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Mechanism of *Vibrio cholerae* Autoinducer-1 Biosynthesis

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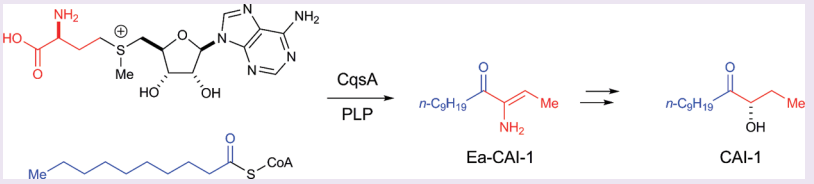
Supporting Information

ABSTRACT: *Vibrio cholerae*, the causative agent of the disease cholera, uses a cell to cell communication process called quorum sensing to control biofilm formation and virulence factor production. The major *V. cholerae* quorum-sensing signal CAI-1 has been identified as (*S*)-3-hydroxytridecan-4-one, and the CqsA protein is required for CAI-1 production. However, the biosynthetic route to CAI-1 remains unclear. Here we report that (*S*)-adenosylmethionine (SAM) is one of the two biosynthetic substrates for CqsA. CqsA couples SAM and decanoyl-coenzyme A to produce a previously unknown but potent quorum-sensing molecule, 3-aminotridec-2-en-4-one (Ea-CAI-1). The CqsA mechanism is unique; it combines two enzymatic transformations, a β,γ-elimination of SAM and an acyltransferase reaction into a single PLP-dependent catalytic process. Ea-CAI-1 is subsequently converted to CAI-1, presumably through the intermediate tridecane-3,4-dione (DK-CAI-1). We propose that the Ea-CAI-1 to DK-CAI-1 conversion occurs spontaneously, and we identify the enzyme responsible for the subsequent step: conversion of DK-CAI-1 into CAI-1. SAM is the substrate for the synthesis of at least three different classes of quorum-sensing signal molecules, indicating that bacteria have evolved a strategy to leverage an abundant substrate for multiple signaling purposes.



Quorum sensing is a cell to cell communication process that bacteria use to coordinately regulate gene expression. Quorum sensing relies on the population-wide production and detection of signaling molecules called autoinducers.[1,2] Quorum sensing controls crucial functions in pathogenesis; thus, disrupting quorum sensing represents a potential method for developing new antimicrobial therapeutics.[3]

Typically, Gram-negative bacteria use acyl-homoserine lactones (acyl-HSL) as their autoinducers.[4] In these cases, the autoinducer synthases (LuxI-type proteins) use (*S*)-adenosylmethionine (SAM) and acylated acyl carrier proteins or acyl-coA thioesters as substrates to produce signals with a variety of acyl tails.[5,6] However, there is no evidence that *Vibrio cholerae*, the causative agent of the disease cholera, and the focus of the present work, produces an acyl-HSL autoinducer,[7] and the genome does not contain a *luxI* homologue. Rather, *V. cholerae* uses two autoinducer-receptor quorum-sensing systems (AI-2/LuxPQ and CAI-1/CqsS[7]) to regulate biofilm formation and virulence factor production.[8,9]

LuxS synthesizes AI-2 by catalyzing the fragmentation of (*S*)-ribosyl-homocysteine (SRH), a byproduct of SAM metabolism, to produce homocysteine and the AI-2 precursor, 4,5-dihydroxy-2,3-pentanedione (DPD). DPD spontaneously cyclizes and chelates borate to yield AI-2, identified as (2*S*,4*S*)-2-methyl-2,3,4-tetrahydroxytetrahydrofuran-borate.[10,11]

CqsA is the CAI-1 synthase, and CAI-1 has previously been identified to be (*S*)-3-hydroxytridecan-4-one (Figure 1).[12,13] CqsA functions as a pyridoxal phosphate (PLP)-dependent aminotransferase-like enzyme.[13,14] *In vitro*, CqsA can use L-aminobutyrate (LAB) and decanoyl-coenzyme A (d-CoA) to produce (*S*)-3-aminotridecan-4-one (Am-CAI-1) (Figure 1) with low efficiency.[13] The *in vivo* pathway for CAI-1 synthesis is unknown.[13,14]

In this report, we demonstrate that, *in vitro*, SAM is a much better CqsA substrate than LAB. CqsA ligates SAM and d-CoA while eliminating (*S*)-methyl-5′-thioadenosine (MTA) and coenzyme A (CoA), resulting in the formation of 3-aminotridec-2-en-4-one (Ea-CAI-1) (Figure 1). Isotope labeling experiments confirm that SAM is used *in vivo* by CqsA to make CAI-1. Our investigation of the conversion of Ea-CAI-1 to CAI-1 suggests that the intermediate molecule, DK-CAI-1 (Figure 1), is formed and that this reaction could happen spontaneously. Consistent with this idea, we have been unable to identify any enzyme capable of catalyzing this reaction. The enzyme VC1059 is required to convert DK-CAI-1 to CAI-1.

