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Quorum sensing differentially regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and III, which are required for pathogenesis

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Pseudomonas aeruginosa harbours three type VI secretion (T6S) loci. Although HSI-I has been partially studied, limited knowledge is available on the homologous loci HSI-II and HSI-III. We show that quorum sensing (QS) differentially regulates the expression of genes at all three loci. HSI-I-associated gene expression is suppressed by both the homoserine lactone transcription factor LasR and the 4-hydroxy-2-alkylquinoline (HAQ) transcriptional regulator MvfR. Conversely, both HSI-II and HSI-III loci are positively controlled by LasR and MvfR. PqsE, a key component of the MvfR regulon, is required for the expression of part of HSI-III but not HSI-II, and previously identified inhibitors of HAQ biosynthesis significantly downregulate HSI-II and -III gene expression. Animal and plant infection studies reveal that both HSI-II and -III play important roles in pathogenesis. Furthermore, analysis of a double ΔHSI-II::III mutant suggests that these loci functionally compensate for one another in virulence. This study illustrates the contribution of the QS systems to T6S gene regulation and reveals the importance of HSI-II and -III in mediating *P. aeruginosa* pathogenesis. Moreover, this work provides new insights into the design and development of selective compounds that may restrict human *P. aeruginosa* and possibly other clinical infections.

INTRODUCTION

Type VI secretion systems (T6SSs) are widely represented throughout the bacterial kingdom and have been implicated in the virulence of several human, animal and plant pathogens (de Bruin *et al.*, 2007; Folkesson *et al.*, 2002; Gray *et al.*, 2002; Mougous *et al.*, 2006; Nano *et al.*, 2004; Parsons & Heffron, 2005; Rao *et al.*, 2004; Schell *et al.*, 2007; Suarez *et al.*, 2008). Gram-negative bacterial genomes commonly encode several T6SSs, which appear to have distinct evolutionary origins rather than having arisen from gene duplication (Bingle *et al.*, 2008). Hallmarks of T6SSs typically include the presence of an AAA+ ATPase usually annotated as ClpV as well as homologues of IcmF, a protein that confers stability upon the *Legionella* type IV secretion apparatus. The *hcp* and *vgrG* genes are also often located within T6SS loci and encode proteins that can be exported extracellularly (Pukatzki *et al.*, 2006). Examples of type VI (T6)-secreted proteins are the three VgrGs from *Vibrio cholerae* (Pukatzki *et al.*, 2006), EvpI (a VgrG homologue) from *Edwardsiella tarda* (Schell *et al.*, 2007; Zheng & Leung, 2007), and Hcp from *V. cholerae* (Pukatzki *et al.*, 2006), *Agrobacterium tumefaciens* (Wu *et al.*, 2008), *Aeromonas hydrophila* (Suarez *et al.*, 2008), *Burkholderia mallei* (Schell *et al.*, 2007) and *Pseudomonas aeruginosa* (Mougous *et al.*, 2006). Interestingly, T6 proteins do not contain canonical signal peptides, indicating that they are not secreted in a Sec- or Tat-dependent manner and probably cross the bacterial cell envelope in a single step

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Abbreviations: 4CABA, 2-amino-4-chlorobenzoic acid; 6CABA, 2-amino-6-chlorobenzoic acid; HAQ, 4-hydroxy-2-alkylquinoline; qRT-PCR, quantitative real-time RT-PCR; QS, quorum sensing; SAM, significance analysis of microarray; T6, type VI; T6S, type VI secretion; T6SS, type VI secretion system.

The GEO series accession number for the microarray data discussed in this paper is GSE17147.

Two supplementary figures, showing a CLUSTAL W alignment of PA14 HSI-I, -II, and -III Hcp and VgrG proteins, and the genomic context of the four *hcp2* homologues in PA14 and a CLUSTAL W alignment of the VgrG proteins adjacent to the hcp2 homologues, are available with the online version of this paper.

(Pallen *et al.*, 2003). A puzzling observation is that several secreted Hcp and VgrG proteins are also apparently essential components of the type VI secretion (T6S) machinery, since *hcp*, *vgrG1* and *vgrG2* mutations in *V. cholerae* have been shown to inhibit Hcp, VgrG1 and VgrG2 secretion (Pukatzki *et al.*, 2007).

T6SSs appear to be tightly regulated components of bacterial systems that respond to a variety of conditions. In *Rhizobium leguminosarum*, expression of T6SSs is temperature-dependent (Bladergroen *et al.*, 2003), while in *V. cholerae*, T6S genes can be induced *in vivo*, e.g. *icmF* (*vasK*), which is upregulated in rabbit ileal loops (Das *et al.*, 2000). *P. aeruginosa* harbours three T6 loci, termed HSI for Hcp secretion island, that appear to be regulated independently of each other (Mougous *et al.*, 2006, 2007). HSI-I has been shown to be transcriptionally regulated by the type III secretion system regulators RetS/LadS and post-translationally regulated by a serine–threonine kinase–phosphatase PpkA/PppA encoded by the locus itself. In contrast, HSI-II is not dependent on RetS/LadS, although this locus does contain a putative serine–threonine kinase–phosphatase. HSI-III neither depends on RetS/LadS nor encodes a serine–threonine kinase–phosphatase (Mougous *et al.*, 2006, 2007).

Our previously published transcriptome studies (Déziel *et al.*, 2005; Lesic *et al.*, 2007) provided indirect evidence that expression of the HSI-II and -III loci is affected by the transcriptional factor MvfR, the regulator of the quorum sensing (QS) 4-hydroxy-2-alkylquinoline (HAQ) system in *P. aeruginosa*. MvfR coligand synthesis is essential for MvfR activation. This synthesis requires two MvfR-regulated operons, *phnAB* and *pqsA–D*. The operon *phnAB* directs production of anthranilic acid, the primary HAQ precursor, while *pqsA–D* directs production of the HAQ congener family (Bredenbruch *et al.*, 2005; Déziel *et al.*, 2004; Ritter & Luckner, 1971). The last gene of the *pqs* operon, *pqsE*, although co-regulated, does not affect HAQs synthesis, although it greatly affects *P. aeruginosa* pathogenesis (Déziel *et al.*, 2004, 2005; Gallagher *et al.*, 2002). Through PqsE and HAQs, MvfR affects the expression of many QS-related genes (Déziel *et al.*, 2005; Lesic *et al.*, 2007; R. Hazan and L. G. Rahme, unpublished data), including many genes involved in *P. aeruginosa* virulence. Indeed, *mvfR*-deficient bacterial cells have greatly reduced pathogenicity in several infection models (Lau *et al.*, 2003; Rahme *et al.*, 1997), which has prompted the study and identification of MvfR regulon inhibitors. Recently we have shown that selective inhibition of MvfR/HAQ regulation greatly restricts *P. aeruginosa* pathogenesis in mice (Lesic *et al.*, 2007).

