

# Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*

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Pathogenesis of *Pseudomonas aeruginosa* is controlled to a major extent by the two quorum-sensing systems *las* and *rhl*. The previously uncharacterized gene *PA2591* was identified as a major virulence regulator, *vqsR*, in the quorum-sensing hierarchy. *vqsR* is a member of the LuxR family and possesses a *las* box in its upstream region. Transposon inactivation of *vqsR* abrogated the production of *N*-acylhomoserine lactones and the secretion of exoproducts and diminished bacterial virulence for *Caenorhabditis elegans*. Cytotoxicity towards macrophages was not affected. *vqsR* mRNA was expressed more strongly in the presence of human serum and oxidative stress than under standard growth conditions. High-density oligonucleotide microarrays were used to compare the global expression profile of a wild-type strain and a *vqsR* mutant. One-hundred-and-fifty-one and 113 genes were significantly differentially expressed in the presence of H<sub>2</sub>O<sub>2</sub> and human serum, respectively. The disruption of *vqsR* repressed the expression of genes that are known to be promoted by quorum sensing and activated the expression of genes that are known to be repressed by quorum sensing. Moreover, the *vqsR* mutant harboured less mRNA transcript for the production of siderophores and membrane-bound elements of antibiotic resistance. The protein encoded by *PA2591* regulates several traits of pathogenicity; hence, the name *vqsR* ('virulence and quorum-sensing regulator') was assigned to *PA2591*.

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## INTRODUCTION

The opportunistic human pathogen *Pseudomonas aeruginosa* is the leading source of Gram-negative nosocomial infections (Van Delden & Iglewski, 1998) and causes chronic lung infections in individuals suffering from cystic fibrosis (Tümmler & Kiewitz, 1999). The analysis of the fully sequenced *P. aeruginosa* genome revealed that more than 9% of the assigned open reading frames (ORFs) encode known or putative transcriptional regulators and two-component systems (Stover *et al.*, 2000). It is thought that the large amount of regulatory factors allows the organism to adapt to various environments and thus represents

the key for the understanding of its enormous metabolic versatility.

Evidence has accumulated over the past few years that for the expression of pathogenic traits a quorum-sensing circuitry that is operating in *P. aeruginosa* is of central importance (for recent reviews, see de Kievit & Iglewski, 2000; Williams *et al.*, 2000; Camara *et al.*, 2002). This form of gene regulation ensures that virulence factors are expressed in a population-density-dependent manner. Two quorum-sensing systems that rely on diffusible *N*-acylhomoserine lactone (AHL) signal molecules have been identified in *P. aeruginosa* and were shown to orchestrate expression of virulence factors and participate in the development of biofilms: the *las* system consisting of the transcriptional activator LasR and the AHL synthase LasI which directs the synthesis of *N*-3-oxo-dodecanoyl-homoserine lactone

Abbreviations: AHL, *N*-acylhomoserine lactone; C<sub>4</sub>-HSL, *N*-butanoyl-homoserine lactone; 3-oxo-C<sub>12</sub>-HSL, *N*-3-oxo-dodecanoyl-homoserine lactone.

(3-oxo-C<sub>12</sub>-HSL), and the *rhl* system consisting of RhlR and RhlI which directs the synthesis of *N*-butanoyl-homoserine lactone (C<sub>4</sub>-HSL). The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two quorum-sensing systems of *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signalling cascade (Latifi *et al.*, 1996; Pesci *et al.*, 1997). This quorum-sensing cascade controls the expression of a battery of extracellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanine, hydrogen cyanide, pyoverdine) and toxins (exotoxin A) (Passador *et al.*, 1993; Winson *et al.*, 1995), as well as the development of biofilms (Davies *et al.*, 1998; Yoon *et al.*, 2002; Hentzer *et al.*, 2003a). The importance of quorum sensing for the pathogenicity of *P. aeruginosa* has been demonstrated in a number of animal models including the neonatal mouse model of pneumonia (Tang *et al.*, 1996), the burned mouse model (Rumbaugh *et al.*, 1999), the mouse agar bead model (Wu *et al.*, 2000) and a *Caenorhabditis elegans* model (Tan *et al.*, 1999). In all these infection models mutants defective in quorum sensing were substantially less pathogenic than their parental strains.

An additional signal molecule, the *P. aeruginosa* quinolone signal (PQS), adds a further level of complexity to the quorum-sensing circuitry, as it provides a link between the *las* and *rhl* systems (Pesci *et al.*, 1999) by modulating expression of *rhlR* and *lasR* (McKnight *et al.*, 2000; Diggle *et al.*, 2003). Furthermore, the quorum-sensing circuitry is subject to regulation by a number of global regulators, including Vfr (Albus *et al.*, 1997), GacA (Reimann *et al.*, 1997), RsaL (de Kievit *et al.*, 1999), MvaT (Diggle *et al.*, 2002), RsmA (Pessi *et al.*, 2001) and DksA (Jude *et al.*, 2003).

In addition to the two AHL signal receptors LasR and RhlR, the *P. aeruginosa* genome encodes a third LuxR homologue, QscR, which has been shown to modulate the timing of quorum-sensing-controlled gene expression by repressing transcription of *lasI* (Chugani *et al.*, 2001).

Here we report on the characterization of a fourth LuxR homologue, which we designated VqsR ('virulence and quorum-sensing regulator'). It is shown that inactivation of the *vqsR* gene abrogates the production of AHL signal molecules, decreases the production of virulence factors and reduces the pathogenicity of *P. aeruginosa* in a nematode infection model system. A transcriptome analysis employing the Affymetrix GeneChip technology provided clear evidence that VqsR is involved in the regulation of the expression of certain sets of virulence and quorum-sensing-controlled genes in *P. aeruginosa*.

