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Altering the Substrate Specificity of RhlI by Directed Evolution

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Quorum sensing regulates biofilm formation and virulence factor production in the human opportunistic pathogen Pseudomonas aeruginosa*. We used directed evolution to engineer RhlI, an enzyme in the RhlI–RhlR quorum-sensing system of* P. aeruginosa*, to alter its substrate specificity and gain insight into the molecular mechanisms of quorum sensing. By using a genetic screen, we identified a mutant with improved production of RhlI's two signaling molecules,* N*-butanoyl- and* N*-hexanoyl-homoserine lactone (BHL and HHL). In particular, production of BHL has been enhanced by more than two-fold, and the synthesis of HHL has been improved from an undetectable level to a level similar to BHL; this change indicates a significant change in substrate specificity. No significant change in the gene expression level was observed. Sequence alignments suggest that the mutations are most likely to facilitate interactions between the enzyme and the two acylated ACP substrates. This work also demonstrates that the genetic screen/selection should be useful in engineering additional quorum-sensing components.*

Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* is present in a wide variety of hospital environments, and contributes to both acute and chronic lung infections in cystic fibrosis patients and patients with compromised immune responses, especially burn victims and patients with AIDS.[1,2] After infection, *P. aeruginosa* grows in the biofilm mode, and exhibits enhanced resistance to antibiotic treatments and the human immune response.[3–5] *P. aeruginosa* contains three quorum-sensing systems to regulate biofilm formation and the production of virulence factors. In particular, the LasI–LasR system regulates the RhlI–RhlR system to form a hierarchical network, though the latter is also controlled by other regulatory networks.[6] The importance of quorum sensing in infections by *P. aeruginosa* is well established, as mutant *P. aeruginosa* with compromised quorum-sensing systems become vulnerable to antibiotic treatments and immune responses.[7] These results also suggest that inhibiting quorum sensing could be an effective strategy to eliminate bacterial biofilms, and a variety of signaling molecule analogues have been synthesized and characterized for their effectiveness in blocking quorum sensing.[8] It also has been proposed that drugs targeting quorum sensing might minimize the development of antibiotic resistance in bacteria[6] and provide new methods for the treatment of antibiotic-resistant bacteria that have become increasingly prevalent.[9] Consequently, antiquorum-sensing drugs might represent a novel class of antimicrobial agents. To develop drugs that specifically and effectively target quorum sensing, an enhanced understanding of bacterial quorum sensing is essential.

The engineering of enzymes can offer unique molecular insights into their functions, and directed evolution frequently reveals amino acid residues remote from the active-center residues that would be difficult to be predicted by rational protein design approaches.[10] Within the enzymatic properties that can be engineered by directed evolution, changing substrate specificity allows for the identification of amino acid residues pertinent to the design of novel inhibitors to disrupt or regulate enzyme function. In a series of studies on the directed evolution of LuxR, the transcriptional activator of the LuxI–LuxR quorum-sensing system in *Vibrio fischeri*, a cluster of amino acid residues important for improved sensitivity towards various acylated homoserine lactones (AHLs) have been identified.[11–14] We have also developed a genetic selection protocol and subsequently used the method for the directed evolution of LuxI to improve its activity; LuxI is an enzyme that is responsible for the synthesis of the signaling molecule 3-oxo-*N*-hexanoyl homoserine lactone (OHHL) of the LuxI–LuxR quorum-sensing system in *V. fischeri*.[15,16] By using a similar method, we report the alteration of the substrate specificity of RhlI, which is an enzyme that is responsible for the synthesis of the signaling molecules of the RhlI–RhlR quorum-sensing system in *P. aeruginosa*.

RhlI mainly catalyzes the synthesis of *N*-butanoyl homoserine lactone (BHL), but also produces *N*-hexanoyl homoserine lactone (HHL) as a minor product at concentrations approximately 1/15 of BHL (Figure 1 A).[17] No other acyl homoserine lactones (AHLs) have been shown to be produced by RhlI. The AHLs are produced from two substrates. One substrate, *S*-ade-

