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A Comparative Analysis of Acyl-Homoserine Lactone Synthase Assays

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Abstract

Quorum sensing is cell-to-cell communication that allows bacteria to coordinate attacks on their host by inducing virulent gene expression, biofilm production, and other cellular functions, including antibiotic resistance. AHL synthase enzymes synthesize N-acyl-L-homoserine lactones, commonly referred to as autoinducers, to facilitate quorum sensing in Gram-negative bacteria. Studying the synthases, however, has shown to be a difficult road. Two assays including a radiolabel and a colorimetric (DCPIP) assay are well documented in literature to study AHL synthases. In this paper, we describe additional methods that include an HPLC-based, C-S bond cleavage, and coupled assays to investigate this class of enzymes. In addition, we compare and contrast each assay for both acyl-CoA and acyl-ACP utilizing synthases. The expanded toolkit described in this study should facilitate mechanistic studies on quorum sensing signal synthases and expedite discovery of antivirulent compounds.

Enzymologist Tool Box

The N-acyl-homoserine lactone synthases are attractive targets to control virulence in Gram-negative bacteria. However, robust assays required to study these enzymes are fairly limited. Here, we have expanded the assay tool kit to expedite mechanistic studies and inhibitor discovery for this important class of enzymes.

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Conflict of Interest Statement

The authors declare no conflict of interests.

Keywords

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INTRODUCTION

Bacteria use a two-component, cell-counting mechanism called quorum sensing (QS) to determine if a minimal subpopulation (quorum) has been reached before making collective decisions on global regulation of gene expression and virulence.^[1] The two-component QS system involves an initiator protein that makes an autoinducer signal and a receptor protein, which perceives and responds to the signal.^[2] The N-acyl-homoserine lactone synthase enzymes in Gram-negative bacteria act as signal initiator proteins to synthesize N-acyl-homoserine lactone (AHL) autoinducers.^[3–5] Depending on the acyl-substrate, AHL synthases fall into one of the two main categories: a) acyl-CoA utilizing synthases such as isovaleryl-CoA utilizing *Bradyrhizobium japonicum* BjaI and b) acyl-ACP utilizing synthases such as *Pseudomonas aeruginosa* RhlI, which uses butyryl-ACP as its acyl-substrate (Figure 1).^[4] The other substrate, S-adenosyl-L-methionine (SAM), is conserved for all AHL synthases discovered so far. Both acyl-CoA and acyl-ACP utilizing synthases use a combination of acylation and lactonization reactions to synthesize AHLs.^[5] Since quorum sensing is a key step in bacterial virulence, QS inhibitors hold significant promise as antivirulent therapeutics.^[6]

Quorum sensing can be interrupted, in principle, by following ways: i) blocking native AHL ligand binding to the AHL receptor ii) preventing native AHL synthesis iii) using AHL-sequestering antibodies iv) using quorum-quenching enzymes.^[7–8] Most quorum sensing inhibitors discovered to date have been molecules that interfere with the AHL binding to the AHL receptor. Since native AHL signals typically associate with nanomolar affinities to these receptor proteins, the rational design of small molecules that can effectively out-compete native AHL ligand binding to the receptor is undoubtedly, a challenging task. A complementary approach to inhibit quorum sensing is to develop AHL synthase inhibitors that would block AHL synthesis. Small molecule inhibitors for AHL synthases should limit signal synthesis, inhibit quorum sensing and thus hold significant promise as novel antimicrobials in treating multidrug resistant bacterial infections.^[9] However, AHL synthase inhibitors are few to none because most synthases still remain uncharacterized.^[10] Furthermore, even if potent synthase inhibitors are discovered, the toxicity profile for these molecules are likely to be high because acyl-ACP and SAM are conserved substrates in many essential enzymes such as enzymes involved in fatty acid biosynthesis. Therefore, developing AHL synthase specific inhibitors remains a significant challenge in this field.

To design quorum sensing selective inhibitors, it is useful to investigate unique mechanistic aspects of AHL synthases that distinguish them from other acyl-ACP and/or SAM utilizing enzymes. A major impediment to meeting this objective is that most AHL synthases remain unexplored, perhaps due to difficulties associated with studying this class of enzymes such as a) substrates required to characterize AHL synthases for routine enzyme assays are

cumbersome and expensive to make b) the turnover number (k_{cat}) for most AHL synthase substrates (including the native substrate) are relatively low and so assays developed to study these enzymes must have higher sensitivity and lower background rates c) lack of robust assays or chemical tools that can probe each enzymatic step in AHL synthesis. In this paper, we specifically address challenges associated with developing assays for acyl-homoserine lactone synthases.

