

# **Supplementary Materials**

## **Sulfane sulfur regulates LasR-mediated quorum sensing and virulence in *Pseudomonas aeruginosa* PAO1**

Guanhua Xuan<sup>1#</sup>, Chuanjuan Lü<sup>1#</sup>, Huangwei Xu<sup>1</sup>, Kai Li<sup>1</sup>, Huawei Liu<sup>1</sup>, Yongzhen Xia<sup>1\*</sup>,  
Luying Xun<sup>1,2\*</sup>

### **Affiliations:**

<sup>1</sup>State Key Laboratory of Microbial Technology, Shandong University, Qingdao, 266237,  
People's Republic of China.

<sup>2</sup>School of Molecular Biosciences, Washington State University, Pullman, WA, 99164-7520,  
USA.

<sup>#</sup> The authors contributed equally. LC started the research; GX completed the research.

\* Corresponding authors:

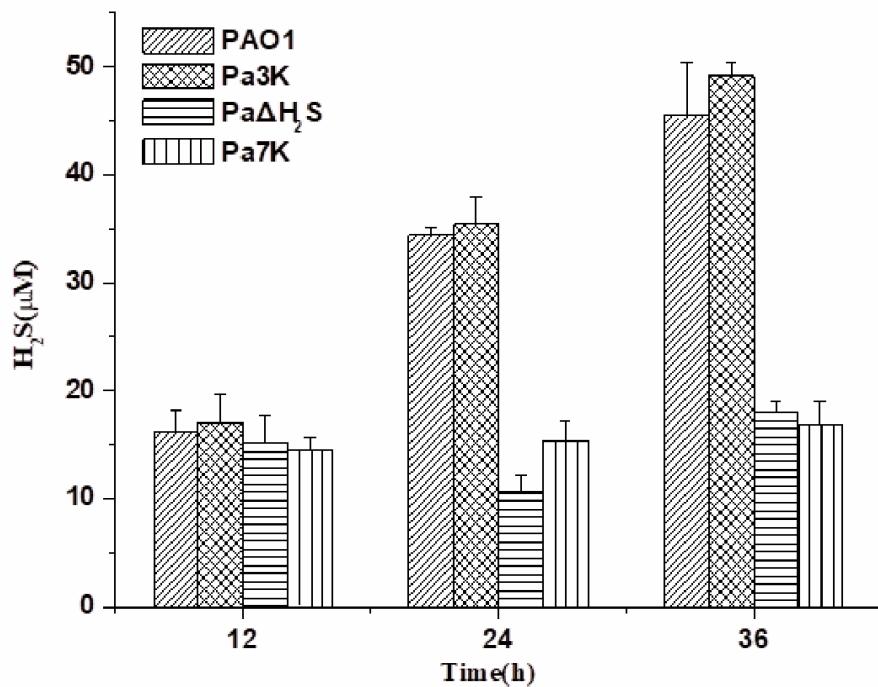
Yongzhen Xia: xiayongzhen2002@email.sdu.edu.cn; Tel. +86 532 58631572.

Luying Xun: luying\_xun@vetmed.wsu.edu; Tel. +1-509-335-2787.

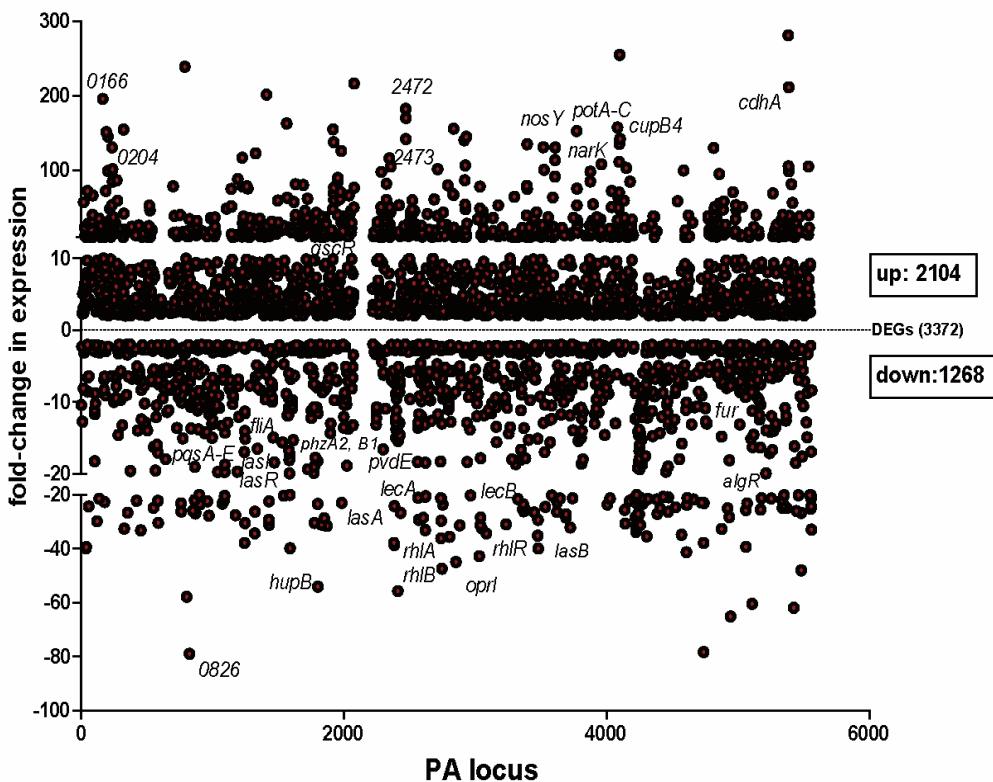
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**Figure S1—Figure S6**