The three T6 loci present in *P. aeruginosa* are a prime example of T6SS diversity within a single genome. While efforts have been undertaken to understand the role of the HSI-I in *P. aeruginosa* pathogenesis, little is known about the other two T6SS loci. Our investigation focused on the regulation of all three HSI loci by investigating their co-dependency and their link to QS. Furthermore, this study also revealed the relevance of HSI-II and -III in *P. aeruginosa* virulence in diverse hosts. This research provides a starting point for the design and development of compounds that may restrict the pathogenesis in humans of *P. aeruginosa* and possibly of other clinically significant bacterial pathogens.

METHODS

Bacterial stains used. The Rif^R *P. aeruginosa* human clinical isolate UCBPP-PA14 (Rahme *et al.*, 1995) and its isogenic mutant derivatives Δ*mvfR* (Cao *et al.*, 2001), Δ*lasR* (Lesic & Rahme, 2008), Δ*pqsE* (Déziel *et al.*, 2005) and ΔHSI-II, deleted from PA14_42880 (*stk1*) to PA14_43100 (Lesic & Rahme, 2008), were grown at 37 °C in Luria–Bertani (LB) broth.

Generation of the ΔHSI-III and ΔHSI-II::III mutants. Lambda Red-based methodology (Lesic & Rahme, 2008) was used to generate the HSI deletion mutants. A 12.4 kb region was deleted in the HSI-III locus, encompassing PA14_33970 and PA14_34130 (*icmF3*). A larger deletion that included *vgrG3* could not be obtained, as PA14 transposon mutants of PA14_33960 (*vgrG3*) or PA14_33940 did not contain the mariner transposon in the same orientation as in *icmF3*. PCR was used to amplify the products necessary to delete the locus. First, genomic DNA of mutants of the PA14 non-redundant transposon library, PA14_33970::MAR2xT7 and *icmF3*::MAR2xT7 were used as templates to generate PCR fragments containing the gentamicin-resistance cassette flanked by the HSI-III loci borders. Primers F_{up}-PA14_33970 (5′-TCGAACAGTACCGTGGAGTAGT-3′) and R_{up}-MAR2xT7 (5′-CAAAGTTAGGTGGCTCAAGTATG-3′) were designed to amplify MAR2xT7 and the 874 nt upstream sequence when inserted into PA14_33970. Primers F_{down}-MAR2xT7 (5′-TTAGGTGGCGGTACTTGGGTCG-3′) and R_{down}-*icmF3* (5′-GATACGTGCATCCAGGGAGGAA-3′) were used to amplify MAR2xT7 and the 782 nt downstream sequence when inserted into *icmF3*. The resulting upstream- and downstream-amplified products were then mixed with the primers F_{up}-PA14_33970 and R_{down}-*icmF3* in a second PCR resulting in a product containing MAR2xT7 flanked by the borders of the HSI-III locus. PCR products were then electroporated into PA14/pUCP18-RedS and recombinants were selected on LB-gentamicin (15 μg ml^{-1}) plates. DNA amplification using the F_{up}-PA14_33970/R_{up}-MAR2xT7 and F_{up}-PA14_33970/R_{down}-*icmF3* primers confirmed deletion of the 12.4 kb region. *P. aeruginosa* recombinants were cured of the pUCP18-RedS plasmid by streaking the mutant strains on NaCl-free LB agar plates supplemented with 10 % sucrose.

The double ΔHSI-II::III mutant was generated by using the Lambda Red technology, as described above (Lesic & Rahme, 2008), to delete 29.6 kb of the HSI-II locus within the ΔHSI-III background. The entire HSI-II locus, ranging from *stk1* to PA14_43100, was deleted and replaced with a tetracycline-resistance cassette from the plasmid pACYC184 (Chang & Cohen, 1978). The upstream PCR product was produced using the primer pair loc2up1 (5′-CTCGCTCTTGTCCTTGACCAG-3′) and loc2up-TU (5′-GTATGGGGCTGACTTCAGGTGGTATAGGAACTTCGGCGTTCTTGCCCGTTGAG-3′); the downstream product was generated with loc2do1 (5′-GTCCAACGCCATCCTCAACTTC-3′) and loc2do-TD (5′-CTGAGTTACAACAGTCCGCACACAAGAACCTAATAGACTTC-3′). These PCR products were mixed with the tetracycline-resistance gene, and primers loc2up-T (5′-CTCAACGGGCAAGAACGCCGAAGTTCCTATACCACCTGAAGTCAGCCCCATAC-3′) and loc2do-T (5′-GAAGTCTATTAGGTTCTTGTGTGCGGACTGTTGTAACTCAG-3′) were used to stitch the fragments together, incorporating the tetracycline-resistance gene between the upstream and downstream sequences.

The final PCR product, amplified by primers loc2up2 (5′-GTTGC-GGATGTGGTTGAACAG-3′) and loc2do2 (5′-CACGCCATTCCT-GGTCGGCGAAC-3′), was electroporated into ΔHSI-III carrying pUCP18-RedS, as above.

Transcriptome analysis. The generation of microarray data for the *mvfR* mutant and for the treatment with anthranilic acid analogues 2-amino-4-chlorobenzoic acid (4CABA) and 2-amino-6-chlorobenzoic acid (6CABA) has been described previously (Déziel *et al.*, 2005; Lesic *et al.*, 2007). For the transcriptome analysis of the *pqsE* mutant, bacteria were cultured at 37 °C to OD_{600} 3.0. The total RNA was isolated with the RNeasy Mini kit (Qiagen) and cDNA synthesis and labelling were performed according to the manufacturer's instructions (Affymetrix). The *P. aeruginosa* PAO1 GeneChip Genome array (Affymetrix) was used for hybridization, staining, washing and scanning according to the manufacturer's instructions. Experiments were independently performed in triplicate. Affymetrix DAT files were processed using the Affymetrix Gene Chip Operating System (GCOS) to create *.cel* files. The raw intensity *.cel* files were normalized by robust multi-chip analysis (RMA) (Bioconductor release 1.7) with PM-only models. Array quality control metrics generated by the Affymetrix Microarray Suite 5.0 were used to assess hybridization quality. Normalized expression values were analysed with SAM (significance analysis of microarray) using the permuted unpaired two-class test. Genes whose transcript levels exhibited a twofold upregulation or downregulation and had a *q* value <6 % (estimated false discovery rate) were further analysed. The results of the GeneChip arrays were imported to GeneSpring 7.3 (Agilent Technologies) and the expression signals of the GeneChip arrays were normalized to the constant value of 1.0 and the ratio cut-off was set to twofold. Data are presented as fold-change ratios compared with wild-type PA14.