In general, enzyme rates can either be quantified by determining the amount of substrate depletion or product accumulation over time. In the past, an accessory reporter system that can form colorimetric or radioactive product was used to study these enzymes. For instance, Greenberg and coworkers used ^{14}C labeled SAM as the acyl-acceptor substrate and followed the enzymatic transfer of this label to acyl-homoserine lactone product to characterize RhII AHL synthase.^[10a] Recently, Tipton and coworkers adopted a colorimetric-based dichlorophenolindophenol (DCPIP) assay to conduct mechanistic investigations on the RhII enzyme.^[11] In this assay, acylation of SAM by acyl-ACP would release holo-ACP thiol, which in turn will reduce the DCPIP dye to DCPIPH₂. The redox reaction is also accompanied by a large decrease in DCPIP extinction coefficient. Despite these advantages, both assays have limitations. The radiolabel assay is laborious to conduct and the DCPIP dye in the colorimetric assay can potentially undergo nonspecific reduction from surface exposed enzyme cysteine residues or other reducing agents in the assay mixture. In this paper, we describe additional methods including a spectrophotometric, nucleosidase coupled and HPLC-based assays to study AHL synthases. The theory behind the development of each assay is discussed below.

C-S bond cleavage spectrophotometric assay

This assay takes advantage of the change in extinction coefficient between the intact and cleaved thioester substrate ($\epsilon_{232} = 4,000 \text{ M}^{-1}\text{cm}^{-1}$).^[12] During the acylation step in AHL synthesis, the thioester C-S bond of the acyl-substrate is cleaved to form CoA or holo-ACP (Figure 2B). Unlike the DCPIP assay described above, this assay does not require any colorimetric or fluorometric reagent for signal detection and provides a direct method for monitoring AHL synthase catalyzed acyl-substrate turnover.

Methylthioadenosine Nucleosidase-Xanthine Oxidase coupled assay

In a coupled enzyme assay, the product of an enzyme reaction of interest is allowed to react with another enzyme to obtain a signal that can be conveniently measured using standard laboratory equipment such as a UV-Visible spectrophotometer. In this assay, the acyl-substrate is incubated with AHL synthase, methylthioadenosine nucleosidase (MTAN) and xanthine oxidase for about 10 minutes prior to initiation with SAM. MTA product from AHL synthesis will first react with methylthioadenosine nucleosidase to form methylthioribose and adenine (Figure 2C). Addition of xanthine oxidase to the assay mixture further increased assay sensitivity because the conversion of adenine to 2,8-dihydroxyadenine is accompanied by a large change in extinction coefficient ($\epsilon_{305} = 15,500 \text{ M}^{-1}\text{cm}^{-1}$ vs $\epsilon_{274} = 1,600 \text{ M}^{-1}\text{cm}^{-1}$ for the nucleosidase reaction).^[13a-e] Furthermore, the nucleosidase enzyme is highly selective for MTA over SAM as the substrate and thus the observed signal corresponds to MTA released from AHL synthesis.

The coupled assay can be generalized using the following scheme

Scheme 1.

Substrates and Products of Nucleosidase Coupled Assay.

In this scheme, v_1 , v_2 and v_3 represent the initial rates for AHL synthase, nucleosidase and xanthine oxidase reactions respectively. In this coupled assay, the concentration of MTA must be low in the beginning. Since the nucleosidase reaction rate is dependent on MTA concentration, the rates for coupling enzymes (v_2, v_3) are lower until MTA concentration reaches steady state levels.^[14] To minimize the initial lag, the concentration of nucleosidase and xanthine oxidase must be adjusted to keep the AHL synthase reaction rate limiting (i.e. $v_1 \ll v_2, v_3$). Under these conditions, MTA and adenine reacts with nucleosidase and xanthine oxidase, respectively, as soon as they are formed and the rate of release of 2,8-dihydroxyadenine signal at 305 nm must therefore reflect the rate of AHL synthesis.

HPLC assay

AHL synthases are bi-ter enzymes that react with two substrates to generate three products. Since both substrates and products differ in molecular size, charge and polarity, a C18 reverse phase column was used to resolve these compounds (Figure 2D). By altering solvent and flow-gradient, we were able to monitor time-dependent changes in holo-ACP, acyl-ACP, SAM and MTA peak areas in the chromatogram to determine reaction rates. To measure AHL synthase reaction rates, the reaction was first initiated by addition of enzyme and then quenched at specific time points using 6 M HCl. The analyte concentration in the quenched reaction mixture was determined from its respective peak area using a standard curve generated for each analyte (determined separately). The rates calculated at each substrate concentration were used to estimate kinetic constants.

