A covariation analysis reveals elements of selectivity in quorum sensing systems

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Abstract

Many bacteria communicate with kin and coordinate group behaviors through a form of cell-cell signaling called acyl-homoserine lactone (AHL) quorum sensing (QS). In these systems, a signal synthase produces an AHL to which its paired receptor selectively responds. Selectivity is fundamental to cell signaling. Despite its importance, it has been challenging to determine how this selectivity is achieved and how AHL QS systems evolve and diversify. We hypothesized that we could use covariation within the protein sequences of AHL synthases and receptors to identify selectivity residues. We began by identifying about 6,000 unique synthase-receptor pairs. We then used the protein sequences of these pairs to identify covariation patterns and mapped the patterns onto the LasI/R system from *Pseudomonas aeruginosa* PAO1. The covarying residues in both proteins cluster around the ligand binding sites. We demonstrate that these residues are involved in system selectivity toward the cognate signal and go on to engineer the Las system to both produce and respond to an alternate AHL signal. We have thus demonstrated a new application for covariation methods and have deepened our understanding of how communication systems evolve and diversify.

14 Quorum sensing (QS) is a widespread form of cell-cell signaling that bacteria use to coordinate
15 the production of public goods including toxins, antibiotics, bioluminescence, and secreted enzymes[1,2].
16 Many Proteobacteria[3] and Nitrospirae[4] employ a form of QS based on acyl-homoserine lactone (AHL)
17 signals. AHL QS systems consist of two proteins: a LuxI-type signal synthase and a LuxR-type receptor
18 (Figure 1a). The signal synthase produces an AHL from S-adenosylmethionine (SAM) and an acyl-acyl
19 carrier protein (ACP) for some LuxI-type synthases or an acyl-coenzyme A (CoA) substrate for others[5]
20 (Fig. 1b). AHL signals can freely diffuse through cell membranes[6,7] and at low cell density the QS
21 system is "off". At high cell density, the signal accumulates and binds the LuxR-type receptor which is a
22 cytosolic transcription factor that regulates gene expression in response to signal binding.
23 AHL signals share a conserved lactone core, but vary in the acyl moiety which can be a fatty
24 acid ranging from 4 to 20 carbons long, with potential oxidation on the C3 carbon and varying degrees
25 of unsaturation, or can have an aromatic or branched structure[8]. This variability in the acyl portion of the
26 signal confers selectivity to the system. Typically, a LuxI-type synthase produces a primary AHL to
27 which its paired LuxR-type receptor selectively responds[9]. Selectivity is critical to cell signaling in order
28 to avoid undesired cross-talk or spurious outputs[10]. In the case of QS, selectivity ensures bacteria
29 cooperate only with kin cells.
30 Despite its importance, we know little about how QS systems achieve selectivity or how they
31 evolve and diversify to use new signals. Although the conserved amino acids essential for synthase and
32 receptor activity are well described[11,12], residues that dictate selectivity are often different from those
33 that are required for activity[13]. Due to the low amino acid sequence identity between LuxI/R
34 homologues, it has been difficult to determine how QS systems discriminate between various AHL
35 signals[14].

36 We hypothesized that we could use covariation patterns to identify QS selectivity residues. Such
37 methods have been used to identify amino acid residues that interact with each other within proteins and
38 between proteins that physically bind each other[15-17]. Here, we endeavored to expand these methods to
39 assess the interaction between AHL synthases and receptors. While AHL synthases and receptors do not
40 physically interact, they interact indirectly via binding to a shared cognate signal and are believed to
41 coevolve to maintain this shared signal recognition[9]. Phylogenetic analyses also support coevolution of
42 synthases and receptors[18,19]. We therefore hypothesized that we could identify amino acid residues that
43 covary between QS synthases and receptors, and further, that the covarying residues would be those
44 responsible for signal selectivity.

45 We used a statistical method, GREMLIN[20], to measure covariation within the sequences of AHL
46 synthase-receptor pairs and mapped the covarying residues onto the LasI/R QS system of *Pseudomonas*
47 *aeruginosa* PAO1. By mutating the top-scoring residues identified by GREMLIN, we demonstrate that
48 they are indeed important for signal selectivity and, further, that these residues can be used to rationally
49 engineer LasI/R to produce and respond to a non-native signal. We thus demonstrate a new application
50 for powerful covariation methods and at the same time identify determinants of QS selectivity.

51 **Results**

52 **Covariation patterns in QS systems.** To begin our analysis, we gathered select protein sequences for
53 known synthase-receptor pairs (Supplementary Table 1) and used these sequences to search the
54 European Nucleotide Archive (ENA) database[21] from the European Bioinformatics Institute and the
55 Integrated Microbial Genomes and Microbiomes (IMG/M) database[22] from the Joint Genome Institute
56 (JGI) for additional synthase-receptor pairs. The genes for synthase-receptor pairs are frequently co-
57 located on the genome, and organisms can harbor more than one complete QS system[14]. To increase the
58 likelihood of identifying true pairs, we required that the two genes be separated by no more than two

59 coding sequences. A total of 6,360 non-identical pairs were identified. We further discarded pairs that
60 were more than 90% identical to another pair, resulting in 3,489 representative AHL synthase-receptor
61 pairs.

