Bacillus megaterium CYP102A1 Oxidation of Acyl Homoserine Lactones and Acyl Homoserines†

Puneet K. Chowdhary,‡ Neela Keshavan,§ Hien Q. Nguyen,‡ Julian A. Peterson,|| Juan E. González,§ and Donovan C. Haines*,‡

Department of Chemistry, University of Texas at Dallas, Richardson, Texas 75080, Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas 75080, and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9038

Received September 25, 2007

ABSTRACT: Quorum sensing, the ability of bacteria to sense their own population density through the synthesis and detection of small molecule signals, has received a great deal of attention in recent years. Acyl homoserine lactones (AHLs) are a major class of quorum sensing signaling molecules. In nature, some bacteria that do not synthesize AHLs themselves have developed the ability to degrade these compounds by cleaving the amide bond or the lactone ring. By inactivating this signal used by competing bacteria, the degrading microbe is believed to gain a competitive advantage. In this work we report that CYP102A1, a widely studied cytochrome P450 from *Bacillus megaterium*, is capable of very efficient oxidation of AHLs and their lactonolysis products acyl homoserines. The previously known substrates for this enzyme, fatty acids, can also be formed in nature by hydrolysis of the amide of AHLs, so CYP102A1 is capable of inactivating the active parent compound and the products of both known pathways for AHL inactivation observed in nature. AHL oxidation primarily takes place at the ω-1, ω-2, and ω-3 carbons of the acyl chain, similar to this enzyme's well-known activity on fatty acids. Acyl homoserines and their lactones are better substrates for CYP102A1 than fatty acids. Bioassay of the quorum sensing activity of oxidation products reveals that the subterminally hydroxylated AHLs exhibit quorum sensing activity, but are 18-fold less active than the parent compound. *In vivo*, *B. megaterium* inactivates AHLs by a CYP102A1 dependent mechanism that must involve additional components that further sequester or metabolize the products, eliminating their quorum sensing activity. Cytochrome P450 oxidation of AHLs represents an important new mechanism of quorum quenching.

The ability of bacteria to sense their own population density, termed quorum sensing, serves an integral role in symbiosis and pathogenesis (*1*, *2*). In recent years the underlying mechanisms have received considerable attention. Perhaps the best characterized class of quorum sensing signal is the acyl homoserine lactone (AHL[1]). AHLs are utilized by many Gram negative bacteria and are composed of the amino acid homoserine cyclized into a lactone and acylated at the amino nitrogen by a fatty acid that may be oxidized at the beta carbon. Members of this class include a variety of acyl chains, but all have the identical homoserine lactone head group (*1*, *2*).

Because the events that take place when a given species of bacteria achieves a quorum give it a competitive advantage, other competing bacteria have evolved to interfere with quorum sensing. For example, acyl homoserine lactonases, which convert the AHL to the acyl homoserine (AH), have been identified in certain species of *Bacillus* and an acylase, which removes the acyl chain from the homoserine nitrogen, in *Ralstonia* (*3*−*7*). Both enzymes destroy the ability of the signaling molecule to turn on quorum sensing regulated gene expression in competing bacteria. This competitive inactivation of quorum sensing signals has been observed in many diverse environs and has been termed "quorum quenching" (*4*, *5*, *8*−*10*).

The excitement surrounding quorum sensing is due in no small part to the demonstration that interference with the ability of bacteria to sense a quorum can prevent infection

† Funding for this work was provided by The University of Texas at Dallas (D.C.H.), the Welch Foundation (AT-1600, D.C.H.), the National Institutes of Health (1R01GM43479, J.A.P.; 1R01GM069925, J.E.G.), and the National Science Foundation (MCB 0517162, J.E.G.).

* Address correspondence to this author at Department of Chemistry, University of Texas at Dallas, Box 830688 M/S BE26, Richardson, TX 75083-0688. Tel: 972-883-4543. Fax: 972-883-2925. E-mail: Haines@utdallas.edu.

‡ Department of Chemistry, University of Texas at Dallas.

§ Department of Molecular and Cell Biology, University of Texas at Dallas.

|| University of Texas Southwestern Medical Center at Dallas.

[1] Abbreviations: AH, acyl homoserine; AHL, acyl homoserine lactone; C12-HSL, *N*-lauroyl-DL-homoserine lactone; C12-HS, *N*-Lauroyl-DL-homoserine; β-oxo-C12-HSL, *N*-2-oxolauroyl-DL-homoserine lactone; β-oxo-C12-HS, *N*-3-oxolauroyl-DL-homoserine; C16-HS, *N*-palmitoyl-DL-homoserine; BSTFA/TMCS, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane; DMSO, dimethyl sulfoxide; GC/MS, gas chromatography/mass spectrometry; KPi, potassium phosphate buffer; LC/MS, liquid chromatography/mass spectrometery; MGM, minimal media; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NMR, nuclear magnetic resonance; NSAID, nonsteroidal anti inflammatory drug; ONPG, *o*-nitrophenyl-β-D-galactopyranose; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; TMS, trimethylsilyl; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

10.1021/bi701945j CCC: $37.00
Published on Web 11/20/2007

of a host by a pathogen. This has been dramatically demonstrated by genetically engineering a plant to produce the *Bacillus* acyl homoserine lactonase, almost entirely preventing the ability of an AHL-utilizing phytopathogen to infect the plant host (*9*). Thus there is great therapeutic potential in the prevention of infection in humans and in the prevention of biofouling by interference with quorum sensing pathways.

The bacterial cytochrome P450 monooxygenase CYP102A1 (traditionally referred to as P450BM-3) from *Bacillus megaterium* has been widely studied due to its similarity to eukaryotic microsomal P450 cytochromes. In *B. megaterium* this enzyme is inducible by barbiturates, fatty acids, and NSAIDs (*11*−*15*), which also makes it a very good bacterial model for inducible xenobiotic metabolizing P450s in mammals. It has been known for quite some time that CYP102A1 is capable of oxidizing fatty acids at the ω-1, ω-2, and ω-3 positions (*16*). We have recently shown that N-fatty acyl amino acids are much better substrates for the enzyme than fatty acids, although the physiological significance of this was unknown (*17*). Because several members of *Bacillus* are known to be able to degrade acyl homoserine lactones to acyl homoserines, which are N-fatty acyl amino acids, we set out to explore whether AHLs and AHs are substrates for the enzyme and whether their oxidation could be of physiological significance. This is the first report that a cytochrome P450 is capable of oxidizing compounds in AHL quorum sensing pathways.

EXPERIMENTAL PROCEDURES

CYP102A1 was prepared as previously described (*18*, *19*). Concentration of P450 was determined by standard carbon monoxide difference spectra (*20*). All UV−vis spectra were collected on an Agilent HP8453 diode array UV−vis spectrophotometer. ^{1}H NMR spectra were recorded on a Varian INOVA 500 MHz and 270 MHz NMR. GC−MS studies were performed on Finnigan MAT GCQ with a J&W scientific high-resolution DB-5MS column. All AHLs synthesized or purchased are racemic at the homoserine α-carbon except where specifically noted otherwise.