In this paper, we compare and contrast each assay for both acyl-CoA and acyl-ACP utilizing enzymes and offer suggestions for future assay development to study quorum sensing signal synthesis enzymes in Gram-negative bacteria.

RESULTS AND DISCUSSION

The AHL synthase enzymes in Gram-negative bacteria use an acyl-substrate and S-adenosyl-L-methionine to synthesize acyl-homoserine lactone autoinducer signals, which are used to relay information on local population density to the bacterium during quorum sensing. The specificity in the signal synthesized by a bacterium is dependent upon the nature of the acyl-chain in the acyl-substrate. Although AHL synthases accept both acyl-CoA and acyl-ACP as their acyl-substrate, their preference for one substrate type over the other classifies the enzyme as either the acyl-CoA or acyl-ACP utilizing synthase.^[4]

AHL synthases are attractive targets to modulate quorum sensing in bacteria. However, assays to study these synthases are fairly limited, which impedes mechanistic studies and inhibitor discovery for this class of enzymes. In addition to radiolabel and colorimetric-

based assays, we have expanded the AHL synthase assay tool kit to include spectrophotometric, HPLC and coupled assays. In this paper, we describe the benefits and limitations of each assay using two model enzymes: the acyl-CoA utilizing *Bradyrhizobium japonicum* BjaI and acyl-ACP utilizing *Pseudomonas aeruginosa* RhII. Results from each of these assays are summarized in Table 1.

DCPIP Colorimetric Assay

The Coenzyme A or holo-ACP product of AHL synthase reaction reduces the blue-colored 2,6-dichlorophenolindophenol (DCPIP) dye in the reaction mixture, which causes large changes in extinction coefficient between the oxidized and reduced forms of this dye ($\epsilon_{600} = 21,000 \text{ M}^{-1}\text{cm}^{-1}$). This assay has thus become the gold standard to study AHL synthases due to its simplicity and high-sensitivity. Indeed, we have also used this assay in the past to study BjaI and BmaI (an octanoyl-homoserine lactone synthase) enzymes in our laboratory.^{[4], [5]} Based on our experience working with the DCPIP assay, we uncovered some of its facets that were not previously reported in literature. For instance, DCPIP undergoes nonspecific reduction from SAM, acyl-CoA and acyl-ACP. The rate of nonspecific reduction is high in the beginning, but tapers off around 10 minutes. Therefore, the dye must be incubated with both substrates for at least 10 minutes before initiating the AHL synthesis reaction with enzyme. We also noticed that the absorbance of DCPIP at 600 nm is pH dependent. For instance, ϵ decreases to $14,000 \text{ M}^{-1}\text{cm}^{-1}$, $4,000 \text{ M}^{-1}\text{cm}^{-1}$ and $2,000 \text{ M}^{-1}\text{cm}^{-1}$, respectively at pH 6, 5 and 4. Consequently, the sensitivity of this assay will be significantly reduced at pHs less than 6. Nonetheless, we note that some thiol detection reagents that work at higher pHs are readily available.^[15c]

Enzyme catalyzed acylation of SAM by acyl-CoA and acyl-ACP will release Coenzyme A and holo-ACP thiols, respectively, to reduce DCPIP in the assay mixture. The change in dye absorbance at 600 nm was used to calculate initial rates and determine kinetic constants for BjaI and RhII catalyzed AHL synthesis (Figure 3). The k_{cat} and K_{M} for isovaleryl-CoA and butyryl-ACP with BjaI and RhII obtained using DCPIP assay is presented in Table 1. We will use these numbers as reference point to compare and contrast other AHL synthase assays discussed below. Although this assay can be used to study both acyl-CoA and acyl-ACP utilizing synthases, one must be aware of the following limitations: a) if the enzyme has one or more surface exposed cysteines, these amino acid residues can potentially reduce DCPIP and inactivate the enzyme b) this assay is unsuitable to conduct inhibition studies with Coenzyme A or holo-ACP or any inhibitor that can potentially reduce DCPIP. Despite these drawbacks, this assay is most popular for its versatility in studying both acyl-CoA and acyl-ACP utilizing AHL synthases. We suspect that other thiol detecting colorimetric reagent (such as Ellman's reagent) or thiol-sensitive fluorescence probes should work equally well in place of DCPIP to study signal synthases.^[15]