## METHODS

**Bacterial strains, plasmids and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* and *Escherichia coli* were in most cases cultivated at 37 °C in Luria broth (LB) medium. Strains carrying pME6010 or pME6010*vqsR* were grown in LB medium containing 50 µg tetracycline ml<sup>-1</sup>. To examine the effect of oxidative stress on gene regulation, 4 ml of stationary-phase culture (LB, OD<sub>600</sub> 5.0) with 1 ml of 5× LB were placed in a dialysis bag (14 kDa) and incubated at 200 r.p.m. on a rotary shaker for 2 h in 600 ml of LB containing 10 mM H<sub>2</sub>O<sub>2</sub> at 37 °C. To examine the effect of blood serum on gene regulation, the pellet from 2 ml of stationary-phase culture was resuspended in 2 ml of RPMI 1640 medium (GibcoBRL), placed into a dialysis bag (300 kDa) and incubated in RPMI 1640 medium containing 10% (v/v) human AB blood serum, at 200 r.p.m. on a rotary shaker for 2 h at 37 °C.

**Table 1.** Bacterial strains and plasmids used in this work

Strains and plasmids	Characteristics	Reference
<b>Strains</b>		
<i>Pseudomonas aeruginosa</i>		
TB	Wild-type, cystic fibrosis airways isolate	Tümmeler <i>et al.</i> (1991)
TB <i>vqsR</i>	Tn5::PA2591 ( <i>vqsR</i> ) mutant of TB	Wiehmann <i>et al.</i> (2002)
TB <i>vqsR</i> (pME6010 <i>vqsR</i> )	Tn5::PA2591 ( <i>vqsR</i> ) mutant of TB complemented with pME6010 <i>vqsR</i>	This study
<i>Pseudomonas putida</i>		
F117	AHL-negative derivative of <i>P. putida</i> IsoF, <i>ppuI</i> <sup>-</sup>	Steidle <i>et al.</i> (2001)
<i>Escherichia coli</i>		
MT102	<i>araD139 (ara-leu)7697 lac thi hsdR</i>	Leo Eberl, lab. collection
JM105	F' <i>traD36 lacI<sup>q</sup> (lacZ)M15 proA<sup>+</sup> B<sup>+</sup> /thi rpsL(Str<sup>r</sup>) endA sbcB15 sbcC hsdR4(r<sub>K</sub> m<sub>K</sub>) (lac-proAB)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>Chromobacterium violaceum</i>		
CV026	AHL-detector strain	McClellan <i>et al.</i> (1997)
<b>Plasmids</b>		
pJBA89	pUC18Not- <i>luxR</i> -P <sub>luxI</sub> -RBSII- <i>gfp</i> (ASV) - T <sub>0</sub> -T <sub>1</sub> ; Ap <sup>r</sup>	Andersen <i>et al.</i> (2001)
pKR-C12	pBBR1MCS-5 carrying P <sub>lasB</sub> - <i>gfp</i> (ASV) P <sub>lac</sub> - <i>lasR</i> ; Gm <sup>r</sup>	Steidle <i>et al.</i> (2001)
pME6010	Shuttle vector replicable in Gram-negative bacteria; Tc <sup>r</sup>	Heeb <i>et al.</i> (2000)
pME6010 <i>vqsR</i>	pME6010 carrying the PA2591 gene	This study
pSB403	Broad-host-range AHL monitor plasmid; Tc <sup>r</sup>	Winson <i>et al.</i> (1998)

**Generation and complementation of the *P. aeruginosa* *vqsR* mutant.** The *P. aeruginosa* *vqsR* mutant was generated by Tn5 transposon mutagenesis as described previously (Wiehlmann *et al.*, 2002). Plasmid pME6010*vqsR*, used for complementation *in trans*, was constructed by cloning the 1254 bp of the PA2591 (*vqsR*) gene generated by PCR with primers 5'-CTTGAACAAGCTTTTCGTCCT-GCGCGTA-3' and 5'-GATTATAGATCTGTGGATATCGCATTGC-AC-3' into the BglIII/EcoRI-restricted shuttle vector pME6010. The plasmid was introduced into *E. coli* OneShotTOP10 chemically competent cells (Invitrogen) by transformation and subsequently into the *P. aeruginosa* TB *vqsR* mutant by electroporation. The amount of exopolysaccharides which may inhibit electroporation was minimized by multiple washings of the bacteria with distilled water.

**RNA isolation.** Cells were harvested from dialysis bags by centrifugation at 3800 g for 2 min at 4 °C. Total RNA was extracted using a modified hot phenol method (Tao *et al.*, 1999). The bacteria were resuspended in water and lysed at 65 °C in a mixture of 5 ml phenol (pH 5.5)/2.5 ml 2% SDS (w/v)/30 mM sodium acetate/3 mM EDTA by vigorous shaking for 10 min. The supernatant was purified by subsequent phenol/chloroform and chloroform extractions. Nucleic acids were precipitated overnight at -20 °C with ethanol and washed with 70% ethanol. DNA was digested by the addition of 40 U DNase I (Roche) and 20 U SUPERaseIn (Ambion) in DNase I buffer (50 mM sodium acetate, 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, pH 6.5) in a total volume of 200 µl for 30 min at 37 °C. RNA purification with RNeasy columns (Qiagen) was followed by quantification of its total yield by UV absorption spectrometry. RNA integrity was monitored by 1.2% agarose gel electrophoresis with addition of 2% (v/v) formaldehyde as denaturing agent.

**Northern blot analysis.** The denatured RNA was separated by formaldehyde/agarose gel electrophoresis and transferred to a positively charged nylon membrane (Hybond-N<sup>+</sup>) using 20× SSC buffer for 20–24 h. The dried and cross-linked membrane was pre-hybridized with 10 ml hybridization buffer (50% formamide, 5× Denhardt's solution, 5× SSC, 1% SDS, 100 µg herring sperm DNA ml<sup>-1</sup>) for 2 h at 42 °C and afterwards hybridized with 10 ml of hybridization buffer containing denatured DIG-labelled probe (615 bp of amplified *vqsR* sequence) for 16–24 h at 42 °C in a hybridization oven. Non-specifically bound probe was removed by several washing steps with 6× SSC+3% SDS/2× SSC+3% SDS/0.2× SSC+3% SDS and hybridization signals were detected by washing of the membrane with anti-DIG-alkaline phosphatase (Roche) and CDP Star as described previously (Allefs *et al.*, 1990).