Synthesis of AHLs and AHs. N-Palmitoyl-DL-homoserine lactone and β-oxo-lauroyl DL-homoserine lactone were synthesized in moderate yield from the corresponding commercially available DL-homoserine lactone and respective acid chloride by methods previously established (*21*, *22*). Purity and identity were confirmed by GC−MS and NMR spectroscopy. *N*-Lauroyl-DL-homoserine lactones were obtained from Fluka. Respective acyl homoserines were then obtained by heating the lactone at 70 °C to 90 °C in basic solution (50 mM potassium carbonate or 10 mM sodium hydroxide) for 30 min.

Binding of AHLs and AHs to CYP102A1. A solution of 2−8 μM CYP102A1 in 50 mM KPi with 50 mM KCl, pH 7.4, was titrated with a solution of 10 mM AHL in DMSO or acyl homoserine in 50 mM K_2CO_3 in a stirred 1.00 cm quartz cuvette to beyond the equivalence point. After addition of each aliquot of substrate, the solution was allowed to equilibrate for 30 s before the UV−visible absorption spectrum was recorded. The data for the absorbance at 418 nm minus the absorbance at 394 nm (to give a maximal change in absorbance) was plotted against inverse of concentration of free substrate (assuming bimolecular association reaction) to obtain the dissociation constant.

Oxidation of AHLs and AHs by CYP102A1. For rate determination, a 1.4 mL solution of 100−250 μM *N*-acylhomoserine and 250 μM NADPH in 50 mM KPi with 100 mM KCl, pH 7.4, were placed in a stirred cuvette at room temp. To this solution, CYP102A1 was added to a final concentration of 100 nM, and the progress of the reaction was monitored by the consumption of NADPH as indicated by the loss of absorbance at 340 nm. The rate of turnover was calculated using an extinction coefficient at 340 nm of 6220 M^{-1} cm^{-1} for NADPH and dividing by enzyme concentration.

For preparative scale reactions for structural analysis, AHL was added to a 178.0 mL solution of 100 nM CYP102A1 in 50 mM KPi, with 100 mM KCl pH 7.4, to a final concentration of 100 μM. To this solution, NADPH was added to 80 μM, and the progress of the reaction was monitored by the consumption of NADPH as indicated by the loss of absorbance at 340 nm. The enzyme mix was acidified (pH 2) after 40 min. This acidified mixture was immediately extracted three times with 20 mL of dichloromethane. The organic extracts were combined, dried with sodium sulfate, and evaporated to dryness.

The preparative scale reaction for AH was prepared and treated essentially the same way as an AHL sample except that the substrate was added to a 70.0 mL solution of 100 nM CYP102A1 in 50 mM KPi, with 100 mM KCl pH 7.4, to a final concentration of 500 μM. To this solution, NADPH was added to 250 μM. The enzyme mix was acidified (pH 2) after 40 min. This acidified mixture was incubated for 6 h and extracted with 3 × 20 mL of dichloromethane. The organic extracts were combined, dried with sodium sulfate, and evaporated to dryness.

Bioassay: Quorum-Sensing Activity of Oxidized Products. The products obtained from metabolizing *N*-lauroyl-DL-homoserine (C12-AH) with CYP102A1 were checked for quorum sensing activity with the *Agrobacterium tumefaciens* NTL4 (pZLR4) indicator strain as described in (*23*). The product extracts containing enzyme oxidized AHLs and AHs were resolved on a normal phase TLC plate (Merck silica 60 F_{254}) and overlaid with top agar containing the indicator strain and 80 μg/mL X-gal and incubated overnight at 30 °C. To examine the activity of the product mixture in lactone form, the product mixture was acidified to pH 2 using oxalic acid and re-extracted in dichloromethane and dried using sodium sulfate. This product extract was spotted on normal phase TLC and developed in 10% (v/v) methanol:dichloromethane, dried, overlaid with top agar containing the indicator strain and X-gal, and incubated overnight at 30 °C. To examine the activity of the product mixture in hydroxy-acid (hydrolyzed lactone) form, the product mixture was adjusted to pH 12 with K_2CO_3 and spotted on a normal phase TLC plate, resolved, dried, and overlaid with top agar containing the indicator strain and X-gal and incubated overnight at 30 °C.

For quantitative assays of quorum activity, the same indicator strain was grown in the presence of AHLs (control) or enzyme metabolized AHLs overnight in 2 mL of MGM media. After incubation overnight at 30 °C with 225 rpm shaking, 200 μL of the cell culture was added to 800 μL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl,

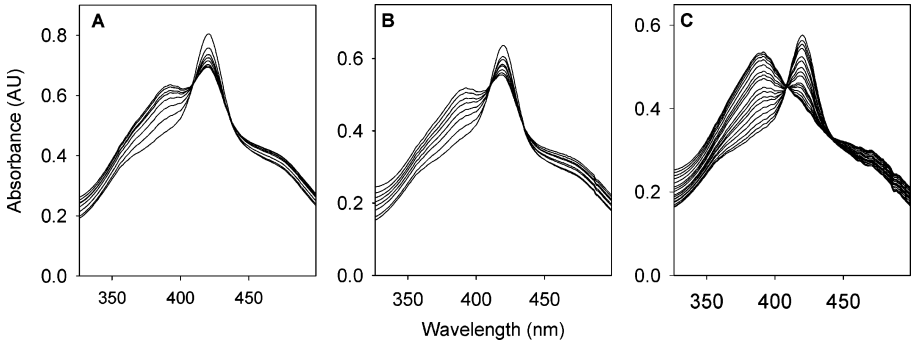


FIGURE 1: Spectral assay of acyl homoserine binding to CYP102A1. Aliquots of (A) 15.5 mM β-oxo-C12-HSL, (B) 11.5 mM β-oxo-C12-HS, or (C) C16-HS were added to a buffered solution of CYP102A1, and the UV−visible spectrum was recorded. Additions were continued until there was no further change in the spectrum. In all titrations the intensity of the Soret band at 418 nm reduces as enzyme converts from free to substrate bound, and the intensity of the peak at 394 nm increases. Concentrations were (A) 5.6 μM CYP102A1 titrated to 0, 27, 55, 82, 110, 137, 164, 192, and 260 μM β-oxo-C12-HSL; (B) 4.4 μM CYP102A1 titrated to 0, 18, 37, 55, 73, 128, 183, and 237 μM β-oxo-C12-HS; and (C) 4.0 μM CYP102A1 titrated to 0.00, 0.06, 0.13, 0.25, 0.50, 0.63, 0.75, 0.88, 1.00, 1.13, 1.25, 1.69, 2.13, 2.56, 3.00, 3.44, and 4.31 μM C16-HS.

1 mM $MgSO_4$, 50 mM β-mercaptoethanol), and permeablized by the addition of 2 drops of chloroform and 1 drop of 0.1% SDS. The chromophoric galactosidase substrate *o*-nitrophenyl-β-D-galactopyranose (ONPG) in Z-buffer was added to 0.4 mg/mL, and the reactions were incubated at 28 °C until significant color development was observed. The incubation time was recorded, the reaction was quenched by the addition of 0.5 mL of 1 M Na_2CO_3, the cells were removed by centrifugation, and the absorbance at 420 nm was recorded. The galactosidase activity, expressed in Miller units, was calculated as $(1000 \times OD_{420nm})/(0.2$ mL culture $\times$ minutes elapsed $\times$ culture $OD_{600nm})$. Optical densities at the time of galactosidase assay were similar for all samples tested.