Quantitative real-time RT-PCR (qRT-PCR). PA14 wild-type and mutant cells were grown in 5 ml LB at 37 °C with agitation in the presence or absence of 6CABA or 4CABA at 6 and 1.5 mM, respectively. 6CABA and 4CABA were dissolved directly in LB media. Samples of three independent cultures were harvested at OD_{600} 2.5; total RNA was purified using the RNAeasy spin column (Qiagen) and cDNA was prepared. qRT-PCR was carried out using the IQ5system (Bio-Rad) and primers *hcp1*-F (5′-GGGTGAGTCCAAGGACAAGA-3′), *hcp1*-R (5′-GGACACCAGGACTTCCTTCA-3′); *hcp2*-F (5′-GGCTTCAACCACGAAGTGAT-3′), *hcp2*-R (5′-AGGTGATCTT-GCGGTAGGTG-3′); *hcp3*-F (5′-GGATGCGATCATTCTCGATT-3′), *hcp3*-R (5′-GGTCGAGGTGTCGATGAACT-3′); and *rpoD*-F (5′-CTGATCCAGGAAGGCAACAT-3′), *rpoD*-R (5′-TGAGCTTG-TTGATCGTCTCG-3′). The *rpoD* or *rplU* gene was used as a housekeeping control, since earlier reports have demonstrated that *rpoD* and *rplU* transcription is independent of the *P. aeruginosa* growth stage or environmental conditions (Kuchma *et al.*, 2005; Mah *et al.*, 2003), and we have found similar results in our various microarray experiments. The data were expressed normalized to *rpoD* or *rplU* transcript as indicated. Both *rplU* and *rpoD* are accepted standards for normalizing *P. aeruginosa* qRT-PCR data, and both genes provided similar results.

Infection models

Mouse acute lung infection model. An acute lung infection model was used as described by Comolli *et al.* (1999). Briefly, *P. aeruginosa* cells were grown to OD_{600} 3.0. The bacteria were then harvested and washed in saline solution, and 5×10^6 bacteria were administered intranasally in 20 µl and survival was monitored for 7 days. Eight animals were used per group. The animal protocol used was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Survival curves were calculated with the Kaplan–Meier procedure (SPSS version 15), see below. The log-rank test was used to compare survival curves, and a *P* value below 0.05 was considered significant.

Mouse burn and infection model. Murine thermal burn injury and infection experiments were performed as described previously (Lesic *et al.*, 2007; Rahme *et al.*, 1995). Briefly, 5×10^5 *P. aeruginosa* cells were injected intradermally into the burn eschar, and mouse survival was monitored post-infection (Rahme *et al.*, 1995). Eight animals 6–8 weeks old were used per group. The parental strain PA14 was used as a control.

Plant infection model. Five-week-old *Arabidopsis thaliana* ecotype Llagostera (Ll-O) were leaf-infiltrated with *P. aeruginosa* ΔHSI-II and ΔHSI-III mutants as described previously (Rahme *et al.*, 1995; Starkey & Rahme, 2009). The parental strain PA14 was used as a control. The plants were kept in a growth chamber during the course of the experiment at 30 °C and at high relative humidity. Bacterial c.f.u. and intensity of water soaking symptoms caused by the mutant strains were assessed and compared with that of the parental strain at 1, 2 and 3 days post-infection. Bacteria were harvested from the leaf intercellular fluid and bacterial counts were determined by plating on Rifampicin LB agar plates (100 µg ml^{-1}). Four different samples were obtained with two leaf discs per sample.

Statistical analysis. Statistical analysis of the animal survival curves was performed using the Kaplan–Meier method (Kaplan & Meier, 1958) in the SPSS software package (version 15, SPSS). Differences between mutant and wild-type survival kinetics were assessed using the log-rank (Mantel–Haenszel) test to compare the Kaplan–Meier survival curves of mutant and wild-type. Two-tailed values of $P \leqslant 0.05$ were considered statistically significant. The Kaplan–Meier survival curve of PA14 was significantly different from that of ΔHSI-II ($P = 0.0159$). The *P* values of plant infection studies were calculated using the *t* test.

RESULTS

Protein sequence analysis of HSI-I, -II and -III reveals commonalities, yet suggests distinct roles for each locus

Nucleotide sequence analysis of the HSI-I, -II and -III loci from strain PA14 showed little overall similarity between the loci; however, significant homologies were identified at the protein level (Fig. 1). The presence of genes in all three loci encoding similar proteins suggests that these gene products might be involved in the assembly of the T6 apparatus and secretion, as has been indicated by Pukatzki *et al.* (2007). Hallmarks of T6SS typically include the presence of ClpV, IcmF, and often Hcp and VgrGs. It is therefore not surprising to find these proteins in all three PA14 HSI loci. Although HSI-I, HSI-II and HSI-III encode Hcp1/VgrG1, Hcp2/VgrG2 and Hcp3/VgrG3, respectively, CLUSTAL W alignment (http://workbench.sdsc.edu/) of the three PA14 Hcp or VgrG proteins shows that the paralogues do not share high sequence similarity (Supplementary Fig. S1). BLASTP analysis indicates that Hcp3 is only 31 % identical and 57 % similar to Hcp1 and 20 % identical and 41 % similar to Hcp2, and that Hcp1 and Hcp2 are not significantly similar to each other.

The three HSI-associated *P. aeruginosa* VgrG proteins share overall about 35 % identity and 50 % similarity. Two