**cDNA generation, labelling and GeneChip hybridization.** RNA from three independent isolations was pooled to minimize the influence of handling. cDNA synthesis, fragmentation and labelling with Biotin-ddUTP were performed as described in the protocol provided by Affymetrix. Briefly, 10 µg of RNA was mixed together with random primers (Invitrogen) and 10 non-pseudomonal control transcripts (kindly provided by S. Lory and co-workers, Harvard) and incubated for 10 min at 70 °C and 10 min at 25 °C. The cDNA mixture [5× first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 25 U SuperScript II µl<sup>-1</sup> (Invitrogen), 0.5 U SUPERaseIn µl<sup>-1</sup> (Ambion)] was added to the reaction mixture and incubated for 10 min at 25 °C, 60 min at 37 °C, 60 min at 42 °C and 10 min at 70 °C. RNA was hydrolysed by the addition of 1 M NaOH and incubation for 30 min at 65 °C, followed by neutralization of the reaction by the addition of 1 M HCl. cDNA was purified with a Qiaquick column (Qiagen) and quantified by UV absorption at 260 nm. The 3–5 µg of cDNA was fragmented with 0.5 U DNase I (µg cDNA)<sup>-1</sup> in One Phor-All buffer (Amersham Pharmacia Biotech) for 10 min at 37 °C and the reaction was stopped by 10 min incubation at 98 °C. Gel electrophoresis and staining with 2% SYBRGreen (Molecular Probes) were performed to ensure that the majority of cDNA fragments were within the required 50–200 bp range. The fragmented

cDNA was biotinylated with the Enzo BioArray Terminal Labelling Kit (Affymetrix) and hybridized to GeneChips in an Affymetrix hybridization oven. The Affymetrix fluidics station was used for washing and staining of GeneChips with streptavidin, antistreptavidin, streptavidin/phycoerythrin solutions. Hybridization signal intensities were scanned using an HP GeneArray scanner.

**GeneChip analysis.** Data analysis was performed with the AFFYMETRIX MICROARRAY SUITE SOFTWARE 5.0 with Affymetrix default parameters. The average microarray hybridization signal intensity was scaled to 150. Experiments were carried out in duplicate; thus, a total of two GeneChips per strain and growth condition were scanned at 570 nm with 3 µm resolution by the Affymetrix scanner. Two GeneChips for each mutant and wild-type were compared by the four-comparison survival method (Chen *et al.*, 2000). The data were imported into the Microsoft ACCESS database and selected by Wilcoxon rank test for genes with significant changes in their expression and at least twofold differential regulation. The arithmetic mean and standard deviation were calculated. Finally, a Bonferroni correction for multiple testing was applied as a rigorous criterion for significantly changed signal intensities. Significantly ( $P < 0.05$ ) differentially expressed ORFs were classified using latest internet annotation from the web site of the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>).

**Detection and characterization of AHLs.** As a simple assay for the production of AHLs, cross-streak experiments using GFP-based AHL biosensors were performed. Two biosensors, which, depending on the components used for construction, respond to different types of AHL molecules, were employed in this study: *Pseudomonas putida* F117(pKR-C12), which is most sensitive for 3-oxo-C<sub>12</sub>-HSL (Steidle *et al.*, 2001), and *E. coli* JM105(pJBA89), which is most sensitive for 3-oxo-C<sub>6</sub>-HSL but detects a broad range of different AHLs (Andersen *et al.*, 2001). The monitor strain and the respective test strains were streaked onto the plates to form a 'T'. Following incubation at 30 °C for 24 h, the plates were illuminated with blue light using an HQ 480/40 filter (AHF-Analysentechnik) in combination with a halogen lamp (Volpi) as a light source. Visualization of GFP fluorescence was accomplished in a dark box equipped with a light-sensitive camera (Hamamatsu Photonics) and an HQ 535/20 filter (AHF-Analysentechnik). For more detailed analysis, the AHL molecules were extracted from spent culture supernatants of the strains, separated by TLC and visualized by overlaying the TLC plates with soft agar seeded with the *luxAB*-based AHL biosensor *E. coli* MT102(pSB403) (Winson *et al.*, 1998) or biosensor *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) as described previously (Geisenberger *et al.*, 2000; Shaw *et al.*, 1997). Bioluminescent spots indicating AHL were detected by exposure to an X-ray film. On the basis of their mobilities ( $R_F$  values) and by including appropriate reference compounds, a tentative identification of AHLs present in the culture extracts was possible.

**Protease and pyocyanine secretion.** In order to investigate their pyocyanine secretion ability, tested bacterial strains were grown in King's A medium, pelleted by centrifugation and the amount of pyocyanine in the supernatant was quantified by measuring the  $A_{695}$  value (King *et al.*, 1954). Protease production was examined by growing the analysed *P. aeruginosa* strains on M9 agar plates supplemented with 0.7% casein (Cowell *et al.*, 2003). Elastase production was tested using the Elastin Congo red assay (Rust *et al.*, 1994).

**Macrophage cytotoxicity assay.** THP-1 monocytes were grown and maintained in ReadyMix RPMI 1640 medium with 10% FCS (PAA Laboratories) and 0.1% antimicrobial/antimycotic solution (Gibco-BRL) and differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA; Calbiochem). Wells of a 96-well plate were seeded with  $3.5 \times 10^5$  cells per well and incubated with 16 nM PMA for 72 h. Prior to use, cells were washed twice with RPMI

1640 medium without FCS and antibiotic/antimycotic solution (PAA Laboratories), and *P. aeruginosa* strains were added to the cells at an m.o.i. of 30 and subsequently incubated in HEPES-buffered RPMI 1640 medium (PAA Laboratories) at 37 °C for 90 min. Two-and-a-half micrograms of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 20 µl RPMI 1640 medium was added to each well and the plate was incubated for another 30 min at 37 °C. The supernatant was removed by suction and the formazan solubilized in 20 µl DMSO and mixed with 80 µl H<sub>2</sub>O. The UV absorption of the mixture was measured at A<sub>572</sub>.

**Nematode killing assays.** All *Caenorhabditis elegans* strains were maintained under standard culturing conditions on nematode growth medium agar with *E. coli* OP50 as a food source (Stiernagle, 1999). Synchronized cultures and staged animals were also obtained as described by Stiernagle (1999). Bristol N2 (wild-type) was used throughout this study and provided by the *Caenorhabditis* Genetics Center (University of Minnesota, St Pauls, MN, USA). *C. elegans* killing experiments were performed in a liquid-medium-based system using 24-well plates (D. Jordan, L. Wiehlmann, I. Ziegler, B. Tümmler, I. Attree & I. Steinmetz, unpublished results). Nematodes were exposed to a suspension (OD<sub>650</sub> 0.5) of bacterial cells for each strain tested.