62 We aligned these sequences to the LasI/R QS system from *P. aeruginosa* PAO1. Not only is *P.*
63 *aeruginosa* a clinically important pathogen, the Las system is well-studied and crystal structures have
64 been solved for both LasI[23] and LasR[24], making this a particularly useful model system for our studies.
65 We connected the sequences of synthase and receptor from each pair and used GREMLIN to analyze
66 covariation patterns in these sequences (Supplementary Fig. 1). We applied Average Product Correction
67 (APC) to the GREMLIN covariance coefficients, a common technique shown to improve the accuracy
68 of coevolution analyses[25]. We performed the same analysis by aligning the synthase-receptor pairs to the
69 LuxI/R system from *Vibrio fischeri* MJ11. The top-ranking coevolving residue pairs overlap
70 significantly between the LasI/R and LuxI/R systems (62.5% in common among the top 0.05% residue
71 pairs) (Supplementary Fig. 1). We integrated the analyses for the LasI/R and LuxI/R systems by using
72 the higher score for each residue pair and the top 10 residue pairs are shown in Fig. 2a. As a control, we
73 randomly paired the synthases and receptors from different species and reanalyzed them using
74 GREMLIN. While top-scoring covarying residues had a minimal GREMLIN score (with APC) of 0.09,
75 the highest score from the randomized control was 0.08 (Fig. 2b). This control provides a guideline for
76 our analysis; residues with a GREMLIN score (with APC) above or near the maximal score for the
77 randomized control are likely to meaningful.

78 **Top-scoring residues cluster near ligand-binding pockets.** For both LasI and LasR, the top-scoring
79 covarying residues cluster around the ligand-binding pocket. For LasR, the top-scoring residues map
80 exclusively to the ligand-binding domain with an average distance of 5.0 Å from the co-crystalized
81 native ligand *N*-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL) (Fig. 2c). In contrast, the

82 residues identified in the randomized control are scattered throughout LasR, including three residues in
83 the DNA-binding domain, and are an average distance of 17.8 Å from 3OC12-HSL (Fig. 2d).
84 In LasI, the top-scoring covarying residues cluster around the hydrophobic pocket thought to bind
85 the fatty acyl substrate (Fig. 2e) and are an average distance of 3.7 Å from an acyl substrate modeled
86 into the LasI structure[23]. As with LasR, the residues identified in the randomized control are scattered
87 throughout LasI, with many of the residues exposed to solvent (Fig. 2f). The randomized control
88 residues in LasI are over three times further from the fatty acyl substrate, mean distance = 11.7Å,
89 compared to the covarying residues.
90 Due to their location near the ligand-binding pockets, several of the covarying residues have been
91 previously studied in various LasI/R homologues. Encouragingly, many of these residues have been
92 reported to be important for protein activity and, in some cases, for selectivity. We have summarized
93 several of these studies in Supplementary Tables 2 and 3.
94 **LasR mutations alter selectivity.** To determine whether residues identified by GREMLIN are involved
95 in LasR selectivity, we mutated a selection of the top-scoring amino acids, G38, R61, A127, S129, and
96 L130, to the most common natural variants at each position (Supplementary Table 4). By expressing
97 LasR in *Escherichia coli* and measuring its activity against a previously reported panel of 19 AHL
98 signals[26], we were able to quickly prioritize mutants for further study. The majority of our LasR mutants
99 retained the ability to respond to AHLs and all mutants had an altered selectivity profile when compared
100 to wild-type (Supplementary Fig. 2).
101 We, and others, have previously demonstrated that compared to native activity, QS receptor
102 sensitivity and selectivity can be altered when in *E. coli*[26,27]. We therefore engineered several mutations
103 into *lasR* on the *P. aeruginosa* PAO-SC4 chromosome to confirm our findings. *P. aeruginosa* PAO-SC4
104 is an AHL synthase-null mutant which we use here to measure LasR activity in response to exogenously

105 provided AHL signals. The *lasR* mutations largely had the same effect on activity and selectivity in *P.*
106 *aeruginosa* as they did when *lasR* was expressed *E. coli* (Supplementary Fig. 3). Of note, $LasR^{A127L}$ had
107 an increased sensitivity to numerous signals (Fig. 3a-d), potentially through increased hydrophobic
108 interactions with the fatty acyl chain of the AHLs. Consistent with its role in a water-mediated hydrogen
109 bond with the C3 oxygen of 3OC12-HSL, and with previous studies[28,29], LasR R61 mutants were less
110 responsive to oxo-substituted AHLs, but maintained wild-type or better levels of activation by
111 unsubstituted AHLs (Fig. 3a-d and Supplementary Fig. 3).

112 The mutations also affected the sensitivity of LasR to 3OC12-HSL (Supplementary Fig. 3).
113 Interestingly, two of our mutants were more sensitive to 3OC12-HSL than wild-type LasR. $LasR^{A127L}$
114 was roughly 3-fold more sensitive and $LasR^{L130F}$ was 2-fold more sensitive (Fig. 3e). This increased
115 sensitivity came at the cost of decreased selectivity for both of these mutations. In fact, many of our
116 single amino acid mutants displayed reduced selectivity compared to wild-type LasR (Supplementary
117 Fig. 2 and Supplementary Fig. 3).

