

# Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence

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## Summary

Indole is an extracellular biofilm signal for *Escherichia coli*, and many bacterial oxygenases readily convert indole to various oxidized compounds including 7-hydroxyindole (7HI). Here we investigate the impact of indole and 7HI on *Pseudomonas aeruginosa* PAO1 virulence and quorum sensing (QS)-regulated phenotypes; this strain does not synthesize these compounds but degrades them rapidly. Indole and 7HI both altered extensively gene expression in a manner opposite that of acylhomoserine lactones; the most repressed genes encode the *mexGHI-opmD* multidrug efflux pump and genes involved in the synthesis of QS-regulated virulence factors including pyocyanin (*phz* operon), 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) signal (*pqs* operon), pyochelin (*pch* operon) and pyoverdine (*pvd* operon). Corroborating these microarray results, indole and 7HI decreased production of pyocyanin, rhamnolipid, PQS and pyoverdine and enhanced antibiotic resistance. In addition, indole affected the utilization of carbon, nitrogen and phosphorus, and 7HI abolished swarming motility. Furthermore, 7HI reduced pulmonary colonization of *P. aeruginosa* in guinea pigs and increased clearance in lungs. Hence, indole-related compounds have potential as a novel antivirulence approach for the recalcitrant pathogen *P. aeruginosa*.

## Introduction

Cell signals can be promiscuous. For example, *Escherichia coli* senses the quorum-sensing (QS) signal

autoinducer-2 (AI-2) that is produced by *Vibrio harveyi* to assess changes in its cell population (Xavier and Bassler, 2005), and *Pseudomonas aeruginosa* responds to AI-2 and modulates its gene expression pattern including pathogenicity, although it does not itself produce AI-2 (Duan *et al.*, 2003). In addition, *Salmonella enterica* and *E. coli* detect acylhomoserine lactones (AHLs) via SdiA, although they do not synthesize AHLs (Michael *et al.*, 2001), and *E. coli* decreases biofilm formation in the presence of AHLs through SdiA (Lee *et al.*, 2007a). *Salmonella enterica* enhances drug resistance in response to indole, although it does not produce indole (Nikaido *et al.*, 2008), and we found that biofilm formation of *P. aeruginosa* and *P. fluorescens* increases in the presence of indole, even though these pseudomonads do not produce indole (Lee *et al.*, 2007a). Furthermore, the large quantities of indole secreted by *E. coli* [up to 0.6 mM in Luria–Bertani (LB) medium] (Domka *et al.*, 2006) may be hydroxylated by other bacteria such as *Burkholderia cepacia* G4 (Rui *et al.*, 2005) to form hydroxyindoles that both increase and decrease biofilm formation in *E. coli* O157:H7 (Lee *et al.*, 2007b).

A variety of bacteria produce indole from L-tryptophan such as *E. coli* (Crawford and Yanofsky, 1958), *Vibrio vulnificus* (Dalsgaard *et al.*, 1999), *Haemophilus influenzae* (Stull *et al.*, 1995), *Pasteurella multocida* (Clemons and Gadberry, 1982), *Klebsiella oxytoca* (Liu *et al.*, 1997) and *Proteus vulgaris* (DeMoss and Moser, 1969); a NCBI BLAST search shows more than 27 genera utilize tryptophanase (*tnaA*) to convert tryptophan into indole, pyruvate and ammonia (Stewart and Yanofsky, 1985). Indole is an extracellular *E. coli* signal (Wang *et al.*, 2001) that inhibits biofilms (Lee *et al.*, 2007a), and it works in a QS fashion (Lee *et al.*, 2007b) primarily at temperatures less than 37°C in *E. coli* (Lee *et al.*, 2008). Indole with *E. coli* also influences motility, acid resistance, chemotaxis and attachment to epithelial cells (Domka *et al.*, 2006; Bansal *et al.*, 2007; Lee *et al.*, 2007a,b). It also controls plasmid stability by delaying cell division in *E. coli* (Chant and Summers, 2007), induces the expression of multidrug exporter genes and increases drug resistance (Hirakawa *et al.*, 2005). Hence, indole is widespread in the environment, controls many phenotypes and may be encountered by other bacteria.

*Pseudomonas aeruginosa* is virulent to a variety of hosts including man, plants and invertebrates (Lewenza

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et al., 2005). *Pseudomonas aeruginosa* captures iron with two endogenous siderophores, pyoverdine and pyochelin (Michel et al., 2005), and both contribute to its virulence (Cox, 1982; Meyer et al., 1996). RhlR-regulated rhamnolipids are glycolipids with biosurfactant properties that are involved in bacterial virulence (Zulianello et al., 2006). Pyocyanin from *P. aeruginosa* has antibiotic activity towards competing bacteria including indole-producing *E. coli* (Hassett et al., 1992), and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) is structurally similar to pyocyanin and possesses antimicrobial activity (Mashburn and Whiteley, 2005). Therefore, *P. aeruginosa* utilizes several virulence factors.

It is likely that *P. aeruginosa* encounters indole because (i) *P. aeruginosa* is ubiquitous (Stover et al., 2000), (ii) *P. aeruginosa* is found in the gut where *E. coli* is dominant (Wang et al., 2006) and produces gut-derived generalized sepsis (Matsumoto et al., 1999), and (iii) *P. aeruginosa* may also encounter *E. coli* outside the human body as *E. coli* exists outside the human host as well. Hence, it is possible *Pseudomonas* encounters indole and 7-hydroxyindole (7HI).

In this study, we used both DNA microarrays and phenotype arrays to investigate the impact of the *E. coli* signal indole and hydroxylated indole (7HI) on global gene expression and QS-regulated phenotypes of *P. aeruginosa* PAO1. Indole and 7HI altered extensively gene expression, decreased the production of various virulence factors, decreased swarming motility and increased antibiotic resistance in *P. aeruginosa*. Furthermore, 7HI reduced colonization of *P. aeruginosa* in guinea pig lungs and increased clearance in the gastrointestinal region.

## Results

### *Indole and 7HI are non-toxic*

To test the toxicity with *P. aeruginosa*, the specific growth rate was measured with indole, 7HI and plant hormone indole-3-acetic acid (IAA, structurally similar plant hormone which served as a negative control). In the absence of indole compounds, the specific growth rate was  $1.5 \pm 0.1 \text{ h}^{-1}$  whereas the growth rate was  $1.51 \pm 0.06 \text{ h}^{-1}$  with 1.0 mM indole,  $1.89 \pm 0.01 \text{ h}^{-1}$  with 0.5 mM 7HI and  $1.64 \pm 0.04 \text{ h}^{-1}$  with 1.0 mM IAA. Hence, indole, 7HI and IAA do not decrease the specific growth rate of *P. aeruginosa* at these concentrations, and the effects of indole and 7HI on *P. aeruginosa* are not due to toxicity. However, higher concentrations (2 mM) decreased the specific growth by 14% for indole ( $1.31 \pm 0.01 \text{ h}^{-1}$ ) and by 47% for 7HI ( $0.8 \pm 0.2 \text{ h}^{-1}$ ).

### *Indole and 7HI stimulate biofilm formation*

As indole and 7HI increased *P. aeruginosa* biofilm formation at 30°C (Lee et al., 2007b), we tested different con-

centrations of indole, 7HI and IAA for their ability to affect *P. aeruginosa* biofilm formation in 96-well plates after 24 h at 37°C (Fig. S1). Indole increased *P. aeruginosa* biofilm formation up to  $1.8 \pm 0.6$ -fold (0–1 mM) compared with no indole addition and did not affect cell growth. 7HI also increased biofilm formation  $2.5 \pm 0.8$ -fold in a dose-response manner (0–0.75 mM) compared with no 7HI addition without affecting cell growth. Notably, 7HI (0.5 mM) increased the liquid/solid (bottom) biofilm formation sixfold ( $0.60 \pm 0.21$  versus  $0.10 \pm 0.07$ ). Therefore, indole and 7HI increase *P. aeruginosa* biofilm formation although *P. aeruginosa* does not synthesize these compounds. However, IAA did not influence the biofilm formation of *P. aeruginosa* (Fig. S1).

### *Indole and 7HI are global regulators*

To investigate the genetic mechanism of biofilm enhancement by indole and 7HI on a global basis, we performed three sets of microarray experiments with 7 h *P. aeruginosa* biofilm cells: (i) a comparison of cells with and without 1.0 mM indole, (ii) a comparison of cells with and without 0.5 mM 7HI, and (iii) a comparison of cells with and without 1.0 mM IAA (negative control). The expression data for the biofilm samples are summarized in Table 1.

Overall, we found the addition of indole regulates significantly 532 genes more than twofold in biofilm cells at 7 h; 88 genes were induced and 444 genes were repressed. Similarly, the addition of 7HI regulates significantly 733 genes more than twofold in biofilm cells at 7 h; 25 genes were induced and 708 genes were repressed. In contrast, the addition of IAA altered expression of 10-fold fewer genes (only 71 genes more than twofold); 57 genes were induced and 14 genes were repressed. Therefore, indole and 7HI primarily repress genes in *P. aeruginosa*. To confirm the presence of indole and 7HI in the microarray samples, the extracellular indole and 7HI were measured using HPLC. The level of indole decreased from 1.0 mM to 0.68 mM in 7 h (the negative control without indole addition showed zero indole). Also, the level of extracellular 7HI decreased from 0.5 mM to 0.154 mM after 7 h.