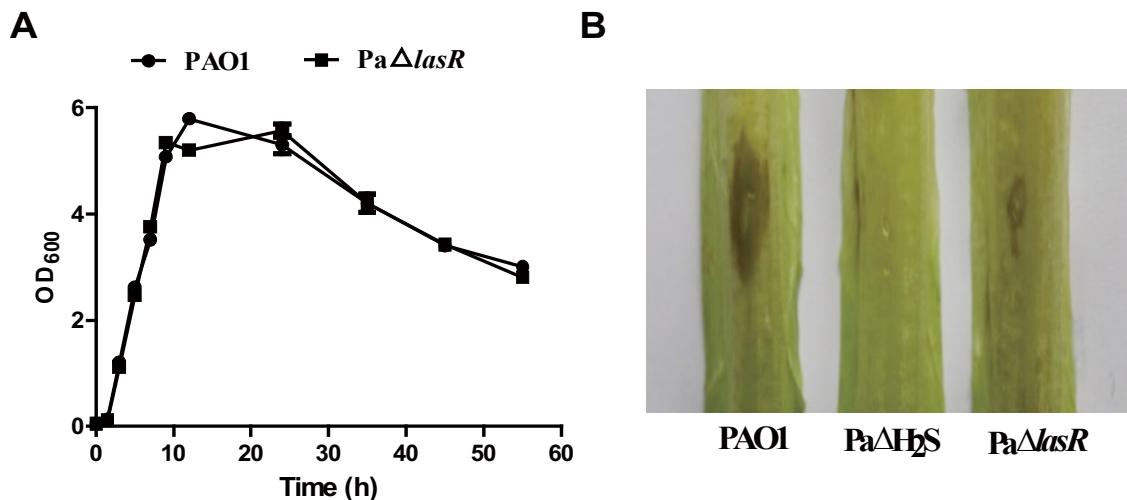
**Table S1—Table S5**



**Figure S1. The production of H<sub>2</sub>S in PAO1 and its mutants.** Overnight cultures were inoculated into LB medium at an initial OD<sub>600</sub> of 0.05. The production of H<sub>2</sub>S was assessed at 12 h, 24 h and 36 h with the mBBr method. All data are averages of three samples with standard deviations (error bars).

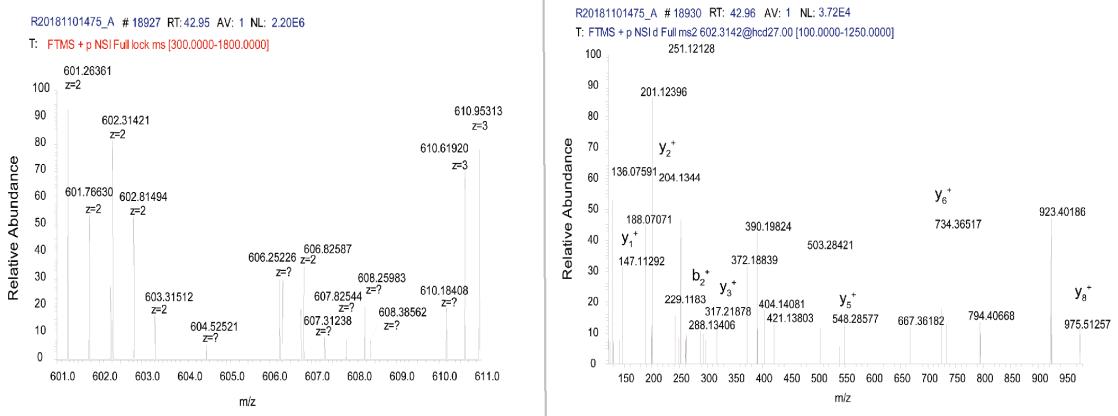
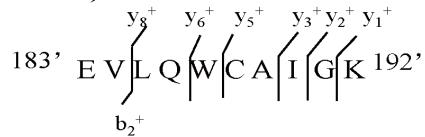


**Figure S2. Transcriptomic analysis of PAO1 and the Pa $\Delta$ H<sub>2</sub>S mutant.** Data are expressed relative to the wild-type strain results. The fold change in expression for each locus tag is indicated. The locus identifier PA\_abcd, where “abcd” represents the locus number. The most representative downregulated genes: PA4944, RNA-binding protein Hfq; PA1804, DNA-binding protein HU; PA2853, outer membrane lipoprotein OprI; PA3479, rhamnosyltransferase subunit A; PA2741, 50S ribosomal protein L20; PA3477, transcriptional regulator RhlR. The most representative upregulated genes: PA5386, 3-hydroxybutyryl-CoA dehydrogenase; PA0166, transporter; PA2472, major facilitator superfamily transporter; PA4083, chaperone CupB4; PA2836, secretion protein; PA0324, ABC transporter permease.



