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A Genetic Circuit System Based on Quorum Sensing Signaling for Directed Evolution of Quorum-Quenching Enzymes

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Quorum sensing is a cell–cell communication mechanism that is involved in the regulation of biological functions such as luminescence, virulence, and biofilm formation. Quorum-quenching enzymes, which interrupt quorum-sensing signaling through degradation of quorum-sensing molecules, have emerged as a new approach to controlling and preventing bacterial virulence and pathogenesis. In an effort to develop quorum-quenching enzymes with improved catalytic activities, a genetic circuit system based on acylhomoserine-lactone (AHL)-mediated quorum-sensing signaling was constructed. The genetic circuit system was composed of lux-R, lux-I promoter, β-lactamase, and β-lactamase inhibitor, and designed to confer antibiotic resistance on host cells expressing an AHL-degrading enzyme, thereby enabling rapid screening of quorum-quenching enzymes. To demonstrate the utility of the genetic circuit system, we attempted the directed evolution of the AHL hydrolase from *Bacillus* sp. The genetic circuit system was shown to be effective in screening of quorum-quenching enzymes with high catalytic efficiency. From these results it is expected that the genetic circuit system can be widely used for the isolation and directed evolution of quorum-quenching enzymes with greater potential.

Introduction

Individual bacteria communicate with each other and alter their physiological behavior in response to changes in the environment in a cell-density-dependent manner through a process that has become known as quorum sensing (QS).[1] Single-celled eukaryotic fungal pathogens such as *Candida albicans* also use quorum sensing to regulate cell physiology.[2] Cell–cell communication mechanisms are believed to play a crucial role in coordinating the concerted expression of genes.[3] Three well-defined signaling molecules or classes of molecules involved in bacterial quorum-sensing are oligopeptides,[4] acylhomoserine lactone (AHL),[5] and autoinducer-2.[6] Of these, the AHL family is the most thoroughly characterized and has been shown to be involved in the regulation of important cellular functions such as bioluminescence, plasmid transfer, motility, virulence, and biofilm formation.[7] A variety of AHLs with different lengths of, and substituents on, their acyl side chains have been found in a range of diverse bacterial species, and these AHLs act as the QS signal in each species.[8] In general, AHL-dependent QS bacteria feature a conserved central mechanism utilizing two major components—the LuxR-type regulator and LuxI-type protein—that serve as the signal receptor and AHL synthase, respectively.[5,7d]

Many bacterial pathogens that cause various diseases both in plant and in animals (including humans) have been shown to use AHL-dependent QS for the expression of virulence factors.[7b,9] Biofilm formation by pathogens such as *Pseudomonas aeruginosa* is also known to be associated with AHL-dependent QS, raising a serious issue with regard to antibiotic resistance.[7c] Over the last decade, quorum-quenching enzymes, which interrupt QS signaling through degradation of quorum-sensing molecules, have emerged as a new approach to controlling and preventing bacterial virulence and pathogenesis,[10] and diverse quorum-quenching enzymes have been identified in many organisms.[11] It was recently reported that quorum-quenching enzymes significantly attenuate the lethality and infectivity of various pathogens, as well as their virulence factor expression and biofilm formation.[10a,11b,12] In order to increase the potential of quorum-quenching enzymes for practical applications, several attempts have been made to enhance their catalytic activities.[13] The evolution or isolation of enzymes with desired catalytic properties is crucial to the development of quorum-quenching enzymes with greater potential. For this, an efficient method to screen for quorum-quenching enzymes is a prerequisite.

Here we report the construction and application of a genetic circuit system based on AHL-mediated (acylhomoserine-lactone-mediated) quorum sensing signaling. The genetic circuit system was designed to confer antibiotic resistance on host cells expressing the AHL-degrading enzyme (Scheme 1), enabling rapid identification of enzymes with AHL-degrading ac-

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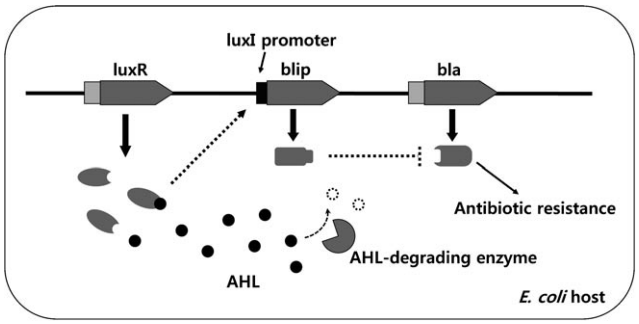