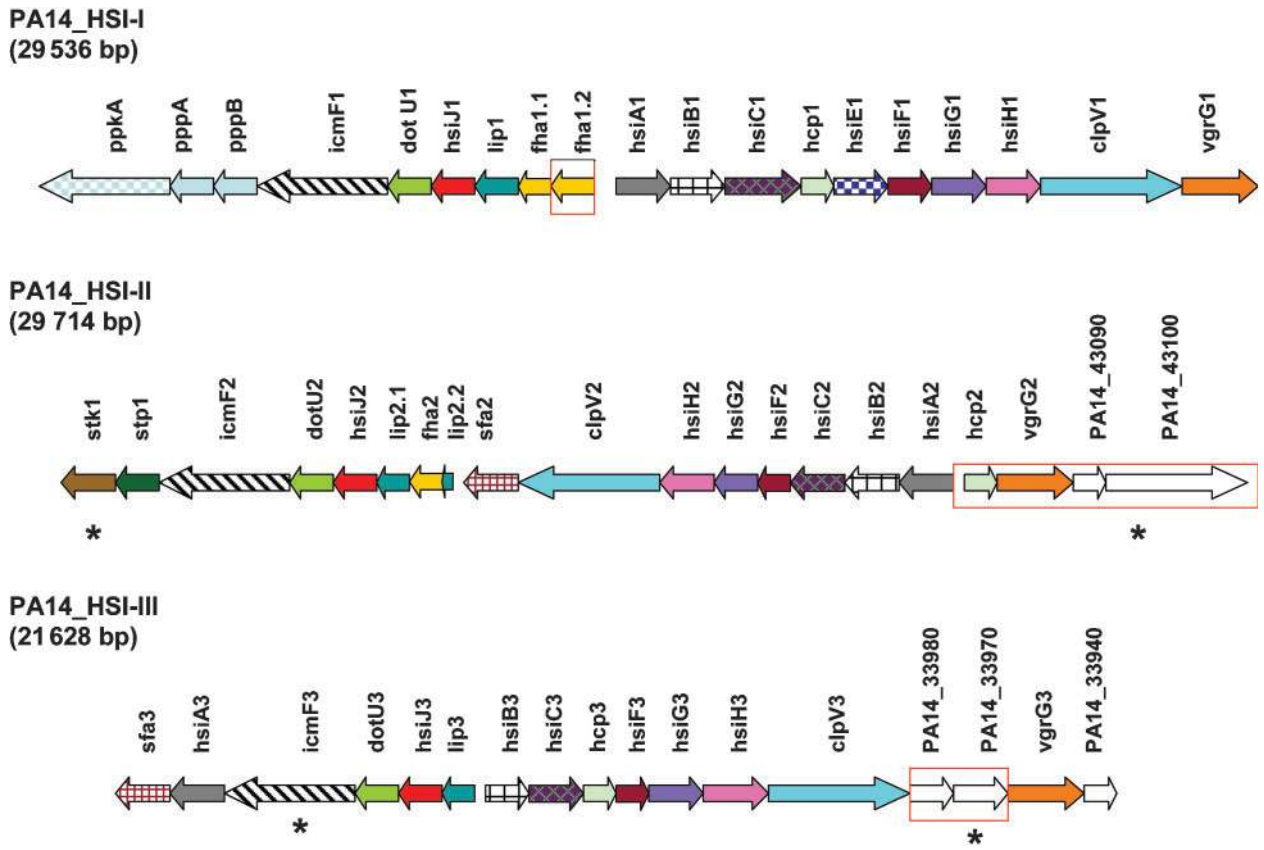

Fig. 1. PA14 HSI loci. Genes were annotated as described by Filloux *et al.* (2008) and Mougous *et al.* (2006). Genes that share homology are similarly designated (colour or pattern). Genes in white do not have paralogues in the other two HSIs and are therefore specific to each HSI. Genes boxed in red are specific to PA14 and absent from the corresponding PAO1 locus. Regions between two asterisks are the regions deleted in the ΔHSI-II and ΔHSI-III mutants.

conserved regions were identified (Supplementary Fig. S1). The first region consists of a conserved stretch of six amino acids (EYCVQY) localized at the N-terminal part of the protein and identified by CDART (Conserved Domain Architecture Retrieval Tool at http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) as resembling GpD, a phage tail protein of the Mu bacteriophage (Morgan *et al.*, 2002). This finding is similar to that for the *V. cholerae* VgrG protein (Pukatzki *et al.*, 2007), in which the N terminus resembles a phage tail protein. The second conserved region includes (i) the DUF586 domain, which is commonly found in VgrG proteins but does not yet have a defined function, and (ii) an extended homologous region 80 amino acids C-terminal to the DUF586 domain (Supplementary Fig. S1). Interestingly, none of the *P. aeruginosa* VgrGs possesses a C-terminal extension similar to the ACD domain of the *V. cholerae* VgrG-1 protein, which is involved in actin cross-linking (Pukatzki *et al.*, 2007).

Finally, the HSI-II and HSI-III loci each appear to encode locus-specific hypothetical proteins which are not present in the other PA14 T6SS loci (Fig. 1). The HSI-II locus-specific gene PA14_43090 encodes a hypothetical conserved protein with 32 % identity to a putative type III effector from *Aeromonas hydrophila*, and PA14_43100 encodes a putative Rhs family protein (based on BLAST homology searches). The HSI-III-specific gene, PA14_33970, encodes a hypothetical conserved protein with 33 % identity to a putative type III effector from *Ralstonia solanacearum*, and PA14_33980 possesses no homology to any known protein contained in the NCBI database. It is worth noting that two of these genes, PA14_43100 and PA14_43090, are specific to HSI-II and -III of PA14, as no orthologues were found within the other three sequenced *P. aeruginosa* strains, PA01, PA7 and LESB58.

QS affects gene expression of all three HSI loci

It has been shown that the three *P. aeruginosa* HSI loci are regulated differently from one another, with HSI-I, but not HSI-II and -III, gene expression being controlled by the RetS/LadS system (Mougous *et al.*, 2006). Furthermore, from independent transcriptome studies it can be appreciated that the homoserine lactone (HSL)-QS system

regulators, LasR and RhlR, also affect HSI gene expression. In Δ*lasRrhlR* or in HSL-induced Δ*lasIrhlI* cells, a large set of HSI-II genes (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003) and several HSI-III genes are downregulated. Our prior MvfR regulon transcriptome studies (Déziel *et al.*, 2005; Lesic *et al.*, 2007) also indicated that this QS pathway affects HSI-II and HSI-III loci gene expression. Several HSI-II and HSI-III genes were found to be downregulated in the Δ*mvfR* mutant (Fig. 2). In fact, almost all HSI-II and HSI-III genes appeared to be repressed when *mvfR* was deleted. We have found recently that *pqsE*, which is part of the operon required for the production of the MvfR ligands, is indispensable for most MvfR-dependent gene expression (R. Hazan and others, unpublished data). Surprisingly, in Δ*pqsE* cells, the expression profiles of HSI-II genes were unchanged compared with those in wild-type cells, while some HSI-III genes were downregulated (Fig. 2). Altogether, these results suggest that the MvfR pathway positively regulates the expression of HSI-II and -III loci and that MvfR/HAQs are required for the expression of HSI-II. PqsE is required for the expression of only five out of 13 HSI-III genes differentially regulated by MvfR.

MvfR and LasR positively regulate *hcp2* and *hcp3* and negatively regulate *hcp1*

To validate that QS regulates HSI transcription, we quantified the transcript levels of three genes representative of each of the HSI loci: *hcp1*, *hcp2* and *hcp3*. We chose the *hcp* genes from each T6S locus as representatives of the loci, since *hcp1* has been shown to be both an effector and an important component of the T6S machinery for HSI-I. We examined transcripts at a growth stage when QS genes are typically fully active (OD_{600} 2.5). Fig. 3(a) shows that *hcp2* and *hcp3* transcripts were expressed in the wild-type strain PA14, while the transcription of *hcp1* was very low. Transcription of *hcp2* and *hcp3* was affected in the QS mutants Δ*mvfR* and Δ*lasR*, demonstrating that both MvfR and LasR control *hcp2* and *hcp3* expression. In contrast, *hcp1* transcript levels were maintained at low levels of expression, which increased in both the Δ*lasR* and Δ*mvfR* mutants, suggesting that LasR and MvfR repress the HSI-I effector *hcp1*.