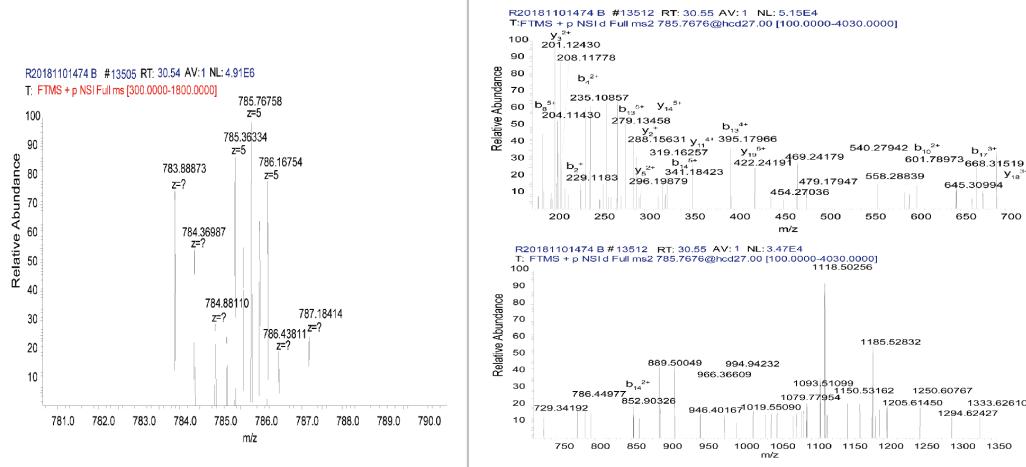
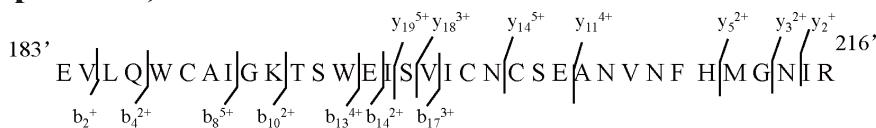
**Figure S3. The effect of *lasR* deletion in *P. aeruginosa* PAO1 on the growth and pathogenicity. (A)** Growth of *P. aeruginosa* PAO1 and its mutant in LB medium at 37°C. Overnight cultures were inoculated into LB medium at an initial OD<sub>600</sub> of 0.05. Data are averages of three samples with standard deviations (error bars). **(B)** Virulence of PAO1 and its mutant strains on lettuce.

## A (Peptide 1a)



Sequence: EVLQWCAIGK, C6-Carbamidomethyl (57.02146 Da) Charge: +2, Monoisotopic m/z: 602.31421 Da (+1.01 mmu/+1.68 ppm), MH<sup>+</sup>: 1203.62114 Da.

## B (Peptide 1b)



Sequence: EVLQWCAIGKT SWEISVICNCSEANVNFMGNIR, C6-S-O-Carbamidomethyl (73.02000 Da), Charge: +5, Monoisotopic m/z: 785.76758 Da (-4.77 mmu/-6.08 ppm), MH<sup>+</sup>: 3924.80878 Da.

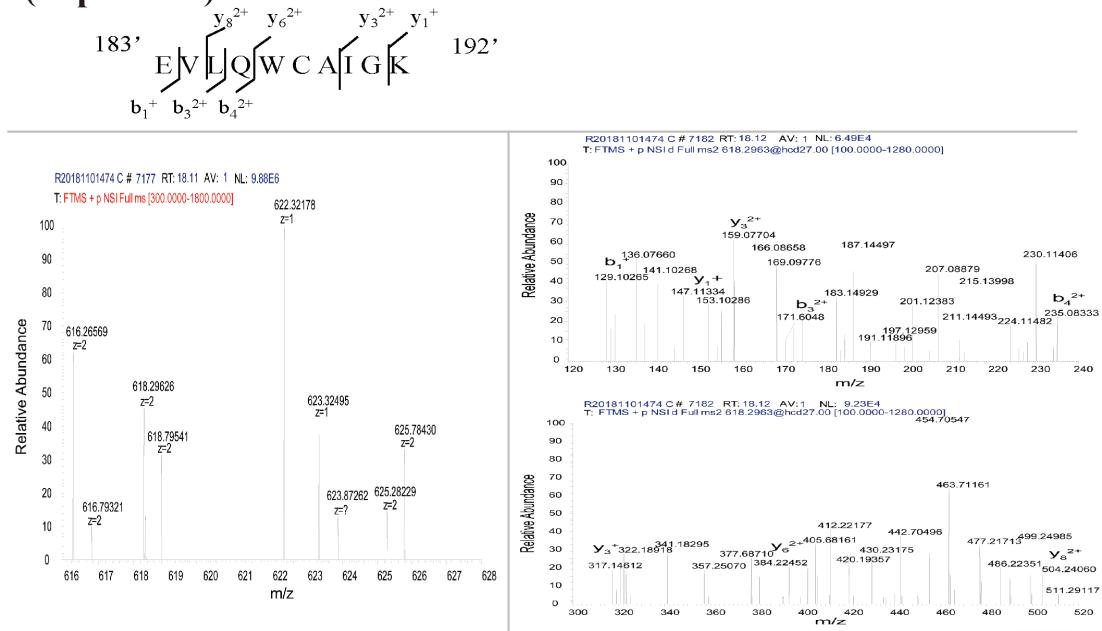
**Figure S4. LTQ-Orbitrap tandem mass spectrometry analysis of LasR (Peptides 1a&1b).**