Scheme 1. Schematic representation of the genetic circuit system for screening of quorum-quenching enzymes. In the absence of the AHL-degrading enzyme, exogenously added AHL binds to LuxR, which leads to the suppression of β-lactamase (Bla) activity, due to the expression of the β-lactamase inhibitor (Blip), and no growth of the host cells occurs. In contrast, in the presence of AHL-degrading enzyme, exogenously added AHL is degraded, and the expression level of the β-lactamase inhibitor remains low. Consequently, the β-lactamase action endows the host cells with antibiotic resistance, resulting in the growth of the host cells even under antibiotic selection pressure.

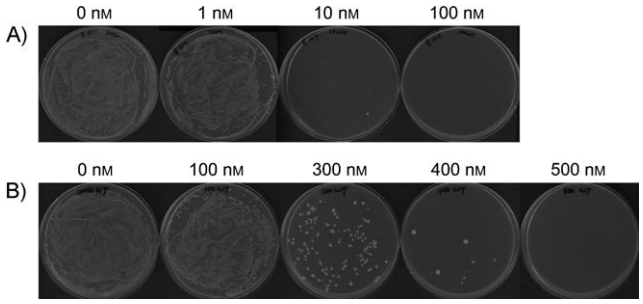

Figure 1. Growth of the cells with and without the expression of wild-type AiiA. A) Cells possessing the pQS and pZS*24Luc plasmids without AiiA. B) Cells harboring the pQS and pZS*24DN plasmids expressing AiiA. Cells were grown at 30 °C for three days on LB agar plates containing a predetermined concentration of C6-AHL. The concentrations of carbenicillin and kanamycin were 100 μg mL^{-1} and 30 μg mL^{-1}, respectively. Experiments were carried out in triplicate, and one of each is shown.

tivity. To demonstrate the utility of the genetic circuit system for screening of quorum-quenching enzymes with improved catalytic activity, we performed the directed evolution of the AHL hydrolase from *Bacillus* sp. The genetic circuit system was shown to be effective in screening of quorum-quenching enzymes with high catalytic efficiency.

Results and Discussion

Genetic circuit system based on quorum sensing signaling

The genetic circuit system is composed of the LuxR transcription activator and luxI promoter (P_{luxI}) of the *Vibrio fisheri* QS operon, a β-lactamase inhibitor (Blip) connected downstream of P_{luxI}, and β-lactamase (Scheme 1). In this system, a quorum-quenching enzyme with high catalytic activity leads to the growth of the host cells even at elevated AHL concentrations, which allows rapid screening of quorum-quenching enzymes with improved catalytic activity toward various AHLs. The genetic circuit components were built into an AHL-response plasmid (pQS), and another plasmid (pZS*24DN) was used for the expression of quorum-quenching enzyme (Figure S1 in the Supporting Information).

We tested the efficacy of the developed genetic circuit system for the screening of quorum-quenching enzymes with the AHL hydrolase (AiiA) from *Bacillus* sp. as a model enzyme. The plasmid pZS*24DN, which possesses the wild-type AiiA, was transformed into the *E. coli* strain DH5α harboring the AHL-response plasmid pQS, and the resulting cells were used as a positive control. DH5α cells containing the pQS and the pZS*24Luc plasmid without AiiA were used as the negative control. Growth of the cells was compared at various concentrations of exogenously added C6-AHL (Figure 1). To allow almost the same amounts of cells to be spread on the test plates, a culture solution of OD_{600} 0.4 was serially diluted 100-fold, and 100 μL of the diluted solution was used. In the case of wild-type AiiA, the growth of host cells decreased gradually as the AHL concentration increased, and no growth was observed at concentrations higher than 500 nM. In contrast, negligible growth was observed even at AHL concentrations of 10 nM when AiiA was not expressed. The number of colonies against a range of AHL concentrations was estimated for the wild-type AiiA, and a similar trend was observed (Figure S2). This result clearly indicates that the constructed genetic circuit system can be effectively used for the rapid screening of quorum-quenching enzymes.