Quorum Quenching in Vivo by Bacillus megaterium. A 2 mL starter culture of *B. megaterium* ATCC 14581 (ATCC) grown for 8 h at 30 °C in LB medium was diluted 100-fold into 100 mL of either LB or *Bacillus* minimal medium (*24*) and grown to an OD600 of ~1.0. Each solution was diluted 50% and divided into aliquots of either 2 mL or 100 mL. For the experiment in LB medium, C12-HSL was added from a 20 mM stock in methanol to a final concentration of 10 μM. For the experiment in minimal medium, the final concentration of C12-HSL was 1 μM. For both experiments, an equal number of samples were prepared with and without the CYP102A1 mechanism-based inhibitor 17-octadecynoic acid (17-ODA) at a final concentration of 25 μM. All samples were then incubated at 30 °C with 225 rpm shaking and aliquots removed at 0, 1, 3, 5, 7, and 24 h. Aliquots were centrifuged to remove the *B. megaterium*, and the spent medium was immediately frozen at −80 °C until ready for analysis by the *A. tumefaciens* bioassay. For bioassay, 100 μL (minimal medium) or 20 μL (LB) of spent medium was used in a 2 mL *A. tumefaciens* overnight culture in MGM and 100 μL of this culture used in each 500 μL galactosidase assay.

RESULTS

Binding of Acyl Homoserine Lactones and Acyl Homoserines. As previous work had shown that acyl amino acids bind to CYP102A1 better than fatty acids, we first measured binding constants for a series of simple AHLs and AHs. Dissociation constants for P450 cytochromes are conveniently measured by monitoring a shift of the Soret band upon substrate binding (*20*, *25*−*27*). In CYP102A1, this shift from 418 nm to 394 nm occurs as the heme iron changes from the low-spin state to the high-spin state due to protein conformational changes that remove the water ligand when substrate binds to the enzyme.

Table 1: Spectral Dissociation Constants for Acyl Homoserines

	CYP102A1 K_D, μM
lauric acid[a]	270
N-lauroyl-DL-homoserine lactone (C12-HSL)	9.9 ± 1.4
N-lauroyl-DL-homoserine (C12-HS)	21 ± 3
β-oxolauroyl-DL-homoserine lactone (β-oxo-C12-HSL)	52 ± 2
β-oxolauroyl-DL-homoserine (β-oxo-C12-HS)	210 ± 20
β-oxolauroyl-L-homoserine lactone (β-oxo-C12-L-HSL)	78 ± 3
N-palmitoyl-DL-homoserine (C16-HS)	0.34 ± 0.10

[a] Dissociation constant reported in reference (*34*).

AHLs with acyl chain lengths of 12 and 16 carbons were assayed. The most widely studied AHLs are the β-oxo-C12 and shorter compounds; CYP102A1 has an established preference for longer chain fatty acids (*16*, *28*−*30*). CYP102A1 will oxidize fatty acids varying from 12 carbons to more than 20 carbons in length, with highest activity for 15 carbons (*28*). AHLs with lengths up to 18 carbons have been characterized in quorum sensing pathways (*31*−*33*). Although numerous AHLs with acyl chains shorter than 12 carbons are known, they would not be expected to be good substrates for CYP102A1 based on the enzyme's known specificity.

We first assayed the binding of the quorum active compounds with a closed homoserine lactone ring. As shown in Figure 1, addition of C12-HSL to a solution of CYP102A1 resulted in efficient conversion of the enzyme from low-spin to high-spin (as measured by the shift of the heme Soret band from 418 nm to 394 nm). The dissociation constants of C12-HSL and β-oxo-C12-HSL were determined to be 9.9 ± 1.4 and 52.4 ± 2.3 μM, respectively (Table 1). These compare favorably to affinities reported in the literature for lauric acid, ~270 μM in (*34*), which is surprising given this enzyme's known preference for anionic substrates. Titrations with the longer C16-HSL were not practical due to the very low solubility of the compound.

We then assayed the binding of the anionic versions of the AHLs where the lactone ring had been hydrolyzed in aqueous sodium carbonate. These acyl homoserines are the expected products of the *Bacillus* quorum quenching AHL lactonases. As can be seen in Figure 1, addition of acyl homoserine also resulted in efficient conversion of the enzyme from low-spin to high-spin. The change was essentially complete by the end of the titration (Figure 1).

Dissociation constants for the binding of the AHs and AHLs studied to CYP102A1 appear in Table 1. It is clear that both the acyl homoserines and their lactones bind very well to the enzyme, with the C16 homoserine binding nearly stoichiometrically under UV−visible conditions. Tighter binding of the C16 acylated compound relative to the C12 acylated compound is consistent with CYP102A1's known specificity for longer chain fatty acids. The ideal saturated fatty acid chain length for CYP102A1 is 15−16 carbons. What is surprising is that for both C12-HSL/C12-HS and β-oxo-C12-HSL/β-oxo-C12-HS the anionic hydrolyzed form binds more weakly than the neutral lactone form of the molecule, in spite of CYP102A1's known preference for negatively charged substrates.

AHLs found in nature normally involve the L-stereoisomer of homoserine. To test whether the enzyme prefers a certain stereochemistry at the α-carbon, we synthesized L-C12-HSL starting from pure L-homoserine. Lack of racemization of the homoserine α-carbon during acylation was confirmed by hydrolysis with human paraoxonase, which is selective for the L-stereoisomer (data not shown). L-C12-HSL bound to CYP102A1 with a slightly lower affinity than the racemic mixture (78 ± 2.9 μM versus 52.4 ± 2.3 μM). This demonstrates a slight preference for the nonphysiological AHL, but clearly both stereoisomers bind better than the comparable fatty acid.

Turnover and Kinetics of Acyl Homoserines. Since it was clear that acyl homoserines bound tightly to the enzyme, we next measured whether the enzyme would efficiently oxidize these potential substrates. Addition of enzyme to a solution of buffer, stoichiometric amounts of the electron source NADPH, and an AHL or AH resulted in the rapid consumption of electrons as monitored by the loss in NADPH absorbance at 340 nm as NADPH is converted to $NADP^+$ (data not shown). Hydrogen peroxide assays revealed only trace amounts of hydrogen peroxide production, indicating that electron consumption is tightly coupled to substrate oxidation as is seen with fatty acid oxidation by CYP102A1 (although some uncoupling, ~10%, can be observed with the short chain fatty acid lauric acid; data not shown).

CYP102A1 can oxidize fatty acids multiple times, as some monohydroxylated products are reasonably good substrates of the enzyme (*35*). Repeating turnover assays with an excess of NADPH relative to acyl homoserine clearly showed a stoichiometry of more than one NADPH per acyl homoserine, with the reaction slowing visibly after the first substrate oxidation as is observed with fatty acid substrates (data not shown). Thus, CYP102A1 is capable of carrying out more than one oxidation per substrate molecule.