**A) Peptide 1a in purified LasR and DTT-treated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 1a. Left, the 2+ charge state (m/z 602.31421) corresponds to a peptide containing Cys<sup>188</sup>

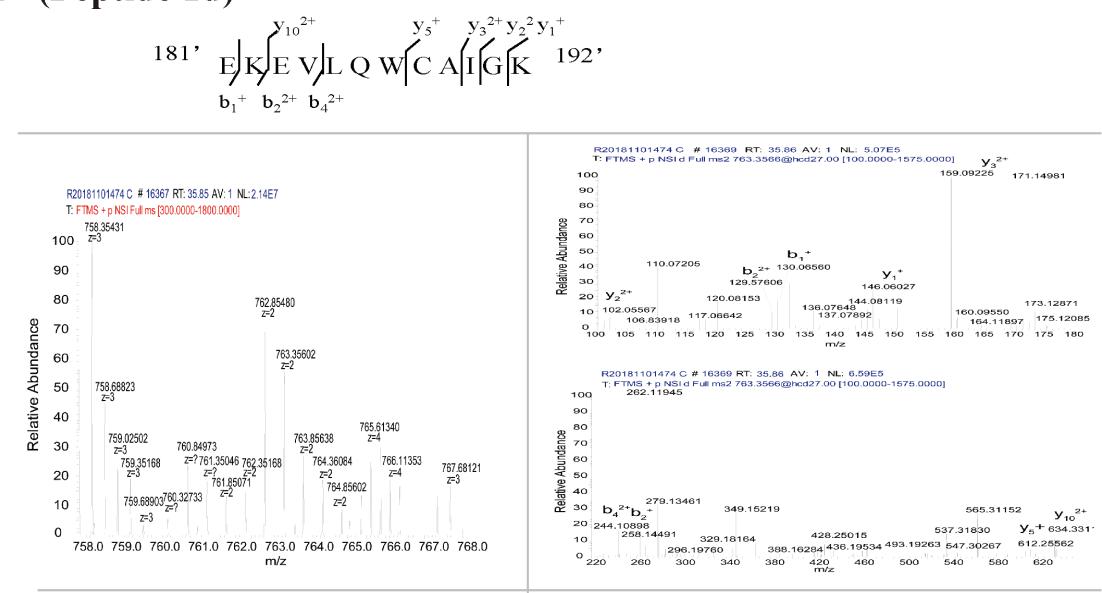
modified by IAM (theoretical molecular mass, 1203.61909). Right, MS/MS fragmentation of the fragment ( $m/z$  602.31421), in which the  $y_1^+$  peak represents the last amino acid residue (K) of Peptide 1a and the  $b_2^+$  peak represents the first two amino acid residues (EV) of Peptide 1a. Other peaks follow the same pattern.

**B) Peptide 1b in untreated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 1b. Left, the 5+ charge state ( $m/z$  785.76758) corresponds to a peptide containing Cys<sup>188</sup>-SOH modified by IAM (theoretical molecular mass, 3924.83249Da). Right, MS/MS fragmentation of the fragment ( $m/z$  785.76758), in which the  $y_2^+$  peak represents the last two amino acid residues (IR) of Peptide 1b and the  $b_2^+$  peak represents the first four amino acid residues (EV) of Peptide 1b. Other peaks follow the same pattern. Since the MS/MS spectrogram is too long, it is separated into two (top and bottom) panels.

## A (Peptide 1c)



## B (Peptide 1d)



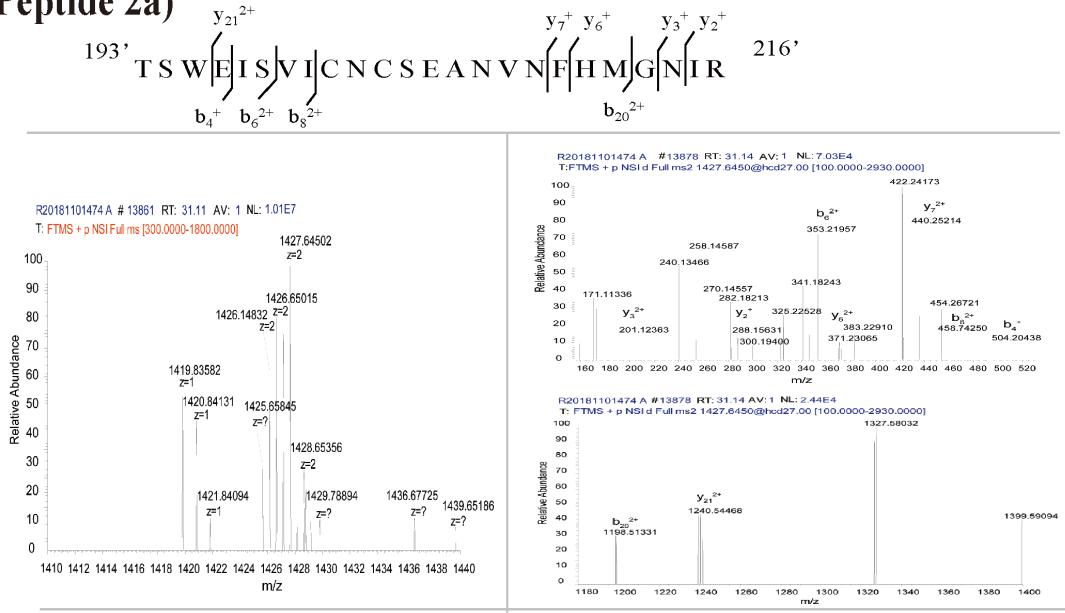
**Figure S5. LTQ-Orbitrap tandem mass spectrometry analysis of LasR (Peptides 1c&1d).**