C-S bond cleavage spectrophotometric assay

We used this assay to determine k_{cat} and K_{M} for isovaleryl-CoA with BjaI. As mentioned earlier, this is a simple and direct assay that measures the rate of cleavage of thioester acyl-substrates by the AHL synthase (Figure 2B). The k_{cat} and K_{M} for isovaleryl-CoA agrees well with the values obtained from the DCPIP assay (Table 1). The C-S bond cleavage assay

also detected weak substrate inhibition of BjaI from isovaleryl-CoA (Figure 4). This suggests that the spectrophotometric assay is a robust assay to study acyl-CoA dependent AHL synthases. Nonetheless, the assay does have some drawbacks: a) the change in extinction coefficient between intact and cleaved C-S bond is fairly low ($\epsilon_{232} = 4,000 \text{ M}^{-1}\text{cm}^{-1}$), which reduces assay sensitivity b) we encountered significant challenges in measuring initial velocities for the reaction of butyryl-ACP with RhII, an acyl-ACP utilizing enzyme. We think both low assay sensitivity and interference from the protein backbone in ACP based substrates contributed to the incompatibility of this assay in studying acyl-ACP utilizing synthases. Therefore, with the exception of a protein-based substrate such as acyl-ACP, we predict that kinetic constants of acyl-substrates with smaller leaving groups such as acyl-N-acetylcysteamine, acyl-pantetheine, acyl-CoA can be determined using this C-S cleavage spectrophotometric method.

Methylthioadenosine Nucleosidase-Xanthine Oxidase coupled assay

We first determined the optimal concentration for methylthioadenosine nucleosidase (MTAN) by reacting methylthioadenosine (MTA, 100 μM) with varied concentrations of nucleosidase enzyme (0.05 μM to 1.0 μM). The K_M and k_{cat} for MTA with *E. coli* MTAN, respectively, are 400 nM and 20 min^{-1} .¹⁶ The minimum MTAN concentration required to achieve highest initial rate for this reaction was determined to be 0.5 μM . Keeping MTAN at 0.5 μM , we then varied xanthine oxidase concentration from 0.5 units/mL to 5 units/mL. We did not observe any appreciable increase in xanthine oxidase activity beyond 2 units/mL and thus the optimum concentration of this enzyme for the coupled assay was fixed at 2 units/mL. The progress curve for nucleosidase-xanthine oxidase coupled assay is presented in Figure 5A.

The kinetic constants for BjaI and RhII AHL synthases are summarized in Table 1. We noticed some difficulties in accurately measuring initial rates at low acyl-substrate concentrations due to higher nonenzymatic background rates from SAM. Since MTA is a common contaminant in commercial SAM samples, the background reaction was unusually high in the beginning, which interfered with accurate determination of low amounts of MTA produced from AHL synthase reaction. Thus, the standard deviation in Michaelis constant for acyl-substrate in both BjaI and RhII catalyzed reactions were little higher than DCPIP or C-S assay (Figure 5, Table 1). We suspect the K_M 's would agree closely with the DCPIP and C-S assays if a higher purity SAM sample were to be used in the coupled assay. Unlike the colorimetric assay, the coupled assay is well suited to study CoA and holo-ACP product inhibition. Nevertheless, this assay is incompatible to conduct inhibition studies from MTA, S-adenosyl-L-homocysteine (SAH) and other compounds that could potentially react with nucleosidase and/or xanthine oxidase. Due to multiple enzymes involved, this assay may not be ideal to conduct inhibition screens and pH dependent measurements.

HPLC assay

Hanzelka and coworkers have previously used HPLC to fractionate AHL product and determined rates of AHL synthesis using radiometry or bioassay.^[13f] In our method, we were able to detect the other analytes namely SAM, MTA, acyl-ACP, holo-ACP etc. The substrates and products of AHL synthase reaction were first resolved using C18 reverse