### *Indole represses the mexGHI-opmD multidrug efflux genes and QS-regulated genes*

The most noticeable change of gene expression with indole was that the *mexGHI-opmD* multidrug efflux genes were highly repressed (6- to 13-fold), and many genes involved in the synthesis of QS-regulated virulence factors were repressed (Table 1). Specifically, the phenazine synthesis operon (*phz*) which is involved in the pyocyanin biosynthesis, the PQS synthesis operon (*pqs*), the

**Table 1.** Indole and 7HI regulate quorum-sensing genes in *P. aeruginosa* PAO1.

PA#	Gene	Fold change <sup>a</sup>			Description
		Indole versus no indole	7HI versus no 7HI	IAA versus no IAA	
<b>Multidrug efflux transporter</b>					
PA4205	<i>mexG</i>	-13.0	-7.0	-1.1	Hypothetical protein, part of the <i>mexGHI-opmD</i> cluster
PA4206	<i>mexH</i>	-11.3	-5.7	-1.3	Efflux membrane fusion protein precursor
PA4207	<i>mexI</i>	-6.1	-4.0	-1.3	Efflux transporter
PA4208	<i>opmD</i>	-7.5	-4.6	-1.1	Probable outer membrane efflux protein
<b>Phenazine (pyocyanin) synthesis</b>					
PA1901	<i>phzC2</i>	-2.5	-2.1	-1.2	Phenazine biosynthesis protein PhzC
PA1902	<i>phzD2</i>	-3.2	-3.0	-1.1	Phenazine biosynthesis protein PhzD
PA1903	<i>phzE2</i>	-3.5	-2.6	-1.2	Phenazine biosynthesis protein PhzE
PA1904	<i>phzF2</i>	-4.0	-4.3	-1.1	Phenazine biosynthesis protein
PA1905	<i>phzG2</i>	-3.2	-3.2	-1.2	Pyridoxamine 5'-phosphate oxidase
PA2274		-4.9	-3.2	-1.1	Possible monooxygenase, involved in phenazine biosynthesis
PA4210	<i>phzA1</i>	-2.5	-3.7	-1.1	Phenazine biosynthesis protein
PA4211	<i>phzB1</i>	-2.3	-2.3	-1.3	Phenazine biosynthesis protein
PA4217	<i>phzS</i>	-2.8	-2.8	-1.1	Flavin-containing monooxygenase
<b>QQS synthesis</b>					
PA0996	<i>pqsA</i>	-1.9	-2.0	-1.1	PqsA, probable coenzyme A ligase
PA0997	<i>pqsB</i>	-2.3	-2.5	-1.1	PqsB, homologous to beta-keto-acyl-carrier protein synthase
PA0998	<i>pqsC</i>	-1.7	-1.9	-1.1	PqsC, homologous to beta-keto-acyl-carrier protein synthase
PA0999	<i>pqsD</i>	-1.3	-1.9	-1.1	PqsD, 3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000	<i>pqsE</i>	-2.0	-2.1	-1.1	PqsE, quinolone signal response protein
PA1003	<i>mvfR</i>	1.3	-1.1	-1.1	MvfR (PqsR), QQS transcriptional regulator
<b>Pyochelin synthesis</b>					
PA4225	<i>pchF</i>	-2.0	-1.1	1.3	Pyochelin synthetase
PA4226	<i>pchE</i>	-2.8	-1.3	1.2	Dihydroaeruginic acid synthetase
PA4227	<i>pchR</i>	-1.5	-1.2	1.1	Transcriptional regulator PchR
PA4228	<i>pchD</i>	-3.2	-1.3	1.2	Pyochelin biosynthesis protein PchD
PA4229	<i>pchC</i>	-2.3	-1.1	1.2	Pyochelin biosynthetic protein PchC
PA4230	<i>pchB</i>	-2.0	-1.2	1.2	Salicylate biosynthesis protein PchB
PA4231	<i>pchA</i>	-2.1	-1.3	1.2	Salicylate biosynthesis isochorismate synthase
<b>Pyoverdine synthesis</b>					
PA2385	<i>pvdQ</i>	-2.1	-1.6	1.1	pyoverdine biosynthesis protein PvdQ
PA2393	<i>pvdM</i>	-2.5	-1.1	2.1	pyoverdine biosynthesis protein PvdM
PA2394	<i>pvdN</i>	-2.5	-1.2	1.3	pyoverdine biosynthesis protein PvdN
PA2395	<i>pvdO</i>	-2.0	-1.1	1.5	pyoverdine biosynthesis protein PvdO
PA2397	<i>pvdE</i>	-2.0	-1.4	1.1	pyoverdine biosynthesis protein PvdE
PA2424	<i>pvdL</i>	-2.3	-1.3	1.9	pyoverdine biosynthesis protein PvdV
PA2426	<i>pvdS</i>	-6.1	-2.6	2.0	Sigma factor PvdS, transcriptional regulator
<b>Metabolism</b>					
PA0283	<i>sbp</i>	-8.6	-1.7	1.3	Sulfate-binding protein precursor
PA1838	<i>cysI</i>	-6.1	-2.1	1.2	Sulfite reductase
PA3441	<i>ssuF</i>	-7.5	-5.7	1.2	SsuF, part of the <i>ssu</i> locus
PA3442	<i>ssuB</i>	-4.6	-2.6	1.3	SsuB, part of the <i>ssu</i> locus
PA3443	<i>ssuC</i>	-3.2	-2.6	1.3	SsuC, part of the <i>ssu</i> locus
PA3444	<i>ssuD</i>	-6.1	-2.1	1.1	SsuD, part of the <i>ssu</i> locus
PA3445	<i>ssuA</i>	-6.5	-2.8	2.8	SsuA, part of the <i>ssu</i> locus
PA3446	<i>ssuE</i>	-6.5	-1.6	1.5	SsuE, part of the <i>ssu</i> locus
PA4442	<i>cysN</i>	-4.6	-2.0	-1.1	ATP sulfurylase GTP-binding subunit
PA4443	<i>cysD</i>	-4.9	-1.7	1.2	ATP sulfurylase small subunit
PA5025	<i>metY</i>	-2.6	-1.3	-1.1	Homocysteine synthase
PA5427	<i>adhA</i>	2.1	2.6	-1.1	Alcohol dehydrogenase
<b>Fimbriae and pili</b>					
PA2131		-1.9	-2.6	1.0	Probable pili assembly chaperone
PA2570	<i>pa1L</i>	1.6	-2.8	1.1	PA-I galactophilic lectin
PA4084	<i>cupB3</i>	-2.8	-4.0	1.5	Probable fimbrial biogenesis usher protein
PA4085	<i>cupB2</i>	-3.0	-2.1	1.1	Probable pili assembly chaperone
PA4297	<i>tadG</i>	2.3	3.5	1.7	TadG
PA4298		1.6	-3.2	1.2	Hypothetical protein
PA4299	<i>tadD</i>	1.7	-2.6	1.1	Flp pilus assembly protein TadD
PA4300	<i>tadC</i>	1.7	-2.3	1.3	Flp pilus assembly protein TadC
PA4301	<i>tadB</i>	1.6	-3.0	1.5	Flp pilus assembly protein TadB
PA4302	<i>tadA</i>	1.9	-2.5	1.2	Flp pilus assembly protein, ATPase CpaF

Table 1. cont.