118 **LasI mutations alter activity and selectivity.** Similar to LasR, we focused our LasI mutations on the
119 top-scoring positions: L102, T142, T145, and L157 (Supplementary Table 5). We expressed wild-type
120 or mutated *lasI* on a low copy number plasmid in the AHL synthase-null *P. aeruginosa* PAO-SC4 and
121 extracted AHLs produced by these bacteria from culture fluid. While bioassays are commonly used for
122 the detection of AHLs[30], they suffer from multiple drawbacks. In particular, bioassays are not equally
123 sensitive to all AHLs and typically cannot be used to determine which AHLs are produced and in what
124 ratio. To screen our LasI mutants for altered activity and selectivity, we developed a thin layer
125 chromatography (TLC) method based on our existing high performance liquid chromatography (HPLC)
126 radiotracer assay[31]. In this method, the C1 position in the homoserine lactone ring is labeled with ^{14}C.
127 The label is incorporated into AHLs at a ratio of one ^{14}C per AHL molecule. This results in unbiased

128 detection of all AHLs produced. While the established method uses HPLC to separate and detect AHLs
129 one sample at a time, we can run nine samples per TLC, resulting in a more high-throughput assay.
130 Using our TLC method, we confirmed that *lasI* directs the synthesis of the same primary product
131 whether it is expressed on a plasmid or from the chromosome (Supplementary Fig. 4). HPLC analysis of
132 matched extracts confirmed that the major LasI product observed by TLC is 3OC12-HSL. As expected,
133 an empty vector control did not produce detectable AHLs, nor did we detect radioactivity in a media-
134 only control. We then screened the activity each *lasI* mutant by TLC (Supplementary Fig. 4). Several
135 mutants produced little or no detectable AHLs, while some appeared to produce more 3OC12-HSL than
136 wild-type LasI. We analyzed select extracts by both TLC and HPLC and found that the results were
137 consistent between the two methods, further validating the TLC method.
138 Based on our TLC results we selected one mutant, $LasI^{L157W}$, for further study by HPLC. We
139 found that $LasI^{L157W}$ produces equal amounts of two ^{14}C-AHLs that elute in the fractions of *N*-3-oxo-
140 decanoyl-L-homoserine lactone (3OC10-HSL) and *N*-3-oxo-octanoyl-L-homoserine lactone (3OC8-
141 HSL) along with a lesser amount of 3OC12-HSL (Fig. 4). These findings demonstrate that the covarying
142 residues influence LasI activity and selectivity, and that a single mutation is sufficient to significantly
143 alter LasI selectivity.
144 **Multiple mutations can "rewire" LasI/R selectivity.** In general, multiple mutations are required to
145 generate a protein with orthogonal selectivity[15,17,28]. In non-QS proteins, altered selectivity has been
146 engineered by swapping the covarying residues in one homolog to the identities in another[15,17]. Here, we
147 seek to "rewire" LasI/R to use an orthogonal signal. We targeted the MupI/R system from *Pseudomonas*
148 *fluorescens* NCIMB 10586, which uses the signal 3OC10-HSL[32]. MupI and MupR share 52% and 39%
149 identity with LasI and LasR, respectively.

150 LasR and MupR differ at eight covariation sites in the ligand-binding domain with a GREMLIN
151 score (with APC) > 0.08 (Supplementary Fig. 5). LasR modified to contain all eight mutations was
152 inactive. However, there were several intermediate mutants that displayed an increased response to
153 3OC10-HSL. We identified three mutations that are sufficient for this increased sensitivity: L125F,
154 A127M, and L130F (Fig. 5a). $LasR^{L125F, A127M, L130F}$ is over 20-fold more sensitive to 3OC10-HSL than
155 wild-type LasR. The L125F mutation appears to be the primary driver of this altered selectivity (Fig.
156 5b,c and Supplementary Fig. 5). All "MupR-like" LasR mutants responded to 3OC12-HSL with similar
157 sensitivity to wild-type LasR (Supplementary Fig. 5).
158 LasI differs from MupI at five high-scoring covariation residues: LasI M125, T145, M152, V159,
159 and N181 (Supplementary Fig. 6), the first 3 of which line the LasI acyl-binding pocket (Fig. 5d).
160 Swapping these three residues for their MupI identities resulted in a synthase that has substantially
161 altered selectivity. $LasI^{M125I, T145S, M152L}$ produces ~2-fold more 3OC10-HSL than 3OC12-HSL (Fig. 5e).
162 The M125I mutation alone was sufficient to relax LasI's selectivity, resulting in a synthase that produces
163 roughly equal amounts of 3OC10-HSL and 3OC12-HSL. As a comparison, we measured the activity of
164 *mupI* expressed in *P. aeruginosa*, and found it produces 9:1 3OC10-HSL:3OC12-HSL (Fig. 5e and
165 Supplementary Fig. 6). All single and double "MupI-like" LasI mutants retained AHL synthase activity,
166 but only those mutants that contain the M125I mutation displayed increased 3OC10-HSL production
167 relative to 3OC12-HSL (Supplementary Fig. 6).

168 **Discussion**

169 Despite decades of study, it has been challenging to determine how AHL QS systems distinguish
170 between signals. We hypothesized that we could identify covariation patterns in AHL QS systems and
171 that these patterns would illuminate residues important for signal selectivity. By analyzing the sequences
172 of 6,360 unique QS systems, we identified amino acids that strongly covary between AHL synthases and

173 receptors. The top-scoring residues in our analysis cluster near the ligand-binding pockets for both
174 proteins and are more than three times closer to the signal molecule compared to top-scoring residues in
175 a randomized control. We focused our study on *P. aeruginosa* LasI/R. Through targeted mutations in the
176 top-scoring covarying residues we demonstrate that these amino acids are indeed determinants of signal
177 selectivity. We have thus validated a new application of covariation analysis for proteins that interact
178 indirectly and not through direct binding to one-another. Additionally, these strong covariation results
179 further support the view that AHL synthases and receptors coevolve.