phase chromatography and the peak areas were then quantified using chromquest software. Since the substrate and product of lactonization and acylation in AHL synthesis, respectively, are small molecules (SAM, MTA) and proteins (acyl-ACP, holo-ACP), two independent methods were developed to monitor each half-reaction (Figure 2D). The standard curves for each analyte showed excellent fit to a linear model (Figure 6A–6D). The reaction mixture was quenched using guanidine (4 M in the reaction mixture) or 6 M hydrochloric acid or pH 4 acetate buffer. The peak areas corresponding to MTA or holo-ACP was closely monitored for 15 min and 30 min after addition of quench to the enzyme reaction. While the peak area for MTA continued to increase even after addition of guanidine, HCl and acetate buffer quenches resulted in less than 1% change in analyte concentration (as determined by peak areas). Although HCl quench worked well for monitoring lactonization (MTA release) in both BjaI and RhII catalyzed AHL synthesis, addition of hydrochloric acid precipitated acyl carrier protein. We therefore used acetate buffer quench specifically to monitor ACP-based analytes (acyl-ACP and holo-ACP) in AHL synthesis. Product formation was linear over a 6-minute time scale and the slope of the line was used to estimate initial rates (Figure 6E). We observed that the substrate-velocity curve for RhII-butyryl-ACP reaction was in excellent agreement obtained from DCPIP and coupled assays (Figures 3, 5 and 6). Since we monitor MTA analyte in HPLC, MTA impurity in SAM commercial sample increased the background rates, which interfered with accurate estimation of initial rates at low concentration of acyl-substrate. For instance, we noticed larger errors in K_M for isovaleryl-CoA with BjaI, which is in-line with our results from coupled assay (Table 1). The Michaelis constant for butyryl-ACP with RhII, however, were in excellent agreement with DCPIP and coupled assays. We monitored changes in MTA (over holo-ACP) concentration to determine RhII activity due to two reasons: a) MTA is a common product in both BjaI and RhII reactions, which helped maintain consistency between the two assays b) a higher extinction coefficient for MTA at 260 nm improved assay sensitivity.

The HPLC assay offers some unique advantages over other assays discussed above. First, HPLC assay provides a direct snapshot of the changes in both substrate and product concentrations in the reaction mixture. Second, this assay does not require an accessory reporter such as a colorimetric dye or coupling enzyme, but is still sensitive and accurate enough to measure enzyme reaction rates. In our hands, the sensitivity limit for detecting holo-ACP and MTA peaks in the standard curve is around 2 μ M. To account for MTA contamination in SAM, we subtracted MTA peak area in SAM (background) from MTA peak area in the assay mixture. Under these conditions, we were able to detect MTA concentration as low as 0.3 μ M in the assay. Unfortunately, the convenience of this enhanced sensitivity using MTA contaminated SAM is offset by increased errors in rate estimation, especially when MTA concentration is low. Therefore, we claim that a higher purity SAM should be used whenever possible, to reduce the margin of error in estimating the true K_M for the acyl-substrate. Third, the acylation and lactonization reactions in AHL synthesis can be *independently* studied using this assay. For instance, the acylation half-reaction can be studied by incubating acyl-substrate with S-adenosyl-L-homocysteine and the lactonization half-reaction can be monitored by reacting SAM with an inert acyl-substrate analog. Overall,

HPLC is an excellent tool to determine the sequence and rates of acylation and lactonization half-reactions of the chemical step in AHL synthesis.

Assays for future development

Alternate assays that can be potentially explored to study acyl-homoserine lactone synthases are discussed below.

1. Native gel assay. In this assay, conformationally sensitive gel electrophoresis may be used to resolve holo-ACP product from acyl-ACP substrate.^[17] Depending on the nature of acyl-ACP (short, medium or long-chain), the acyl-chain is sequestered inside the protein core to different extents, which gives distinct conformation and unique R_f value for each acyl-ACP in a native gel.^[18] An AHL synthase reaction quenched at specific times will have varied amounts of holo-ACP that can be quantified using a standard curve (using serial dilutions of holo-ACP or acyl-ACP) run on the same gel.
2. Other coupled assays. Schramm and coworkers used a luciferase assay to characterize a DNA methyltransferase enzyme, which can be adopted to study AHL synthase enzymes.^[19] In this assay, adenine product from the MTAN coupled assay should first be enzymatically converted to AMP (adenine phosphoribosyltransferase) and then to ATP (pyruvate orthophosphate dikinase). The ATP thus made *in situ* is subsequently utilized by the luciferase enzyme to produce light that can be quantified using chemiluminescence.^[19] A nucleosidase-deaminase assay is also a good alternative to explore.^[20] The acyl-homoserine lactone product can also be coupled with a quorum-quenching lactonase enzyme to characterize AHL synthases.^[21] Finally, a colorimetric method that utilizes hydroxylamine to detect AHLs can be used in lieu of a coupling enzyme to assay AHL signal synthases.^[21a]

Conclusion

AHL synthase enzymes play a crucial role in bacterial quorum sensing by synthesizing signals required for intercellular communication. Although more than 40 AHL synthases have been identified so far, published enzyme data on synthases are few in number. There is a growing interest to develop inhibitors for AHL synthases to inhibit quorum sensing due to their potential application in antiviral therapy and in designing chemical probes to investigate social behavior in bacteria.^{[6–10], [22]} Unfortunately, the lack of robust chemical tools necessary to investigate these slow enzymes (that do not appear to have been selected for catalytic efficiencies) impedes mechanistic studies. To address this need, we have developed multiple assays to investigate and characterize both acyl-CoA and acyl-ACP utilizing signal synthases. Overall, the DCPIP method is a versatile assay to study both types of synthases as long as the enzyme cysteines do not cross-react with the dye. The C-S assay is best suited to study acyl-CoA utilizing synthases while the nucleosidase assay is well suited to conduct inhibition studies with thiol-based compounds such as CoA or holo-ACP