Evolution of a quorum-quenching enzyme

In order to test the utility of the genetic circuit system for the improvement of enzyme activity, we attempted the directed evolution of AiiA as a model quorum-quenching enzyme. Host cells expressing the AiiA mutants with enhanced activity would be expected to grow even at higher AHL concentrations than those expressing wild-type enzyme. A library of AiiA mutants was generated by error-prone PCR to give rise to an average mutation frequency of two to three substitutions in 250 amino acids. The PCR products were cloned into pZS*24DN, and the resulting pZS*24DN plasmids were inserted into the *E. coli* DH5α host cell harboring pQS (Figure S1).

In the screening of quorum-quenching enzymes with improved catalytic activities with the genetic circuit system, the expression level or catalytic activity of a quorum-quenching enzyme might influence the sensitivity and selectable scale of the system. In our preliminary tests, the use of a plasmid with a high expression resulted in the dense growth of host cells harboring wild-type AiiA even at high AHL concentrations. A plasmid with a stringently low expression thus seems to be suitable for an enzyme with a relatively high activity. In the case of an enzyme with a relatively low activity, however, a high-expression plasmid is desirable. We employed a pZS*24-derived plasmid with a low-copy pSC101* origin and a weak $P_{lac/ara-1}$ promoter for directed evolution of wild-type AiiA, because wild-type AiiA has a relatively high catalytic activity.

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Because host cells expressing wild-type AiiA showed no growth at C6-AHL concentrations higher than 500 nM (Figures 1 and S2), the transformants were spread on LB agar plates containing 500 nM C6-AHL to screen the variants with increased catalytic activity. The library size was around 5×10^5. In the primary screen, approximately 30 colonies grew on the screening plates. To exclude false positives, the pZS*24DN plasmids were prepared from a mixture of the selected colonies and retransformed into new DH5α host cells harboring pQS. Following overnight culture in LB medium, cells were replated on the agar plates containing 500 nM C6-AHL. As a result, the best mutant, designated 1R1, was selected in the first round of directed evolution, and was subjected to characterization. Sequencing of the 1R1 revealed a single amino acid change: from Val to Leu at position 69. The mutant enzyme V69L was expressed in the pMAL-c2x vector in *E. coli* XL1-blue and purified in the maltose-binding-protein (MBP)-fused form. It was previously reported that fusion of MBP at the N terminus of AiiA had no effect on the catalytic activity of the enzyme.[14] Kinetic analysis showed that the k_{cat}/K_M value of the V69L for C6-AHL was $3.7\times10^4\,M^{-1}s^{-1}$, approximately four times higher than that of the wild-type enzyme (Table 1).

Table 1. Amino acid substitutions and kinetic parameters of the selected mutants toward C6-AHL.

Enzyme (mutations)	k_{cat} [s^{-1}][a]	K_M [mM][a]	k_{cat}/K_M [$M^{-1}s^{-1}$]	Fold increase
wild-type AiiA	85 ± 17	8.1 ± 1.4	1.0×10^4	
1R1 (V69L)	77 ± 11	2.1 ± 0.44	3.7×10^4	3.7
2R1 (V69L/I190F)	66 ± 5.2	0.9 ± 0.12	7.0×10^4	7.0
2R2 (V69L/I190F/G207V)	93 ± 10	1.5 ± 0.28	6.1×10^4	6.1

[a] Each value represents mean and standard deviation in triplicate experiments.

The second round of evolution was performed with the best mutant—1R1—from the first round of evolution. The experimental conditions were the same as those of the first round of evolution, except for an increased concentration of C6-AHL, to 1 μM. As a result, two mutants—2R1 and 2R2—were selected. Sequencing of these mutants revealed that 2R1 had an additional mutation, I190F on the V69L template, and that 2R2 had two mutations, I190F and G207V on the V69L template (Table 1). The two mutants were expressed and purified in the MBP-fused form and were subjected to kinetic analysis. As shown in Table 1, both 2R1 and 2R2 exhibited increased catalytic efficiency toward C6-AHL, and their k_{cat}/K_M values were estimated as $7.0\times10^4\,M^{-1}s^{-1}$ and $6.1\times10^4\,M^{-1}s^{-1}$, respectively. The catalytic efficiency of 2R1 was higher than that of 2R2, so the G207V mutation is likely to have a negligible effect on the catalytic activity of the enzyme. The increased catalytic efficiency of these mutants seemed to be the result mainly of a decrease in K_M rather than an increase in k_{cat}. The AHL concentration used to screen the AiiA mutants was as low as 1/10000 of the K_M value of the wild-type enzyme, so it is likely that the mutants with increased affinity for the substrate were primarily selected by the screening system during the directed evolution process. The growth of the best mutant—2R1—at the elevated C6-AHL concentration was confirmed (Figures 2 and S2). This result indicates that our system can be effectively used for rapid screening of quorum-quenching enzymes with high catalytic activity.