A full substrate kinetic study was carried out to quantify the dissociation and turnover numbers of CYP102A1 for representative AHL and AH substrates. For reference, laurate was also characterized under identical conditions. K_M and V_{max} values appear in Table 2. As was seen from spectroscopic dissociation constants, it is clear that the homoserine moiety increases the compound's affinity for the enzyme (decreased K_M) in a manner similar to that observed for other acylated amino acids. Presumably this tighter binding of acyl homoserines is due to the same interactions with the B′-helix that were observed for *N*-palmitoylglycine (*17*). Also consistent with the spectroscopic measurement, the neutral lactone form of β-oxo-C12-HSL had a significantly lower K_M than the negatively charged hydroxy acid form. The turnover numbers for the AHLs and AHs are all equal to or significantly larger than that for lauric acid. The combination of higher turnover number and lower K_M establishes AHLs and AHs as much better substrates for CYP102A1 than the corresponding fatty acids.

Table 2: Kinetic Parameters for the CYP102A1 Oxidation of Substrates with C12 Acyl Chains

	K_M, μM	turnover number, min^{-1}	catalytic efficiency, min^{-1} μM^{-1}
lauric acid	250 ± 30	1700 ± 180	6.8 ± 1.1
N-lauroyl-DL-homoserine lactone (C12-HSL)	ND[a]	ND	ND
N-lauroyl-DL-homoserine (C12-HS)	140 ± 30	2700 ± 300	19 ± 5
N-β-oxolauroyl-DL-homoserine lactone (β-oxo-C12-HSL)	34 ± 6	1660 ± 70	49 ± 9
N-β-oxolauroyl-DL-homoserine (β-oxo-C12-HS)	210 ± 30	1700 ± 180	5.9 ± 1.1

[a] ND = not determined due to solubility issues.

Product Identification. Previous experience has shown that CYP102A1 oxidized both saturated fatty acids and fatty acyl amino acids at the ω-1, ω-2, and ω-3 positions. These were clearly the expected products for AHL and AH oxidation also, and this was verified by performing a larger scale turnover experiment under conditions where half of the substrate molecules are expected to be oxidized (thus eliminating complexities of both single oxidation and multiple oxidation products being present in the sample to be analyzed). The products were extracted, derivatized with BSTFA/TMCS and analyzed by GC/MS. The chromatogram (Figure 2) shows three major products. TMS derivatives of subterminal hydroxyl groups tend to fragment at either side of the carbinol carbon, and this allows the identification of the three major products as ω-3, ω-2, and ω-1 in order of elution. Key fragments identifying these products are *m*/*z* 145/328 for ω-3, 131/342 for ω-2, and 117/356 for ω-1. A trace amount of ω-4 and ω-5 hydroxylated products can also be detected, but they amount to less than one percent of total products. Full mass spectra and product identification analysis can be found in the accompanying Supporting Information. Comparing the integrations of the total ion current for each peak provides an estimate of the abundance of the different products of 43:47:11 ω-3:ω-2:ω-1. Similar LC/MS analysis of the recyclized products of C12-HS oxidation as well as ^{1}H NMR analysis of the product extract providing further confirmation of the identity of the products can be found in the Supporting Information. In both cases, the only significant products were the ω-1, ω-2, and ω-3 hydroxylated AHLs. The hydroxyacyl homoserines produced by CYP102A1 are novel products not previously reported in the literature.

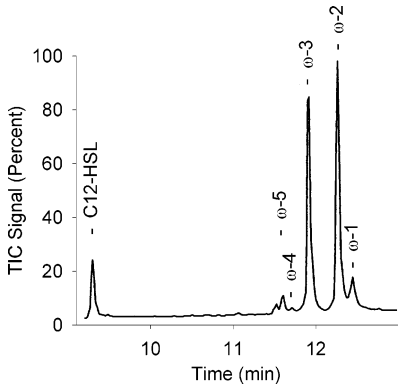


FIGURE 2: Chromatographic analysis of reaction products. Analysis by GC/MS. A sample of C12-HSL was oxidized by CYP102A1 under conditions that would give 60% conversion to products. The starting material and products were extracted into ethyl acetate, taken up in $CDCl_3$ for NMR analysis, and a portion of the NMR sample was derivatized with BSTFA/TMCS. This was then resolved on a Finnigan GCQ GC/MS with a DB-5MS column. Only the region of the chromatogram where starting material and products eluted is shown, starting material eluted prior to products.

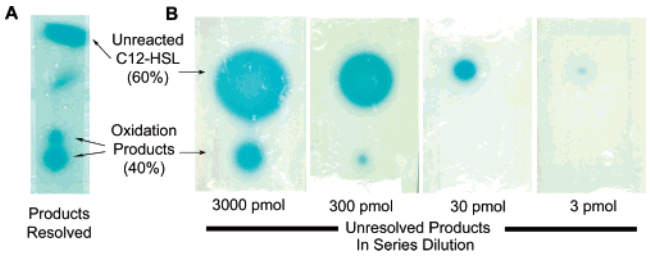


FIGURE 3: Bioassay determination of the quorum sensing activity of oxidized AHLs. Panel A: A TLC spotted with product extract (~40% turnover of substrate) and developed in 1:10 methanol: chloroform showing multiple active products (lower blue spots). Products partially resolve in this solvent. The TLC was then dried and overlaid with *A. tumefaciens* NTL4 (pZLR4) indicator strain in agar and allowed to grow overnight at 30 °C. Panel B: A TLC spotted with product extract (~40% turnover of substrate) in a 10-fold series dilution (starting ~3 nmol C12-HSL) and developed in 0.1:10 methanol:chloroform. The less polar solvent in this case and short run distance causes all products to coelute but resolve from starting material, assisting visual comparison of starting material and (total) product quorum sensing activities. The TLC was dried and overlaid with *A. tumefaciens* NTL4 (pZLR4) indicator strain in agar and allowed to grow overnight at 30 °C.

The data clearly establish formation of ω-1, ω-2, and ω-3 hydroxylated products from CYP102A1 oxidation of C12-HSL or C12-HS. There appears to be more ω-3 and less ω-1 oxidation than occurs with lauric acid, but we have observed this in previous studies with the comparable compound *N*-lauroylglycine (unpublished results). It appears that short chain acyl amino acids give slightly altered regiospecificity relative to fatty acids with the same acyl chain length. Otherwise the regiospecificity is identical to that observed for fatty acid and fatty acyl amino acid substrates.

CYP102A1 will oxidize fatty acids more than once, as some of the products of the initial oxidation are also substrates for the reaction. This results in the formation of diols and hydroxyl-ketones (*19*, *29*, *36*), for example. AHLs could also undergo multiple rounds of oxidation as indicated by the consumption of a large excess NADPH relative to the amount of AHL or AH provided in the enzymatic reaction. The complex mixtures of compounds that result were not structurally characterized, but are expected to be similar to the products formed in fatty acid oxidation.

Quorum Activity of Hydroxylated AHLs. To test whether the oxidation was a novel mechanism for quorum quenching (destruction of the ability of the molecules to turn on quorum sensing signaling pathways), we carried out quorum sensing bioassays on the enzymatic products. Bioassays were carried out by spotting reaction products on a normal phase TLC plate and overlaying with the commonly used *A. tumefaciens* NTL4 (pZLR4) indicator strain (*37*). With this bioassay, activation of quorum sensing induces the TraF gene which has been fused to the lacZ gene. Production of the galactosidase fusion results in the breakdown of X-gal and the release of a blue dye. The result is appearance of blue color where quorum sensing is activated.