180 For both the synthase, LasI, and the receptor, LasR, a single amino acid substitution is sufficient
181 to significantly alter selectivity. Interestingly, our mutations also revealed that LasR is not optimized to
182 be as sensitive as possible to its native 3OC12-HSL signal. The increase in sensitivity of specific
183 mutants came at the cost of decreased selectivity, which suggests that QS systems may evolve to balance
184 these two properties. Furthermore, increased sensitivity to the native signal may lead to premature
185 activation of the QS regulon, which would likely decrease fitness[33]. The mutants generated in our study
186 provide us with the tools to directly address these questions and assess the impact of sensitivity and
187 selectivity on QS function.

188 We also demonstrated that we can use covarying residues to rationally engineer a QS system to
189 produce and respond to a signal of our choosing. By mutating the covarying residues in LasI/R, we
190 improved the sensitivity of LasR to 3OC10-HSL over 20-fold and increased the production of 3OC10-
191 HSL by LasI roughly 15-fold. For both the synthase and receptor, a single amino acid substitution was
192 the primary driver of the altered selectivity. This was surprising given the low sequence identity between
193 LasI/R and MupI/R. These findings suggest new QS systems might evolve with relative ease. Further,
194 the ability to engineer QS selectivity could be beneficial to synthetic biology where AHL signaling is a
195 powerful tool to build biological circuits[34].

196 Though we were able to substantially increase the 3OC10-HSL activity of LasI/R, our mutants
197 retained their native 3OC12-HSL activity. We have thus generated a promiscuous system with
198 broadened selectivity. Similarly, a directed evolution study of the AHL receptor LuxR found that it
199 evolves through promiscuous intermediates[13]. This has also been observed in other systems, such as
200 toxin-antitoxin systems[17]. Proteins tend to evolve through broadly active intermediates before gaining
201 new specificity. In this way, the system maintains functionality *en route* to altered selectivity. Quorum
202 sensing systems appear to follow these same trends.

203 One limitation we faced is a lack of close LasI/R homologs with known signals. It has been
204 demonstrated that "supporting" residues, i.e. residues within a protein that covary with the selectivity
205 residues, may indirectly impact selectivity by influencing the orientation of selectivity residues[17]. Thus,
206 given the large differences in sequence identity between the Las and Mup systems, there are likely other
207 residues that must be mutated to fully swap selectivity. The identification of a more closely related
208 system to LasI/R may provide a better starting point for engineering altered selectivity. Alternatively,
209 our mutants could be further evolved though saturating mutagenesis and/or *in vitro* evolution.

210 Collectively, our results provide insight into AHL QS selectivity and will help us predict signal
211 selectivity in newly identified QS systems, in metagenomes, and in naturally occurring QS variants such
212 as those found in clinical isolates. More broadly, we have gained insight into how AHL QS systems
213 evolve and diversify.

214 **Methods**

215 **Identification of quorum sensing systems.** Starting from 24 pairs of manually curated QS synthases
216 and receptors (Supplementary Table 1), we searched for homologs in complete bacterial genomes using
217 BLAST (e-value <0.01)[35]. We filtered the BLAST hits by sequence identity (>30%) to the query and the
218 alignment coverage (query coverage >0.75 and hit coverage >0.75), and the filtered hits were aligned by

219 Clustal Omega[36]. We selected the LasI/R system from *Pseudomonas aeruginosa* PAO1 as the target and
220 mapped the Multiple Sequence Alignments (MSA) to the target system. We built sequence profiles from
221 the MSA with HMMER[37] and hmmbuild for the QS synthases and receptors, respectively. The sequence
222 profiles were then used to search against the European Nucleotide Archive database[21] and the Integrated
223 Microbial Genomes and Microbiomes database[22] from Joint Genome Institute using HMMER
224 hmmsearch. A total of 149,837 and 5,046,620 homologs were found in these databases for the QS
225 synthase and receptor, receptively. Because the synthases and receptors of the known QS systems
226 frequently locate near each other in the genome, we kept synthase-receptor gene pairs that are separated
227 by no more than two other open reading frames (ORFs) in the genome or contig. A total of 14,980
228 synthase-receptor gene pairs were identified and they represent 6,360 non-identical QS systems. In
229 another attempt, we carried out the same procedure using the LuxI/R system from *Vibrio fischeri* MJ11
230 as the target system. A similar number of QS systems were identified.
231 **Identification of covarying residues.** We connected the synthase and receptor protein sequences for
232 each QS system we found in the databases and derived the alignments between these QS systems to the
233 target QS system (LasI/R) from the hmmsearch result. We filtered the MSA for synthase-receptor pairs
234 by sequence identity (maximal identify for remaining sequences <=90%) and gap ratio in each sequence
235 (maximal gap ratio <=25%). We applied GREMLIN to analyze the covariation in the MSA[20], and the
236 GREMLIN coefficients were normalized using Average Product Correction (APC)[25] as we described
237 previously[16]. The GREMLIN coefficients after APC were used as measures for covariation signals
238 between synthase and receptor amino acid residues. As a control, we connected each synthase sequence
239 with a randomly selected receptor sequence and performed the covariation analysis in the same way.
240 We mapped the top-scoring covarying residues in the LasI/R system onto the crystal structures
241 for each protein. Reported distances between residues and ligands are the shortest distance between any

242 non-hydrogen atoms. For LasR, distances were calculated using PDB 6V7X. For LasI, distances were
243 calculated using a LasI structure with 3-oxo-C12-acyl-phosphopantetheine modeled into the acyl-
244 binding pocket[23]. Reported distances for LasI are between residues and the acyl portion of the modeled
245 substrate.

