001). Survival of *Escherichia coli* during long-term starvation: effects of aeration, NaCl, and the *rpoS* and *osmC* gene products. *Res Microbiol* 152, 17–26.
- Davis, S. J., Vener, A. V. & Vierstra, R. D. (1999). Bacteriophytochromes: phytochrome-like photoreceptors from nonphotosynthetic eubacteria. *Science* 286, 2517–2520.
- de Kievit, T. R. & Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infect Immun* 68, 4839–4849.
- de Lorenzo, V. & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol* 235, 386–405.
- Dubern, J. F. & Diggle, S. P. (2008). Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol Biosyst* 4, 882–888.
- Dunn, N. W. & Holloway, B. W. (1971). Pleiotrophy of *p*-fluorophenylalanine-resistant and antibiotic hypersensitive mutants of *Pseudomonas aeruginosa*. *Genet Res* 18, 185–197.
- Eymann, C., Dreisbach, A., Albrecht, D., Bernhardt, J., Becher, D., Gentner, S., Tam, T., Büttner, K., Buurman, G. & other authors (2004). A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics* 4, 2849–2876.
- Firoved, A. M. & Deretic, V. (2003). Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* 185, 1071–1081.
- Froehlich, A. C., Noh, B., Vierstra, R. D., Loros, J. & Dunlap, J. C. (2005). Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*. *Eukaryot Cell* 4, 2140–2152.
- Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y. & other authors (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5, R80.
- Gilbert, K. B., Kim, T. H., Gupta, R., Greenberg, E. P. & Schuster, M. (2009). Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. *Mol Microbiol* 73, 1072–1085.
- Giraud, E., Fardoux, J., Fourier, N., Hannibal, L., Genty, B., Bouyer, P., Dreyfus, B. & Verméglio, A. (2002). Bacteriophytochrome controls photosystem synthesis in anoxygenic bacteria. *Nature* 417, 202–205.
- Giraud, E., Zappa, S., Vuillet, L., Adriano, J. M., Hannibal, L., Fardoux, J., Berthomieu, C., Bouyer, P., Pignol, D. & Verméglio, A. (2005). A new type of bacteriophytochrome acts in tandem with a classical bacteriophytochrome to control the antennae synthesis in *Rhodospseudomonas palustris*. *J Biol Chem* 280, 32389–32397.
- Gutierrez, C. & Devedjian, J. C. (1991). Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. *J Mol Biol* 220, 959–973.
- Gutierrez, C., Gordia, S. & Bonnassie, S. (1995). Characterization of the osmotically inducible gene *osmE* of *Escherichia coli* K-12. *Mol Microbiol* 16, 553–563.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166, 557–580.
- Hatfield, G. W., Hung, S. P. & Baldi, P. (2003). Differential analysis of DNA microarray gene expression data. *Mol Microbiol* 47, 871–877.
- Häussler, S. & Becker, T. (2008). The pseudomonas quinolone signal (PQS) balances life and death in *Pseudomonas aeruginosa* populations. *PLoS Pathog* 4, e1000166.