Initial crude assays verified that starting material with the lactone intact was active and starting material with the lactone hydrolyzed was not. Because of their enhanced solubility in large scale enzyme turnover assays, we performed initial experiments on oxidized AHs recyclized to lactone. Initial assays of partially metabolized C12-HSL clearly showed that the hydroxylated acyl homoserine lactones thus formed retain significant quorum sensing activity as indicated by the blue spots near the bottom of the TLC in Figure 3A.

To perform a more sensitive assay for change in activity, a reaction with 50% conversion of C12-AH to products was carried out. The products were then incubated in acid to reclose the lactone ring and varying amounts were spotted on a TLC plate and resolved just enough to separate starting material from products. The bioassay was then carried out. In the NTL4 bioassay, semiquantitative information can be obtained from the size of the blue spot obtained. In multiple experiments and for every concentration that gave activity for both samples, the recyclized starting material gave a significantly larger blue spot (zone of activation) than the spot corresponding to the hydroxyacyl homoserine lactones (Figure 3B). Therefore, oxidation of the acyl homoserine lactones decreases their quorum sensing activity, but not as much as lactonolysis. Although quantitation in this assay is crude, spot sizes have been used to estimate degree of quenching. We estimate from the sizes of the spots that it would take approximately 20- to 40-fold more AHL oxidization products to achieve the same activity as the parent AHL. This estimate should be considered crude due to the predicted higher aqueous solubility, and therefore diffusibility, of hydroxylated product.

For a more precise measurement of the degree of reduction of quorum sensing activity of oxidized products that avoids this problem, we performed *in vitro* β-galactosidase assays using the *A. tumefaciens* indicator strain. To mimic as closely as possible the potential physiological situation, we chose to use the most commonly studied β-oxo-C12-HSL and to let it be oxidized to the maximum extent possible. Cultures of indicator were induced by addition of varying amounts of a solution composed of 50 μM β-oxoC12-HSL in 50 mM KPi pH 7.4 incubated with 250 μM NADPH and normal ~250 μM oxygen present in air equilibrated buffer. The sample to be metabolized was incubated for 20 min in the presence of 100 nM CYP102A1, and a control was incubated similarly but without the addition of enzyme. The metabolized sample was monitored by UV−visible spectroscopy to ensure the reaction was complete (the reduction of the

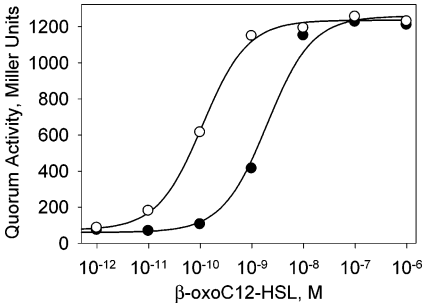


FIGURE 4: Quantitative bioassay determination of the EC50 of oxidized AHLs. Aliquots of a 20 min incubation of 50 μM β-oxo-C12-HSL with 250 μM (large excess) NADPH in 50 mM KPi pH 7.4 with (closed circles) or without (open circles) 100 nM CYP102A1 were used to induce the *Agrobacterium* indicator strain overnight and β-galactosidase activity was measured by UV−vis using the substrate ONPG as described in Experimental Procedures. Various concentrations were obtained by series dilutions of cultures just after addition of AHL or oxidized AHL. Lines represent the best fit of the data to a simple hyperbolic saturation model.

peak at 340 nm, representing NADPH conversion to NADP, slowed to a halt). As expected, the NADPH was not entirely consumed, but multiple turnovers occurred to the average substrate molecule (not all single oxidation products are normally substrates for subsequent oxidation). Aliquots of metabolized sample or control were added to 2 mL cultures of the *Agrobacterium* indicator strain in series dilution to generate a range of concentration as shown in Figure 4. After overnight incubation the degree of induction was measured in Miller Units as described in the Experimental Procedures.

As can be seen in the figure, the quorum sensing activity at saturation was similar for both control and sample at extremely high concentrations of AHL (or total AHL metabolites). The data were fit to a hyperbolic equation to determine EC50s, the concentrations that gave half-maximal induction. For control, an EC50 of 0.110 ± 0.009 nM AHL was obtained. The enzyme metabolized sample, however, had a much higher EC50 of 2.0 ± 0.4 nM total AHL metabolites. At AHL concentrations around 0.1 nM, this represents loss of 80% of the quorum activity of the AHL. It would take 18× more AHL to produce the same threshold of quorum activity for concentrations in the active region if the AHL has been metabolized by CYP102A1.

Both assays yield approximately the same degree of quenching of the quorum signal. This confirms that the effect is largely independent of the presence or absence of the β-oxo group and occurs whether the lactone form is oxidized or the hydroxy-acid form is oxidized and then recyclized. It is also similar for singly oxidized and multiply oxidized products.

In Vivo Quorum Quenching by Bacillus megaterium. Although the evidence that CYP102A1 is capable of significantly quenching quorum signals is clear, it does not necessarily mean that this reaction occurs appreciably *in vivo* or is physiologically significant. To test whether the reaction can occur *in vivo*, cultures of *B. megaterium* were incubated with 1 μM (for cultures in *Bacillus* minimal medium) or 10 μM (for cultures in LB medium) C12-HSL. Aliquots were removed at various time points and analyzed by bioassay, which required a 20-fold (minimal media) or 100-fold (LB) dilution of spent media into the bioassay culture. Larger volumes of media in the bioassay cultures resulted in artifacts