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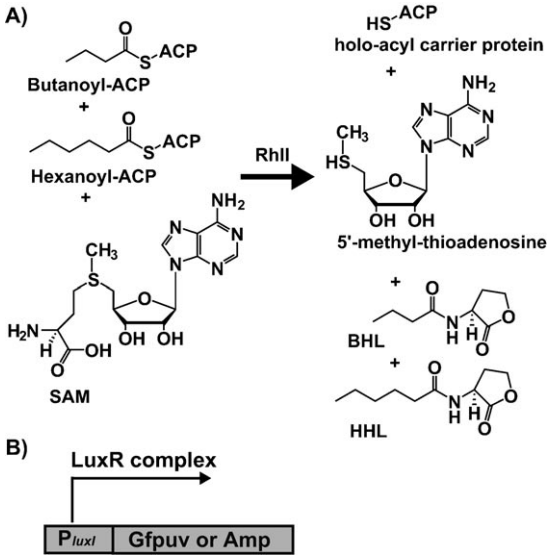

Figure 1. A) Chemical reactions catalyzed by RhlI. B) Principle of the genetic screen and selection. In the presence of sufficiently high concentrations of OHHL, LuxR activates the P_{luxI} promoter; this results in expression of GFPuv and production of fluorescence upon UV excitation in the genetic screen, and β-lactamase (ampicillin-resistance gene) to rescue cells in the genetic selection.

nosyl methionine (SAM), provides the homoserine lactone moiety, and the other substrate, butanoyl or hexanoyl acyl carrier protein (ACP), donates the acyl sidechain.[18–21] The reaction is initialized by SAM binding, and the subsequent recruitment of butanoyl ACP results in the formation of the intermediate *N*-butanoyl-*S*-adenosylmethionine, which then lactonizes to release BHL.[20,22,23] Site-directed and random mutagenesis have been used to gain understanding of RhlI's function by exploring its protein sequence, and a number of amino acid residues critical for the enzyme activity at the N and C termini have been identified.[24] However, amino acid residues that determine the substrate specificity of RhlI have never been explored, and the molecular mechanism responsible for binding acylated ACPs largely remains unknown. Because a large number of amino acid sequences of RhlI homologues are available, sequence alignments might seem to be a reasonable approach for identifying amino acid residues by determining the substrate specificity. However, despite the presence of highly conserved amino acid residues across AHL synthases,[24] rational alteration of conserved residues has only been successful for highly homologous AHL synthases.[25] In this study, we have used directed evolution to identify the amino acid residues that are important for determining the acyl chain length specificity of RhlI, and these findings provide unique insights into the substrate specificity of the enzyme.

Results and Discussion

Directed evolution of RhlI for producing OHHL

From our previous experiences with improving the activity of LuxI by using a genetic selection method,[16] we rationalized that a similar selection could be used to introduce OHHL synthesis activity into RhlI by directed evolution. In modifying the selection for RhlI evolution, we first needed to determine the antibiotic concentration to use for mutant selection. Due to leaky expression from the P_{luxI} promoter, a high concentration of ampicillin (1000 $\mu g\,mL^{-1}$) was used in the first round of the directed evolution of LuxI. Because there is no detectable OHHL synthesis activity in the wild-type RhlI, such a high selection pressure might prevent the identification of RhlI mutants with improved but much weaker OHHL synthesis activity compared to LuxI, especially in the initial rounds of evolution. As a result, we first used a genetic screen to identify RhlI mutants with low OHHL synthesis activity. In this genetic screen, the accumulation of OHHL produced by the mutant RhlI proteins leads to formation of LuxR–OHHL complexes, which then activate expression of GFPuv from the P_{luxI} promoter; this results in fluorescence (Figure 1 B).