**A) Peptide 1c in HS<sub>n</sub>-treated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 1c. Left, the 2+ charge state (m/z 618.29626) corresponds to Peptide 1c containing Cys<sup>188</sup>-SSH persulfidation

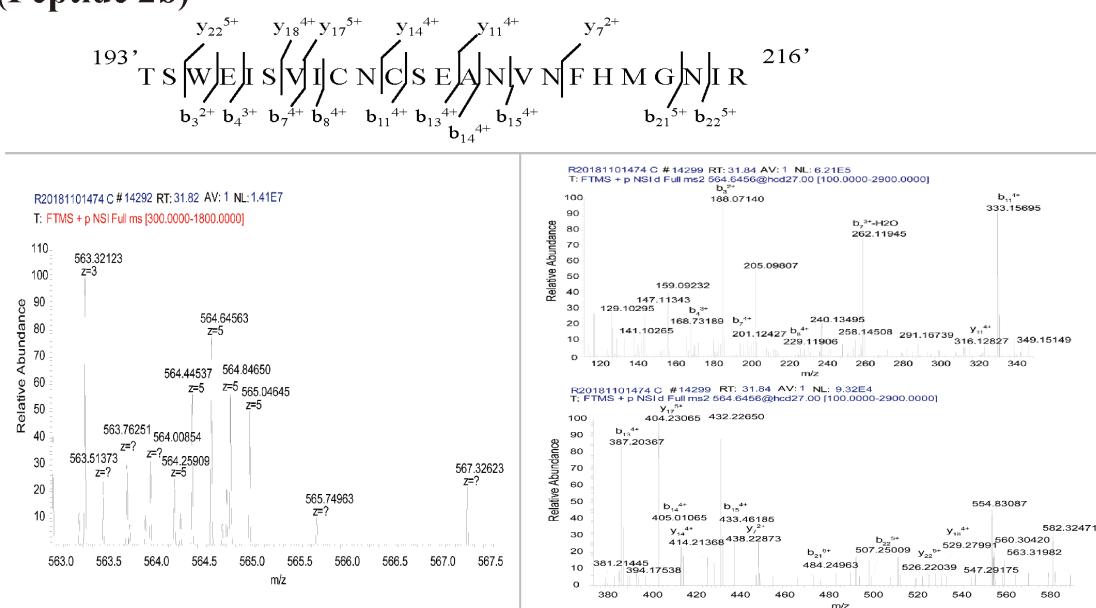
modified by IAM (theoretical molecular mass, 1235.59063Da). Right, MS/MS fragmentation of the fragment ( $m/z$  618.29626), in which the  $y_1^+$  peak represents the last amino acid residue (K) of Peptide 1c and the  $b_1^+$  peak represents the first four amino acid residues (E) of Peptide 1c. Other peaks follow the same pattern. Since the MS/MS spectrogram is too long, it is separated into two (top and bottom) panels.

**B) Peptide 1d in  $\text{HS}_n^-$ -treated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 1d. Left, the 2+ charge state ( $m/z$  762.85480) corresponds to Peptide 1d containing  $\text{Cys}^{188}$ -SSSH trisulfidation modified by IAM (theoretical molecular mass, 1524.69819Da). Right, MS/MS fragmentation of the fragment ( $m/z$  762.85480), in which the  $y_a^+$  peak represents the last amino acid residue (K) of Peptide 1d and the  $b_1^+$  peak represents the first four amino acid residues (E) of Peptide 1d. Other peaks follow the same pattern. Since the MS/MS spectrogram is too long, it is separated into two (top and bottom) panels.

## A (Peptide 2a)



## B (Peptide 2b)



**Figure S6. LTQ-Orbitrap tandem mass spectrometry analysis of LasR (Peptides 2a&2b).**

**A) Peptide 2a in DTT-treated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 2a in DTT-treated LasR. Left, the 2+ charge state (m/z 1427.64502) corresponds to Peptide 2a containing Cys<sup>201</sup> and

Cys<sup>203</sup> modified by iodoacetamide (IAM) (theoretical molecular mass, 2854.27058Da). Right, MS/MS fragmentation of the fragment ( $m/z$  1427.64502), in which the  $y_2^+$  peak represents the last two amino acid residues (IR) of Peptide 2a and the  $b_4^+$  peak represents the first four amino acid residues (TSWE) of Peptide 2a. Other peaks follow the same pattern. Since the MS/MS spectrogram is too long, it is separated into two (top and bottom) panels.