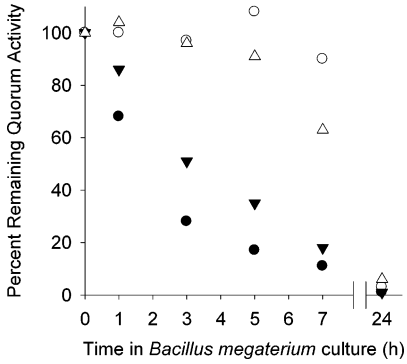


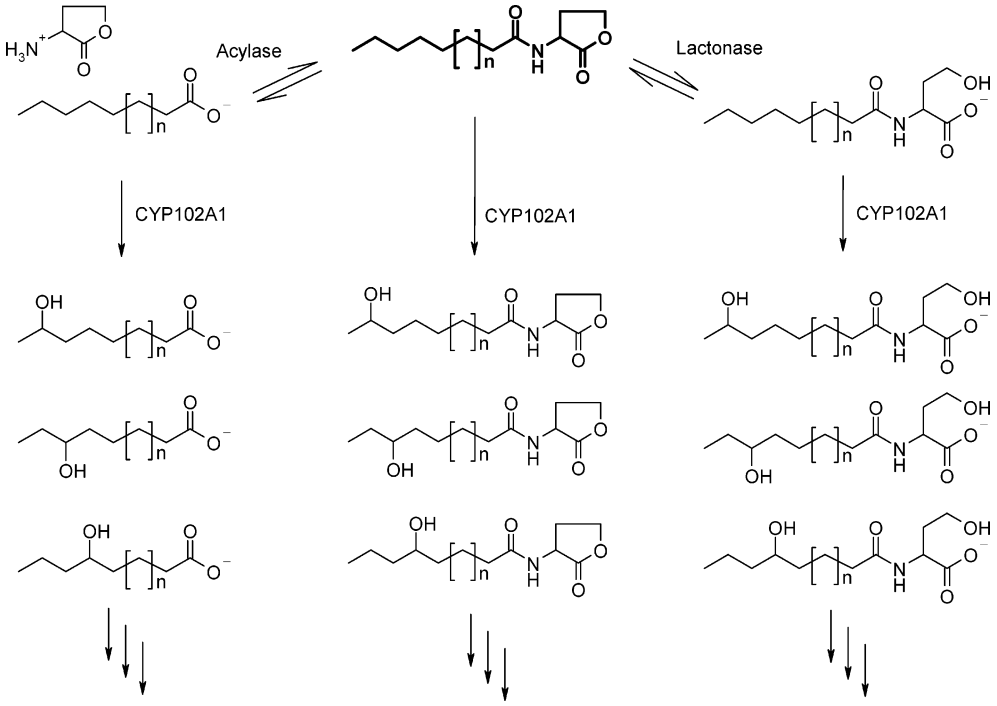
FIGURE 5: Time Dependence of Quorum Quenching *in vivo*. A *B. megaterium* culture with an OD600 of 0.5 in LB medium (triangles) or *Bacillus* minimal medium (circles) was incubated with C12-HSL in the presence (open symbols) or absence (closed symbols) of the CYP102A1 mechanism-based inhibitor 17-ODA. At 0, 1, 3, 5, 7, and 24 h aliquots were removed and the ability to activate the *A. tumefaciens* quorum sensing bioassay was measured. For the LB culture the initial C12-HSL concentration was 10 μM (100 nM after dilution into the bioassay) and for the minimal media the initial concentration was 1 μM (50 nM after dilution into the bioassay).

resulting from the presence of significant amounts of *B. megaterium* culture medium, so these were the lowest dilution factors that could be used to provide reliable assay results. The results (Figure 5, filled symbols) clearly show a reduction in quorum activity over the course of several hours. The rate of inactivation was comparable to, but slightly (~50%) slower than, that reported for lactonase expressing *Bacillus* spp. (*3*) (this will be discussed in detail in the Discussion section). The half-life of C12-HSL in *B. megaterium* culture was approximately 2 h in minimal media and 3 h in LB medium. Interestingly, in both cultures the extent of quenching was more than 80% by 7 h and essentially 100% by 24 h. Because *in vitro* experiments with purified CYP102A1 were never able to achieve such a high degree of quenching even with extensive turnover of AHL, this requires that other components must also contribute to quenching *in vivo*.

To determine if the observed quenching was due to CYP102A1, and test if the additional degree of quenching observed *in vivo* was dependent on the function of CYP102A1, the experiment was repeated in the presence of 25 μM 17-octadecynoic acid (17-ODA). This compound is a known suicide inhibitor of CYP102A1 (*38*) that has been used previously to chemically knock out the enzyme's activity *in vivo* (*39*). It is not expected to inhibit hydrolytic enzymes (lactonases or acylases) as it is nearly identical in structure to simple saturated fatty acids. As seen in Figure 5 (open symbols), the presence of the suicide inhibitor blocked nearly all (~90%) of the time dependent reduction in C12-HSL activity. It should be noted that the inhibitor may not have eliminated 100% of the CYP102A1 activity, and its inhibitory potential may last for only a limited time (likely several hours). Thus reactivation of quenching after a lag time of more than several hours is not unexpected and may simply indicate that the inhibitor is no longer active. CYP102A1 appears to be absolutely required for the quenching activity observed, and appears to be prerequisite to the other quenching processes in the cell.

From the quorum activity remaining and the starting concentration of C12-HSL, it is possible to estimate the final concentration of AHL that was achieved in the *B. megaterium*

Scheme 1: CYP102A1 Involvement in AHL Metabolism[a]



[a] Known AHL quorum quenching pathways with products that can be metabolized by CYP102A1 are shown.

culture. In minimal media, only 2−3% of the quorum activity remained after 24 h. This results in an estimate of 20−30 nM C12-HSL, which has an EC50 in this bioassay in our hands of 40−50 nM. Thus, in a physiologically meaningful time frame *B. megaterium* was able to metabolize 1 μM AHL to levels that would prevent significant activation of quorum regulated genes in a competing bacterium.

DISCUSSION

In what may be a curious coincidence in nature, CYP102A1 is able to oxidize active AHLs plus the products of both known pathways of quorum quenching (Scheme 1). A huge body of literature has characterized CYP102A1's ability to oxidize long chain saturated and unsaturated fatty acids, and work with *B. megaterium* cell culture has characterized the enzyme's ability to protect its host from the toxicity of polyunsaturated fatty acids (*15*, *39*). Long chain fatty acyl homoserine lactones have only fairly recently been discovered, and further reports continue to emerge on their role in the symbiosis of rhizosphere bacteria with plant hosts and in regulation of bacterial pathogenicity (*1*, *2*). Amide hydrolysis, which has been observed in *Ralstonia eutropha* and some other bacteria (*5*), produces the long chain fatty acids. Lactonolysis, which has been characterized in some *Bacillus* spp. (*3*, *4*), produces the acyl homoserines, which are significantly more potent substrates for the enzyme. In an environment of soil with its diverse mixture of microbes, AHLs have been shown to undergo rapid degradation (*40*).

CYP102A1 is a member of a P450 family that is rapidly growing (due to the extraordinary increase in sequenced microbial genomes) and currently includes more than twenty members. Members of the CYP102 family are rare in nature but are found in organisms containing both quenching systems. For example, CYP102A5 is found in *B. cereus* ATCC 14579 which has an AHL lactonase, and CYP102E1 is found in *Ralstonia metallidurans*, which contains an AHL acylase. Some CYP102s, including CYP102A1, are found in organisms that have not yet been found to be capable of degrading AHLs.

Is a micromolar affinity for AHLs, sensitive signaling molecules that can be detected in subnanomolar concentrations in bioassays, enough for this reaction to be of physiological significance at low nanomolar AHL concentrations? On this point, a comparison with the properties of the AHL lactonases is appropriate. As mentioned earlier, the AHL lactonases provide a nearly complete disruption of the AHL signal and this has been shown to be very useful *in vivo* in preventing the establishment of pathogenic infection, so clearly their reaction is of physiological significance. Surprisingly, work by Wang et al. (*41*) on the substrate specificity of this enzyme from *Bacillus* spp. indicates it has a very low affinity for AHLs. For β-oxo-C12-HSL, for example, it is reported that the "minimal concentration required to detect activity" is 1 mM. In fact, K_M values for all AHLs tested range from 1.4 to 7.5 mM. With K_M values ranging from 0.034 to 0.21 mM, the affinity of CYP102A1 for AHLs is 1−2 orders of magnitude higher than those of the AHL lactonase. The affinity would seem to be more than high enough to operate in the physiological environment based on this comparison. The turnover rates of the two enzymes are nearly identical. The *in vivo* rates of AHL inactivation observed here are approximately 50% of those observed previously for lactonase expressing *Bacillus* spp. under nearly identical conditions (*3*). Calculation of the initial rate of oxidation of subnanomolar concentrations of AHL in the cell by even 1 nM CYP102A1 based on the K_M and k_{cat} measured in this study (and assuming normal Michaelis−Menten kinetics) reveals that the AHL would be eliminated in a matter of 30 min even at concentrations which are many

orders of magnitude lower than the K_M. CYP102A1 is a quite capable quenching system under these conditions.