In the first round of directed-evolution experiments, screening a random mutagenesis library generated by error-prone PCR[26] allowed us to identify four RhlI mutants that caused development of fluorescence after 20 h on screening plates, whereas the reference that expressed wild-type RhlI did not develop fluorescence. The genetic selection was also used to screen the same library, but it failed to identify any mutants; this confirms the low sensitivity of the selection method. To verify the mutants, we determined the fluorescence development of cells that contain these mutants with time, a method that we had developed previously to verify our LuxI mutants.[16] Wild-type RhlI and LuxI cultures were used as references. All of the recovered mutants exhibited weak but increased fluorescence at high cell densities compared to cells that express the wild-type RhlI, and similar growth rates (data not shown). We then used StEP,[27] an in vitro gene recombination method that is particularly efficient for recombining homologous genes, to recombine these four mutants and recover mutant R1 which developed fluorescence significantly faster than all of the parent mutants or wild-type RhlI on screening plates. Significant improvements in fluorescence intensities were also observed in mutant R1 cultures (Figure 2 A). In particular, wild-type RhlI cell cultures remained at background levels for high cell densities ($OD_{595} > 1.0$), but the R1 cell cultures exhibited fluorescence levels comparable to LuxI cell cultures at mid-log-phase cell densities; this indicates a strong putative OHHL synthesis activity. Due to this significant putative OHHL synthesis activity, we again attempted a genetic selection of a random mutagenesis library generated by using mutant R1 as the template to identify RhlI mutants with further increased OHHL activity, but no mutants were identified. Screening the same library with the genetic screen allowed us to identify mutant R2, and cells containing R2 exhibited similar fluorescence levels to cells that harbored LuxI (Figure 2 A), but significantly higher levels than cells harboring R1 at all of the cell densities measured; this indicates that the putative OHHL synthesis activity of mutant R2 is comparable to the wild-type LuxI. Further evolution by using R2 as the template did not generate any additional improvements in activity.

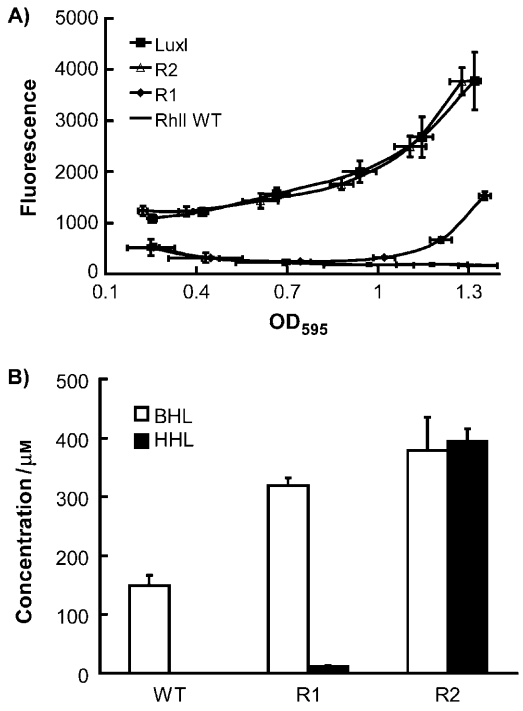

Figure 2. A) Fluorescence development in the cultures containing wild-type RhlI, LuxI, mutant R1, and mutant R2. Fluorescence was normalized to cell optical densities. B) Concentrations of BHL and HHL in cell cultures expressing wild-type and mutant RhlI after extraction and concentration. All data are presented as mean ± s.d. (standard deviation).

RhlI mutants synthesize BHL and HHL but not OHHL

RhlI uses SAM[18] and acylated ACPs as substrates, and the difficulty of preparing the required acylated ACPs makes the determination of RhlI variant kinetic parameters problematic.[20,23] As a result, we used HPLC–MS/MS to determine the overall product yields of the wild-type and mutant RhlI proteins to characterize their relative activity. A similar method has been used for characterizing LuxI mutants with improved OHHL synthesis activity.[16] Because cell cultures containing the R2 mutant exhibited similar fluorescence levels compared to cells expressing a wild-type LuxI, R2 was expected to produce OHHL at a level similar to LuxI. Because the wild-type LuxI generates OHHL at quantifiable levels under the culture conditions,[16] OHHL produced by R2 was expected to be detected and quantified. Surprisingly, no OHHL was detected by HPLC–MS/MS from any of the mutant extracts. Further quantifications indicated that both mutants demonstrated more than a two-fold improvement in BHL yields compared to the wild-type RhlI, and the BHL levels from cell cultures containing mutant R2 were slightly higher than those from cells harboring mutant R1 (Figure 2B). Despite no detectable production of HHL in wild-type RhlI cell cultures, significant levels of HHL were produced in both mutant RhlI cell cultures. Cells expressing mutant R1 generated a detectable level of HHL (about 10 μM), but cells expressing mutant R2 yielded a HHL level (395 μM after extraction and concentration) comparable to the BHL concentration (379 μM after extraction and concentration). These results indicate that the substrate specificity of RhlI has been changed dramatically: the mutant R2 accepts both butanoyl-ACP and hexanoyl-ACP as substrates to a similar degree, whereas the wild-type RhlI predominately favors butanoyl-ACP.