(which would have reacted with DCPIP dye in colorimetric assay). In addition, the nucleosidase coupled assay could be considered if DCPIP inactivates the enzyme or MTA inhibition interfered with accurate determination of initial rates. Finally, the HPLC assay, although tedious, is the best method to independently investigate acylation and lactonization half-reactions that occur at the chemical step in AHL synthesis. The assays described in this paper should open new avenues for investigators to explore more signal synthases in the future. It is our hope that these efforts would expedite inhibitor discovery for this important class of enzymes.

EXPERIMENTAL SECTION

Grade I bovine milk xanthine oxidase, isovaleryl-CoA and SAM are purchased from Sigma Aldrich Company. Prof. Michael Burkart (University of California, San Diego, Prof. Satish Nair (UIUC), Prof. Peter Tipton (University of Missouri-Columbia) and Prof. Ken Cornell (Boise State University), respectively, provided the enzyme clones for Sfp, BjaI, RhlI and MTAN. Enzymes and butyryl-ACP are purified as described previously.^{[4], [5], [23]} Graphpad Prism 6 was used to estimate kinetic constants in each assay. UV-Visible data were acquired using a Thermo Scientific Evolution 260 spectrophotometer and HPLC data were obtained using an Accela600 instrument from Thermoscientific.

General Note

In the assays discussed below, each data point was repeated in triplicate and the average initial rate obtained from slope of progress curves was fit to either Michaelis-Menten (RhlI-butyryl-ACP; Equation 1) or substrate inhibition equation (BjaI-isovaleryl-CoA; Equation 2) to obtain kinetic constants. Furthermore, some assays were repeated by two or more different experimenters to ensure data reproducibility.

$$v_0 = \frac{k_{cat}[E_t][S]}{K_M + [S]} \quad \text{Equation 1}$$

$$v_0 = \frac{k_{cat}[E_t][S]}{K_M + [S] \left(1 + \frac{[S]}{K_i}\right)} \quad \text{Equation 2}$$

In the above equations k_{cat} , K_M and K_i refer respectively, to catalytic constant, Michaelis constant and substrate inhibition constant.

DCPIP assay

The rate of enzyme-catalyzed AHL synthesis was determined by monitoring the colorimetric reduction of dichlorophenolindophenol (DCPIP) dye by Coenzyme A or holo-ACP thiol at 600 nm. The assay mixture consisted of DCPIP (30 μ M), SAM (300–400 μ M), enzyme (0.3–0.6 μ M) and varied concentrations of acyl-substrate in 100 mM HEPES buffer at pH 7.3. DCPIP was first incubated with substrates for about 10 minutes prior to enzyme

addition. The concentration of isovaleryl-CoA was varied between 3–150 μM . For the RhII assay, butyryl-ACP concentration was varied from 1.5 μM to 50 μM while the enzyme concentration was maintained at 0.3 μM . The initial rate for each enzymatic reaction was calculated from the initial slope in the progress curve. To calculate nonenzymatic background rates, the assay mixture was incubated for 10 minutes as described above and the change in absorbance at 600 nm was followed after adding an aliquot of water in place of the enzyme. The net rate calculated by subtracting background rate from enzymatic rate was used to determine kinetic constants.

C-S bond cleavage spectrophotometric assay

This spectrophotometric assay was conducted similar to DCPIP assay except that the reaction was monitored at 232 nm and DCPIP was excluded from the assay mixture. A typical C-S bond cleavage assay mixture consisted of 100 mM HEPES in pH 7.3, SAM (300–400 μM) and acyl-substrate (3–150 μM), which were incubated for 10 minutes before initiation with 0.5 μM BjaI. The kinetic constants were determined by fitting the average initial rates obtained from triplicate or more measurements to substrate inhibition equation.