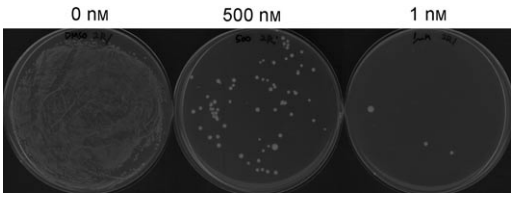

Figure 2. Growth of host cells expressing the mutant 2R1 (V69L/I190F) on agar plates containing the indicated concentrations of C6-AHL. Experiments were carried out in triplicate, and one of each is shown.

Structural analysis of mutations

In order to gain further insight into the effect of the mutations on the catalytic efficiency, we analyzed the active sites of the mutants in complexation with the target substrate C6-AHL through a docking simulation. A known product-bound structure of AiiA (PDB ID: 3DHB) was used as a template. Flexible side chains at Val69 (wild-type) and Leu69 (substituted) were used in AutoDock modeling. The most energetically stable conformations found for each case are collectively shown in Figure 3. The predicted binding modes indicate that substitution of Val with Leu (with a side chain one carbon atom longer) at position 69 shortened the distance between the amino acid chain and the acyl chain of C6-AHL (5.0→3.7 Å). This change appears to result in a stronger hydrophobic interaction between the mutant and C6-AHL, increasing the affinity

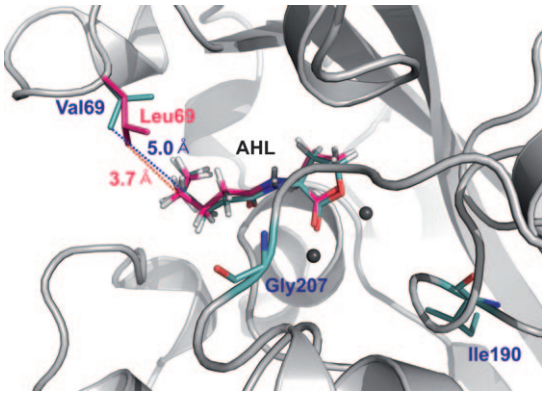

Figure 3. The predicted binding modes of C6-AHL at the active site of AiiA. The most energetically stable conformations are superimposed. At position 69, flexible amino acid side chains were used during the AutoDock modeling. The involved amino acid residues and C6-AHL from docking simulation of wild type and substrate are represented as light blue carbon sticks. The Leu69 and C6-AHL from docking of V69L mutant and substrate are displayed as pink carbon sticks. Two zinc ions are displayed as gray spheres. Oxygen is shown in red, nitrogen in blue, and hydrogen in white. The dashed lines indicate the predicted closest distances between amino acid chains at position 69 and the acyl chain of C6-AHL.

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of the enzyme toward C6-AHL. The kinetic analysis of the V69L mutation supports this presumption: the K_M value of the V69L mutant decreased to one fourth of that of the wild-type enzyme. Meanwhile, a negligible change in k_{cat} was observed, as shown in Table 1.

The double mutant (V69L/I190F) exhibited an increased catalytic efficiency over the single mutant V69L; this implies a beneficial effect of the second mutation (I190F; Table 1). To analyze the effect of I190F in more detail, we constructed a single mutant (I190F) from the wild-type enzyme and measured its catalytic activity. Interestingly, the I190F mutant showed a slightly enhanced affinity for substrate, but decreased catalytic turnover, even though the mutation resulted in a catalytic efficiency (k_{cat}/K_M) similar to that of wild-type AiiA (Table S1). The structural analysis revealed that Ile190 is located beside Asp191, which is a key residue involved in the coordination of two Zn cofactors and organization of the catalytic core at the active site.[15] It has been shown that highly conserved residue homologous to Asp191 in the metallo-β-lactamase superfamily is important for catalytic composition.[16] Mutation near Asp191 is thus more likely to influence the activity of the enzyme. We verified that the I190F mutation has no significant effect on the expression level (data not shown).