We searched the intergenic regions of the T6S loci for the LasR consensus sequence using the DNA motif search tool at http://www.pseudomonas.com/tools.jsp (Schuster & Greenberg, 2007) to examine whether QS control of these

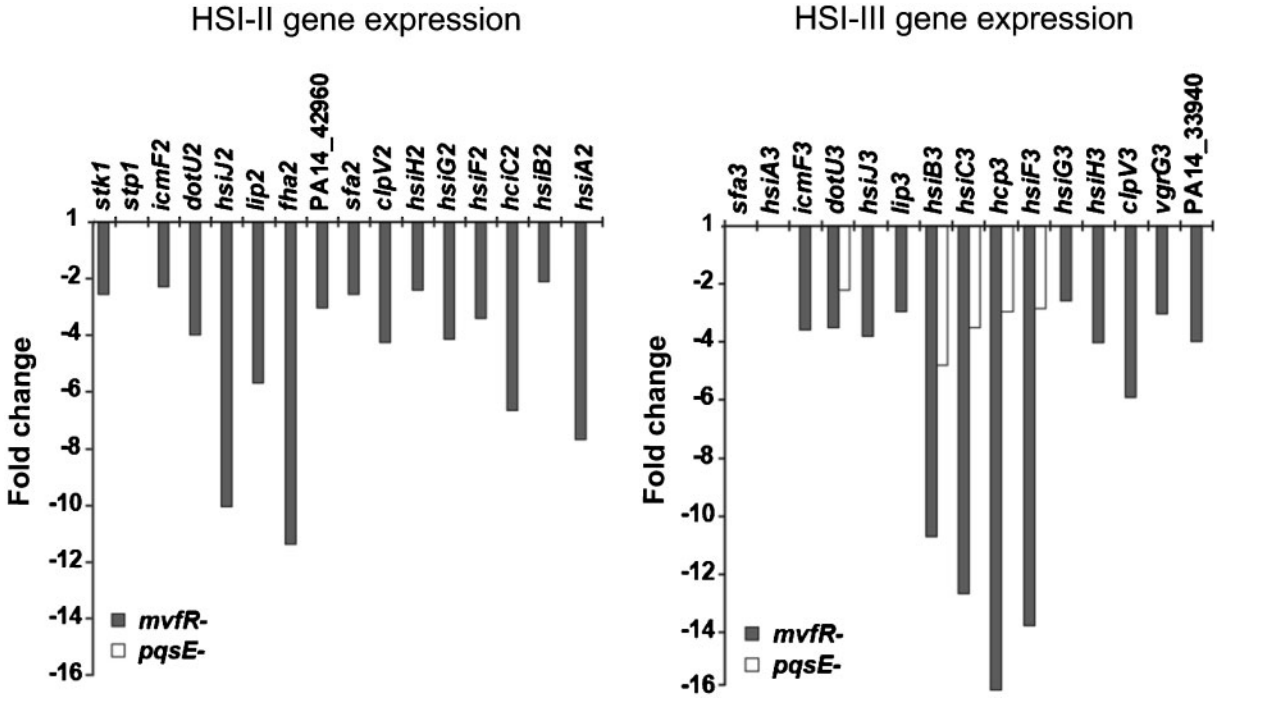

Fig. 2. Differential expression ratios of HSI-II and HSI-III genes in Δ*mvfR* and Δ*pqsE* cells. Differential gene expression from microarray data is expressed as fold-change values for Δ*mvfR* (Déziel *et al.*, 2005) and Δ*pqsE* (R. Hazan and others, unpublished data) strains versus the wild-type controls. Bacteria were grown in biological triplicates to OD_{600} 2.5 for the microarray experiments, and the data were analysed with SAM software with a twofold cut-off and a *q* value <6 % (estimated false discovery rate). Negative ratios indicate that genes are repressed in the mutant. Two groups of genes: *hcp2*, *vgrG2*, PA14_43090, PA14_43100; and PA14_33980, PA14_33970 are not present in the PAO1 genome and are thus not available on the *P. aeruginosa* Affymetrix chip.

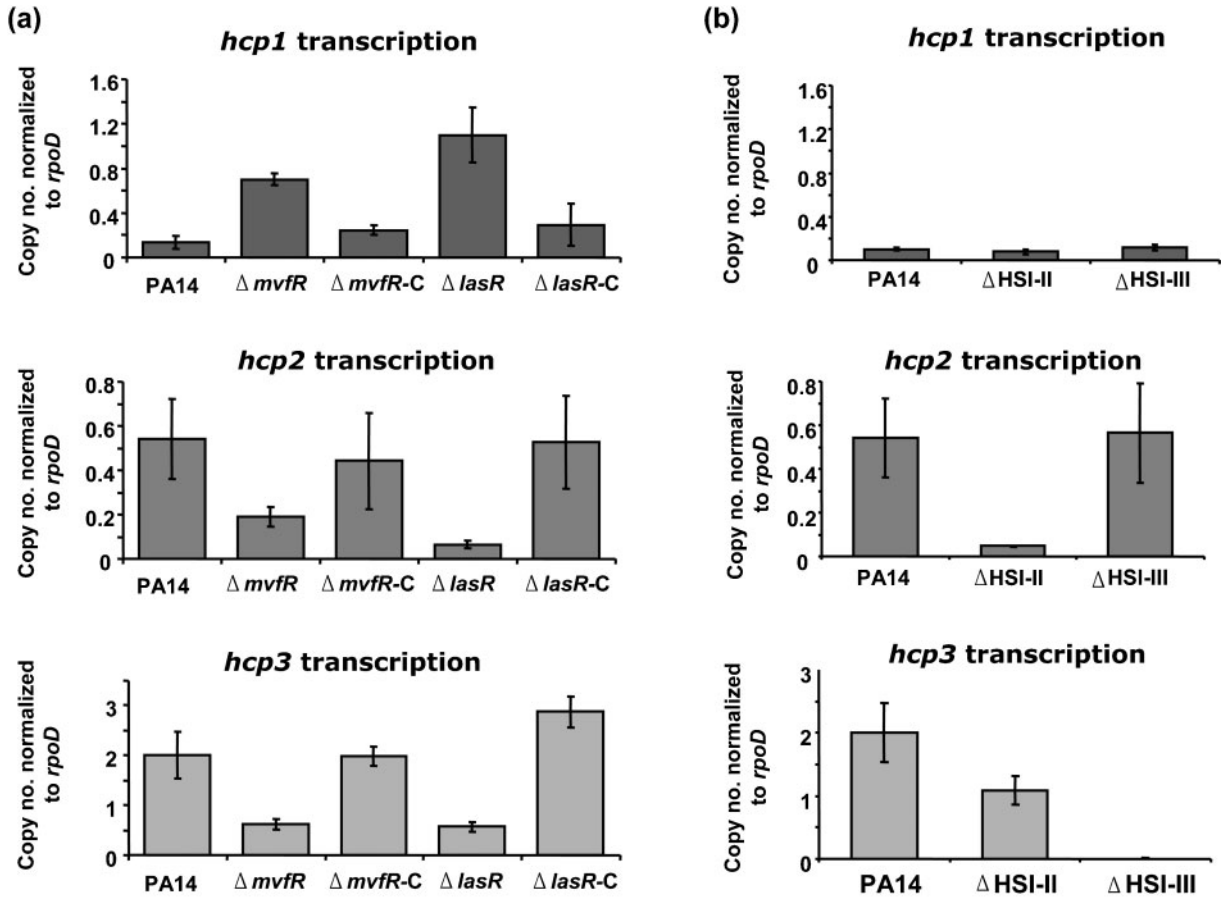

Fig. 3. Regulation of *hcp* transcription in different genetic backgrounds. PA14 wild-type and mutant cells were harvested at OD_{600} 2.5 and cDNA was prepared for qRT-PCR. The qRT-PCR data represent the mean obtained from three biological samples of *P. aeruginosa* strains, as indicated. (a) QS mutants Δ*mvfR* and Δ*lasR* alter transcription of *hcp1*, *2* and *3*. The mutants can be complemented back to wild-type expression levels by constitutively expressing MvfR (Δ*mvfR*-C) or LasR (Δ*lasR*-C), respectively, using plasmids pMT1 and pDN18-MvfR (Déziel *et al.*, 2004; Medina *et al.*, 2003). (b) Transcription levels of *hcp1*, *2* and *3* in the T6S loci deletion mutants ΔHSI-II and ΔHSI-III. While neither *hcp1* nor *hcp2* expression levels are altered in either ΔHSI-II or ΔHSI-III backgrounds, *hcp3* expression is reduced in the ΔHSI-II mutant. Expression for all samples was normalized relative to the housekeeping gene *rpoD* transcript levels. Error bars, SD.