The residual quorum sensing activity of subterminally hydroxylated AHLs is not surprising. The crystal structure of the transcriptional regulator TraR from *A. tumefaciens* in complex with β-oxo-C8-HSL has been determined (*42*, *43*). In this structure, the acyl chain is buried within TraR but with the terminal part of the chain just below the protein surface pointing outward. Thus, a longer compound like β-oxo-C12-HSL would either have to protrude into solvent or require a structural rearrangement within the protein. If it protrudes into solvent, oxidation at the ω-1, ω-2, or ω-3 position would reduce problematic exposed hydrophobic surface of the acyl chain. For the same reason, it may be expected that other quorum sensing regulators that have evolved to bind longer hydrophobic acyl chains might be more negatively affected by the oxidation. The *A. tumefaciens* based indicator used in the current study is the most widely used indicator, but is not necessarily the most physiologically relevant for quorum quenching by *Bacillus* spp..

There are several potential reasons why oxidation of AHLs and AHs may be advantageous for *B. megaterium.* The first, and most obvious, is the reduction in the ability of the cyclized compound to activate quorum sensing pathways. Oxidation near the omega end of the fatty acid may make the acyl homoserine more membrane permeable, preventing buildup of the lactonolysis product inside the cell. It would also make the compound more water soluble, leading to increased diffusion away from the *Bacillus* bacterium. It has been reported that β-oxoacyl homoserine lactones can spontaneously convert to tetramic acid compounds highly toxic to *Bacillus* spp. (*44*). It may be that oxidation of the acyl chain may help to detoxify the compound before it converts to the active toxin. Finally, oxidation by CYP102A1 may merely be the first step of a degradation pathway, and the work reported here clearly demonstrates additional metabolism or sequestration must be taking place in the cell. Further work clearly needs to be done to explore the roles of the novel hydroxyacyl homoserine lactones in bacteria.

This is not the only known case of CYP102A1 metabolism of a quorum sensing compound. Although not discussed in relation to quorum sensing, farnesol has been shown to be a substrate *in vitro* for this enzyme (*45*). Farnesol is a quorum sensing signal used by the yeast *Candida albicans* (*46*, *47*). The oxidation of farnesol has been shown *in vitro*, but the potential physiological significance of this reaction has not been explored. In the case of farnesol, presence of the signal inhibits biofilm formation by the microbe, unlike acyl homoserine lactones (*47*). Degradation of farnesol may be harmful to *C. albicans*, however, as the presence of the farnesol quorum sensing signal has been shown to protect the yeast from oxidative stress (*48*).

The novel subterminally hydroxylated acyl homoserine lactones and their hydroxy-acid analogues will provide an interesting area of study as we pursue the potential physiological role of these enzymatic products *in vivo* in *B. megaterium* and related CYP102 expressing *Bacillus* spp.. We are currently examining the extent to which these products may be produced by other P450s (including human enzymes very closely related to CYP102A1) and the ability of these compounds to interfere with quorum systems other than the *A. tumefaciens* NTL4 (pZLR4) strain, as well as attempting to identify the subsequent metabolic steps in *B. megaterium* that enhance the P450 mediated quenching.

SUPPORTING INFORMATION AVAILABLE

Determination of the products of oxidation of C12-HS by CYP102A1 using LC/MS and ^{1}H NMR; determination of the products of oxidation of C12-HSL by GC/MS. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Gonzalez, J. E., and Marketon, M. M. (2003) Quorum sensing in nitrogen-fixing rhizobia, *Microbiol. Mol. Biol. Rev. 67*, 574−592.
2. Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., and Salmond, G. P. (2001) Quorum-sensing in Gram-negative bacteria, *FEMS Microbiol. Rev. 25*, 365−404.
3. Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., and Zhang, L. H. (2002) Identification of quorum-quenching N-acyl homoserine lactonases from Bacillus species, *Appl. Environ. Microbiol. 68*, 1754−1759.
4. Dong, Y. H., Zhang, X. F., Xu, J. L., and Zhang, L. H. (2004) Insecticidal Bacillus thuringiensis silences Erwinia carotovora virulence by a new form of microbial antagonism, signal interference, *Appl. Environ. Microbiol. 70*, 954−960.
5. Lin, Y. H., Xu, J. L., Hu, J., Wang, L. H., Ong, S. L., Leadbetter, J. R., and Zhang, L. H. (2003) Acyl-homoserine lactone acylase from Ralstonia strain XJ12B represents a novel and potent class of quorum-quenching enzymes, *Mol. Microbiol. 47*, 849−860.
6. Liu, D., Lepore, B. W., Petsko, G. A., Thomas, P. W., Stone, E. M., Fast, W., and Ringe, D. (2005) Three-dimensional structure of the quorum-quenching N-acyl homoserine lactone hydrolase from Bacillus thuringiensis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 11882−11887.
7. Thomas, P. W., Stone, E. M., Costello, A. L., Tierney, D. L., and Fast, W. (2005) The quorum-quenching lactonase from Bacillus thuringiensis is a metalloprotein, *Biochemistry 44*, 7559−7569.
8. Tait, K., Joint, I., Daykin, M., Milton, D. L., Williams, P., and Camara, M. (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga Ulva to bacterial biofilms, *Environ. Microbiol. 7*, 229−240.
9. Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase, *Nature 411*, 813−817.
10. Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Defago, G., and Haas, D. (2002) Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1, *Microbiology 148*, 923−932.
11. Rajnarayanan, R. V., Rowley, C. W., Hopkins, N. E., and Alworth, W. L. (2001) Regulation of phenobarbital-mediated induction of CYP102 (cytochrome P450(BM-3)) in Bacillus megaterium by phytochemicals from soy and green tea, *J. Agric. Food Chem. 49*, 4930−4936.
12. Kemper, B. (1998) Regulation of cytochrome P450 gene transcription by phenobarbital, *Prog. Nucleic Acid Res. Mol. Biol. 61*, 23−64.
13. English, N. T., and Rankin, L. C. (1997) Antioxidant-mediated attenuation of the induction of cytochrome P450BM-3(CYP102) by ibuprofen in Bacillus megaterium ATCC 14581, *Biochem. Pharmacol. 54*, 443−450.
14. English, N., Hughes, V., and Wolf, C. R. (1996) Induction of cytochrome P-450 BM-3 (CYP 102) by non-steroidal anti-inflammatory drugs in Bacillus megaterium, *Biochem. J.* 316 (Pt 1), 279−283.
15. English, N., Palmer, C. N., Alworth, W. L., Kang, L., Hughes, V., and Wolf, C. R. (1997) Fatty acid signals in Bacillus megaterium are attenuated by cytochrome P-450-mediated hydroxylation, *Biochem. J.* 327 (Pt 2), 363−368.
16. Ho, P. P., and Fulco, A. J. (1976) Involvement of a single hydroxylase species in the hydroxylation of palmitate at the omega-1, omega-2 and omega-3 positions by a preparation from Bacillus megaterium, *Biochim. Biophys. Acta 431*, 249−256.