**Sequence analysis.** Codon usage and the codon adaptation index were calculated as described previously (Kiewitz *et al.*, 2002). The bendability/curvature propensity plot was calculated with the BEND.IT server ([http://hydra.icgeb.trieste.it/~kristian/dna/bend\\_it.html](http://hydra.icgeb.trieste.it/~kristian/dna/bend_it.html)), using DNase I-based bendability parameters and consensus bendability scale as described previously (Brukner *et al.*, 1995).

## RESULTS AND DISCUSSION

### Features of the *vqsR* gene

The sequence of *vqsR* corresponds to the ORF PA2591 of the *P. aeruginosa* PAO1 genome (<http://www.pseudomonas.com>). The G+C content of this gene (58.33 mol%) is lower than the average G+C content (66.6 mol%) of the *P. aeruginosa* genome (Stover *et al.*, 2000), but its codon adaptation index (0.688) calculated from codon usage is similar to that typically found for *P. aeruginosa* transcriptional regulators (Kiewitz *et al.*, 2002). This suggests that despite its lower G+C content, *vqsR* was not acquired by phylogenetically recent horizontal transfer, but is rather a part of the core *P. aeruginosa* genome.

*vqsR* possesses a *las* box (5'-AACTACCAGTTCTGGTAG-GT-3') in its -157 to -138 upstream region (Wagner *et al.*, 2003) which exhibits homology with the palindromic *lux* box DNA elements identified in *Vibrio fischeri*. *las* boxes are usually located upstream of LuxR-regulated genes and serve as binding sites for regulatory protein–autoinducer complexes. A bendability/curvature propensity plot revealed a low curvature DNA sequence in the upstream region of *vqsR* (data not shown). Hence, binding of a protein–autoinducer complex could promote more extensive bending of the upstream sequence leading to the transcriptional activation of this gene. Moreover, *vqsR* harbours a DNA-binding domain in its carboxy terminus that is typical for the LuxR group of transcriptional regulators (Fuqua *et al.*, 1996). These *in silico* findings suggest that the

*vqsR* gene plays an important role in the quorum-sensing cascade of *P. aeruginosa*.

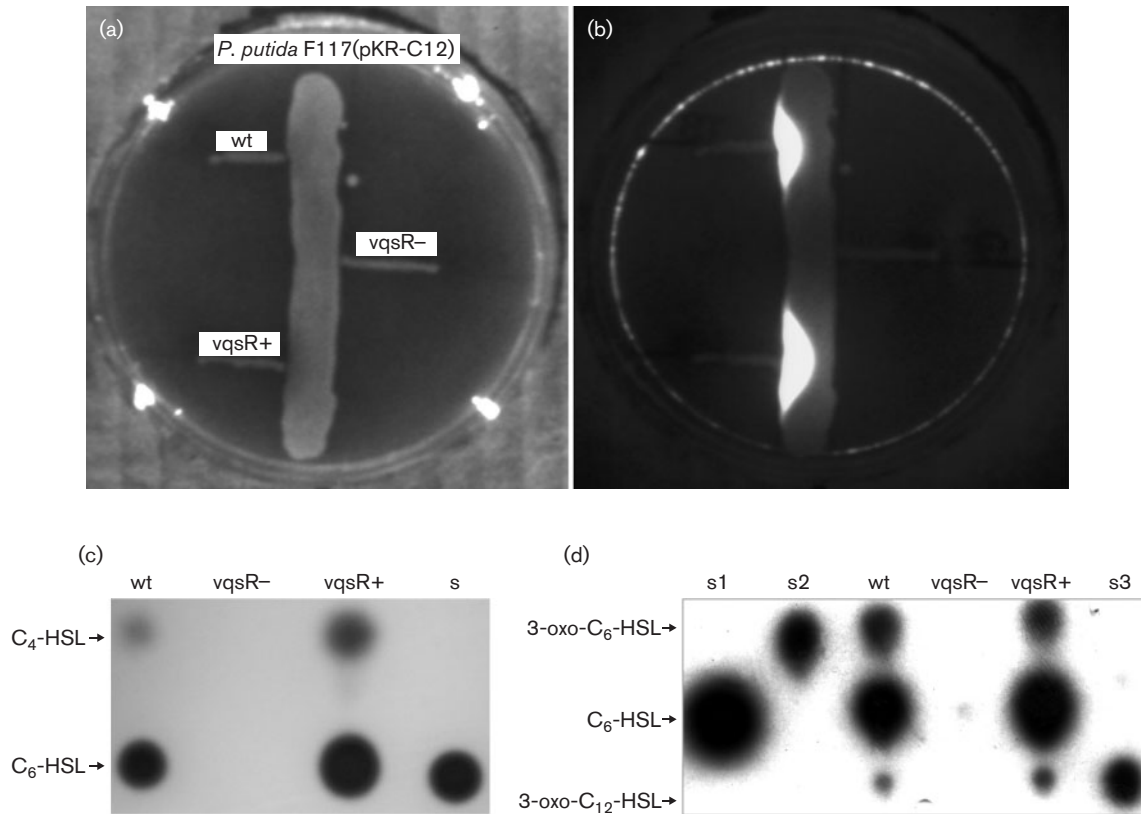
### VqsR is essential for the production of autoinducer molecules