PA#	Gene	Fold change <sup>a</sup>			Description
		Indole versus no indole	7HI versus no 7HI	IAA versus no IAA	
PA4303	<i>tadZ</i>	1.7	-2.3	1.2	Flp pilus assembly protein, ATPase CpaE
PA4304	<i>rcpA</i>	2.0	-1.9	1.0	Flp pilus assembly protein, secretin CpaC
PA4305	<i>rcpC</i>	1.6	-2.5	1.1	Flp pilus assembly protein CpaB
PA4306	<i>flp</i>	1.5	-2.8	1.1	Flp pilus assembly protein, pilin Flp
PA4651		2.0	-3.5	1.3	Probable pili assembly chaperone
<b>Transport of small molecules</b>					
PA1108		-1.9	-5.7	1.5	Probable MFS transporter
PA2092		-2.6	-4.0	1.4	Probable MFS transporter
PA2204		-9.8	-1.9	1.1	Binding protein component of ABC transporter
PA2328		-6.1	-5.3	1.1	Hypothetical protein in the cluster of ABC transporter
PA2329		-5.7	-5.7	1.0	Probable ATP-binding component of ABC transporter
PA2330		-4.9	-4.3	1.1	Hypothetical protein in the cluster of ABC transporter
PA2331		-5.7	-4.0	-1.1	Hypothetical protein in the cluster of ABC transporter
PA3531	<i>bfrB</i>	3.2	2.3	-1.2	Bacterioferritin, transport of small molecules
PA3926		-1.7	-2.0	1.3	Probable MFS transporter
PA4514	<i>piuA</i>	-4.3	-2.5	1.1	Probable outer membrane receptor for iron transport
<b>Oxygenases</b>					
PA2512	<i>antA</i>	5.3	-2.0	9.2	Anthranilate dioxygenase large subunit
PA2513	<i>antB</i>	6.1	-2.6	1.6	Anthranilate dioxygenase small subunit
PA2514	<i>antC</i>	3.0	-2.8	1.6	Anthranilate dioxygenase reductase
PA0106	<i>coxA</i>	3.2	1.4	1.0	Cytochrome <i>c</i> oxidase, subunit I
PA0107		2.8	1.3	-1.1	Predicted cytochrome oxidase assembly factor
PA0108	<i>coIII</i>	3.2	1.2	-1.1	Cytochrome <i>c</i> oxidase, subunit III
PA0109		2.0	-1.1	1.1	Hypothetical protein in the cluster of oxidase
PA0110		2.6	1.4	1.1	Hypothetical protein in the cluster of oxidase
PA0111		3.0	1.3	1.1	Hypothetical protein in the cluster of oxidase
PA0112		3.0	1.1	1.1	Hypothetical protein in the cluster of oxidase
PA0113		3.0	1.4	-1.3	Probable cytochrome <i>c</i> oxidase assembly
<b>Others</b>					
PA0715		3.7	1.4	-2.0	Probable bacteriophage protein
PA0716		5.7	7.0	-1.3	Probable bacteriophage protein
PA0740	<i>sdsA1</i>	-8.6	-3.0	1.1	Probable beta-lactamase
PA1109		-2.6	-5.7	2.5	Transcriptional regulator
PA2258	<i>ptxR</i>	-1.6	-3.2	1.9	Transcriptional regulator PtxR
PA3153	<i>wzx</i>	2.6	1.0	-1.4	O-antigen translocase
PA3154	<i>wzy</i>	3.5	1.0	-1.3	B-band O-antigen polymerase
PA3155	<i>wbpE</i>	2.8	1.5	-1.2	Probable aminotransferase WbpE
PA3156	<i>wbpD</i>	2.3	1.4	-1.1	Probable acetyltransferase
PA3234	<i>yjcG</i>	4.3	2.0	-1.1	Probable sodium:solute symporter
PA3337	<i>rfaD</i>	2.0	2.5	1.1	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3450	<i>IsfA</i>	-6.1	-1.4	1.1	Probable antioxidant protein
PA3721	<i>nalC</i>	3.0	-1.1	1.6	Transcriptional regulator NalC, induced by MvfR
PA4563	<i>rpsT</i>	2.8	2.8	-1.1	30S ribosomal protein S20
<b>Hypothetical proteins</b>					
PA0284		-13.9	-1.6	1.2	Hypothetical protein, induced by MvfR
PA0492		-4.3	-3.5	1.9	Hypothetical protein
PA0696		-4.3	-2.5	1.3	Hypothetical protein
PA0939		-1.1	-5.3	1.6	Hypothetical protein
PA1190		5.7	1.6	1.2	Hypothetical protein
PA1837		-4.6	-2.1	1.1	Hypothetical protein
PA1914		1.1	-6.1	1.1	Hypothetical protein
PA1953		-3.2	-6.1	1.5	Hypothetical protein
PA2036		-2.0	-7.0	1.1	Hypothetical protein
PA2078		-3.0	-5.7	1.6	Hypothetical protein
PA2419		-1.6	6.1	1.5	Hypothetical protein
PA2805		2.3	2.1	1.1	Hypothetical protein
PA3080		3.2	1.1	-1.1	Hypothetical protein
PA3235		4.0	2.5	1.1	Hypothetical protein
PA3237		-1.9	-6.1	1.9	Hypothetical protein
PA3572		2.0	2.5	1.1	Hypothetical protein
PA3719		4.6	1.3	1.3	Hypothetical protein
PA3720		2.6	1.2	1.6	Hypothetical protein
PA3931		-5.3	-1.7	1.0	Hypothetical protein

Table 1. *cont.*

PA#	Gene	Fold change <sup>a</sup>			Description
		Indole versus no indole	7HI versus no 7HI	IAA versus no IAA	
PA4087		<b>-2.5</b>	<b>-3.5</b>	1.0	Hypothetical protein
PA4359		<b>-4.0</b>	-1.1	1.3	Hypothetical protein
PA4377		<b>3.7</b>	1.7	1.4	Hypothetical protein
PA4614		<b>3.5</b>	1.4	1.1	Hypothetical protein
PA4638		<b>6.5</b>	-1.6	-1.2	Hypothetical protein
PA4683		<b>-2.8</b>	-1.3	1.1	Hypothetical protein

a. Partial list of differentially expressed genes in LB medium after 7 h for (i) biofilm cells grown with 1.0 mM indole versus no indole, (ii) biofilm cells grown with 0.5 mM 7-hydroxyindole (7HI) versus no 7HI and (iii) biofilm cells grown with 1.0 mM indole-3-acetic acid (IAA, control compound) versus no IAA. Most significant changes are shown in bold.

pyochelin synthesis operon (*pch*) and the pyoverdine synthesis operon (*pvd*) were repressed (two- to sixfold) by indole. Also, several genes involved in the transport of small molecules were repressed by indole. Among genes involved in metabolism, genes involved in sulfur metabolism, such as *sbp*, the whole *ssuFBCDAE* locus that plays a key role in organosulfur uptake (Kahnert *et al.*, 2000), and *cysI* and *cysND*, were significantly repressed (three- to eightfold) by indole (Table 1). This suggests that indole downregulates sulfur uptake of *P. aeruginosa*. In contrast, several oxidase genes and *antABC*-encoding anthranilate dioxygenase genes were induced (three- to sixfold) by indole, while 7HI repressed the *antABC* operon. However, the transcriptional level of the QS signal regulators, such as RhlRI, LasRI and MvfR (PqsR), was not altered by indole or 7HI.

#### 7HI represses the *mexGHI-opmD* multidrug efflux genes and QS-regulated genes

Like indole, 7HI repressed (four- to sevenfold) the four *mexGHI-opmD* multidrug efflux genes and genes involved in the synthesis of QS-regulated virulence factors (e.g. *phz* operon, *pqs* operon and *pvdS*) (Table 1). Unlike indole, 7HI did not change the expression of pyochelin synthesis genes.

Note, the control IAA did not change the transcription of nearly all the genes involved in virulence (Table 1); therefore, the impact on virulence is specific for indole and 7HI. The most induced gene (9.2-fold) in the presence of IAA was *antA* (encodes the anthranilate dioxygenase large subunit). Also induced were six genes involved in metabolism (two- to threefold) and 12 genes involved in the transport of small molecules (two- to eightfold).

#### Verification of DNA microarray results

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to verify gene expression for

several of the highly differentially expressed loci in the two sets of the DNA microarray experiments (response to indole and 7HI). Using independent cultures, RT-PCR showed differential changes in expression comparable to the DNA microarrays. For the indole experiments, both techniques (RT-PCR versus microarray) showed the genes were repressed in a similar manner: 14.1- versus 13.0-fold for *mexG*, 26.4- versus 11.3-fold for *mexH*, 1.9- versus 6.1-fold for *mexI*, 5.6- versus 6.1-fold for *pvdS*, 10.0- versus 7.5-fold for *ssuF* and 12.7- versus 13.9-fold for PA0284. Similarly, for the 7HI experiments, both techniques (RT-PCR versus microarray) showed the genes were repressed in a similar manner: 54.7- versus 7.0-fold for *mexG*, 4.0- versus 5.7-fold for *mexH*, 2.8- versus 4.0-fold for *mexI*, 3.8- versus 2.6-fold for *pvdS*, 3.0- versus 5.7-fold for *ssuF* and 2.6- versus 1.6-fold for PA0284.

#### Indole and 7HI decrease QS-regulated virulence phenotypes

As the microarray data showed that indole and 7HI repressed genes involved in the synthesis of QS-regulated virulence factors (Table 1), we assayed phenotypes related to the three QS systems of *P. aeruginosa* including the RhlR-regulated phenotypes [pyocyanin (Gallagher *et al.*, 2002), rhamnolipid production (Ochsner and Reiser, 1995) and swarming motility (Déziel *et al.*, 2003)], LasR- and RhlR-regulated PQS production (Diggle *et al.*, 2006) and LasR-regulated elastase production (Gambello and Iglewski, 1991). Production of the siderophore pyoverdine, a virulence factor in *P. aeruginosa* (Lamont and Martin, 2003), was also measured as indole and 7HI repressed the pyoverdine synthesis genes and a transcriptional regulator *pvdS* (Table 1).