genes is direct or indirect. No LasR binding sites in either HSI-I or HSI-III were found, suggesting that LasR does not directly bind to these loci. A LasR box (AACTACCTGTTTTGGTAGGG) was found in HSI-II, 303 bp upstream of the beginning of *hsiA2* (PA14_43050), suggesting that LasR directly positively regulates this gene. We also searched for putative MvfR binding sites using the only known MvfR binding site to date, which lies upstream of *pqsA*: TTCGGACTCCGAA (Xiao *et al.*, 2006). A BLASTN search of the PA14 genome using this sequence with an E-value cutoff of 1000 did not yield any significant hits within the HSI loci. Collectively, these results demonstrate that the expression of all three PA14 T6S loci was affected by QS, although the regulation may not be due to direct binding of LasR or MvfR. Both the LasR and MvfR systems negatively affect HSI-I transcription and positively affect transcription of HSI-II and HSI-III.

Gene regulation by the HSI-II or HSI-III locus shows some overlap

Since PA14 contains three distinct T6S loci, we examined whether the expression of HSI-II or HSI-III affects the gene expression of the other T6SS loci. In order to further understand the regulatory properties of each locus, we generated large deletions in the PA14 HSI-II and HSI-III loci by removing 24.3 and 12.4 kb DNA segments, respectively (Fig. 1). qRT-PCR revealed that a deletion of HSI-II or HSI-III had no significant effect on transcription of *hcp1* (Fig. 3b). Similarly, an HSI-III deletion did not

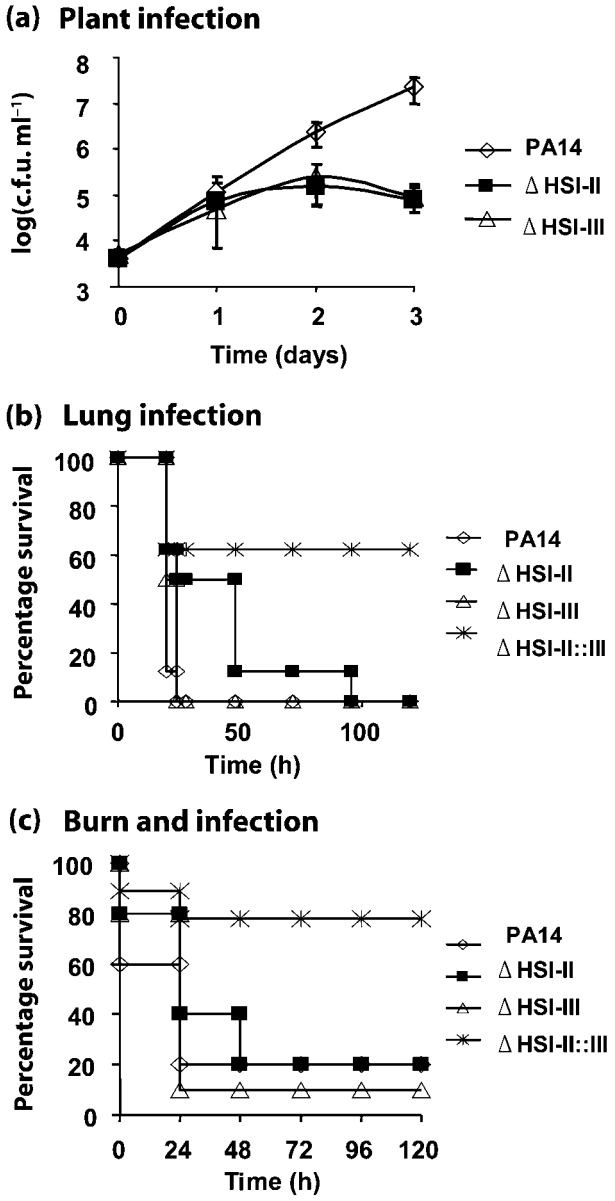

Fig. 4. HSI-II and HSI-III are important for *P. aeruginosa* virulence. (a) *Arabidopsis thaliana* ecotype Llagostera (Ll-O) was leaf-infiltrated with wild-type *P. aeruginosa* or with the isogenic deletion mutants ΔHSI-II or ΔHSI-III. c.f.u. obtained from leaves infected with the mutants were significantly lower than those from leaves infected with PA14 at both days 2 and 3. *P* values were calculated by comparing each mutant to PA14 using the *t* test: day 2 ΔHSI-II $P=0.024$, ΔHSI-III $P=0.028$; day 3 ΔHSI-II $P=0.023$, ΔHSI-III $P=0.008$. Error bars, SD. The murine lung (b) and burn (c) infection models were used to assess bacterial pathogenicity. In the lung infection model (b), mice were intranasally infected with 5×10^6 wild-type *P. aeruginosa* or deletion mutants, while in the burn model (c), 5×10^5 cells were injected intradermally in burn eschar. Data are expressed as percentage survival over a five-day period (eight mice per group). The Kaplan–Meier survival curve of PA14 was significantly different from that of ΔHSI-II ($P=0.0159$). Neither single mutant (ΔHSI-II or ΔHSI-III) altered virulence; however, when burned and infected with a double ΔHSI-II : : III mutant, 80 % of mice survived. Two independent experiments ($n=16$ total per group for lung or burn infection) gave similar survival kinetics.

influence *hcp2* transcription. However, *hcp3* transcription was reduced in the HSI-II mutant.

HSI-II and HSI-III are required for *P. aeruginosa* pathogenesis

HSI-I has been implicated in *P. aeruginosa* virulence (Mougous *et al.*, 2006), and since we have shown that HSI loci are regulated by both LasR and MvfR, we tested whether HSI-II and HSI-III also play a role in *P. aeruginosa* pathogenesis. Since MvfR was initially identified and shown to play an important role in plant pathogenesis (Rahme *et al.*, 1997, 2000) and MvfR function is required for HSI-II and -III gene expression, we first assessed the phenotype of the HSI-II and HSI-III deletion mutants in an *Arabidopsis thaliana* infection model (Rahme *et al.*, 1995; Starkey & Rahme, 2009). Reduced water-soaked symptoms were noted in leaves infected with the T6SS mutants compared with the wild-type-infected controls (data not shown), and bacterial cell counts three days post-infection were approximately two logs lower than in wild-type-infected controls (Fig. 4a). Both ΔHSI-II and ΔHSI-III mutants exhibited significantly decreased growth at 2 and 3 days post-infection.