246 **Bacterial strains, plasmids, and culture conditions**. Bacterial strains and plasmids are listed in
247 Supplementary Table 6. Unless otherwise specified, *Pseudomonas aeruginosa* and *Escherichia coli*
248 were grown in lysogeny broth (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) buffered with 50
249 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH 7) (LB/MOPS) or on LB agar (LB plus 1.5%
250 Bacto agar)[26]. Liquid cultures were grown at 37°C with shaking. For radiotracer thin layer
251 chromatography (TLC) experiments, *P. aeruginosa* was grown in Jensen's medium with 0.3%
252 glycerol[31].

253 For plasmid selection and maintenance, antibiotics were used at the following concentrations: *P.*
254 *aeruginosa*, 30 μg per mL gentamicin (Gm) and 150 μg per mL carbenicillin (Cb); *E. coli* 10 μg per mL
255 Gm and 100 μg per mL ampicillin (Ap). BD Difco Pseudomonas Isolation Agar (PIA) was prepared
256 according to manufacturer directions and supplemented with 100 μg per mL Gm as needed. Where
257 needed for gene expression L-arabinose (0.4% w/v) was added.

258 All chemicals and reagents were obtained from commercial sources. AHLs were dissolved either
259 in dimethyl sulfoxide (DMSO) or in ethyl acetate (EtAc) acidified with glacial acetic acid (0.01% v/v).
260 AHLs in DMSO were used at <=1% of the final culture volume and AHLs dissolved in EtAc were dried
261 on the bottom of the culture vessel prior to addition of the bacterial culture. DMSO or acidified EtAc
262 was used as a vehicle control where appropriate.

263 **Plasmid and strain construction.** pJN-lasI and pJN-RBSmupI were constructed using *E. coli*-mediated
264 DNA assembly[38]. Briefly, for pJN-lasI, *lasI* was amplified from *P. aeruginosa* PAO1 genomic DNA

265 (gDNA) using primers lasI-pJN-F and lasI-pJN-R (Supplementary Table 6). pJN105 was amplified
266 using the reverse complement of these primers. The resulting PCR products were treated with the
267 restriction enzyme DpnI to remove the parent template. Both PCR products were then used to transform
268 *E. coli* (NEB 5alpha). The resulting constructs were confirmed by Sanger sequencing. For pJN-
269 RBSmupI, we began by amplifying *mupI* from *Pseudomonas fluorescens* Migula (ATCC 49323) gDNA
270 using primers mupI-F and mupI-R. We then used primers mupI-pJN-F and mupI-pJN-R to amplify the
271 *mupI* PCR product and used the reverse complement of these two primers to amplify pJN-RBSlasI. The
272 resulting PCR products were treated the same as for pJN-lasI. We constructed pJN-RBSlasI using
273 restriction digestion. The *lasI* gene, including its upstream ribosomal binding site (RBS), was amplified
274 from *P. aeruginosa* PAO1 gDNA using primers RBS-lasI-F and lasI-pJN-R. pJN-lasI and the RBS-*lasI*
275 PCR product were digested using NheI and SacI, gel or column purified respectively, ligated by T4
276 DNA ligase, and transformed into NEB 5alpha. The resulting constructs were confirmed by Sanger
277 sequencing. Plasmids were introduced into *E. coli* by using heat shock and were introduced into *P.*
278 *aeruginosa* by electroporation.

279 Point-mutations were introduced to *lasI* and *lasR* on pJN-lasI and JNL or pEXG2-lasR,
280 respectively, using site directed mutagenesis by PCR. Primers were designed to amplify each plasmid
281 while introducing the desired mutation(s). The resulting PCR products were treated with DpnI and were
282 then used to transform NEB 5alpha. Plasmids from the resulting colonies were screened for the desired
283 mutations by Sanger sequencing. To mutate *lasR* on the *P. aeruginosa* PAO-SC4 chromosome, *E. coli*
284 S17-1 was used to deliver pEXG2-lasR containing various *lasR* mutations to PAO-SC4 via conjugation
285 and potential mutants were isolated as previously described[39]. All mutations were confirmed by PCR
286 amplification of *lasR* from the genome followed by Sanger sequencing.