Quorum sensing responses were triggered by HHL

To verify that the increased fluorescent responses of the cells expressing the RhlI mutants were the result of enhanced HHL production, we added exogenous HHL into wild-type RhlI cultures to induce fluorescence development. It has been shown that LuxR responds to HHL much less efficiently than to its cognate signaling molecule OHHL and does not interact with BHL to elicit a response.[13] As shown in Figure 3A, strong fluorescence was observed starting at the middle log-phase in the presence of 4 or 40 μM HHL, and the strength of the fluorescent responses increased with HHL concentration. These results further confirmed that the observed fluorescence was contributed by the increased HHL that was produced by the mutant RhlI enzymes.

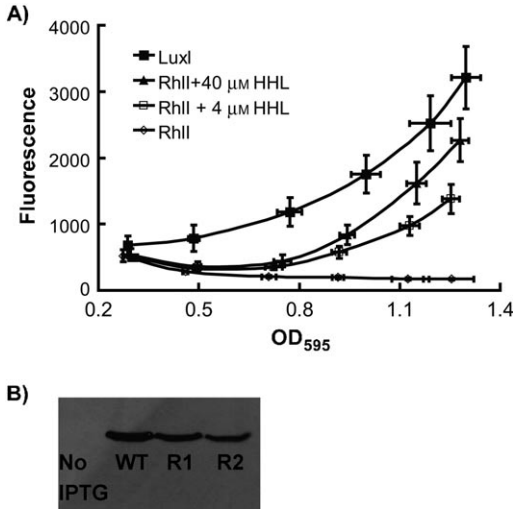

Figure 3. A) Fluorescence development in the wild-type RhlI cultures with exogenous HHL. All data are presented as mean ± s.d. Fluorescence was normalized to cell optical densities. B) Western blotting of RhlI variants. No significant changes in gene expression were observed. Uninduced samples were used as the negative control (first lane).

No HHL was detected in wild-type *E. coli* cultures

There was no detectable HHL in *E. coli* cells expressing wild-type RhlI under our experimental conditions, and the BHL concentration was 150 μM after extraction and concentration. However, it has been shown that the wild-type RhlI produces HHL at levels approximately 1/15 of BHL in vivo, and 1/20 in vitro in the presence of saturated acylated ACPs.[17,20] As a result, the concentration of HHL in the wild-type RhlI culture is estimated to be about 10 μM, a level which should be detectable by our HPLC–MS/MS system. One reason for the discrepancy could be the different extraction efficiencies of dichlorome-

thane for different AHLs; this might result in the extraction of higher concentrations of BHL over HHL. However, the structural difference (one methyl group) of the two AHLs is insignificant; this indicates that it is unlikely that the extraction efficiencies contribute significantly to the discrepancy between the predicted and observed levels of HHL. Parsek and co-workers have demonstrated that RhlI is able to discriminate butanoyl-ACP and hexanoyl-ACP (prepared with the *E. coli* ACP) despite that the kinetic parameters of the enzyme for the two substrates are fairly comparable.[20] In addition, long-chain Acyl ACPs are more abundant than short-chain ACPs in *E. coli*.[28] Taken together, the pool sizes of the two acylated ACPs are unlikely to contribute to the absence of HHL in the wild-type RhlI culture. More studies are required to determine the cause of the low levels of HHL observed in the wild-type RhlI culture.

Increased productions of BHL and HHL were not caused by enhanced gene expression

To determine whether the increased production of BHL and HHL by mutant RhlI is caused by enhanced activity and altered substrate specificity, or from a general improvement in the enzyme expression levels we measured their relative expression levels by western blotting. Previously, it has been shown that less than 1% of the recombinant RhlI in *E. coli* is soluble.[21] As shown in Figure 3B, the expression levels of the wild-type and mutant RhlI in the soluble fraction are comparable; this indicates that improved enzymatic activities primarily contribute to the observed increased production of BHL and HHL. As a result, the mutations do not cause increased solubility of the recombinant RhlI.