Quorum sensing in *P. aeruginosa* employs AHLs as auto-inducer signalling molecules (Pearson *et al.*, 1994). In order to verify the effect of *vqsR* on AHL synthesis, we streaked the *P. aeruginosa* TB wild-type strain and its Tn5::*vqsR* transposon mutant close to the GFP-based AHL sensor *E. coli* JM105(pJBA89). No AHL production was observed with the *P. aeruginosa* Tn5::*vqsR* transposon mutant (data not shown). *P. aeruginosa* possesses two different quorum-sensing acylhomoserine lactones: 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL, which are the major components of the *las* and *rhl* systems, respectively (Wagner *et al.*, 2003). Cross-streak experiments were also performed with the biosensor *P. putida* F117(pKR-C12), which only detects a narrow range of long-chain AHLs, being most sensitive for 3-oxo-C<sub>12</sub>-HSL. As with *E. coli* JM105(pJBA89), the *vqsR* mutant did not provoke a positive signal (Fig. 1a, b), supporting the view that VqsR plays a crucial role in the *P. aeruginosa* quorum-sensing cascade. Complementation of Tn5::*vqsR* *in trans* was performed in order to verify that the observed phenotype of the mutant was caused by the transposon mutation and not by any other secondary genetic event. For this purpose, the gene was cloned in the shuttle plasmid pME6010. This plasmid, based on the minimal *pVS1* replicon, has been demonstrated to express proteins in both *E. coli* and *P. aeruginosa* (Heeb *et al.*, 2000). pME6010 was transformed into *E. coli* and subsequently introduced into the *P. aeruginosa* *vqsR* mutant via electroporation. Complementation *in trans* restored the AHL molecules secretion ability of the *vqsR* mutant to levels comparable to the wild-type (Fig. 1a, b). For more detailed analysis, TLC was performed. Using this sensitive technique, the *vqsR* mutant was confirmed to be impaired in the production of AHLs, including C<sub>4</sub>-HSL (Fig. 1c) and 3-oxo-C<sub>12</sub>-HSL (Fig. 1d). Hence, our experiments revealed the important role of VqsR in the initial steps of the quorum-sensing cascade: in the production and secretion of quorum-sensing autoinducers.

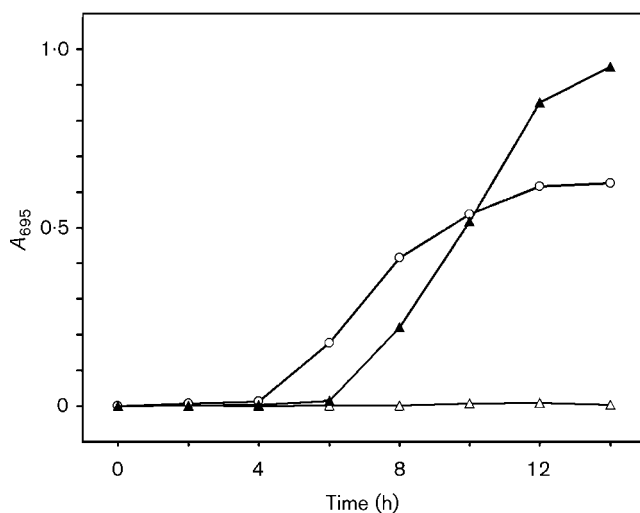
### VqsR affects protease and pyocyanine synthesis

Quorum sensing modulates the expression of a broad spectrum of virulence genes in *P. aeruginosa* (Passador *et al.*, 1993). To analyse the effect of the *vqsR* mutation on the production of bacterial virulence factors, we investigated its impact on protease and pyocyanine synthesis. Measuring the amount of pyocyanine in the supernatants of the tested bacterial strains revealed a dramatic decrease of pyocyanine secretion in the *vqsR* mutant when compared to the wild-type and the complemented mutant (Fig. 2).

The halo on casein agar plates, indicating proteolytic activity mainly due to LasB, alkaline protease and proteinase IV (Cowell *et al.*, 2003), was observed for the TB wild-type,