- He, J., Baldini, R. L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N. T., Lee, D., Urbach, J., Goodman, H. M. & Rahme, L. G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci U S A* **101**, 2530–2535.
- Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Responses*, pp. 161–178. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z. & other authors (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* **22**, 3803–3815.
- Hentzer, M., Eberl, L. & Givskov, M. (2005). Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. *Biofilms* **2**, 37–61.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Hübschmann, T., Yamamoto, H., Gieler, T., Murata, N. & Börner, T. (2005). Red and far-red light alter the transcript profile in the cyanobacterium *Synechocystis* sp. PCC 6803: impact of cyanobacterial phytochromes. *FEBS Lett* **579**, 1613–1618.
- Hung, S. P., Baldi, P. & Hatfield, G. W. (2002). Global gene expression profiling in *Escherichia coli* K12. The effects of leucine-responsive regulatory protein. *J Biol Chem* **277**, 40309–40323.
- Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B. & Speed, T. P. (2003a). Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* **31**, e15.
- Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U. & Speed, T. P. (2003b). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264.
- Ishihama, A. (2010). Prokaryotic genome regulation: multifactor promoters, multitarget regulators and hierarchic networks. *FEMS Microbiol Rev* **34**, 628–645.
- Kang, Y., Nguyen, D. T., Son, M. S. & Hoang, T. T. (2008). The *Pseudomonas aeruginosa* PsaA responds to long-chain fatty acid signals to regulate the *fadBA5* β -oxidation operon. *Microbiology* **154**, 1584–1598.
- Kojic, M., Jovcic, B., Vindigni, A., Odreman, F. & Venturi, V. (2005). Novel target genes of PsaA transcriptional regulator of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **246**, 175–181.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Kromina, K., Ignatov, A. & Abdeeva, I. (2008). Role of peptidyl-prolyl-*cis/trans*-isomerases in pathologic processes. *Biochemistry (Moscow): Supplemental Series A: Membrane and Cell Biology* **2**, 195–202.
- Lacour, S. & Landini, P. (2004). σ^S -dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of σ^S -dependent genes and identification of their promoter sequences. *J Bacteriol* **186**, 7186–7195.
- Lamanda, A., Zahn, A., Röder, D. & Langen, H. (2004). Improved Ruthenium II tris (bathophenanthroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. *Proteomics* **4**, 599–608.
- Lamparter, T. (2006). A computational approach to discovering the functions of bacterial phytochromes by analysis of homolog distributions. *BMC Bioinformatics* **7**, 141.
- Lamparter, T., Michael, N., Mittmann, F. & Esteban, B. (2002). Phytochrome from *Agrobacterium tumefaciens* has unusual spectral properties and reveals an N-terminal chromophore attachment site. *Proc Natl Acad Sci U S A* **99**, 11628–11633.
- Latifi, A., Foglino, M., Tanaka, K., Williams, P. & Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* **21**, 1137–1146.
- Li, L. & Lagarias, J. C. (1992). Phytochrome assembly. Defining chromophore structural requirements for covalent attachment and photoreversibility. *J Biol Chem* **267**, 19204–19210.
- Liechty, A., Chen, J. & Jain, M. K. (2000). Origin of antibacterial stasis by polymyxin B in *Escherichia coli*. *Biochim Biophys Acta* **1463**, 55–64.
- Medina, G., Juárez, K., Díaz, R. & Soberón-Chávez, G. (2003). Transcriptional regulation of *Pseudomonas aeruginosa* *rhlR*, encoding a quorum-sensing regulatory protein. *Microbiology* **149**, 3073–3081.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Montgomery, B. L. & Lagarias, J. C. (2002). Phytochrome ancestry: sensors of bilins and light. *Trends Plant Sci* **7**, 357–366.
- Münch, R., Hiller, K., Barg, H., Heldt, D., Linz, S., Wingender, E. & Jahn, D. (2003). PRODORIC: prokaryotic database of gene regulation. *Nucleic Acids Res* **31**, 266–269.
- Oberpichler, I., Molina, I., Neubauer, O. & Lamparter, T. (2006). Phytochromes from *Agrobacterium tumefaciens*: difference spectroscopy with extracts of wild type and knockout mutants. *FEBS Lett* **580**, 437–442.
- Oh, J.-T., Cajal, Y., Skowronska, E. M., Belkin, S., Chen, J., Van Dyk, T. K., Sasser, M. & Jain, M. K. (2000). Cationic peptide antimicrobials induce selective transcription of *micF* and *osmY* in *Escherichia coli*. *Biochim Biophys Acta* **1463**, 43–54.
- Pesci, E. C., Pearson, J. P., Seed, P. C. & Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3127–3132.
- Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229–11234.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.
- Prithiviraj, B., Weir, T., Bais, H. P., Schweizer, H. P. & Vivanco, J. M. (2005). Plant models for animal pathogenesis. *Cell Microbiol* **7**, 315–324.
- Ramakers, C., Ruijter, J. M., Deprez, R. H. & Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**, 62–66.
- Ratliff, M., Zhu, W., Deshmukh, R., Wilks, A. & Stojilkovic, I. (2001). Homologues of neisserial heme oxygenase in Gram-negative bacteria: degradation of heme by the product of the *pigA* gene of *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6394–6403.
- Rockwell, N. C., Su, Y. S. & Lagarias, J. C. (2006). Phytochrome structure and signaling mechanisms. *Annu Rev Plant Biol* **57**, 837–858.
- Rodrigue, A., Quentin, Y., Lazdunski, A., Méjean, V. & Foglino, M. (2000). Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8**, 498–504.
- Schuster, M. & Greenberg, E. P. (2007). Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics* **8**, 287.
- Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa*

quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066–2079.

Schuster, M., Hawkins, A. C., Harwood, C. S. & Greenberg, E. P. (2004). The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* **51**, 973–985.

Smith, H. (2000). Phytochromes and light signal perception by plants – an emerging synthesis. *Nature* **407**, 585–591.

Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E. & Ohman, D. E. (1999). Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 3890–3897.

Tasler, R., Moises, T. & Frankenberg-Dinkel, N. (2005). Biochemical and spectroscopic characterization of the bacterial phytochrome of *Pseudomonas aeruginosa*. *FEBS J* **272**, 1927–1936.

Trunk, K., Benkert, B., Quäck, N., Münch, R., Scheer, M., Garbe, J., Jänsch, L., Trost, M., Wehland, J. & other authors (2010). Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ Microbiol* **12**, 1719–1733.

Ventre, I., Filloux, A. & Lazdunski, A. (2004). Two-component signal transduction systems: A key to the adaptive potential of *Pseudomonas aeruginosa*. In *Pseudomonas*, vol. 2, *Virulence and Gene Regulation*, pp. 257–288. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.

Vinckx, T., Wei, Q., Matthijs, S., Noben, J. P., Daniels, R. & Cornelis, P. (2011). A proteome analysis of the response of a *Pseudomonas aeruginosa* *oxyR* mutant to iron limitation. *Biometals*. doi:10.1007/s10534-010-9403-4.

Wagner, V. E. & Iglewski, B. H. (2008). *P. aeruginosa* biofilms in CF infection. *Clin Rev Allergy Immunol* **35**, 124–134.

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I. & Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**, 2080–2095.

Waite, R. D., Paccanaro, A., Papakonstantinou, A., Hurst, J. M., Saqi, M., Littler, E. & Curtis, M. A. (2006). Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. *BMC Genomics* **7**, 162.

Wegele, R., Tasler, R., Zeng, Y., Rivera, M. & Frankenberg-Dinkel, N. (2004). The heme oxygenase(s)-phytochrome system of *Pseudomonas aeruginosa*. *J Biol Chem* **279**, 45791–45802.

Whiteley, M., Parsek, M. R. & Greenberg, E. P. (2000). Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 4356–4360.

Williams, P. & Cámara, M. (2009). Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* **12**, 182–191.

Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* **63**, 230–262.

Yeats, C. & Bateman, A. (2003). The BON domain: a putative membrane-binding domain. *Trends Biochem Sci* **28**, 352–355.

Yim, H. H. & Villarejo, M. (1992). *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. *J Bacteriol* **174**, 3637–3644.

Edited by: P. Cornelis