Methylthioadenosine Nucleosidase-Xanthine Oxidase coupled assay

The activity of BjaI and RhII AHL synthases were determined by measuring the rate of release of 2,8-dihydroxyadenine in a methylthioadenosine nucleosidase (MTAN) and xanthine oxidase coupled reaction (Figure 5). In a quartz cuvette, 50 mM KCl, 100 mM HEPES buffer at pH 7.3, 0.5 μM MTAN, 2 units of xanthine oxidase and BjaI (0.5 μM) or RhII (0.3 μM) were incubated for 10 minutes and the reaction was then initiated by addition of SAM (300 μM). Formation of 2,8-dihydroxyadenine was followed as increase in absorbance at 305 nm ($\epsilon_{305} = 15,500 \text{ M}^{-1}\text{cm}^{-1}$). To calculate background rates, AHL synthase was excluded from the reaction mixture and the assay was set up as described above. The net rate after background subtraction was fitted to the Michaelis-Menten equation (equation 1) or the substrate inhibition equation (equation 2) to calculate k_{cat} and K_{M} for the acyl-substrate.

HPLC assay

Both acylation and lactonization steps in AHL synthesis can be independently studied using the HPLC method. A gradient beginning at 75% solvent A and ending with 25% A over a period of 10 min, at a flow of 600 $\mu\text{L}/\text{min}$ was used to monitor acylation half-reaction (acylation method). To monitor lactonization half-reaction, we used a gradient with a flow rate of 500 $\mu\text{L}/\text{min}$, beginning at 0% B and ending at 10% B over a 10 minute interval (lactonization method). Solvent A is 0.1% TFA (trifluoroacetic acid) in nanopure water and solvent B is 0.1% TFA in acetonitrile. To generate standard curves, SAM, MTA, adenine, holo-ACP and acyl-ACP were serially diluted in the buffer with the quench and the peak areas at each analyte concentration were determined from chromatograms run at 260 nm (for SAM, MTA and adenine) or at 220 nm (for holo-ACP and acyl-ACP). The lactonization and acylation HPLC methods, respectively, were used to determine standard curves for small molecules and ACP based analytes. In a typical enzyme assay, 100 mM HEPES at pH 7.3, SAM (300 μM) and acyl-substrate were incubated for 10 minutes. After the incubation period, the reaction was initiated with BjaI (0.5 μM) or RhII (0.3 μM) and the assay mixture

was split to three equal portions for quenching at 2.5, 4 and 6 min using 6 M hydrochloric acid (6% by volume). To monitor holo-ACP, we used acetate buffer at pH 4 to quench the enzyme. MTA and holo-ACP peak areas for quenched reactions were determined from the chromatogram. The slope of progress curves in peak area per minute was converted to micromolar per minute using standard curves. The rate thus obtained was fitted to the Michaelis-Menten (equation 1) or the substrate inhibition equation (equation 2) to estimate kinetic constants for each enzyme-catalyzed reaction. The peak areas were quantified using chromquest software supplied by Thermoscientific.

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Figure 1. AHL Synthase Reaction

A) Acylation and lactonization steps in AHL synthesis. Holo-ACP/CoA and MTA, respectively, are derived from acylation and lactonization half-reactions in AHL synthesis.

B) Substrates and products for BjaI and RhII catalyzed AHL synthesis. BjaI and RhII, respectively, are representative enzymes of acyl-CoA and acyl-ACP utilizing synthase families.

Figure 2. Assay Principle

A) DCPIP Assay. Acylation of acyl-substrate by SAM-amine releases holo-ACP/CoA thiol, which reduces DCPIP dye in the assay mixture. B) C-S Assay. In the 230 nm region, UV absorption of acyl-CoA (due to the intact thioester C-S bond) is significantly higher than CoA. Therefore, acylation induced thioester bond cleavage in the acyl-substrate can be monitored by following the change in absorbance at 232 nm. C) MTAN-XO Coupled Assay. Methylthioadenosine, a product of lactonization step in AHL synthesis reacts with nucleosidase to form adenine and methylthioribose. The conversion of adenine to 2,8-dihydroxyadenine by xanthine oxidase is monitored at 305 nm. The changes in extinction coefficient for nucleosidase (274 nm) and xanthine oxidase (305 nm) coupled reactions, respectively, are 1,600 and 15,500 M⁻¹ cm⁻¹. Assay conditions are optimized to keep AHL synthase reaction rate limiting. D) HPLC Assay. Products of acylation and lactonization half-reactions are conveniently monitored using C18 reverse phase chromatography. Green, red and blue traces correspond to reactions quenched at 0, 15 and 30 minutes, respectively. In the lactonization half-reaction, peak at 0 min correspond to MTA impurity from

commercially available SAM (Sigma). The background MTA peak area was always subtracted to estimate MTA concentration from AHL synthase reaction. The overlaid MTA peaks at different reaction times are magnified and displayed in the inset. In the acylation reaction, increase in holo-ACP peak area was concomitant with decrease in butyryl-ACP peak area. The gradient used for monitoring acylation and lactonization half-reactions is described in experimental section.