287 **LasR activity measurements.** LasR activity was measured in *E. coli* containing pJNL and pPROBE-
288 P_{rsaL} or in *P. aeruginosa* PAO-SC4 containing pPROBE-P_{rsaL} using previously reported methods[26].
289 Briefly, overnight-grown cultures were diluted 1:100 and grown back to log-phase. For *E. coli*, cultures
290 were grown to an optical density at 600 nm (OD) of 0.3, treated with L-arabinose (0.4%), and incubated
291 with AHLs for 4 h. For *P. aeruginosa*, cultures were grown to an OD between 0.05 and 0.3, were diluted
292 to an OD of 0.01 and then incubated with AHLs for 16 to 18 h. LasR activity was measured as GFP
293 fluorescence (excitation 490 nm, emission 520 nm, gain 50) using a Synergy H1 microplate reader
294 (Biotek Instruments). Activity measurements were normalized by dividing by OD and subtracting
295 background values (fluorescence per OD for cultures incubated with vehicle control). Concentrations of
296 half maximal activation, EC_{50}, were calculated using GraphPad Prism.
297 **TLC screening for AHLs.** Cultures of *P. aeruginosa* PAO1Δ*rhlI* or of *P. aeruginosa* PAO-SC4 with
298 pJN-empty or with wild-type or mutated pJN-lasI were grown overnight in Jensen's medium with 0.3%
299 glycerol. Overnight cultures were used to inoculate fresh medium (1% v/v). When the OD reached 0.5,
300 *lasI* expression was induced with arabinose (0.4%) and 1.1 mL cultures were incubated with 1.1 μCi/mL
301 L-[1-^{14}C]-methionine (^{14}C-methionine) for 90 min[31]. Cells were pelleted by centrifugation and 1 mL of
302 supernatant fluid was extracted twice with 2 mL acidified EtAc. The extracts were dried under N_2 and
303 resuspended in 15 μL acidified EtAc. Five μL of each extract was spotted on an aluminum backed C18-
304 W-silica TLC plate (Sorbtech). AHLs were separated using 70% methanol in water, then the TLC plate
305 was dried and exposed to a phosphor screen for at least 16 h. Phosphor screens were imaged with a
306 Sapphire Biomolecular Imager (Azure Biosystems). To confirm TLC findings, select extracts were
307 dried, suspended in methanol and analyzed by C18-reversed-phase high performance liquid
308 chromatography (HPLC) using a previously reported method[31].

309 **HPLC radiotracer assays for LasI activity.** For better detection of AHLs, we slightly modified the
310 radiolabeling protocol detailed above, modeling it after a previously published method[40]. Cultures of *P.*
311 *aeruginosa* PAO-SC4 with wild-type or mutated pJN-RBSlasI were grown overnight in LB/MOPS.
312 Overnight cultures were used to inoculate 5 mL LB/MOPS (1% v/v). After 2 h, *lasI* expression was
313 induced with arabinose (0.4%) and cultures were grown to OD 0.7. Cells were centrifuged at 5,000 rpm
314 for 10 min, and pellets were suspended in 1.1 mL phosphate buffered saline (PBS) with 10 mM glucose.
315 After shaking incubation at 37°C for 10 min, 1.1 μCi ^{14}C-methionine was added to the cell suspension.
316 Cell suspensions were incubated with radiolabel for 2 h, after which cells were pelleted by
317 centrifugation and 1 mL supernatant fluid was extracted twice with 2 mL acidified EtAc. Radiolabeled
318 AHLs were dried under N_2 and suspended in methanol. One-third of each extract was analyzed by
319 reversed-phase HPLC using a gradient of 10 to 100% methanol-in-water[31].

320 **References**
321

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Author contributions

SWM, QC, DB, and EPG conceived of and designed the investigation. QC carried out bioinformatic analyses. SWM, EM, and AZ constructed mutants and measured their activity. SWM and ALS developed and conducted radioassays. SWM together with QC, ALS, EPG, and DB interpreted data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

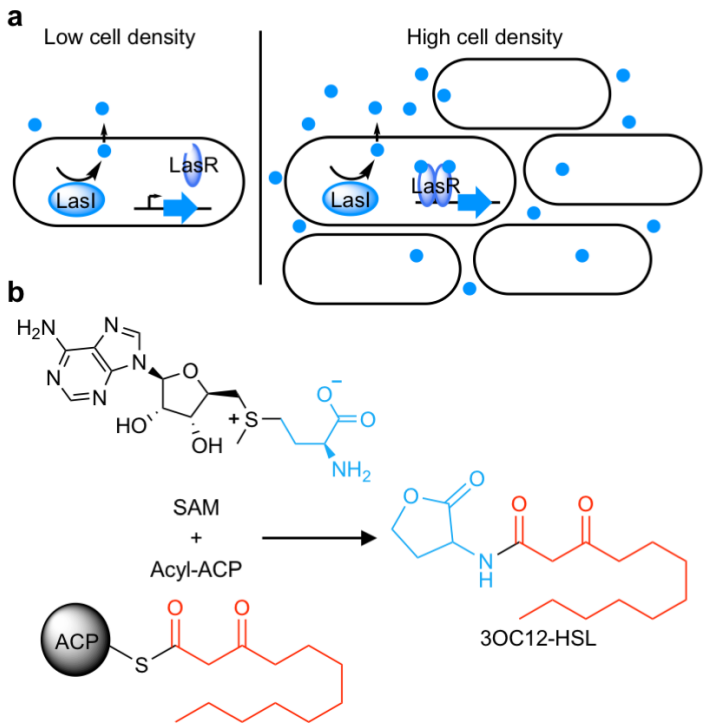


Fig. 1 | Schematic of acyl-homoserine lactone (AHL) quorum sensing (QS). a) AHL QS circuits consist of a signal synthase, a LuxI homolog (LasI in this cartoon), that produces an AHL signal. At low cell densities, the system exists in an "off" state. At high cell densities, the AHL concentration increases and the signal binds its cytosolic receptor, a LuxR homolog (LasR in this cartoon), which functions as a transcription factor. **b)** LuxI-type synthases produce AHL signals from two substrates: S-adenosyl methionine (SAM) and an activated organic acid in the form of an acyl-acyl carrier protein (ACP) or acyl-coenzyme A (CoA). SAM provides the lactone core, which is conserved across all AHL signals, while the acyl-ACP (shown here) provides an acyl moiety which varies between signaling systems. In this example, the synthase LasI produces *N*-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL).