The kinetic analysis of 2R2 showed that the effect of the G207V mutation on the catalytic efficiency of the V69L/I190F mutant (Table 1) is negligible. To confirm this, we constructed a single mutant (G207V) and determined its catalytic parameters. As a result, the G207V mutation displayed a minor change in relation to wild-type AiiA (Table S1), even though this residue was presumed to interact with an acyl chain of AHL.[17]

Conclusions

In conclusion, we have demonstrated that the genetic circuit system we present here can be effectively used for the rapid screening of AHL-degrading enzymes with high catalytic activity. Our selection system relies on the growth of host cells, depending on the catalytic efficiency of the quorum-quenching enzyme, and enables high-throughput screening of the enzyme. Our system thus offers a distinct advantage over other methods.[18] The genetic circuit system might be expected to be a useful tool for the rapid isolation and directed evolution of quorum-quenching enzymes with greater potential.

Experimental Section

Reagents: The *N*-hexanoyl-L-homoserine lactone (C6-AHL) was purchased from Cayman Chemical (Ann Arbor, MI), and its chemical formula is shown in Figure S3. The antibiotics, carbenicillin and kanamycin, were obtained from Sigma–Aldrich. Luria–Bertani (LB) agar and broth were purchased from Duchefa Biochemie (Haarlem, the Netherlands). The restriction enzymes were purchased from New England Biolabs. The T4 DNA ligase, thermophilic polymerases, and other PCR reagents were purchased from TaKaRa Bio (Kyoto, Japan). The plasmid pZS*24Luc was purchased from Expressys (Ruelzheim, Germany), and the plasmid pMAL-c2x was obtained from New England Biolabs. The oligonucleotides were synthesized by Bioneer (Daejeon, Korea). All other molecular biology reagents were purchased from commercial sources and were of analytic grade.

Construction of a genetic circuit system: For the construction of a genetic circuit system based on AHL-dependent quorum sensing signaling, the AHL-response plasmid pQS was constructed. Briefly, the plasmid pluxBLIP was first modified by replacement of colE1 ori with p15A ori from pLuxR through the use of the restriction endonucleases SacI and XbaI. The $P_{lac/ara-1}$-LuxR fragment containing the promoter and luxR gene was amplified from pLuxR with use of PrimeSTAR DNA polymerase and primers (5′-AAT AAC TAG TTG TGT GGA ATT GTG AGC GGA-3′) and (5′-AAT AAC TAG TTC TAG GGC GGC GGA TTT GTC-3′). The reaction temperature was 95 °C for 3 min, followed by 20 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 90 s, and 72 °C for 90 s. The amplified fragment was inserted into the ori-modified pluxBLIP by SpeI restriction reaction, resulting in the AHL-response plasmid pQS. The plasmids pLuxR and pluxBLIP were donated by Dr. Frances Arnold's lab.[19] The plasmid pZS*24DN for the expression of quorum-quenching enzyme was constructed from pZS*24Luc by silent deletion of the NcoI endonuclease site of the kan^r gene. The plasmids used in this study are shown in Figure S4.

Generation of a mutant library: Host cells for library generation were constructed by insertion of the plasmids pQS and pZS*24DN into *E. coli* DH5α. The AHL hydrolase (AiiA) of *Bacillus* sp. obtained from Dr. Jung-Kee Lee's lab was used as a model quorum-quenching enzyme.[15a] A random library of AiiA was generated by error-prone PCR at a mutation frequency of two to three substitutions per 250 amino acids as described previously.[20] The PCR mixture contained Tris·HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (7 mM), $MnCl_2$ (0.1 mM), dGTP (0.2 mM), dATP (0.2 mM), dCTP (1 mM), dTTP (1 mM), template plasmid (~100 pg per 50 μL reaction), Taq DNA Polymerase (2.5 unit per 50 μL reaction), and primers (each 0.5 μM). The primers used were N-(5′-AAT TAC CAT GGG AAC AGT AAA GAA ACT TTA TTT CAT C-3′) and C-(5′-AAT TAT CTA GAT TAT ATA TAT TCC GGG AAC ACT CTA C-3′). The reaction was conducted at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 50 s, and finally at 72 °C for 50 s. The PCR products were cloned into the pZS*24DN plasmid by using the restriction endonucleases NcoI and XbaI. The resulting plasmids were transformed into *E. coli* DH5α harboring the AHL-response plasmid—pQS—by electroporation.