Amino acid substitutions likely contribute to the substrate–enzyme interactions

The amino acid sequences of the RhlI mutants show the amino acid substitutions responsible for their increased activities and altered substrate specificities (Table 1). There are two amino acid substitutions (E7K and F147L) and two synonymous (T92T and G21G) mutations in mutant R1. Two additional amino acid substitutions (E182G and P159E) are present in mutant R2. Because there is no change in expression levels for all RhlI mutants, the synonymous mutations do not seem to contribute to the mutant properties. Crystal structures of EsaI, an OHHL synthase, and LasI, an *N*-3-oxododecanoyl homoserine lactone synthase, reveal that the substrate specificity of AHL synthases are commonly determined by a tunnel that accommodates the length of the acyl sidechain.[29,30] Sequence alignments of EsaI, LasI, and RhlI reveal that two of the substitutions (see Figure S1 in the Supporting Information), F147L (L150 in EsaI), and P159E (E162 in EsaI), are present in EsaI; this suggests that these two mutations are likely to facilitate the interactions with the hexanoyl sidechain. A substitution of a charged amino acid (Glu) with a hydrophobic one (Gly) occurred at position 182, which corresponds to hydrophobic amino acid residues V191 and A187 in EsaI and LasI respectively; this implies that a hydrophobic amino acid residue is preferred for long-chain AHLs at this position. In addition, the crystal structure of EsaI (Figure S2) shows that L150 (F147 in RhlI) has indirect contact with the acyl moiety that shapes the tunnel to accommodate a specific acyl sidechain, while the crystal structure of LasI reveals that the corresponding residue (M152) forms the tunnel with 17 other amino acid residues (Figure S2). Consequently, these results support the conclusion that the F147L mutation has an important role in improving the enzyme–substrate interactions. Mutation E182G is located adjacent to a residue (M183 in RhlI and L188 in LasI) that forms the tunnel in LasI, and presumably contributes to the formation of the desired conformation of the tunnel. Mutation P159E is located in a loop that has contact with β6 and β7, which comprise many amino acid residues that form the tunnel. As a result, this mutation might also contribute to the reshaping of the tunnel for improved activity and altered substrate specificity. Functions of mutation E7K and V201M were unclear, as they might be introduced as sequence errors of the primers used for error-prone PCR. In addition, V201M is not present in mutant R2; this indicates its function for the observed activities is not significant.

Table 1. Nucleotide and amino acid substitutions in RhlI variants.

RhlI variants	Nucleotide substitutions	Amino acid substitutions
WT	None	None
R1	G19A, A63G, C276A, T439C, G601A	E7K, G21G, T92T, F147L, V201M
R2	G19A, A63G, C276A, T439C, C476A, A545G,	E7K, G21G, T92T, F147L, P159E, E182G

Fine-tuning of the genetic screen/selection is necessary for further improving RhlI activity and introducing OHHL synthesis activity

Using R2 as the template for further directed evolution of RhlI did not result in any mutants, and the reason remains unclear. One possible reason is the weak activity of the promoter (P_{luxI}), which could fail to express sufficient amount of β-lactamase to rescue the cells. However, in our previous LuxI engineering study, a much higher concentration (1400 $\mu g\,mL^{-1}$ vs. 1000 $\mu g\,mL^{-1}$ in the third generation in this research) of ampicillin was used in the genetic selection; this indicates that the promoter is sufficiently strong enough to rescue the cells once it is induced. The most probable reason is the insensitivity of LuxR towards HHL; this insensitivity could lead to slow activation of gene expression even in the presence of saturating concentrations of HHL, and therefore, could fail to generate sufficient fluorescence or rescue cells within the desired time periods. It has been demonstrated that genetic screen or selection becomes less efficient when the enzyme becomes very active or its expression level is high, and the fine-tuning of enzyme expression levels is frequently required to identify more active mutants.[32] As a result, by reducing expression levels of RhlI, further improvement of the enzyme properties by genetic screen or selection would be possible.

Our initial goal was to generate RhlI mutants with OHHL synthesis activity, and our failure in identifying these mutants was obviously due to the nonspecific response of LuxR. However, we have shown that high concentrations of HHL can be produced by introducing a small number of amino acid substitutions; this indicates that AHL synthases seem to be flexible for changes in substrate specificity, which is also supported by a site-directed mutagenesis study.[25] Consequently, with a LuxR variant specific to OHHL but not to HHL,[31] it would be possible to introduce OHHL synthesis activity into RhlI.