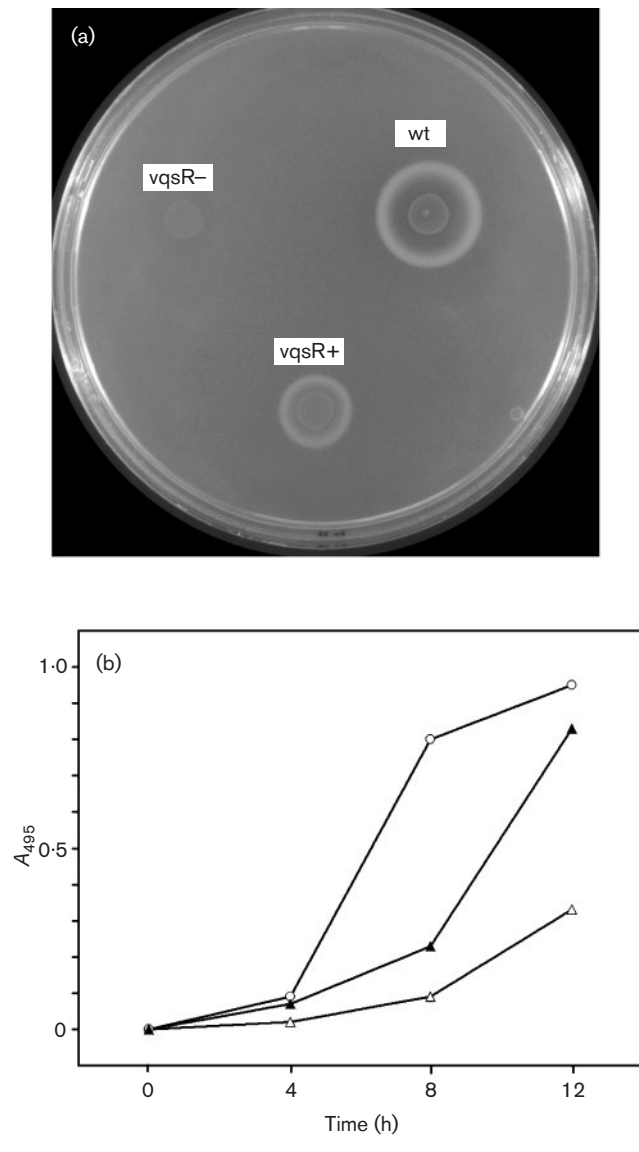


**Fig. 1.** Secretion of AHLs by the *P. aeruginosa* TB wild-type (wt) strain, its Tn5::vqsR transposon mutant (vqsR<sup>-</sup>) and the complemented mutant (vqsR<sup>+</sup>). (a) Tested *P. aeruginosa* strains were cultivated with *P. putida* F117 harbouring plasmid pKR-C12, which contains a translational fusion of the *lasB* promoter to *gfp* and the *lasR* gene under control of a *lac*-type promoter, and illuminated with normal light. (b) Tested strains illuminated with blue light exciting green fluorescent protein. Notice the full restoration of the ability to produce autoinducer molecules in the Tn5::vqsR complemented mutant (vqsR<sup>+</sup>). (c) TLC analysis of AHLs secreted by tested *P. aeruginosa* strains using the AHL biosensor *Chromobacterium violaceum* CV026 which is able to detect C<sub>4</sub>-HSL. Note the significantly diminished ability to secrete AHLs, including C<sub>4</sub>-HSL (upper spot) in the vqsR mutant (vqsR<sup>-</sup>). (d) TLC analysis of AHLs secreted by tested *P. aeruginosa* strains using AHL biosensor *E. coli* MT102(pSB403) which is able to detect 3-oxo-C<sub>12</sub>-HSL. Notice the remarkably diminished ability to secrete AHLs, including 3-oxo-C<sub>12</sub>-HSL, in the vqsR mutant (vqsR<sup>-</sup>). In (c, d), lanes with AHL standards are indicated by s.



but not for its vqsR mutant (Fig. 3a). Proteolytic activity of the vqsR mutant was not inducible even after exogenous addition of 0.3 μM 3-oxo-C<sub>12</sub>-HSL and 0.4 μM C<sub>4</sub>-HSL. Using the same concentrations, the extracellular proteolytic activity of a *lasI rhII* PAO1 double mutant was restored to the level of the wild-type (data not shown). These data indicate that, in contrast to the inactivation of *lasI* and *rhII*, the lack of AHL synthesis does not completely explain the phenotype of protease deficiency in the vqsR mutant.

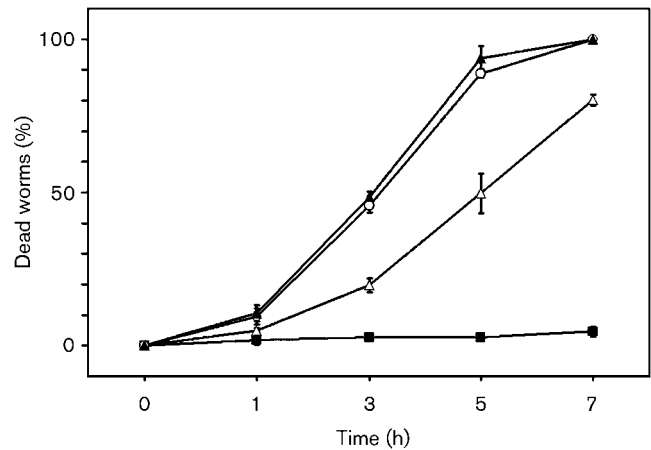
**Fig. 2.** Kinetics of the secretion of pyocyanine by the *P. aeruginosa* TB wild-type (wt, ○) strain, its Tn5::vqsR transposon mutant (vqsR<sup>-</sup>, △) and the complemented mutant (vqsR<sup>+</sup>, ▲). Strains were cultivated in King's A medium and the amount of pyocyanine in the supernatant was quantified by measuring absorbance at 695 nm (A<sub>695</sub>).



**Fig. 3.** Secretion of proteases by the *P. aeruginosa* TB wild-type (wt) strain, its Tn5::vqsR transposon mutant (vqsR-) and the complemented mutant (vqsR+). (a) No proteolytic activity of the mutant strain was detected on casein agar. (b) The amount of elastase secreted by the mutant was reduced when compared to the wild-type strain. ○, wt; △, vqsR-; ▲, vqsR+.

Using the Elastin Congo red assay, the vqsR mutant was found to be impaired in the ability to produce elastase which is known to be LasR-3-oxo-C<sub>12</sub>-HSL-dependent (Fig. 3b). In the complemented mutant, protease as well as pyocyanine secretion ability were restored according to our expectations. Thus, VqsR was proven to be crucial for protease and pyocyanine secretion in *P. aeruginosa*.

However, when we examined the cytotoxicity of *P. aeruginosa* towards human macrophages, no difference was seen among the wild-type, the vqsR mutant and the complemented strain, indicating that under the tested



**Fig. 4.** Kinetics of the killing of *Caenorhabditis elegans* by the *P. aeruginosa* TB wild-type (wt) strain, its Tn5::vqsR transposon mutant (vqsR-) and the complemented mutant (vqsR+). Forty-five to 60 L4 larvae were placed in each well and scored for dead worms by microscopic examination. *E. coli* DH5 $\alpha$  served as a negative control. Values are the mean  $\pm$  SD of a representative experiment with triplicate values. ○, wt; △, vqsR-; ▲, vqsR+; ■, *E. coli*.

conditions VqsR does not play a role in the modulation of cytotoxicity.

### VqsR has an impact on virulence in a *C. elegans* model

The nematode *C. elegans* has been used as a bacterial pathogenesis model for the identification of virulence-attenuated mutants in *P. aeruginosa* (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999). In fact, it has been shown that the analysis of the interaction with this invertebrate host has the potential to predict disease outcome in the mammalian host. Testing of the wild-type and its Tn5::vqsR mutant revealed a significant attenuation of the vqsR mutant compared to the wild-type (Fig. 4). The killing activity of the complemented mutant was restored to wild-type levels (Fig. 4), indicating an important role of the vqsR gene in virulence towards *C. elegans*.

### mRNA expression profiling of vqsR

The expression of vqsR mRNA was analysed by Northern blots under various growth conditions (early- and late-exponential growth phase in LB medium, minimal mineral medium, presence of oxidative stress generated by H<sub>2</sub>O<sub>2</sub>, presence of serum and polymorphonuclear leukocytes). Hybridization with a genomic vqsR probe gave a clear signal when *P. aeruginosa* TB had been cultured in the presence of H<sub>2</sub>O<sub>2</sub> or human serum, but only barely detectable signals under the other tested conditions. The Tn5::vqsR mutant did not produce any detectable VqsR transcript under all chosen conditions.