a

Covarying residues

Synthase amino acid position	Receptor amino acid position	Top GREMLIN score
142	127	0.109
145	36	0.103
102	38	0.100
145	61	0.096
157	129	0.096
102	61	0.092
140	130	0.091
103	91	0.091
152	36	0.090
144	76	0.090

b

Randomized control

Synthase amino acid position	Receptor amino acid position	GREMLIN score
137	203	0.080
123	175	0.079
183	28	0.070
62	58	0.068
38	102	0.067
93	114	0.065
110	151	0.066
180	138	0.065
175	193	0.065
183	59	0.065

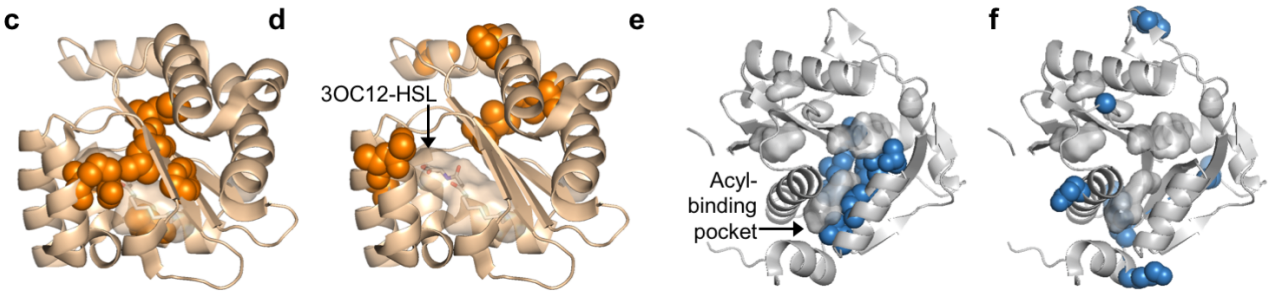
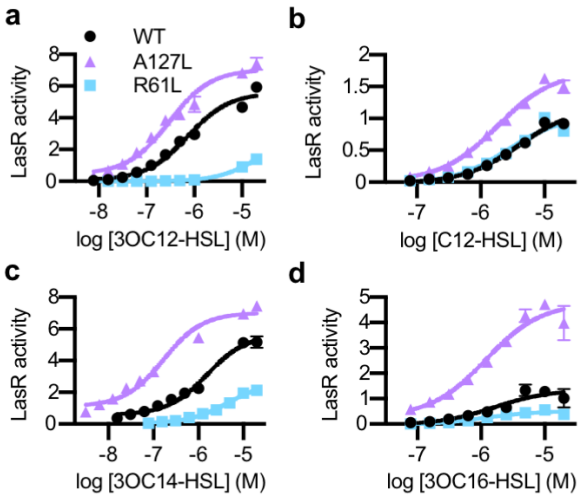


Fig. 2 | Covarying residues identified in LasI/R. a) Top-scoring covarying residues in LasI (synthase) and LasR (receptor) along with the top GREMLIN score (with APC) for each residue pair based an integrated analysis of the Las and Lux systems. **b)** Top-scoring residues in a randomized control, mapped onto LasI/R, along with the GREMLIN score (with APC) for each pair. **c)** Top-scoring covarying residues mapped onto LasR (covarying residues in orange, 3OC12-HSL in silver; PDB 3IX3) and **e)** LasI (covarying residues in blue; PDB 1RO5). Top-scoring residues in the randomized control are mapped onto **d)** LasR and **f)** LasI as in panels **c** and **e**.



e

LasR sequence	3OC12-HSL EC_{50}	Sensitivity vs WT
WT	847 ± 78 nM	1.0X
A127L	306 ± 27 nM	2.8X
A127M	930 ± 177 nM	0.9X
R61L	> 20 μM	< 0.1X
R61M	> 20 μM	< 0.1X
S129N	8.3 ± 0.8 μM	0.1X
L130F	461 ± 92 nM	1.8X
G38A	977 ± 115 nM	0.9X
G38V	> 20 μM	< 0.1X

Fig. 3 | Activity of LasR mutants. Activity of chromosomal *lasR* mutants in *P. aeruginosa* PAO-SC4 pPROBE-P_{rsaL} in response to **a)** 3OC12-HSL, **b)** *N*-dodecanoyl-L-homoserine lactone (C12-HSL), **c)** *N*-3-oxo-tetradecanoyl-L-homoserine lactone (3OC14-HSL) or **d)** *N*-3-oxo-hexadecanoyl-L-homoserine lactone (3OC16-HSL). Indicated mutations are amino acid substitutions. Wild-type (WT) is shown in black, $LasR^{A127L}$ in purple, and $LasR^{R61L}$ in blue. The horizontal axis indicates AHL concentration. LasR activity is reported on the vertical axis as relative fluorescence units normalized by optical density at 600 nm (RFU/OD x 1,000). Data are the mean and standard deviation of three biological replicates and are representative of three independent experiments. **e)** Concentration for half-maximal activation (EC_{50}) of 3OC12-HSL for *P. aeruginosa* PAO-SC4 LasR mutants calculated from data in Supplementary Fig. 3. Data are the mean and SEM of three (mutants) or four (wild-type) independent experiments. Sensitivity of the mutants compared to $LasR^{WT}$ is calculated by dividing WT EC_{50} by mutant EC_{50}.