As expected, indole and 7HI decreased PQS production by  $5.0 \pm 2$ -fold and  $10 \pm 5$ -fold, decreased pyocyanin production by  $7.1 \pm 0.6$ -fold and  $11 \pm 3$ -fold, decreased rhamnolipid production by  $2.2 \pm 0.7$ -fold and  $2.0 \pm 0.5$ -fold, and decreased pyoverdine production by

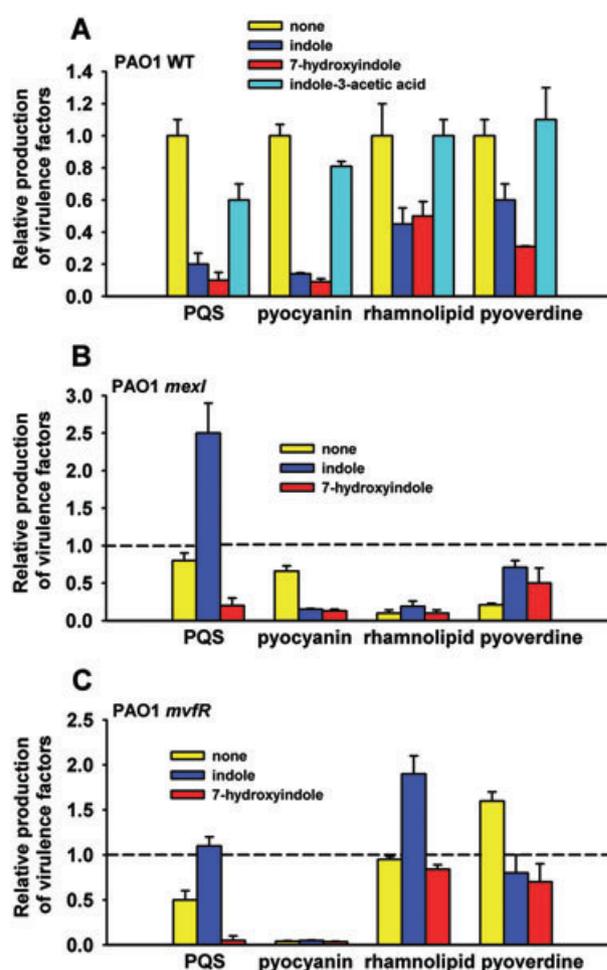
1.7 ± 0.3-fold and 3.2 ± 0.3-fold respectively (Figs 1A and S2 for PQS production); however, indole and 7HI did not alter elastase production. Therefore, the decreased production of these QS-regulated virulence factors upon addition of indole and 7HI corroborated the microarray data (Table 1). Notably, the impact of 7HI on the production of PQS and pyoverdine was more significant than that with indole. In contrast, IAA did not significantly change the production of the four virulence factors; hence the changes in the regulated phenotypes are specific for indole and 7HI.

#### 7HI abolishes swarming

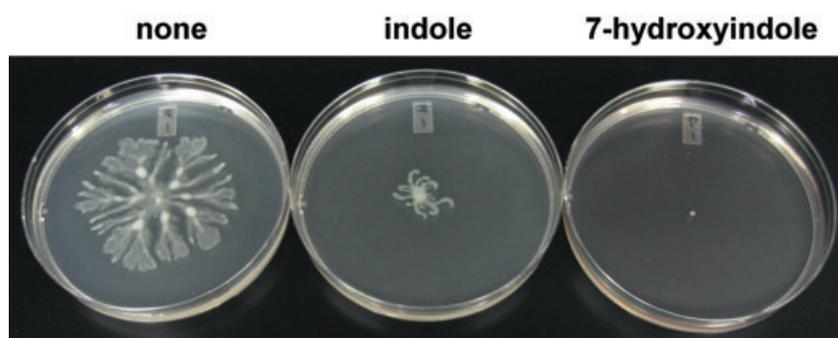
The impact of indole and 7HI on the swimming, swarming and twitching motility of *P. aeruginosa* was tested as swarming motility plays an important role in *P. aeruginosa* biofilm formation (Overhage *et al.*, 2007), swarming is related to QS (Köhler *et al.*, 2000), and our microarray data showed that QS genes (e.g. *phz* operon, *pqs* operon and *pvdS*) were repressed by 7HI (Table 1). Indole and 7HI both decreased slightly swimming motility; the swimming halo diameters at 24 h were 3.24 ± 0.08 cm (no addition), 2.75 ± 0.01 cm (indole) and 2.28 ± 0.01 cm (7HI). Twitching motility was not significantly changed with indole and 7HI. However, indole significantly decreased swarming motility, and 7HI abolished swarming motility (Fig. 2).

#### Indole and 7HI increase antibiotic resistance

As MexGHI-OpmD efflux pump mutants of *P. aeruginosa* show enhanced antibiotic resistance (Aendekerk *et al.*, 2005) and as our microarray data showed that these efflux genes were the most repressed genes by indole and 7HI (Table 1), we assayed the antibiotic resistance of *P. aeruginosa* upon addition of indole and 7HI with five antibiotics. Four antibiotics (tetracycline, gentamicin, kanamycin and vanadyl sulfate) were investigated by



**Fig. 1.** Reduction of virulence factors by indole and 7HI. Production of virulence factors with 1.0 mM indole, 0.5 mM 7HI and 1.0 mM IAA (negative control) with *P. aeruginosa* PAO1 (A), with *P. aeruginosa* PAO1 *mexI* (B) and *P. aeruginosa* PAO1 *mvfR* (C). For clarity, wild-type values are not shown in (B) and (C) so bars indicate the relative amount of each compound made compared with the wild-type strain without indole or 7HI added. Each experiment was performed with at least two independent cultures. Data show the average of the replicates, and one standard deviation is shown.



**Fig. 2.** Inhibition of swarming motility by indole and 7HI. Swarming motility of *P. aeruginosa* on BM2 medium with 0.5% agar with 1.0 mM indole and 0.5 mM 7HI after 28 h. Each experiment was performed with at least two independent cultures and one representative data set is shown.

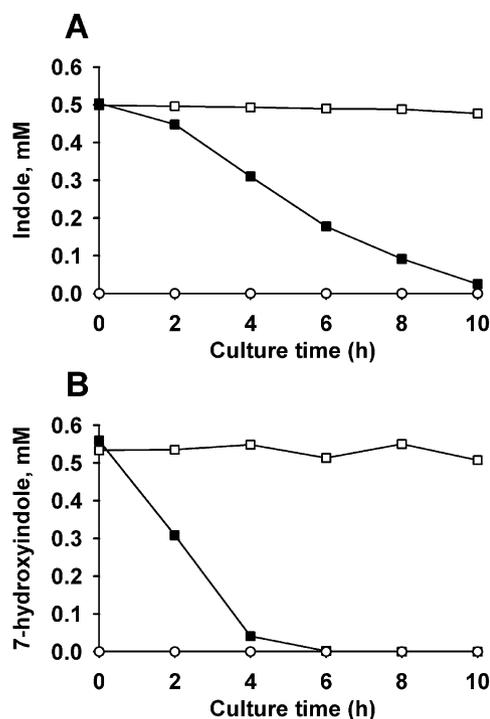
measuring the survival rate, and ampicillin was investigated by using a minimal inhibition concentration (MIC) method. Indole and 7HI increased tetracycline resistance by  $5 \pm 3$ -fold and  $4 \pm 3$ -fold, respectively, and increased gentamicin resistance by  $2 \pm 2$ -fold and  $3 \pm 2$ -fold, respectively, and 7HI increased kanamycin resistance by  $2.5 \pm 0.5$ -fold (Fig. S3). Hence, our results agreed well with previous results using *mexI* and *opmD* mutants with kanamycin and tetracycline (Aendekerck *et al.*, 2005) in that repression of MexGHI-OpmD by indole and 7HI enhanced antibiotic resistance consistently. However, indole and 7HI did not change the survival rates ( $\sim 55\%$ ) in the presence of 5 mM vanadyl sulfate (Aendekerck *et al.*, 2002). Similarly, the MIC of ampicillin was increased in the presence of indole and 7HI ( $300 \mu\text{g ml}^{-1}$  for no indole or 7HI,  $500 \mu\text{g ml}^{-1}$  with indole and  $600 \mu\text{g ml}^{-1}$  with 7HI). Therefore, indole and 7HI enhance *P. aeruginosa* antibiotic resistance.

#### Indole affects many cell phenotypes

As the regulation of 532 genes were altered by adding indole as shown by the DNA microarrays (Table 1), we explored the impact of adding indole to *P. aeruginosa* on 756 phenotypes using phenotype arrays (Table S1). The phenotype arrays showed that the addition of indole severely suppressed growth with many carbon sources (e.g. D-saccharic acid, L-alanine, glucuronamide, M-tartaric acid, D-aspartic acid and  $\alpha$ -methyl-D-galactoside), many nitrogen sources (e.g. xanthosine, tyramine, cytidine, cytosine, guanine and D-lysine), many phosphorus sources (e.g. D-mannose-1-phosphate, D-2-phospho-glyceric acid and cytidine-2-monophosphate) and three sulfur sources (lanthionine, glutathione, and L-cysteine). The reduced cell growth in the presence of indole with three sulfur sources corroborates our microarray data in that indole repressed genes involved in sulfur uptake (*sbp*, *ssuFBCDAE*, *cysI* and *cysND*) (Table 1), which suggests that indole decreases sulfur uptake in *P. aeruginosa*. Overall, indole alters the carbon, nitrogen, sulfur and phosphorus metabolism of *P. aeruginosa*.

#### *P. aeruginosa* degrades indole and 7HI

In the microarray samples of *P. aeruginosa*, the concentration of indole and 7HI decreased significantly after 7 h (1.0 mM indole decreased to 0.68 mM and 0.5 mM 7HI decreased to 0.154 mM); hence, the degradation of indole and 7HI was quantified by measuring the concentration of extracellular indole and 7HI using HPLC. It was found that *P. aeruginosa* degrades indole in LB medium at  $1.04 \pm 0.04 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  and degrades 7HI in LB medium at  $2.6 \pm 0.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  while there was no significant change of indole and 7HI con-



**Fig. 3.** Degradation of indole and 7HI by *P. aeruginosa*. *Pseudomonas aeruginosa* degrades 0.5 mM indole (A) and 0.5 mM 7HI (B) in LB. The initial turbidity of cells was 1.0 at 600 nm. Closed square data (■) are from live cells, open square data (□) are from autoclaved cells (dead cell control) and open circle data (○) are from live cells that lack added indole or 7HI. Each experiment was performed using two independent cultures, and one representative data set is shown.

centrations with the negative control (dead cells) (Fig. 3A and B). Furthermore, *P. aeruginosa* PAO1 does not utilize either indole (1 or 2 mM) or 7HI (0.5 or 2 mM) as a sole source of carbon and energy (data not shown) although *P. aeruginosa* Gs isolated from mangrove sediments (Yin *et al.*, 2005) and *Pseudomonas* sp. ST-200 from soil (Doukyu and Aono, 1997) grow on indole.