Figure 3. DCPIP Assay

A) Representative Progress Curve. The reduction of DCPIP dye by CoA released upon reaction of isovaleryl-CoA (6 μM), SAM (300 μM) and BjaI (0.44 μM) was followed at 600 nm. B) Rate Curve for RhII (0.3 μM). SAM concentration was fixed at 300 μM. C) BjaI Rate Curve. The enzyme and SAM concentrations are fixed respectively, at 0.44 μM and 331 μM.

Figure 4. C-S Assay

A) Representative Progress Curve. The change in absorbance at 232 nm for SAM (352 μM) and isovaleryl-CoA (16 μM) reacting with BjaI (0.51 μM). B) Rate Curve for BjaI-isovaleryl-CoA Reaction. BjaI and SAM concentrations in this experiment are fixed respectively, at 0.51 μM and 352 μM .

Figure 5. Methylthioadenosine Nucleosidase-Xanthine Oxidase Coupled Assay

A) Representative Progress Curve for RhII (0.3 μM) reacting with SAM (300 μM) and butyryl-ACP (50 μM). The concentrations of MTAN and xanthine oxidase are respectively at, 0.5 μM and 2 units/ml. Slopes from steady state phase were used to determine initial rates and kinetic constants. B) Substrate-Velocity Curve for BjaI (0.5 μM) reacting with fixed concentration of SAM (300 μM) and varied concentrations of isovaleryl-CoA. C) Substrate-Velocity Curve for RhII (0.3 μM) reacting with butyryl-ACP. The concentration of SAM in this assay was fixed at 300 μM .

Figure 6. HPLC Assay

A–D) Standard Curves. Each analyte was serially diluted and their respective peak areas in chromatogram were determined. While SAM and MTA were monitored at 260 nm, protein peaks (acyl-ACP and holo-ACP) were followed at 220 nm. The peak areas are reported in arbitrary units (a.u.). E) RhII Progress curves. Enzyme reactions were quenched at 2.5, 4 and 6 minutes using 6 M hydrochloric acid. MTA concentration at each of these time points was calculated using standard curve as described in Panel B in this figure. F) Rate Curve for RhII Catalyzed Butyryl-Homoserine Lactone Synthesis. The initial rate at each substrate concentration was fitted to Michaelis-Menten equation to estimate kinetic constants. Enzyme and SAM concentration were fixed, respectively, at 0.3 μM and 300 μM .

Table 1

Comprehensive analysis of AHL synthase assays

Enzyme	Substrate ^a	Assay	[SAM] μM	K_M μM	k_{cat} min^{-1}	k_{cat}/K_M $\text{min}^{-1}\mu\text{M}^{-1}$
BjaI ^d	IV-CoA	DCPIP	331	2.1 ± 0.4^b	2.7 ± 0.2^b	1.3 ± 0.3
BjaI ^d	IV-CoA	C-S	352	1.5 ± 0.3	2.4 ± 0.1	1.6 ± 0.3
BjaI ^d	IV-CoA	MTAN-XO	300	6.5 ± 4.1	3.0 ± 0.9	0.5 ± 0.3
BjaI ^e	IV-CoA	HPLC ^c	300	7.1 ± 2.3	3.8 ± 0.4	0.5 ± 0.2
RhlI ^e	C4-ACP	DCPIP	300	7.3 ± 2.1	2.7 ± 0.3	0.4 ± 0.1
RhlI ^e	C4-ACP	MTAN-XO	300	6.8 ± 3.0	2.6 ± 0.3	0.4 ± 0.2
RhlI ^e	C4-ACP	HPLC ^c	300	7.6 ± 2.0	2.4 ± 0.3	0.3 ± 0.1

^aIV-CoA is isovaleryl-CoA, C4-ACP is butyryl-ACP^bThe k_{cat} and K_M values for isovaleryl-CoA with BjaI are little different from values reported in reference 4. We attribute these minor variations in kinetic constants to differences in assay conditions and in the type of SAM salt (tosylate vs chloride) used in the study.^cMTA peak area was monitored in this assay. The concentration of IV-CoA in the HPLC assay was capped at 20 μM .^dSubstrate Inhibition equation was used to fit substrate-velocity data for determining the kinetic constants of IV-CoA with BjaI. The following K_i values were obtained from each assay: 192 ± 57 μM (DCPIP), 495 ± 184 μM (C-S) and 112 ± 120 μM (MTAN-XO).^eMichaelis-Menten equation was used to determine kinetic constants.