*P. aeruginosa* GeneChips were used to investigate the effect of VqsR on global changes in the gene expression profile. Total RNA, extracted from bacterial cultures cultivated in the presence of 10 % human blood serum or 10 mM H<sub>2</sub>O<sub>2</sub>, was hybridized on the DNA microarrays in duplicate. Numerous genes were differentially regulated in the *vqsR* mutant compared to the wild-type strain. By applying the stringent criteria outlined in Methods, the mutation of *vqsR* significantly influenced the expression of 151 genes in the presence of H<sub>2</sub>O<sub>2</sub> and of 113 genes in the presence of human blood serum (Fig. 5). Only 26 genes were significantly regulated under both conditions. The products of these genes are involved in the transport of small molecules, pyochelin biosynthesis, pyoverdinin synthesis, rhamnolipid biosynthesis, hydrogen cyanide biosynthesis and protease secretion (Fig. 6).

The majority of the affected mRNA species are under the control of quorum sensing (Hentzer *et al.*, 2003b; Pesci *et al.*, 1997; Pessi *et al.*, 2001; Schuster *et al.*, 2003b; Wagner *et al.*, 2003; Winzer *et al.*, 2000; Fig. 5). As shown previously (Wagner *et al.*, 2003), variable responses in the expression of quorum-sensing-regulated genes to different environmental stimuli may be caused by alterations in the expression of the regulators themselves. When comparing mRNA chip expression data of *vqsR* with those of the known major regulators of AHL-mediated quorum sensing (*lasR*, *lasI*, *rhIR*, *rhII* and *qscR*), *vqsR* was the only gene with a comparatively higher expression (2.5-fold) in the presence of serum than in LB medium. The GeneChip data confirm the results of the Northern blot experiments showing that the expression of *vqsR* is more strongly activated by an environment that contains human serum, as happens in *Pseudomonas* septicemia or burn wounds.

### Expression of a wide variety of quorum-sensing-regulated genes and virulence factors is modulated by VqsR

VqsR modulates the expression of a broad spectrum of quorum-sensing and virulence genes. Fig. 5 lists the genes whose expression was found to be significantly up- and down-regulated in the Tn5:: *vqsR* mutant according to the results of our GeneChip analysis. The mutant produced significantly less mRNA species of genes that are necessary for the synthesis of quorum-sensing quinolones and AHLs and of virulence factors under control thereof (Fig. 6). Nearly half of the remaining genes belong to the class of conserved hypotheticals of unknown function. The genes with ascribed function predominantly encode enzymes required for siderophore biosynthesis (Lamont & Martin, 2003; Ravel & Cornelis, 2003) or membrane proteins, the latter of which are almost all known to be involved in antimicrobial resistance (Hancock & Speert, 2000; Lambert, 2002; Poole, 2001).

In the presence of serum, the *vqsR* mutant overexpressed genes that belong either to the category of amino acid metabolism or to the category of conserved hypotheticals,

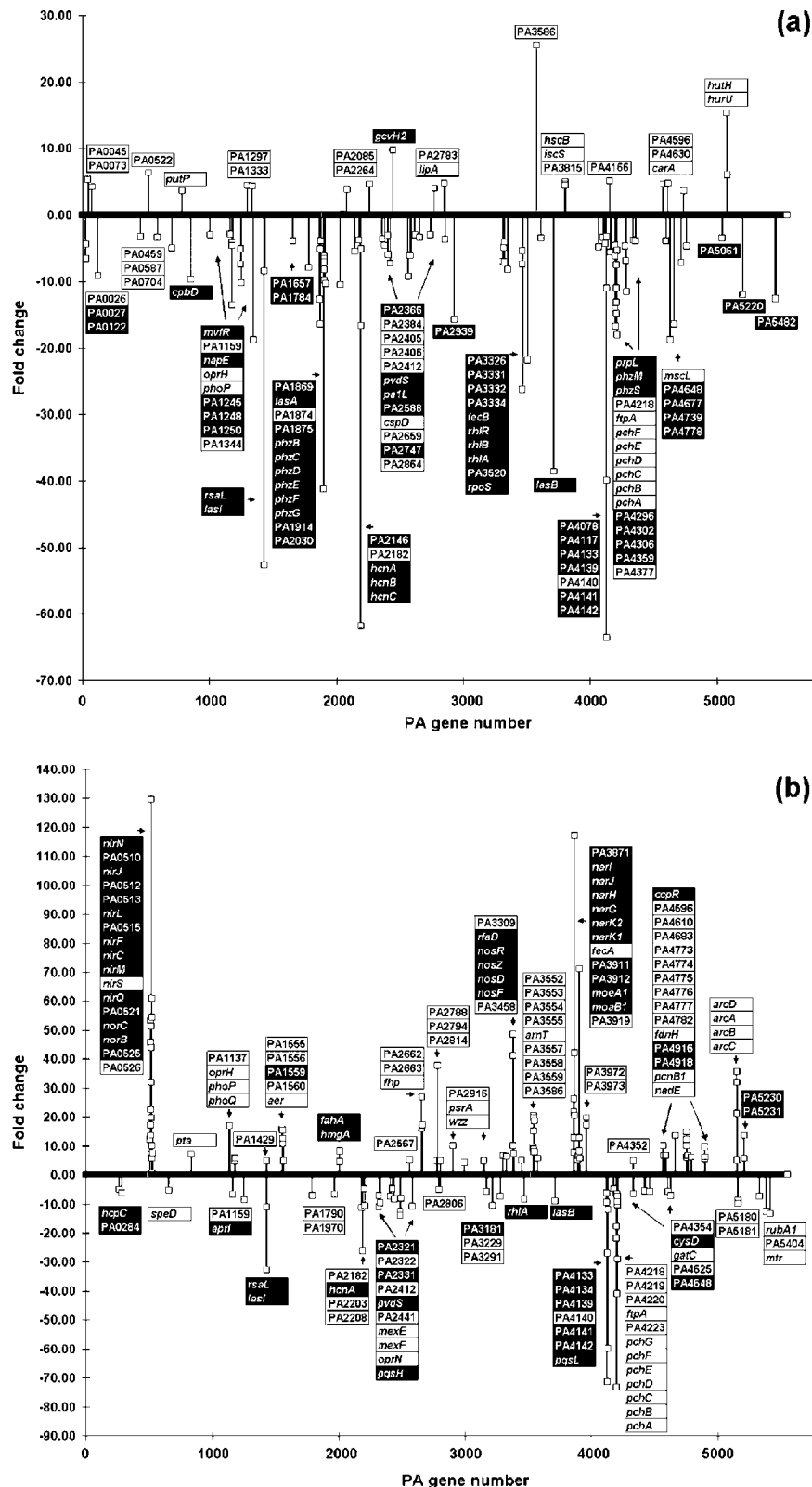
presumably just reflecting the serum as a protein-rich carbon source. Exposure to H<sub>2</sub>O<sub>2</sub> activated genes by more than 10-fold in the mutant that are either conserved hypotheticals or encode components of anaerobic energy metabolism. Nitrite and nitrate respiration have been reported to be repressed by quorum sensing (Wagner *et al.*, 2003).