To determine the role of these loci in mammalian virulence we tested ΔHSI-II and ΔHSI-III mutants in an acute mouse lung infection model. As presented in Fig. 4(b), mortality was delayed when mice were infected with the ΔHSI-II mutant intranasally but not when infected with the ΔHSI-III deletion mutant. To rule out a possible compensation between the functions of the HSI-II and HSI-III loci, we generated a double deletion mutant ΔHSI-II : : HSI-III. Indeed, Fig. 4(b) shows that infection with the ΔHSI-II : : HSI-III cells increased survival dramatically. Interestingly, both single mutants were equally as virulent as the wild-type strain in an acute mouse burn infection model (Fig. 4c) (Stevens *et al.*, 1994). However, the double ΔHSI-II : : HSI-III mutant also exhibited dramatically reduced virulence in this model, strongly suggesting potential compensation *in vivo* between the two loci when one is non-functional (Fig. 4c). Altogether, these results indicate that HSI-II and HSI-III play important roles in *P. aeruginosa* virulence.

Inhibition of HSI-II and HSI-III gene expression

We have previously demonstrated that anthranilic acid analogues including 4CABA and 6CABA can inhibit MvfR activity and the production of HAQs in *P. aeruginosa* (Lesic *et al.*, 2007). We therefore determined whether 4CABA or 6CABA could affect the expression of the HSI-II and HSI-III genes, further investigating MvfR control of these loci.

Fig. 5(a) shows a range of 1.5- to 3.9-fold change in gene expression from microarray data for wild-type cells grown in the presence of 4CABA and 6CABA compared with untreated controls. 4CABA and 6CABA repressed transcription of about half of the HSI-II-associated genes and almost all of the HSI-III-associated genes (Fig. 5a). These results are consistent with the notion that the MvfR pathway controls the expression of T6S loci. The downregulation of *hcp2* and *hcp3* transcription by 4CABA and 6CABA was further confirmed by qRT-PCR. Fig. 5(b) shows that both compounds significantly reduced *hcp2* and *hcp3* gene transcription.

DISCUSSION

Many bacterial genomes possess several T6SS loci but the reason for this redundancy remains unknown. Our data suggest that the redundancy is due to differential regulation of the HSI loci. HSI-I, as represented by *hcp1* expression, was repressed by the QS transcriptional regulators LasR and MvfR, while HSI-II and -III were activated by both LasR and MvfR. Therefore, in environments that stimulate high levels of QS activity, HSI-II and -III-associated genes are expressed, while HSI-I-associated genes are repressed and vice versa. Since MvfR controls the production of multiple secreted *P. aeruginosa* products (Cao *et al.*, 2001; Déziel *et al.*, 2005), it is plausible that some of these products are secreted via the T6SS, though there is no direct evidence to support this hypothesis. We speculate that an additional level of regulation exists via the putative transcriptional factor *sfa*, which is embedded in each locus (Fig. 1). In HSI-III, *sfa3* was upregulated, while the other genes in the locus were downregulated when PA14 cells

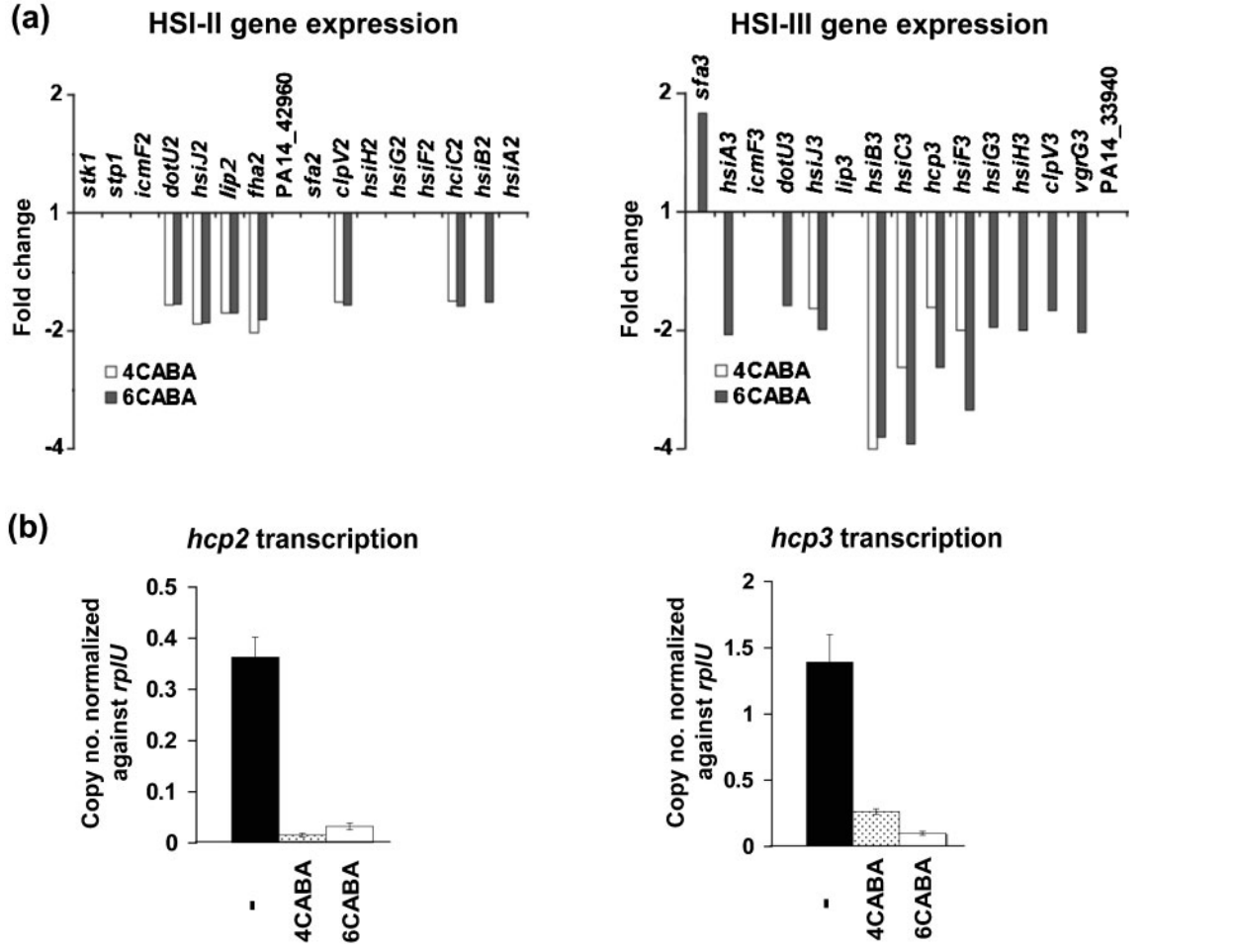

Fig. 5. MvfR inhibitors 4CABA and 6CABA alter the HSI-II and HSI-III expression profile. (a) Differential expression ratios of HSI-II- and HSI-III-associated genes in response to MvfR inhibitors 4CABA and 6CABA were obtained from microarray data. The ratios correspond to the fold-change in differential gene expression for PA14 cells grown in the presence or absence of 6CABA or 4CABA to OD_{600} 2.5 (Lesic *et al.*, 2007). Negative ratios indicate that compounds repressed gene expression. Data represent biological triplicates. (b) Wild-type PA14 was grown to OD_{600} 2.5 in the absence or presence of 6CABA or 4CABA for qRT-PCR analysis of *hcp2* and *hcp3* genes. The qRT-PCR data represent the mean obtained for three duplicate samples. Levels detected were normalized relative to *rplU* transcript levels. Error bars, SD.

were exposed to the MvfR/HAQ inhibitor 6CABA. Since *sfa3* is a predicted transcription factor and regulated oppositely compared with the rest of HSI-III, *sfa3* could potentially negatively regulate the HSI-III genes.