**B) Peptide 2b in HS<sub>n</sub><sup>-</sup>-treated LasR.** A Graphical fragment map correlating the relevant peptide sequence with the observed fragmentation ions of Peptide 2b. Left, the 5+ charge state ( $m/z$  564.44537) corresponds to the pentasulfide-containing peptide of Peptide 2b without modification by IAM (theoretical molecular mass, 2818.20481Da). Right, MS/MS fragmentation of the fragment ( $m/z$  564.44537), in which the  $y_7^+$  peak represents the last amino acid residue (FHMGNIR) of Peptide 2b and the  $b_3^+$  peak represents the first four amino acid residues (TSW) of Peptide 2b. Other peaks follow the same pattern. Since the MS/MS spectrogram is too long, it is separated into two (top and bottom) panels.

**Table S1. Strains and plasmids used in this study.**

Strain/plasmid	Characteristic	Source
<b><i>Escherichia coli</i> strains</b>		
DH5α	Cloning strain	Invitrogen
BL21(DE3)	Cloning strain	Invitrogen
S17-1	Transfer strain	Teng <sup>a</sup>
<b><i>P.aeruginosa</i> strains</b>		
PAO1	Wild type	ATCC 15692
PaΔH <sub>2</sub> S	PAO1 mutant with <i>cbs</i> , <i>cse</i> , <i>mst</i> and <i>cysI</i> genes disrupted	This study
Pa3K	PAO1 mutant with <i>pdo</i> and <i>sqr</i> genes disrupted	This study
Pa7K	PAO1 mutant with <i>cbs</i> , <i>cse</i> , <i>mst</i> , <i>cysI</i> , <i>pdo</i> and <i>sqr</i> genes disrupted	This study
ΔlasR	PAO1 mutant with <i>lasR</i> gene disrupted	This study
<b>Plasmids</b>		
pK18mobsacBtet	Km <sup>r</sup> and Tet <sup>r</sup> , <i>sacB</i> , RP4 oriT, ColE1 ori; suicide vector	This study
Ptrc99a	Amp, broad host range	Our lab
pBBR1mcs2	Kmr, broad host range	Kovach <sup>b</sup>
pBBR1mcs5	Gm, broad host range	Kovach <sup>b</sup>
pBBR2-Ppsqr	pBBR1mcs2 containing <i>sqr</i> from <i>Pseudomonas putida</i> S16	Our lab
Ptrc- <i>P<sub>lacI</sub>-lasR-P<sub>rhlR</sub>-mkate</i>	The trc promotor of Ptrc99a was replaced by lac pro motor and with <i>lasR</i> and <i>rhlR</i> operon	This study
Ptrc- <i>P<sub>lacI</sub>-lasR/C79S-P<sub>rhlR</sub>-mkate</i>	Ptrc- <i>P<sub>lacI</sub>-lasR-P<sub>rhlR</sub>-mkate</i> with Cys79Ser	This study
Ptrc- <i>P<sub>lacI</sub>-lasR/C188S-P<sub>rhlR</sub>-mkate</i>	Ptrc- <i>P<sub>lacI</sub>-lasR-P<sub>rhlR</sub>-mkate</i> with Cys188Ser	This study
Ptrc- <i>P<sub>lacI</sub>-lasR/C201S-P<sub>rhlR</sub>-mkate</i>	Ptrc- <i>P<sub>lacI</sub>-lasR-P<sub>rhlR</sub>-mkate</i> with Cys201Ser	This study
Ptrc- <i>P<sub>lacI</sub>-lasR/C203S-P<sub>rhlR</sub>-mkate</i>	Ptrc- <i>P<sub>lacI</sub>-lasR-P<sub>rhlR</sub>-mkate</i> Cys203Ser	This study
pET30a	Kmr, expression vector	Invitrogen
pET30-LasR	pET30a containing LasR with N terminal his-tag	This study

<sup>a</sup> Teng F, Murray BE, Weinstock GM. 1998. Conjugal transfer of plasmid DNA from *Escherichia coli* to enterococci: a method to make insertion mutations. Plasmid 39:182–186. doi:10.1006/plas.1998.1336.

<sup>b</sup> Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 166:175-176.

<sup>c</sup> An rbs sequence (aaagaggagaaa) was inserted before *mkate*.