In summary, the disruption of *vqsR* repressed the expression of genes that are known to be promoted by quorum sensing and activated the expression of genes that are known to be repressed by quorum sensing. Moreover, the *vqsR* mutant harboured less mRNA transcript for the production of siderophores and membrane-bound elements of antibiotic resistance. Besides the cytotoxicity caused by elements of the type III secretion system (Frank, 1997), antibiotic resistance and extracellular virulence factors are the major determinants for morbidity and prognosis of infections with *P. aeruginosa* in humans. The protein encoded by *PA2591* regulates several traits of pathogenicity; hence, we assigned the name *vqsR* ('virulence and quorum-sensing regulator') to *PA2591*.

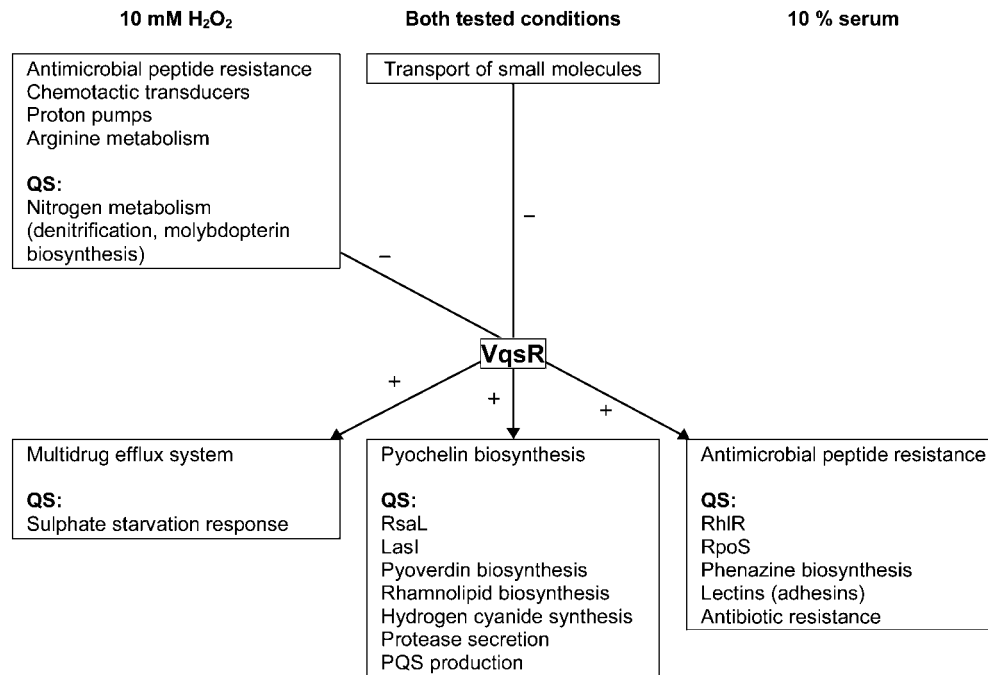
Inactivation of *vqsR* abrogated the production of AHLs and decreased *LasI* mRNA by about 10-fold with *LasR* mRNA levels being unaffected. In addition, *vqsR* contains a *las* box in its upstream region. Considering these data, we should like to conclude that *vqsR* is an essential element of the quorum-sensing hierarchy whose inactivation disrupts the production of AHLs, including 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL. On the other hand, the presence of the *las* box located upstream of *vqsR* places this gene under the direct control of the regulatory *LasR*-3-oxo-C<sub>12</sub>-HSL complex, thus creating an autoinducer feedback loop. This hypothesis is also supported by the GeneChip experiment performed by Wagner *et al.* (2003) where transcription of the *vqsR* gene (*PA2591*) was found to be reduced 5.7-fold in the *P. aeruginosa* mutant strain deficient in 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL synthesis. As 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL are involved in the initiation of quorum sensing, the disruption of 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL production and secretion in the *vqsR* mutant inevitably has a negative effect on the whole quorum-sensing cascade. Indeed, our GeneChip experiments revealed significant down-regulation of the whole battery of quorum-sensing-regulated genes in the *vqsR* transposon mutant. Correspondingly, the mutant was compromised in phenotypic traits that are under quorum-sensing control such as elastase secretion and virulence in the *C. elegans* infection model. In summary, VqsR is a new member of the LuxR family that is involved in cell-to-cell communication and virulence of *P. aeruginosa*.

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**Fig. 5.** Differential gene expression of the Tn5::vqsR transposon mutant compared to *P. aeruginosa* wild-type strain TB. Positive values represent genes whose expression is up-regulated in the mutant and negative values represent genes whose expression is down-regulated in the mutant compared to the TB wild-type strain. Only genes whose expression is significantly regulated are shown. Quorum-sensing-regulated genes are highlighted black (Hentzer *et al.*, 2003b; Schuster *et al.*, 2003; Wagner *et al.*, 2003). (a) In the presence of 10% human blood serum. (b) In the presence of 10 mM H<sub>2</sub>O<sub>2</sub>.



**Fig. 6.** Major gene categories and metabolic pathways regulated by VqsR in the presence of 10 mM H<sub>2</sub>O<sub>2</sub> and 10% serum. VqsR is an essential element of the quorum-sensing hierarchy, hence it activates the expression of genes that are known to be promoted by quorum sensing and negatively regulates the expression of genes that are known to be repressed by quorum sensing. Moreover, VqsR was proven to be involved in the regulation of siderophores and membrane-bound elements of antibiotic resistance.

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