Conclusions

In summary, we have generated RhlI mutants with increased activities and altered substrate specificities by using directed evolution coupled with a genetic screen. Alignment of the mutant RhlIs to the amino acid sequences of two homologues with known crystal structures showed that the three amino acid substitutions are likely to contribute to the binding of the substrate by the enzyme by reshaping the tunnel that accommodates the acyl sidechain of acylated ACPs. Finally, this work and our previous reports demonstrated that the genetic screen and selection that we developed could be used to engineer the components of quorum-sensing systems to change various properties, and these engineered quorum-sensing systems are expected to be useful in synthetic biology and metabolic engineering.[33]

Experimental Section

Bacterial strains, media, chemicals and culture conditions: *E. coli* strain Top10F′ was used for all experiments. Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA). Overnight cultures (3 mL) were grown in Luria–Bertani (LB) medium at 37 °C with a shaking speed of 225 RPM for 16 h. Kanamycin (50 $\mu g\,mL^{-1}$), chloramphenicol (100 $\mu g\,mL^{-1}$), and IPTG (isopropyl-β-D-thiogalactopyranoside, 1 mM) were used for all cell cultures and LB-agar plates unless otherwise indicated. For in vivo semi-quantification, cells were grown in M9 minimal medium (250 mL) supplemented with casamino acids (0.1%) at 30 °C with a shaking speed of 180 rpm.

Plasmid construction: The *rhlI* gene was cloned into EcoRI and BamHI-digested pLuxIR plasmid;[16] this resulted in a PRR plasmid harboring *rhlI* and *luxR* genes regulated by a $P_{lac/ara}$ promoter and conferring conferred resistance to kanamycin. The *rhlI* gene was amplified from plasmid pJPP6[34] by using primers RhlIF (5′-ATA GAA TTC **AAG GAG ATA TAC C**CA TAT GAT GAT CGA ATT GCT CTC T-3′) and RhlIR (5′-ATA GGA TCC TCA CAC CGC CAT CGA CAG-3′), where the DNA sequences recognized by restriction enzymes EcoRI, BamHI, and NdeI are indicated with underlines. An NdeI restriction site was incorporated into the forward primer downstream of the RBS and linker sequence (in bold). Plux-GFPuv, which harbored a *gfpuv* gene regulated by a P_{luxI} promoter, and Plux-AMP, which harbored an ampicillin resistance gene that was regulated by a P_{luxI} promoter were generated in a previous study, and both contain a chloramphenicol-resistance gene.[16] All primers were ordered from Invitrogen (Carlsbad, CA, USA) and all the restriction enzymes were from New England BioLabs (Ipswich, MA, USA).

Directed evolution of RhlI: RhlI random mutagenesis libraries were created by using error-prone PCR as described previously with primer RhlIF and RhlIR, except that 0.2 mM $MnCl_2$ were used for all reactions.[16] Amplified DNA fragments and plasmid pLuxIR were digested with NdeI and BamHI and purified with a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). The purified insert and plasmid fragments were ligated with a T4 DNA ligase for overnight at 16 °C. The ligated products were transformed into *E. coli* Top10F′ cells containing plasmid plux-GFPuv (genetic screen) or plux-Amp (genetic selection) through electroporation, and the transformed cells were plated onto LB-agar screening plates supplemented with IPTG, kanamycin, and chloramphenicol or selection plates containing IPTG, kanamycin, chloramphenicol, and ampicillin (1000 $\mu g\,mL^{-1}$). Cells on screening plates were checked for florescence development upon UV excitation, and single colonies that developed fluorescence faster than a reference (cells expressing the parental gene) were collected for plasmid purification and further analysis. Single colonies on selection plates were also collected for plasmid purification and further analysis (see below). Approximately 20000 mutants were screened in each round.