**Table S2. Primers used in this study.**

Primers	Sequence (5'-3')	Usage
Cu11	CTATGACATGATTACGAATTGGCGGCAGCACCAGCCTACAG	Flanking up region for <i>cbs/cse</i> disruption
Cu12	CGATCCTTGATGGAGCCGCCG	
Cd11	GGCGGCTCCATCAAGGATCG	Flanking down region for <i>cbs/cse</i>
	GGGCATTGGTACTCGCTGATCC	disruption
Cd12	CCGGGTACCGAGCTCGAATTCTGGTGGAACAGAGCGGGCGTGC	
Yu11	CTATGACATGATTACGAATTCTGTGCTGACCAAGGTGC	Flanking up region for <i>cysI</i> disruption
Yu12	TAGAGACCCTCTGCAGGCGC	
Yd11	CGCCTGCAGAACGGTCTCTACCGGACCGAGGAAGAACCC	Flanking down region for <i>cysI</i> disruption
Yd12	CCGGGTACCGAGCTCGAATTCAAGCTACATAGACTGGGTCGTGG	
Mu11	CTATGACATGATTACGAATTGCCTTCACCGACGACCTCGAC	Flanking up region for <i>mst</i> disruption
Mu12	CGGCAGTCGAGGACCAGCAG	
Md11	CTGCTGGTCCTCGACTGCCGTCGCTTACTGCGGCTCCGGC	Flanking down region for <i>mst</i> disruption
Md12	CCGGGTACCGAGCTCGAATTGACCACTTCCCGGCCGTG	
lasR-del-1	AGCTATGACATGATTACGAATTCTGGGAGCTGGAGCATGG	Flanking up region for <i>lasR</i> disruption
lasR-del-2	ACTGACCGTCGGGTCGAC	
lasR-del-3	GCGGGTCGACCCGACGGTCAGTCCAATGTGAACCTCCATATGG	Flanking down region for <i>lasR</i> disruption
lasR-del-4	CCCCGGGTACCGAGCTCGAATTGAGTTCGATGCGCAAGGC	
his-lasR-1	GTATGAAAGAAACCGCTGCTATGGCCTGGTTGACGGTTTCTTGA	LasR recombinant expression
his-lasR-2	TCAGAGAGTAATAAGACCCAAATTACGGC	
pET30a-1	AGCAGCGGTTCTTCATACCA	
pET30a-2	TTGGGTCTTATTACTCTCTGACACCACCACTGAGATCCGGC	
Ptrc-F	CGAAATTAGGACATCGTTGATGCGTTCTACAAACTCTTTGTT	
Ptrc-R	AAAAGGCCATCCGTCAAGGAT	
P <sub>rhl</sub> R-mkate-1	ATCCTGACGGATGGCCTTTGTTCATGGAATTGTACAAACCG	
P <sub>rhl</sub> R-mkate-2	TCAACGATGTCCTAATTGACGG	
lasR-1	TCTCATCCGCCAAACAGCCTCAGAGAGTAATAAGACCCAAATTAAACGG	Primers for construction of <i>Ptrc-P<sub>lacI</sub>-lasR-P<sub>rhl</sub>R-mkate</i>
lasR-2	TTTCACACAGGAAACAGACCATGCCCTGGTTGACGGTT	
Ptrc-F1	GGTCTGTTCTGTGTGAAATTG	
Ptrc-F2	GGCTGTTTGGCGGATGAGA	
Ptrc-lac-1	GCAGTGATTACGACCTGCAC	
Ptrc-lac-2	GGCTCGTATGTTGTGGAATTTCACACAGGAAACAGACCATG	
lac-1	TGCAGGTCGAAATCACTGCTATGCCCTTGTGATGGCTTC	
lac-2	AAATTCCACACAACATACGAGCCGG	
qPCR-lasB-F	ACCATGTTCTATCCGCTGGT	Primers for RT-qPCR
qPCR-lasB-R	AGAACGCTTCGTTATTCCG	
qPCR-lasI-F	AAGTTGCGTGCCTAAGTGT	
qPCR-lasI-R	ATCGAGAATTGCCAGCAC	
qPCR-rplS-F	ATACCGTGATCGTCCAGGTC	
qPCR-rplS-R	GGCTGTAGGTCTGGAAAGGTA	
qPCR-rhlR-R	CTGGGCTTCGATTACTACGC	
qPCR-rhlR-R	CCCGTAGTTCTGCATCTGGT	

**Table S3. List of genes significantly down regulated in PaΔH2S ( $\geq$ 5-fold) relative to the wild-type strain PAO1.**

Gene_id	Gene name	log <sub>2</sub> -Fold_change	Description	"Regulated by LasR?"
PA2853	oprI	5.4888	outer membrane lipoprotein OprI	
PA3479	rhlA	5.3183	rhamnosyltransferase subunit A	Yes
PA4306	flp	5.1465	type IVb pilin Flp	
PA4224	pchG	5.0771	pyochelin biosynthetic protein PchG	
PA4944	hfq	6.0236	RNA-binding protein Hfq	
PA3477	rhlR	5.135	transcriptional regulator RhlR	Yes
PA4224	pchG	5.0771	pyochelin biosynthetic protein PchG	
PA3724	lasB	5.0053	elastase LasB	Yes
PA1871	lasA	4.9773	protease LasA	
PA4230	pchB	5.0255	isochorismate-pyruvate lyase	
PA1431	rsaL	4.9585	regulatory protein RsaL	Yes
PA3478	rhlB	4.8727	rhamnosyltransferase subunit B	Yes
PA1432	lasI	4.4812	acyl-homoserine-lactone synthase	Yes
PA2591	vqsR	3.3081	transcriptional regulator	Yes
PA1003	MvfR	3.704	transcriptional regulator MvfR	Yes
PA1092	fliC	4.4516	B-type flagellin	
PA1095	-	4.2259	B-type flagellar protein FliS	
PA4525	pilA	4.621	type 4 fimbrial protein PilA	
PA1899	phzA2	3.8406	phenazine biosynthesis protein PhzA	
PA1900	phzB2	3.7006	phenazine biosynthesis protein PhzB	
PA0996	pqsA	3.1703	anthranilate--CoA ligase	Yes
PA0997	pqsB	3.9034	hypothetical protein	Yes
PA0998	pqsC	3.7062	hypothetical protein	Yes
PA0999	pqsD	3.9007	3-oxoacyl-ACP synthase	Yes
PA1000	pqsE	3.6412	thioesterase PqsE	Yes
PA1130	rhlC	3.03	rhamnosyltransferase	
PA2426	pvdS	2.9271	extracytoplasmic-function sigma-70 factor	Yes
PA2587	pqsH	2.2108	2-heptyl-3-hydroxy-4(1H)-quinolone synthase	Yes
PA1754	cysB	3.6132	transcriptional regulator CysB	
PA5366	pstB	3.5758	ABC transporter ATP-binding protein	
PA1246	aprD	3.811	alkaline protease secretion ATP-binding protein AprD	Yes
PA1247	aprE	3.5344	alkaline protease secretion protein AprE	Yes
PA1248	aprF	3.5018	alkaline protease secretion protein AprF	Yes
PA3584	glpD	4.3362	glycerol-3-phosphate dehydrogenase	
PA1584	sdhB	4.0662	succinate dehydrogenase iron-sulfur subunit	
PA5521	-	3.6808	short-chain dehydrogenase	
PA2302	ambE	2.5615	protein AmbE	Yes
PA2303	ambD	2.5755	protein AmbD	Yes
PA2304	ambC	2.6372	protein AmbC	Yes
PA3326	-	4.4329	ATP-dependent Clp protease proteolytic subunit	Yes
PA0426	MexB	2.6353	multidrug resistance protein MexB	
PA0427	OprM	2.3323	outer membrane protein OprM	
PA3384	phnC	2.6233	phosphonate ABC transporter ATP-binding protein	Yes
PA0425	MexA	2.7473	multidrug resistance protein MexA	