P. aeruginosa contains three distinct homologous T6SS loci which encode ClpV, IcmF, VgrG and Hcp-like proteins. T6SSs in *P. aeruginosa* were originally described in the PAO1 strain (Mougous *et al.*, 2006). Although the HSI-I loci are very similar in PAO1 and PA14, there are some differences between these strains at the HSI-II and HSI-III loci (Fig. 3), implying that the function of HSI-II and -III loci in virulence is different in PAO1 and PA14. First, HSI-II contains an additional region of four genes (*hcp2*, *vgrG2*, PA14_43090 and PA14_43100) which is absent from PAO1 and which contains both *hcp2* and *vgrG2*. Second, the HSI-III loci of PA14 and PAO1 harbour different nucleotide sequences between the *clpV3* and *vgrG3* genes, with two PA14-specific genes, PA14_33980 and PA14_33970. That several PA14-specific genes show sequence homology to effector proteins in other systems suggests that these are effector molecules for T6S. In contrast to PAO1, all three HSI loci in PA14 contain the *hcp* and *vgrG* genes. Although Hcp and VgrG paralogues have low sequence similarities, suggesting that these paralogues play distinct roles in *P. aeruginosa* virulence, additional experiments would be needed to assess whether the proteins are functionally similar, as low sequence similarity may not necessarily reflect different functions. Nevertheless, the animal experiment with the double knockout suggests that one paralogue can complement the other, as the double mutant is far less virulent than either single mutant.

Interestingly, the Hcp2 (*hcpD*) sequence is found four times within the genome of PA14 (annotated as *hcpA*, *B*, *C* and *D*) and three times within PAO1 (annotated as *hcpA*, *B* and *C*). Furthermore, the *hcpA*, *B* and *C* genes are not located adjacent to T6S clusters. Although there are slight nucleotide variations between the four PA14 *hcp2* homologues, they are identical at the protein level. It is noteworthy that all four PA14 *hcp2* homologues are located directly adjacent to *vrgG* genes (Supplementary Fig. S2a), which do not encode identical proteins. PA14_43080 (*vrgG* adjacent to *hcp2*; PA14_43070) is 98 % identical to PA14_69550 (*vrgG* adjacent to *hcpB*; PA14_69560), while PA14_44900 (*vrgG* adjacent to *hcpA*; PA14_44890) is 94 % identical to PA14_03220 (*vrgG* adjacent to *hcpC*; PA14_03240), and together these proteins share only 37 % identity (see Supplementary Fig. S2b for protein sequence alignment). The reason for this redundancy and the functional consequences are unknown, though the additional *hcp–vgrG* clusters are not surrounded by other genes typically associated with T6SS (Supplementary Fig. S2a).

The T6 HSI-I locus has been implicated in *P. aeruginosa* pathogenicity (Mougous *et al.*, 2006), and in this study we unravelled the importance of the other two T6SS loci in *P. aeruginosa* virulence. Various genes encoded in HSI-I have been shown to be important for chronic *P. aeruginosa* infections of the rat lung (Lehoux *et al.*, 2002; Potvin *et al.*, 2003), and anti-Hcp1 antibodies have detected Hcp1 in sputum of long-term-infected cystic fibrosis (CF) patients (Mougous *et al.*, 2006). An independent study has shown that long-term versus short-term infections in CF patients often accumulate Δ*lasR* bacterial cells (Smith *et al.*, 2006), and we have found that *hcp1* is upregulated in the Δ*lasR* background (Fig. 3a). These data together with our results provide for the first time, to our knowledge, an insight into the mechanism of Hcp1 expression in CF patients, suggesting that Hcp1 secretion is triggered by the accumulation of Δ*lasR* cells.

We showed that this opportunistic pathogen is armed with distinct T6SSs that facilitate different types of infection. Our data suggest that HSI-II and possibly HSI-III encode effector(s) that, like HSI-I, find their target in mammalian lung tissues (Mougous *et al.*, 2006). Interestingly, both HSI-I and HSI-II appeared to be involved in lung infection, with the former being relevant in chronic lung disease (Lehoux *et al.*, 2002; Mougous *et al.*, 2006; Potvin *et al.*, 2003), while the latter is important in acute infection. Nevertheless, we could not find proteins specifically encoded by HSI-I and HSI-II and not by HSI-III that would explain the lung specificity. This suggests that other proteins associated with the HSI loci or related loci are also involved and transcriptionally upregulated in lung pathology. In addition, we showed that HSI-II and HSI-III are important in plant pathogenesis. Although the effector proteins encoded by the HSI-II and HSI-III loci could not be identified, effectors mediating plant infections could be protein(s) present in all three loci, since the role of HSI-I in plant infection remains to be determined.

We suggest that HSI-II and HSI-III can functionally compensate for one another when one system is non-functional, a novel observation. Although neither HSI-II nor HSI-III had a virulence defect in the burn and infection model, a double deletion had a nearly avirulent phenotype. Likewise in the lung infection model, the double deletion drastically reduced mortality compared with either single deletion alone. It is possible that HSI-II and HSI-III encode distinct secretion apparatuses that can interchangeably be used to secrete effectors produced from either locus. The contribution of HSI-I to this phenomenon of apparent complementation has yet to be determined.

Our study showed that in addition to HSI-I, the other two T6SSs, HSI-II and -III, are essential components of *P. aeruginosa* virulence, making T6SSs attractive targets for the development of antimicrobial therapies. To this end we demonstrated that the newly described MvfR regulon inhibitors 4CABA and 6CABA (Lesic *et al.*, 2007) repressed transcription of both loci, and in particular the transcription of *hcp2* and *hcp3*, two putative T6-secreted proteins. Other important bacterial pathogens, such as *Burkholderia* species, also synthesize HAQs (Diggle *et al.*, 2006), and we have shown that production of these compounds could

also be inhibited by 4CABA and 6CABA (Lesic *et al.*, 2007). It is still unknown whether the *Burkholderia* T6S genes are dependent on HAQs or whether their transcription can be inhibited by 6CABA or 4CABA. Nevertheless, these compounds provide a starting point for the design and development of selective anti-infectives that may restrict human *P. aeruginosa* pathogenesis.

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