17. Haines, D. C., Tomchick, D. R., Machius, M., and Peterson, J. A. (2001) Pivotal role of water in the mechanism of P450BM-3, *Biochemistry 40*, 13456−13465.
18. Govindaraj, S., and Poulos, T. L. (1997) The domain architecture of cytochrome P450BM-3, *J. Biol. Chem. 272*, 7915−7921.
19. Boddupalli, S. S., Oster, T., Estabrook, R. W., and Peterson, J. A. (1992) Reconstitution of the fatty acid hydroxylation function of cytochrome P-450BM-3 utilizing its individual recombinant hemo- and flavoprotein domains, *J. Biol. Chem. 267*, 10375−10380.
20. Omura, T., and Sato, R. (1962) A new cytochrome in liver microsomes, *J. Biol. Chem. 237*, 1375−1376.
21. Lapidot, Y., Rappoport, S., and Wolman, Y. (1967) Use of esters of N-hydroxysuccinimide in the synthesis of N-acylamino acids, *J. Lipid Res. 8*, 142−145.
22. Lapidot, Y., de Groot, N., and Fry-Shafrir, I. (1967) Modified aminoacyl-tRNA. II. A general method for the preparation of acylaminoacyl-tRNA, *Biochim. Biophys. Acta 145*, 292−299.
23. Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Jr., Rinehart, K. L., and Farrand, S. K. (1997) Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography, *Proc. Natl. Acad. Sci. U.S.A. 94*, 6036−6041.
24. Todar, K. http://www.textbookofbacteriology.net/Bacillus.html.
25. Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1965) Spectrophotometric properties of a Triton-clarified steroid 21-hydroxylase system of adrenocortical microsomes, *Life Sci. 4*, 2101−2107.
26. Imai, Y., and Sato, R. (1967) Studies on the substrate interactions with P-450 in drug hydroxylation by liver microsomes, *J. Biochem. (Tokyo) 62*, 239−249.
27. Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967) Spectral studies of drug interaction with hepatic microsomal cytochrome, *Mol. Pharmacol. 3*, 113−123.
28. Miura, Y., and Fulco, A. J. (1975) Omega-1, Omega-2 and Omega-3 hydroxylation of long-chain fatty acids, amides and alcohols by a soluble enzyme system from Bacillus megaterium, *Biochim. Biophys. Acta 388*, 305−317.
29. Boddupalli, S. S., Estabrook, R. W., and Peterson, J. A. (1990) Fatty acid monooxygenation by cytochrome P-450BM-3, *J. Biol. Chem. 265*, 4233−4239.
30. Fulco, A. J. (1991) P450BM-3 and other inducible bacterial P450 cytochromes: biochemistry and regulation, *Annu. Rev. Pharmacol. Toxicol. 31*, 177−203.
31. Llamas, I., Keshavan, N., and Gonzalez, J. E. (2004) Use of Sinorhizobium meliloti as an indicator for specific detection of long-chain N-acyl homoserine lactones, *Appl. Environ. Microbiol. 70*, 3715−3723.
32. Piper, K. R., Beck von Bodman, S., and Farrand, S. K. (1993) Conjugation factor of Agrobacterium tumefaciens regulates Ti plasmid transfer by autoinduction, *Nature 362*, 448−450.
33. Marketon, M. M., Gronquist, M. R., Eberhard, A., and Gonzalez, J. E. (2002) Characterization of the Sinorhizobium meliloti sinR/sinI locus and the production of novel N-acyl homoserine lactones, *J. Bacteriol. 184*, 5686−5695.
34. Modi, S., Sutcliffe, M. J., Primrose, W. U., Lian, L. Y., and Roberts, G. C. (1996) The catalytic mechanism of cytochrome P450 BM3 involves a 6 A movement of the bound substrate on reduction, *Nat. Struct. Biol. 3*, 414−417.
35. Capdevila, J. H., Wei, S., Helvig, C., Falck, J. R., Belosludtsev, Y., Truan, G., Graham-Lorence, S. E., and Peterson, J. A. (1996) The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3, *J. Biol. Chem. 271*, 22663−22671.
36. Truan, G., Komandla, M. R., Falck, J. R., and Peterson, J. A. (1999) P450BM-3: absolute configuration of the primary metabolites of palmitic acid, *Arch. Biochem. Biophys. 366*, 192−198.
37. Luo, Z. Q., Clemente, T. E., and Farrand, S. K. (2001) Construction of a derivative of Agrobacterium tumefaciens C58 that does not mutate to tetracycline resistance, *Mol. Plant-Microbe Interact. 14*, 98−103.
38. Shirane, N., Sui, Z., Peterson, J. A., and Ortiz de Montellano, P. R. (1993) Cytochrome P450BM-3 (CYP102): regiospecificity of oxidation of omega-unsaturated fatty acids and mechanism-based inactivation, *Biochemistry 32*, 13732−13741.
39. Palmer, C. N., Axen, E., Hughes, V., and Wolf, C. R. (1998) The repressor protein, Bm3R1, mediates an adaptive response to toxic fatty acids in Bacillus megaterium, *J. Biol. Chem. 273*, 18109−18116.
40. Wang, Y. J., and Leadbetter, J. R. (2005) Rapid acyl-homoserine lactone quorum signal biodegradation in diverse soils, *Appl. Environ. Microbiol. 71*, 1291−1299.
41. Wang, L. H., Weng, L. X., Dong, Y. H., and Zhang, L. H. (2004) Specificity and enzyme kinetics of the quorum-quenching N-Acyl homoserine lactone lactonase (AHL-lactonase), *J. Biol. Chem. 279*, 13645−13651.
42. Zhang, R. G., Pappas, T., Brace, J. L., Miller, P. C., Oulmassov, T., Molyneaux, J. M., Anderson, J. C., Bashkin, J. K., Winans, S. C., and Joachimiak, A. (2002) Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA, *Nature 417*, 971−974.
43. Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De, Francesco, R., Neddermann, P., and Marco, S. D. (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA, *EMBO J. 21*, 4393−4401.
44. Kaufmann, G. F., Sartorio, R., Lee, S. H., Rogers, C. J., Meijler, M. M., Moss, J. A., Clapham, B., Brogan, A. P., Dickerson, T. J., and Janda, K. D. (2005) Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones, *Proc. Natl. Acad. Sci. U.S.A. 102*, 309−314.
45. Murataliev, M. B., Trinh, L. N., Moser, L. V., Bates, R. B., Feyereisen, R., and Walker, F. A. (2004) Chimeragenesis of the fatty acid binding site of cytochrome P450BM3. Replacement of residues 73−84 with the homologous residues from the insect cytochrome P450 CYP4C7, *Biochemistry 43*, 1771−1780.
46. Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault, P., and Nickerson, K. W. (2001) Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol, *Appl. Environ. Microbiol. 67*, 2982−2992.
47. Ramage, G., Saville, S. P., Wickes, B. L., and Lopez-Ribot, J. L. (2002) Inhibition of Candida albicans biofilm formation by farnesol, a quorum-sensing molecule, *Appl. Environ. Microbiol. 68*, 5459−5463.
48. Westwater, C., Balish, E., and Schofield, D. A. (2005) Candida albicans-Conditioned Medium Protects Yeast Cells from Oxidative Stress: a Possible Link between Quorum Sensing and Oxidative Stress Resistance, *Eukaryotic Cell 4*, 1654−1661.

BI701945J