In the whole-transcriptome analysis, *antABC* was the most induced locus upon indole addition (Table 1); hence, we investigated if anthranilate dioxygenase is necessary for indole degradation. We found that the *P. aeruginosa* PAO1 *antA* mutant degraded indole to the same extent as the wild-type strain; therefore, indole degradation is not related to anthranilate metabolism. Furthermore, as addition of the negative control IAA highly induced *antA* (Table 1), it appears both IAA and indole induce the *ant* locus due to some non-specific interaction with a regulatory protein rather as substrates.

#### Indole and 7HI are less effective in the *mexI* and *mvfR* mutants

As the most highly repressed locus in the presence of indole and 7HI was *mexGHI-opmD* (Table 1) and as muta-

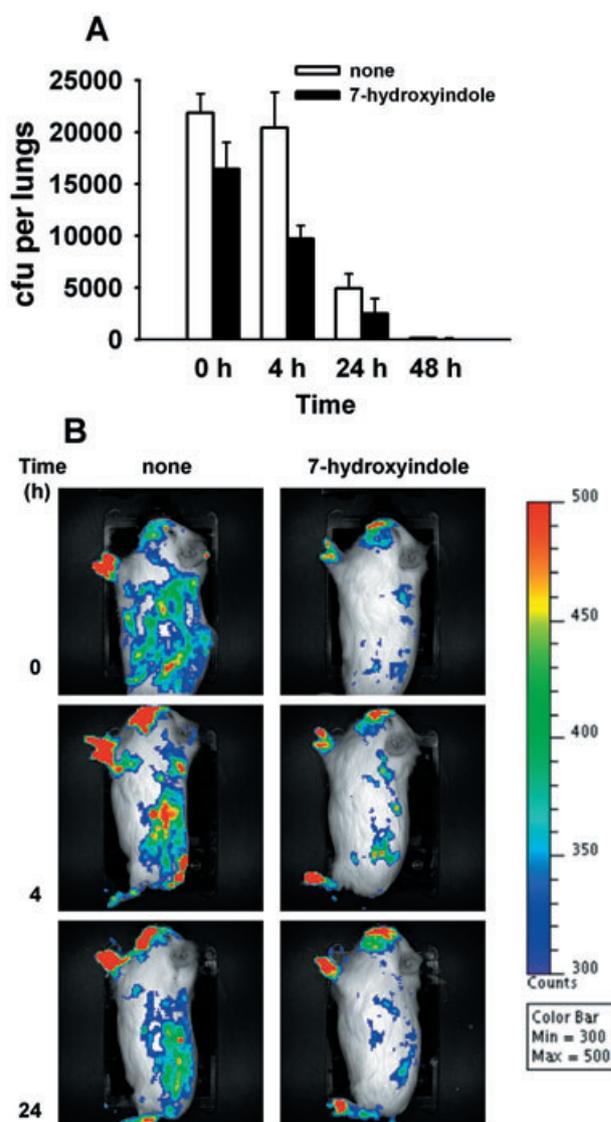
tion of *mexI* decreased the production of rhamnolipid and pyoverdine (Aendekerk *et al.*, 2002), the effect of indole and 7HI was further investigated with *P. aeruginosa* PAO1 *mexI*. Consistent with a previous report (Aendekerk *et al.*, 2002), the *mexI* mutation reduced rhamnolipid and pyoverdine by 10- and 5-fold respectively (Fig. 1B). However, unlike with the wild-type strain, indole did not decrease the production of PQS, rhamnolipid and pyoverdine in the *mexI* strain (Fig. 1B), and 7HI also did not decrease the production of rhamnolipid and pyoverdine in the *mexI* strain. These results suggest *mexI* is involved in a complex manner with the decrease of virulence factors with indole and 7HI. In contrast, indole and 7HI significantly decreased pyocyanin production in the *mexI* strain probably due to the presence of two different pathways for pyocyanin synthesis, the *aro* pathway and the *phz* pathway (Lau *et al.*, 2004).

As the transcriptional regulator MvfR is required for full *P. aeruginosa* virulence and induces both *mexGHI-opmD* and phenazine transcription (*phz* operon that is involved in pyocyanin biosynthesis) (Déziel *et al.*, 2005), the effect of indole and 7HI was also investigated with the *P. aeruginosa* *mvfR* mutant. As expected, PQS and pyocyanin were decreased in the *mvfR* mutant compared with the wild-type strain (Fig. 1C). However, unlike with the wild-type strain, the addition of indole did not decrease the production of PQS, rhamnolipid and pyocyanin. These results suggest that indole requires MvfR to fully reduce virulence factors.

In addition, the *P. aeruginosa* PAO1 *antA* mutant was investigated as indole diminished PQS production (Fig. 1A) while inducing the *antABC* (Table 1); *antABC* encode proteins involved in the degradation of anthranilate, a precursor of PQS. As expected, the *antA* mutant produced about 1.8-fold more PQS (Fig. S4) (as there is a larger pool of anthranilate for PQS). However, the response of the *antA* mutant upon addition of indole was similar to that of the wild-type strain. Hence, *antA* is not directly involved in the indole mechanism to control virulence factors.

#### 7HI reduces *P. aeruginosa* virulence in guinea pigs

As 7HI diminished virulence factors in *P. aeruginosa* more than indole (Table 1 and Fig. 1) and as 7HI abolished swarming motility (Fig. 2), we evaluated the ability of *P. aeruginosa* pre-treated with 7HI or diluent dimethylformamide (DMF) as a control to colonize guinea pig lungs after infection. Pulmonary colonization was examined immediately after infection until 48 h post infection (Fig. 4A). Colonization of the lung by *P. aeruginosa* treated with 7HI was reduced by 25%, and 7HI-treated bacteria are cleared more easily from the lungs for the first 4 h post infection, leading to a greater than 50% reduction in pulmonary bacteria by this time point. In the acute model, both the 7HI-



**Fig. 4.** Reduction of virulence of *P. aeruginosa* in guinea pigs. **A.** Colonization and clearance of *P. aeruginosa* pre-treated with 7HI or solvent (DMF) prior to infection of guinea pigs by aerosol with  $\sim 2 \times 10^5$  cfu. Average of five replicates, and one standard deviation is shown.

**B.** Real-time analysis of *P. aeruginosa* pre-treated with 7HI or solvent (DMF) in the acute guinea pig infection model (representative guinea pigs are shown for each group and are imaged laterally) using the Xenogen IVIS CCD camera. Colour bar represents the intensity of luminescent signal in photons  $s^{-1} cm^{-2}$  from low (blue) to high (red).

and DMF-treated *P. aeruginosa* continue to be expelled from the lungs, resulting in nearly complete clearance of the bacterial pneumonia by 48 h. As it was not possible to maintain 7HI levels with the bacteria *in vivo*, the differences between DMF- and 7HI-treated bacteria decrease over the course of this experiment, as expected.

We used *in vivo* bioluminescence imaging to obtain a more comprehensive picture of the tissue colonization by DMF- and 7HI-treated *P. aeruginosa* and to confirm

differences in pulmonary colonization in guinea pigs (Fig. 4B). Guinea pigs infected by aerosol were colonized in the nasopharynx, lungs, liver and gastrointestinal area (Fig. 4B), similar to previous studies in mice (DiGiandomenico *et al.*, 2007). As 7HI did not affect bioluminescence and as equal amounts of *P. aeruginosa* cells was used to infect the guinea pigs, the difference in colonization of *P. aeruginosa* was due to the treatment of 7HI. Similar to data obtained by counting colony-forming units (cfu) in the guinea pig lungs, pulmonary clearance occurs rapidly for 7HI-treated *P. aeruginosa*, with low bioluminescence signal observed in the pulmonary region by 4 h in this group ( $2.05 \pm 0.04 \times 10^5$  photons  $s^{-1} cm^{-2}$ ) as compared with the DMF-treated group ( $3.59 \pm 0.47 \times 10^5$  photons  $s^{-1} cm^{-2}$ ). Lower colonization ( $P < 0.03$ ) of all organs was observed for animals infected with 7HI-treated bacteria ( $3.5 \pm 0.1 \times 10^6$  photons  $s^{-1} cm^{-2}$ ) as compared with DMF-treated bacteria ( $4.19 \pm 0.08 \times 10^6$  photons  $s^{-1} cm^{-2}$ ). In addition, regional lymph nodes do not appear to be significantly involved, primarily with colonization of the gastrointestinal region. These observations could indicate that dissemination of *P. aeruginosa* to extrapulmonary and extra-intestinal sites is also reduced, in addition to initial colonization. Further studies are needed to definitively identify the sites and tissues infected, despite these intriguing observations. Therefore, both cfu and bioluminescence imaging confirm that 7HI pre-treatment of *P. aeruginosa* decreases colonization of guinea pig tissues.