<sup>a</sup> We identify the genes that are regulated by LasR protein based on a published paper (28).

**Table S4. List of genes significantly up regulated in PaΔH<sub>2</sub>S ( $\geq$  5-fold) relative to the wild-type strain PAO1.**

Gene_id	Gene name	log <sub>2</sub> . Fold_change	Description
PA0324	-	7.2716	ABC transporter permease
PA0205	-	7.1812	ABC transporter permease
PA3609	potC	7.026	polyamine ABC transporter permease PotC
PA2924	hisQ	6.7349	histidine ABC transporter permease HisQ
PA0204	-	6.6281	ABC transporter permease
PA4859	-	6.5656	ABC transporter permease
PA4860	-	5.7844	ABC transporter permease
PA5383	-	8.1357	hypothetical protein
PA4099	-	7.9936	hypothetical protein
PA0790	-	7.8997	hypothetical protein
PA0166	-	7.6124	transporter
PA2923	hisJ	5.7473	histidine ABC transporter substrate-binding protein HisJ
PA5386	cdhA	7.7244	3-hydroxybutyryl-CoA dehydrogenase
PA2295	-	5.4593	ABC transporter permease
PA1113	-	3.0211	ABC transporter ATP-binding protein/permease
PA1386	-	3.1932	ABC transporter ATP-binding protein
PA4064	-	3.1915	ABC transporter ATP-binding protein
PA3394	nosF	6.3041	copper ABC transporter ATP-binding protein
PA1635	kdpC	5.0389	potassium-transporting ATPase subunit C
PA3039	-	5.0173	transporter
PA2350	-	4.7686	methionine ABC transporter ATP-binding protein
PA0220	-	5.5798	amino acid APC family transporter
PA1908	-	3.7616	major facilitator superfamily transporter
PA3000	aroP1	2.7517	aromatic amino acid transporter AroP
PA5512	mifS	4.1193	sensor histidine kinase MifS
PA2356	msuD	6.7111	methanesulfonate monooxygenase
PA1724	pscK	5.9492	type III export protein PscK
PA1695	pscP	4.0634	translocation protein in type III secretion
PA1971	braZ	5.9384	branched-chain amino acid transport system 3 carrier protein
PA1146	-	5.671	iron-containing alcohol dehydrogenase
PA1284	-	4.1207	acyl-CoA dehydrogenase
PA3061	peld	5.4548	pellicle/biofilm biosynthesis protein Peld
PA1927	metE	3.4388	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
PA5294	-	4.7242	multidrug efflux protein NorA
PA2493	mexE	4.0596	resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein MexE
PA2494	mexF	3.5557	resistance-nodulation-cell division (RND) multidrug efflux transporter MexF
PA4599	mexC	4.2725	resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein MexC
PA4598	mexD	3.1978	resistance-nodulation-cell division (RND) multidrug efflux transporter MexD
PA4597	oprJ	3.3622	multidrug efflux outer membrane protein OprJ

**Table S5. Mass data from LTQ-Orbitrap tandem mass spectrometry.**

	Modification	Observed mass	Calculated mass
		(MH <sup>+</sup> )	(MH <sup>+</sup> )
Peptide 1a	RS-CAM	1203.62114	1203.61909
Peptide 1b	RS(+O)	3924.80878	3924.83249
Peptide 1c	RS(+S-CAM)	1235.58525	1235.59063
Peptide 1d	RS(+S-S-CAM )	1524.70232	1524.69819
Peptide 2a	RS-CAM	2854.28276	2854.27058
Peptide 2b	RS-SSS-SR'	2818.19776	2818.20481

Peptide mass was calculated on the website:

<http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html>