In vivo quantification: Single colonies recovered from the screen were inoculated into LB medium (3 mL) with kanamycin and chloramphenicol and grown overnight for plasmid purification. The purified plasmid mixture contained both a PRR plasmid harboring a mutant *rhlI* gene and a plux-GFPuv plasmid. The plasmid mixture was co-transformed into *E. coli* Top10F′ cells and plated onto LB-agar plates supplemented with kanamycin and chloramphenicol. Single colonies were inoculated into LB medium (3 mL) containing kanamycin and chloramphenicol and grown overnight. The overnight cultures were then inoculated (0.5 mL) into M9 medium (250 mL) supplemented with casamino acids (0.1%), and grown at 30 °C and 180 RPM while measuring cell densities and florescence intensities at various times to determine fluorescence–cell densities curves. Cell densities were measured with a spectrophotometer (UV1101, WPA, Cambridge, UK) at the absorbance of 595 nm (OD_{595}), and fluorescent measurements were taken by using a microtiter plate reader (SPECTRAmax GEMINI Xs, Molecular Devices, Union City, CA, USA). Wild-type RhlI and LuxI were used as references. For spiking experiments, overnight cultures (0.5 mL) containing the wild-type *rhlI* gene and PluxI-GFPuv plasmids were inoculated into LB medium (250 mL) with appropriate antibiotics and IPTG as well as HHL (4 μM or 40 μM). All cultures exhibited a similar growth rate.

Western blotting: To prepare c-*myc*-tagged RhlI enzymes, a wild-type gene was cloned into the pBAD/myc-His A plasmid (Invitrogen, Carlsbad, CA, USA) by using primers rhlIF(XhoI) (5′-ATA CTC GAG AAT CGA ATT GCT CTC TGA ATC G-3′) and rhlIR(HindIII) (5′-ATA AAG CTT CAC CGC CAT CGA CAG-3′) where the DNA sequences recognized by the XhoI and *Hind*III restriction enzymes are underlined. Similar primers that contained the desired mutations were used to clone mutant R1 and R1 genes into the plasmid. The resulting plasmids were transformed into Top10F′ by using electroporation, and a single colony was inoculated into LB medium (3 mL) with appropriate antibiotics and grown overnight at 37 °C. The overnight culture (20 μL) was then inoculated into fresh LB medium (3 mL) with appropriate antibiotics and the sample was grown for 3 h at 30 °C, followed by an additional 4 h introduction with arabinose (0.2%) to induce gene expression. Cells were collected by centrifugation at 13200 rpm and the cell pellet was resuspended with 1× fast break lysis buffer (250 μL; Promega, San Luis Obispo, CA, USA), phenylmethylsulphonyl fluoride (PMSF, 1 mM) and lysozyme (20 $mg\,mL^{-1}$ 10 μL; Fischer Scientific). After in-

cubation for 20 min, DNase (1 μL; Promega) was added, and the sample was incubated for additional 5 min. Cell debris was then removed by centrifugation at 13200 rpm for 10 min, and the resulting supernatant was mixed with an equal volume of SDS sample buffer followed by boiling for 3 min. The sample was then centrifuged for 10 min at 13200 rpm, and the proteins were fractioned by standard SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare Bio-Sciences Corp.) by using electrophoresis (BioRad Trans-Blot Cell Apparatus, Hercules, CA, USA). The membrane was incubated with blocking buffer PBST (10 mL of PBS with 0.05% Tween-20 and 5% nonfat dry milk) for 1 h, and then was washed twice with PBST followed by an overnight incubation with blocking buffer (10 mL) containing anti-*Myc*-His HRP antibody (2 μL, 1:5000 dilution rate). The membrane was washed twice with PBST and chemiluminescence was developed by using an ECL detection kit (GE Healthcare Bio-Sciences Corp.) and recorded on a film (Krackeler, Albany, NY, USA).

AHL Extraction and HPLC-MS/MS: PRR plasmids harboring wild-type or mutant *rhlI* genes were transformed into *E. coli* Top10F′ and inoculated in overnight cultures from single colonies. Each overnight culture (0.5 mL) was then used to inoculate two growth cultures of M9 medium (500 mL) supplemented with casamino acids (0.1%) and grown at 30 °C for 11 h (OD 1.3). From these samples, equal volumes of CH_2Cl_2 were used to extract AHLs, which were pooled, and the organic solvents were evaporated. Extracts were then dissolved into doubly distilled H_2O (1 mL), and analyzed by using HPLC–MS/MS. Detailed procedures for extracting AHL and HPLC–MS/MS quantitation have been described previously.[16]

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Keywords: directed evolution · genetic engineering · high-throughput screening · metabolism · quorum sensing

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