Additionally, indole- and 7HI-treated *P. aeruginosa* decreased cytotoxicity in human red blood cells by 40% and 57% respectively. These results imply that indole and 7HI made *P. aeruginosa* less virulent to red blood cells.

## Discussion

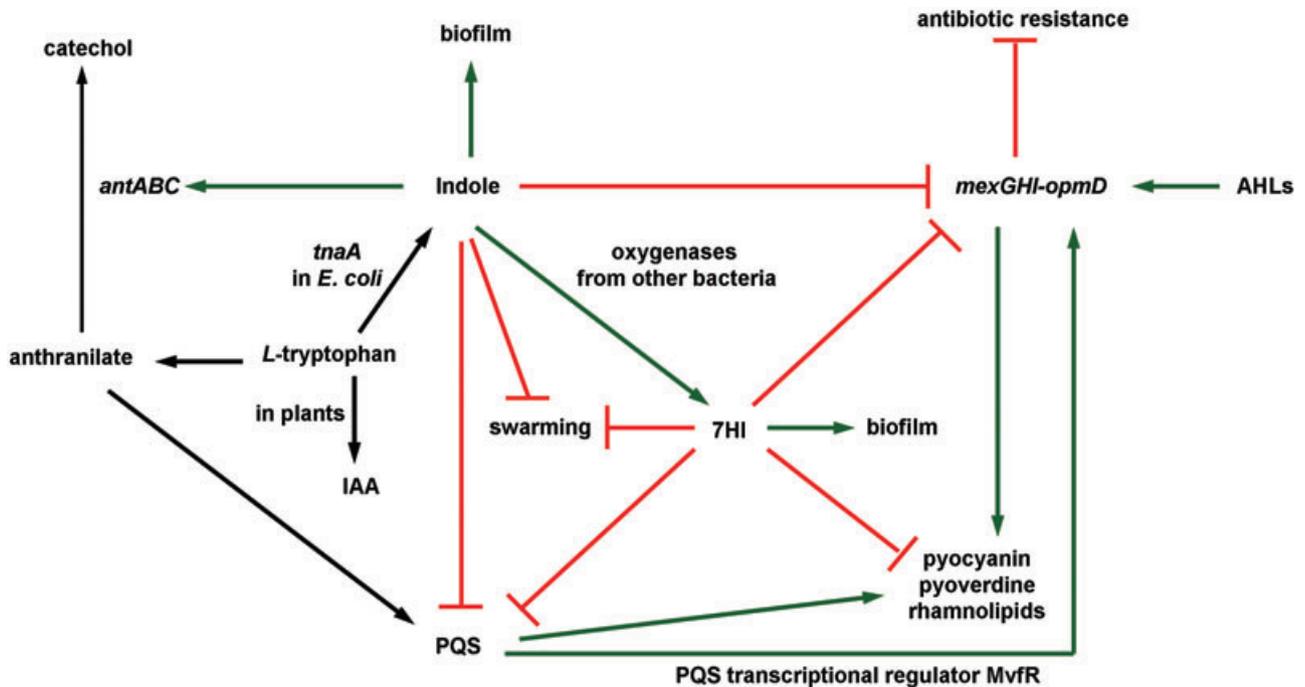
Quorum sensing is related to virulence as has been shown by whole-transcriptome studies of *P. aeruginosa* with 3O-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL (Schuster *et al.*, 2003; Wagner *et al.*, 2003) and PQS (Bredenbruch *et al.*, 2006). Here, the addition of indole and 7HI resulted in an opposite pattern of gene expression of the *mexGHI-opmD* multidrug efflux genes and many virulence genes compared with gene expression with the two exogenous AHLs. For example, the addition of the AHLs consistently induced the *mexGHI-opmD* genes, genes involved in phenazine synthesis and PQS synthesis, and the *flp-tad-rcp* gene cluster (Schuster *et al.*, 2003; Wagner *et al.*, 2003), while indole and 7HI repressed these genes (Table 1). Similarly, the addition of PQS most significantly induced virulence genes involved in pyochelin synthesis (*pch* operon) (Bredenbruch *et al.*, 2006) whereas indole repressed the *pch* operon. Genes involved in small molecule transport (PA2328, PA2329, PA2330 and PA2331)

were also induced by AHLs (Schuster *et al.*, 2003), while indole repressed them. Furthermore, the addition of pyocyanin, a physiological terminal signal molecule for the upregulation of QS-controlled genes (Dietrich *et al.*, 2006), induced *mexGHI-opmD* and a possible regulator/putative monooxygenase for pyocyanin synthesis, PA2274 (Dietrich *et al.*, 2006), while indole and 7HI repressed PA2274 (Table 1). In addition to the whole-transcriptome data, our virulence factor assays (Fig. 1) clearly show that indole and 7HI inhibit QS-controlled virulence factors. Taken together, indole and 7HI decrease the production of antimicrobial compounds and virulence factors in *P. aeruginosa*. A conceptual model of the interaction of indole and 7HI on cellular phenotypes of *P. aeruginosa* is shown in Fig. 5.

Genes encoding the MexGHI-OpmD efflux pump were the most highly repressed genes in *P. aeruginosa* upon addition of indole and 7HI (Table 1), and antibiotic resistance was clearly enhanced in the presence of indole and 7HI (Fig. S3). With *P. aeruginosa*, inactivation of MexGHI-OpmD reduces production of rhamnolipids, pyocyanin, pyoverdine, elastase and swarming motility (Aendekerk *et al.*, 2002), impairs cell growth (Aendekerk *et al.*, 2005), enhances antibiotic resistance (Aendekerk *et al.*, 2005) and attenuates virulence via PQS (Aendekerk *et al.*, 2005). As observed with *mexI* and *opmD* mutants (Aendekerk *et al.*, 2005), indole and 7HI decreased the production of virulence factors without affecting the transcriptional levels of *lasI* and *rhlI*; hence, indole and 7HI probably inhibit virulence factors due to a similar mechanism, through PQS regulation at the post-transcriptional level (Aendekerk *et al.*, 2005). Therefore, the reduction of virulence factors, swarming motility and antibiotic sensitivity may be partially explained by the repression of this locus by indole and 7HI.

Also, the PQS transcriptional regulator MvfR induces the *mexGHI-opmD* genes, pyocyanin synthesis (*phz* operon), PA0284, PA1914, PA2329 and PA2331 (Déziel *et al.*, 2005), while both indole and 7HI repressed the *mexGHI-opmD* genes and repressed PA2328, PA2329, PA2330 and PA2331, and indole repressed PA0284 (14-fold) and 7HI repressed PA1914 (sixfold, Table 1). Interestingly, indole and 7HI were less effective for the production of virulence factors in the *mvfR* mutants (Fig. 1C), which suggested that MvfR was also required for the indole mechanism.

Swarming motility is related to QS (Köhler *et al.*, 2000) and plays a role in the biofilm development of *P. aeruginosa* (Caiazza *et al.*, 2007; Overhage *et al.*, 2007). Overhage and colleagues (2007) showed a direct relationship between swarming motility and biofilm formation from the study of 5000 *P. aeruginosa* insertion mutants. In contrast, Caiazza and colleagues (2007) showed an inverse regulation of biofilm formation and swarming motility. In the



**Fig. 5.** Summary of indole-affected processes in *P. aeruginosa*. → indicates induction of gene expression or stimulation of a phenotype, ⊥ indicates repression of gene expression or repression of a phenotype, and black arrows indicate reactions.

present study, both indole and 7HI decrease slightly swimming motility, 7HI abolishes swarming motility (Fig. 2) and both lead to more biofilm formation (Fig. S1). Hence, we also observed an inverse relationship between biofilm formation and swarming motility with 7HI. In addition, the reduction of rhamnolipids by indole and 7HI (Fig. 1A) may be a partial reason for the reduced swarming motility (Fig. 2) as rhamnolipids modulate swarming motility patterns in *P. aeruginosa* (Caiazza *et al.*, 2005).

Decreasing rhamnolipid concentrations also promotes the clearance of *P. aeruginosa* biofilms *in vivo* (Jensen *et al.*, 2007), which suggests a role for rhamnolipids in the reduced pulmonary colonization and increased clearance of *P. aeruginosa* by 7HI in the guinea pigs (Fig. 4). Colonization of multiple tissues by *P. aeruginosa* after aerosol infection is consistent with previous observations in mice demonstrating the greatest degree of infection as shown by *in vivo* imaging (DiGiandomenico *et al.*, 2007). Both the number of bacteria present in the lungs and the bioluminescent signal produced after infecting guinea pigs with *P. aeruginosa* that have been pre-treated with 7HI support this conclusion. Our *in vivo* bioluminescence imaging also suggests that dissemination of *P. aeruginosa* to extrapulmonary sites may be affected by 7HI pre-treatment, but further studies are needed before this conclusion can be made. These observations suggest that hydroxylated indole represents a potential therapeutic approach or preventative measure for pulmonary infections caused by *P. aeruginosa*.