Screening of improved mutant enzymes: The transformants of the mutant library were directly plated onto LB agar plates containing carbenicillin (100 μg mL^{-1}), kanamycin (30 μg mL^{-1}), $ZnSO_4$ (0.1 mM), and a predetermined concentration of C6-AHL, followed by incubation at 30 °C for three days. The average library size of the transformants was 5×10^5. To eliminate false positives, the pZS*24DN plasmids were exclusively purified from a mixture of the primary screened colonies and pQS-specific degradation by using the restriction endonuclease AseI, and retransformed into new DH5α host cells harboring pQS. After overnight culture at 37 °C in LB medium and dilution to OD_{600} 0.004, the cells (100 μL) were replated on agar plates containing the same AHL concentration. The growing colonies were individually reconfirmed by the same procedure.

Characterization of selected mutant enzymes: The selected mutant enzymes were expressed and investigated as MBP-tagged forms by amplification with use of PrimeSTAR™ DNA polymerase and the primers. The sequences of the primers were: N-(5′-AAT ATG AAT TCA TGG GAA CAG TAA AGA AAC TTT AT-3′) and C-(5′-AAT TAT CTA GAT TAT ATA TAT TCC GGG AAC ACT CTA C-3′). The ex-

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perimental conditions were: 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 50 s, and 72 °C for 50 s. The PCR products were inserted into the expression vector pMAL-c2x by using the restriction endonucleases EcoRI and XbaI and T4 DNA ligase, followed by cloning in *E. coli* strain XL1-blue. The transformed cells were grown at 37 °C in LB medium containing carbenicillin (100 μg mL^{-1}), $ZnSO_4$ (0.2 mM), and glucose (0.2%, *w*/*v*). When the OD_{600} reached 0.4, isopropyl β-D-1-thiogalactopyranoside (0.3 mM) was added, followed by further incubation at 30 °C for 6 h. The MBP-fused proteins were purified by using the MBP Excellose® SPIN Kit (TaKaRa Bio), binding buffer [Tris-HCl (50 mM, pH 7.0), NaCl (100 mM), and $ZnCl_2$ (0.2 mM)], and elution buffer [binding buffer containing maltose (10 mM)]. The enzyme activity was determined by measuring the decrease in absorbance at 557 nm as reported previously.[14] The reaction mixture (1 mL) contained deionized water (480 μL), 2× dye solution [HEPES (2 mM, pH 7.5), Na_2SO_4 (200 mM), $ZnSO_4$ (0.4 mM), and Phenol Red (80 μM), 500 μL], enzyme (10 μL of 0.2 mg mL^{-1}), and C6-AHL (10 μL of 50~500 mM) dissolved in DMSO. The initial rates were corrected for spontaneous hydrolysis of AHL.[21] The kinetic parameters were calculated from triplicate experiments by using the double reciprocal plot of the substrate concentration and the initial reaction rate of enzyme.

Docking simulation: To gain some insight into the effect of substituted amino acid residues in the mutant enzymes, docking simulation of the enzyme in complex with the target substrate C6-AHL were performed with AutoDock 4.0.[22] The 3D structure of AiiA was obtained from the Protein Data Bank (PDB ID: 3DHB).[15b] The 3D structure of C6-AHL was generated and energy-minimized with Chem3D Ultra 8.0 (Cambridge Soft). We determined the docking parameters through preliminary docking of the substrate to the wild-type enzyme structure. Initially a large grid was placed around the two zinc ions and was manually adjusted in two additional rounds according to the results. To maximize the likelihood of finding optimal orientations, we allowed for 50 million evaluations and increased the maximum number of generations to 50000. To mimic the variance that exists among the molecular ensemble of actual enzyme structures and to provide stable mutant models for the AutoDock program, we generated each ten optimized structures of the wild-type and the V69L mutant with MODELLER 9v7,[23] a homology modeling software package. Flexible residues (Val69 and Leu69) were assigned with the aid of the AutoDockTools. Ten parallel sessions of flexible docking were performed under the parameters determined during the preliminary docking. The results from all models were collected, sorted for estimated total free energy of binding, and visually examined in PyMol (DeLano Scientific LLC).[24]

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