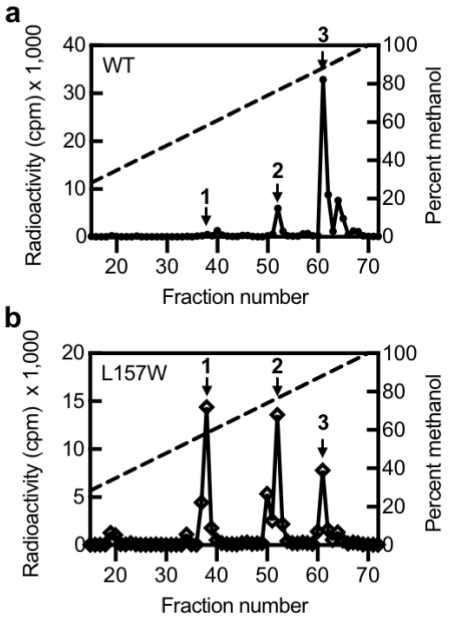
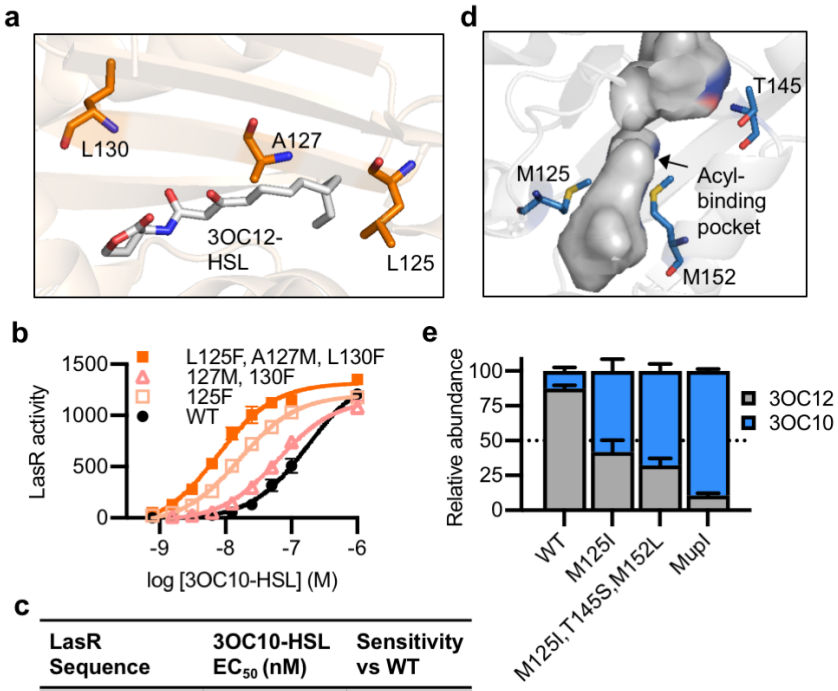


Fig. 4 | Activity of LasI mutants. HPLC analysis of radiolabeled AHLs extracted from *P. aeruginosa* PAO-SC4 harboring **a)** pJN-RBSlasI^{WT} or **b)** pJN-RBSlasI^{L157W}. The horizontal axis denotes the HPLC fraction number (fractions 1-14 are not shown). The methanol gradient is indicated as a dashed line plotted on the right vertical axis. The left vertical axis indicates the amount of radioactivity (counts per minute (cpm)) in each fraction. Data are representative of two (L157W) or three (WT) independent experiments. Arrow 1 indicates the fraction in which 3OC8-HSL elutes, arrow 2 indicates the fraction in which 3OC10-HSL elutes, and arrow 3 indicates the fraction in which 3OC12-HSL elutes.



c

LasR Sequence	3OC10-HSL EC_{50} (nM)	Sensitivity vs WT
WT	134.5 ± 12.2	1.0X
L125F	21.2 ± 2.5	6.3X
A127M, L130F	66.7 ± 19.4	2X
L125F, A127M, L130F	5.9 ± 1.1	22.8X

Fig. 5 | Reprogramming LasI/R selectivity. a) Residues mutated in LasR shown as orange sticks in the LasR structure (PDB 3IX3). 3OC12-HSL is shown in grey. **b)** LasR activity in response to 3OC10-HSL measured in *E. coli* harboring pJNL (wild-type, WT, or with indicated mutations) and pPROBE-P_{rsaL}. Data are the mean and standard deviation of three biological replicates and are representative of three independent experiments. **c)** Concentration of half maximal activity (EC_{50}) of 3OC10-HSL for LasR, calculated from data shown in panel **b**. Data are mean and SEM. Sensitivity of mutants compared to $LasR^{WT}$ is calculated by dividing the WT EC_{50} by mutant EC_{50}. **d)** Residues mutated in LasI shown as blue sticks in the LasI structure (PDB 1ROH). **e)** Relative amount of AHLs produced by *P. aeruginosa* PAO-SC4 harboring pJN-RBSlasI (WT or with the indicated mutations) or pJN-RBSmupI. Ratios were calculated from HPLC data shown in Supplementary Fig. 6. Bars show mean and standard deviation. The dashed line indicates equal production of 3OC10-HSL and 3OC12-HSL.