Previously, we reported that several hydroxyindoles affect biofilm formation of a pathogenic *E. coli* via different mechanisms; for example, doubly hydroxylated indole, isatin, enhanced *E. coli* O157:H7 biofilm by inducing flagellar genes and repressing genes encoding AI-2 transporters *IsrABCDGK* (reminiscent of effects caused by AI-2) and by repressing indole synthesis genes *tnaABC*, but indole, 5-hydroxyindole and 7HI inhibited *E. coli* biofilm through cysteine (sulfur) metabolism (Lee *et al.*, 2007b). Here, both indole and 7HI stimulate biofilm formation in *P. aeruginosa*, inhibit production of QS-derived virulence factors, and enhance antibiotic resistance via similar mechanisms (Fig. 5) as both compounds differentially induced or repressed many common genes (Table 1). However, the impact of 7HI on the production of virulence factors was more significant than that with indole. Also, indole alone specifically induced several oxygenases, and 7HI alone repressed the type IVb pilus *flp-tad-rcp* gene cluster and abolished swarming motility of *P. aeruginosa*, suggesting a different physiological role of indole and hydroxylated indole. This result is additional support of the hypothesis that hydroxylation of abundant indole by non-specific oxygenases (which are probably present in consortia) can lead to the formation of hydroxyindoles that alter cellular functions in *P. aeruginosa* as well as in *E. coli* in a manner different from indole (Lee *et al.*, 2007b). The pattern of the modification of indole is important as the control compound IAA did not influence

the expression of QS-related genes (Table 1) or influence the production of virulence factors (Fig. 1).

In this study, we have demonstrated that the *E. coli* cell signal indole and hydroxylated indole (7HI) diminish several QS-controlled virulence factors in *P. aeruginosa*, reduce its virulence in guinea pigs, enhance its biofilm formation, enhance its antibiotic resistance and are degraded readily (Fig. 5). Hence, we have shown that there is possibly interference of the *E. coli* signal indole by *P. aeruginosa* in that *P. aeruginosa* has a defence system to degrade indole. In a manner similar to the way *P. aeruginosa* may utilize AHLs as an interference strategy to preclude encroachment by competing bacteria (Kaufmann *et al.*, 2005), *E. coli* may utilize indole to compete against *P. aeruginosa* (a pathogen that threatens its host) by decreasing the production of its siderophores (pyoverdine and pyochelin) and virulence factors. Although highly speculative, another possibility is that *P. aeruginosa* may use the indole to increase its biofilm formation in the intestinal tract to initially form a colony to avoid washout from the host; at this point the opportunistic pathogen downregulates virulence factors with the prevalent indole to avoid detection until a suitable cell density is reached (Williams, 2007). As AHLs have been shown to have other functions other than QS (Kaufmann *et al.*, 2005), indole and 7HI may have functions other than inhibiting virulence factors in *P. aeruginosa*.

Given our results showing that indole diminishes virulence factors and virulence, that an indole derivative (CBR-4830) has been shown to inhibit *P. aeruginosa* growth through a multidrug efflux pump, *mexAB-oprM* (Robertson *et al.*, 2007), and that some natural indole derivatives, such as indole-3-carbinol and 3,3'-diindolylmethane derived from Cruciferous vegetables, show antimicrobial, antiviral and anticancer activity (Higdon *et al.*, 2007), indole and indole derivatives appear to have pharmaceutical potential as treatments against pathogenic *P. aeruginosa*. Most importantly, indole and 7HI present an opportunity for antivirulence therapies (Lesic *et al.*, 2007; Cegelski *et al.*, 2008) which are also known as antipathogenic drugs (Rasmussen and Givskov, 2006). Antivirulence compounds are an important way to fight infectious diseases because unlike antimicrobials, antivirulence compounds like indole do not affect growth and so there is less chance of developing resistance (Hentzer *et al.*, 2002; Lesic *et al.*, 2007).

## Experimental procedures

### *Bacterial strains, materials and growth rate measurements*

All experiments were conducted at 37°C, and LB medium (Sambrook *et al.*, 1989) was used except for the pyoverdine assay and swarming motility assay. *Pseudomonas aerugi-*

*nosa* PAO1 used in this study was the sequenced Holloway strain (Stover *et al.*, 2000), and *P. aeruginosa* PAO1 isogenic *antA*, *mexI* and *mvfR* mutants (Jacobs *et al.*, 2003) were obtained from Professor Colin Manoil. *Pseudomonas aeruginosa* PA14 *phzS*, *rhIR*, *pqsA* and *lasB* mutants (Liberati *et al.*, 2006) were used only as negative controls in virulence factor assays. The transposon mutations were confirmed (*antA* by us, *mexI* and *mvfR* by the source) by four PCR reactions using two primers in the IS*phoA*/hah or IS*lacZ*/hah transposon (5'-CGGGTGCAGTAATATCGCCCT-3' and 5'-GGGTAACGCCAGGGTTTTCC-3') and two primers in the target gene (5'-GTGAGAACGCATGAACGCTA-3' and 5'-CTGACGATCTCGGTACGGTT-3' for *antA*, 5'-ATCCGCCGCAACAAC TAC-3' and 5'-GTAGACCTGGTCGAGCTTGC-3' for *mexI*, and 5'-CTGCATGCTGGAATTGCTC-3' and 5'-ACTGAA GATCTCCCGCTTCA-3' for *mvfR* respectively).

Indole, 7HI and IAA were used at 1.0, 0.5 and 1.0 mM, respectively, except for the biofilm dose–response and biodegradation experiments. Indole and 7HI were purchased from Fisher Scientific (Pittsburg, PA). IAA was purchased from USB Biochemicals (Cleveland, OH). The toxicity of indole, 7HI and IAA was evaluated using the specific growth rate with two independent cultures; these compounds were dissolved in DMF, and DMF was added as a control at 0.1 vol% for all experiments. To test for utilization of indole and 7HI as a carbon source by *P. aeruginosa*, cell numbers were measured for 4 days in M9 minimal medium (Rodriguez and Tait, 1983) with 1.0 and 2.0 mM indole and 0.5 and 2.0 mM 7HI.

### *Total RNA isolation and microarray analysis*

For the microarray experiments, 10 g of glass wool (Corning Glass Works, Corning, NY) was used to form biofilms (Ren *et al.*, 2004a) for 7 h in 250 ml in 1 l Erlenmeyer shake flasks which were inoculated with overnight cultures that were diluted 1:100. Indole (1 mM), 7HI (0.5 mM) or IAA (1.0 mM) in 250 µl of DMF, or 250 µl of DMF alone was added. Glass wool was used to increase the surface area so that RNA could be readily obtained for the microarrays. RNA was isolated from the biofilm cells as described previously (Ren *et al.*, 2004a).

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5500 of the 5570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation and hybridizations were as described previously (González Barrios *et al.*, 2006). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artefact. Background values, noise values and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labelling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*P*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher

(twofold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004b) (1.5-fold for indole, 1.6-fold for 7HI and 1.1-fold for IAA). Gene functions were obtained from the Affymetrix-NetAffx Analysis Center (<https://www.affymetrix.com/analysis/netaffx/index.affx>).

#### Reverse transcription polymerase chain reaction (RT-PCR)

To corroborate the DNA microarray data, the transcription level of six prominent genes was quantified using RT-PCR: *mexG* (forward primer 5'-GGCGAAGCTGTTCTCGACTATC-3'; reverse primer 5'-AGAAGGTGTGGACGATGAGG-3'), *mexH* (forward primer 5'-GAAAAGCAATTTCTCCCTGGA C-3'; reverse primer 5'-GTTGATCTGTCCGGAAGTCACTA-3'), *mexI* (forward primer 5'-CTCTACCGGACCATGGAAGA-3'; reverse primer 5'-AGCGGTTGACGTAGTTCTCG-3'), *pvdS* (forward primer 5'-TAACCGTACGATCCTGGTGAAGA-3'; reverse primer 5'-ACGATCTGGAACAGGTAGCTGAG-3'), *ssuF* (forward primer 5'-CATCAACGTTTCGTAACCAAGTTCA-3'; reverse primer 5'-GATGGAGACCTCGGTGGACTT-3') and PA0284 (forward primer 5'-ACCCTCAGAAGCCTGGATG-3'; reverse primer 5'-GTTGCTGCAGACGGAATTTT-3'). The expression level of the housekeeping gene *proC* (forward primer 5'-CAGGCCGGCAGTTGCTGTC-3'; reverse primer 5'-GGTCAGGCGCAGGCTGTCT-3') (Savli *et al.*, 2003) was used to normalize the expression data of interesting genes. An independent RNA sample using identical DNA microarray conditions were used for these studies (63 RT-PCR reactions based on three RT-PCR reactions for each of seven genes including the *proC* housekeeping gene, with DMF, indole and 7HI). RT-PCR was performed in triplicate using a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). The relative fold changes for RT-PCR were calculated from threshold cycle numbers measured using StepOne software v2.0 (Applied Biosystems). Using another housekeeping gene *rpoN* (forward primer 5'-GAGCTCGACGAAGTGAAGT-3'; reverse primer 5'-CGCATCAATTGGCTGTAGTC-3') and nine RT-PCR reactions, similar results were obtained.

#### Crystal violet biofilm assay

This assay was adapted (Pratt and Kolter, 1998); overnight cultures diluted to an optical density at 600 nm of 0.05 and were incubated in polystyrene 96-well plates for 24 h without shaking. The dye staining the biofilms (air-liquid interface biofilm as well as bottom liquid-solid biofilm) was dissolved in 95% ethanol, and an absorbance at 540 nm ( $OD_{540}$ ) was measured to quantify the total biofilm mass. Each data point was averaged from more than 12 replicate wells (six wells from two independent cultures).

#### Virulence factor assays

Except for the pyoverdine assay, overnight cultures were diluted 1:100 and contacted with indole (1 mM), 7HI (0.5 mM), IAA (1.0 mM) or diluent DMF (negative control). The pyocyanin assay was adapted (Essar *et al.*, 1990); after growth for 7 h, supernatants were extracted with chloroform

and 0.2 N HCl, and analysed spectrophotometrically. The *phzS* mutant was used as a negative control. The rhamnolipid assay was adapted (Wilhelm *et al.*, 2007); after growth for 7 h, supernatants were assayed for rhamnolipids using the orcinol colorimetric assay (Wilhelm *et al.*, 2007). The *rhIR* mutant was used as a negative control. The PQS assay was adapted (Attila *et al.*, 2008a); after growth for 7 h, supernatants were extracted with acidified ethyl acetate and analysed by thin-layer chromatography. As the standard, a synthetic PQS obtained from Professor Marvin Whiteley was used (Fig. S2), and the *pqsA* mutant was used as a negative control. The elastase assay was adapted (Ohman *et al.*, 1980); after growth to a turbidity of 2 at 600 nm, supernatants were incubated with elastin-Congo Red (MP Biomedicals, 101637), and the absorbance was measured at 495 nm to determine the elastase activity. The *lasB* mutant was used as a negative control. The pyoverdine assay was adapted (Ren *et al.*, 2005); after growth in minimal succinate medium (Ren *et al.*, 2005), cells were diluted to a turbidity of 0.05 at 600 nm in fresh minimal succinate medium and were grown for 8 h. The pyoverdine concentration was measured spectrophotometrically at 405 nm (Stintzi *et al.*, 1998). Each experiment was performed using at least two independent cultures.

#### Antibiotic resistance assays

Overnight cultures were diluted 1:100 and grown to a turbidity of 1.5 at 600 nm with indole, 7HI or DMF. Antibiotics (0.06 mg ml<sup>-1</sup> gentamicin, 10 mg ml<sup>-1</sup> kanamycin, 0.4 mg ml<sup>-1</sup> tetracycline and 5 mM VOSO<sub>4</sub> · 2H<sub>2</sub>O) were mixed with cells and incubated for 60 min without shaking; cells were enumerated with LB agar plates. To test the antibiotic ampicillin, the MIC of ampicillin was determined using an LB agar dilution technique (Andrews, 2001). Cells were grown to mid-log phase (turbidity of 1.5 at 600 nm) and 10 µl of diluted cells at 10<sup>4</sup> cells ml<sup>-1</sup> were added to the surface of LB agar plates with indole or 7HI and 100–800 µg ml<sup>-1</sup> ampicillin. All plates were incubated for 18 h. Two independent cultures were used for each strain.

#### Swimming, swarming and twitching motility

For swimming motility, 0.3% agar with 1% tryptone and 0.25% NaCl was used (Sperandio *et al.*, 2002); for swarming motility, BM2 swarming medium (62 mM potassium phosphate buffer at pH 7, 2 mM MgSO<sub>4</sub>, 10 µM FeSO<sub>4</sub>, 0.4% glucose, 0.1% casamino acids and 0.5% agar) was used (Overhage *et al.*, 2007); and for twitching motility, LB with 1.0% agar was used (Overhage *et al.*, 2007). Briefly, strains were grown from diluted overnight cultures to a turbidity of 1.0 at 600 nm. Indole and 7HI dissolved in DMF were added to the motility agar. DMF (0.1%) was added as the negative control. The halo diameter at 24 h was measured for swimming motility. Each experiment was performed using two independent cultures.

#### Degradation and detection of indole and 7HI

For the degradation of indole and 7HI, overnight cultures were diluted to a turbidity of 1.0 at 600 nm and were re-grown

with indole (0.5 mM) and 7HI (0.5 mM) at 250 r.p.m. As a negative control, autoclaved cells (turbidity of 1.0 at 600 nm) were contacted with indole or 7HI at the same conditions to confirm that there is no evaporation or adsorption of indole or 7HI. Extracellular concentrations of indole or 7HI were measured directly from filtered supernatants with reverse-phase HPLC using a 100 × 4.6 mM Chromolith Performance RP-18e column (Merck KGaA, Darmstadt, Germany) and gradient elution with H<sub>2</sub>O-0.1% formic acid and acetonitrile as the mobile phases at a flow rate of 1 ml min<sup>-1</sup> (65:35 for 0–5 min, 35:65 for 5–12 min and 65:35 at 12 min) (Lee *et al.*, 2007b). Under these conditions, the retention times and the absorbance maxima were 3.6 min/264 nm for 7HI and 5.9 min/271 nm for indole. Each experiment was performed with two independent cultures. To determine the total protein, the Modified Lowry Protein Assay Kit from Pierce Biotechnology (Rockford, IL) was used. The protein content of *P. aeruginosa* was 0.255 mg protein (ml OD)<sup>-1</sup>.

#### Phenotype microarray

Phenotype PM1-8 microarray plates (12111, 12112, 12121, 12131, 12141, 12181, 12182, 12183) (Biolog, Hayward, CA) were used to investigate 756 different phenotypes. Briefly, overnight cells were removed from BUG+B agar plates with a sterile swab and placed into IF-O base buffer, and the cell turbidity at 600 nm was adjusted to 0.065. Bacterial inocula were prepared, and 100 µl of each cell suspension was inoculated into the plates.

#### Acute model of *Pseudomonas* infection in guinea pigs

Random bred Hartley strain guinea pigs weighing 200–300 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). The animals were housed individually in polycarbonate cages in a temperature- and humidity-controlled environment; ambient lighting was automatically controlled to provide 12 h light and 12 h dark cycles. Animals were given commercial chow and tap water *ad libitum*. All procedures were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee. *Pseudomonas aeruginosa* was cultured with 7HI or DMF alone as a control in LB for 7 h, and guinea pigs were infected by aerosol with equal amounts of washed cells using a Madison chamber aerosol generation device calibrated to deliver between 10<sup>4</sup> and 10<sup>5</sup> cfu of *P. aeruginosa* into the lungs. At each time point (immediately after infection, 4, 24 and 48 h), five guinea pigs in each group were euthanized by overdose with sodium pentobarbital and necropsy was performed to obtain total lung mass. Lungs were homogenized in saline and dilutions plated to determine the total cfu present at each time point.

#### In vivo infection imaging

In order to confirm differences observed in colonization of guinea pig tissues, four guinea pigs were infected in the same manner as for the acute model of infection and two animals from each group were examined by quantitative *in vivo* imaging. Dorsal and lateral images were acquired for each

animal immediately after infection, at 4 h and at 24 h. *Pseudomonas aeruginosa* with the *Photobacterium luminescens luxCDABE* operon stably inserted into the chromosome (single copy) was used (strain Xen41 from Xenogen, Alameda, CA); addition of 7HI did not change luminescence. Images were acquired using the luminescence settings on the Spectrum In Vivo Imaging System (IVIS) CCD camera and analysed with Living Image 3.0 software (Xenogen). Colonization of all organs was quantified by measurement of total photons from each animal and from the pulmonary region by measurement of total photons from the pulmonary region of each animal.

#### Cytotoxicity

Assays to determine cytotoxicity to red blood cells with indole and 7HI versus DMF alone were conducted as described previously (Attila *et al.*, 2008b) with *P. aeruginosa* cultured for 7 h.

#### Microarray accession numbers

The expression data for biofilm samples with and without indole 7HI, and IAA are summarized in Table 1 and have been deposited in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through Accession No. GSE10065 (Edgar *et al.*, 2002).

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Indole influences *P. aeruginosa* metabolism. Metabolic phenotype based on Biolog arrays upon adding 1.0 mM indole versus no indole addition at 24 h. Optical density at 590 nm (OD<sub>590</sub>) indicates growth under these conditions.

**Fig. S1.** Indole and 7HI increase *P. aeruginosa* biofilm formation. Biofilm formation in LB after 24 h in 96-well plates with indole (A), 7HI (B), and IAA (negative control) (C). Each experiment was performed two to four times with 6 wells each, and one standard deviation is shown. Structures of compounds are indicated as insets.

**Fig. S2.** Indole and 7HI decrease PQS production. Culture components were purified and analyzed by thin layer chromatography. Included on the TLC plate are 500 ng of chemically synthesized PQS (arrow at lane 1), extract of *P. aeruginosa* wild-type culture (2), extract of *P. aeruginosa* wild-type culture with indole (1.0 mM) (3), extract of *P. aeruginosa* wild-type with 7HI (0.5 mM) (4), extract of *P. aeruginosa mexI* culture (5), and extract of *P. aeruginosa* PA14 *pqsA* as a negative control (6).

**Fig. S3.** Indole and 7HI increase *P. aeruginosa* antibiotic resistance. Antibiotic resistance with 1.0 mM indole and 0.5 mM 7HI. Final concentrations were 0.4 mg/mL tetracycline, 0.06 mg/mL gentamicin, and 10 mg/mL kanamycin. Each experiment was performed with at least two independent cultures. Data show the average of the replicates, and one standard deviation is shown.

**Fig. S4.** The *ant* locus is not responsible for indole signaling. Production of virulence factors with 1.0 mM indole with *P. aeruginosa* PAO1 *antA*. For clarity, values of the wild-type strain are not shown so bars indicate the relative amount of each compound made compared to no indole addition to the wild-type strain. Each experiment was performed with at least two independent cultures. Data show the average of the replicates and one standard deviation is shown.

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