RESULTS AND DISCUSSION

SAM Is an Efficient Substrate for CqsA. Purified CqsA acts as a PLP-dependent aminotranferase, mediating carbon−carbon bond formation between LAB and d-CoA to produce Am-CAI-1.[13] We proposed Am-CAI-1 to be an intermediate in the synthesis of the *V. cholerae* quorum-sensing molecule CAI-1. To identify additional enzymes involved in the pathway leading to CAI-1, we examined a library of transposon insertion mutants for those

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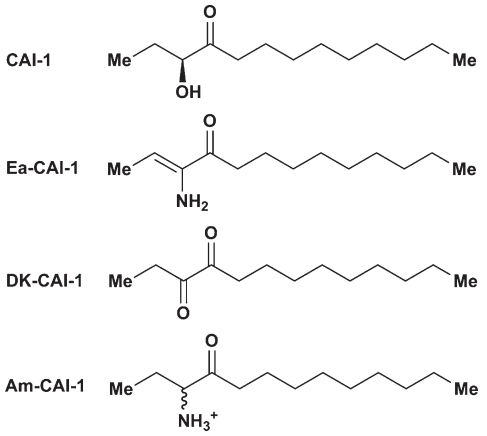


Figure 1. Structures of CAI-1-type molecules. CAI-1, (*S*)-3-hydroxytridecan-4-one; Am-CAI-1, (*S*)-3-aminotridecan-4-one; Ea-CAI-1, 3-aminotridec-2-en-4-one; and DK-CAI-1, tridecane-3,4-dione are shown.

exhibiting altered CAI-1 production. A mutant harboring a transposon insertion in the *pfs* gene displays increased CAI-1 activity. Pfs plays a key role in SAM metabolism. Following methyl-transfer from SAM to substrates, the product (*S*)-adenosyl-L-homocysteine (SAH) is cleaved by Pfs to produce SRH and adenine.[10,15] We were unable to construct a Δ*pfs* strain presumably because *pfs* is essential or causes a severe growth defect.[16] We reasoned that the mutant harboring the Tn*5* in *pfs* must have acquired a suppressor mutation that enabled growth and affected CAI-1 production by changing the levels of metabolites related to SAM. Finding that CAI-1 production was altered when this process was impaired suggested that SAM or a SAM byproduct could be involved in CAI-1 production. Accordingly, we tested SAM, SAH, L-methionine, and homocysteine together with d-CoA as substrates for purified CqsA. Reaction with SAM and d-CoA produced a molecule with significant quorum-sensing activity, whereas no active compound was produced from the reactions with any of the other substrates (Figure 2a).

The above experiment together with our previous results show that both SAM and LAB are substrates for CqsA. To quantify their respective efficiencies, we used a coupled enzyme assay, measuring release of CoA following consumption of d-CoA (see Methods). Consistent with the earlier report, the measured K_m for LAB is 3,700 μM (Figure 2b).[13] The K_m for SAM is 100-fold lower, 28.7 μM (Figure 2c). Likewise, SAM has a 12-fold higher k_{cat} (0.1 s^{-1}) compared with that of LAB (0.008 s^{-1}) (Figure 2b,c). Importantly, the K_m for SAM is lower than the reported SAM concentration in bacterial cells.[17] Together, these results suggest that SAM is a much better CqsA substrate than LAB and therefore that SAM may play an important role in *in vivo* CAI-1 synthesis.

CqsA Produces Ea-CAI-1 by Combining Two Distinct PLP-Dependent Reaction Mechanisms. The products of the CqsA-mediated reaction of d-CoA and SAM were characterized using a bioluminescence bioassay exploiting a *V. cholerae* strain that produces light in response to CAI-1 and by high resolution mass spectrometry (HRMS). Following extraction, products from reactions with purified CqsA, SAM, and d-CoA were assayed. We observed significant activity in the bioassay when molecular ions 212.2009 and 298.0969 are observed in HRMS. The 298.0969 molecular ion corresponds to MTA. The 212.2009 ion correlates to the molecular formula of $C_{13}H_{25}NO$ (calculated 212.2014 m/z [M + H]), consistent with the structure of Am-CAI-1 bearing one additional degree of unsaturation. Because we employed fully saturated d-CoA in this reaction, we reasoned that the additional degree of unsaturation in this product must arise from the SAM substrate. Furthermore, the appearance of MTA by HRMS suggested to us that SAM had undergone a fragmentation reaction in addition to coupling with d-CoA.

We identified the 212.2009 molecular ion as 3-aminotridec-2-en-4-one (Ea-CAI-1) using a combination of proton-NMR and carbon-NMR studies (Figure 1 and Supplementary Figure 1). The structure of Ea-CAI-1 was confirmed through total synthesis. Ea-CAI-1 is also present in cell-free culture fluids of *V. cholerae*.[18] We analyzed Ea-CAI-1 for its quorum-sensing activity and determined that it is a slightly more potent agonist than are CAI-1 and Am-CAI-1 for the CqsS receptor (Figure 3).

The ability of purified CqsA to not only catalyze an acyltransferase reaction with d-CoA but, additionally, to facilitate the fragmentation of SAM was unexpected. While there are numerous examples of PLP-dependent acyltransferase enzymes and examples of PLP-mediated enzymatic transformations of SAM, the combination of these two chemical processes has not been reported to proceed through a single enzyme-catalyzed step. We accordingly examined the sequence of reactions catalyzed by CqsA. Using order of addition reactions, we found that CqsA is capable of directly processing SAM as a substrate in the absence of d-CoA. HRMS shows a product with molecular ions of 102.0545, presumably vinyl-glycine (V-Gly) or 1-aminocyclopropyl-glycine (Cy-Gly) and 298.0970 (MTA) (Supplementary Figure 2). Cy-Gly is the reported elimination product of SAM by the enzyme 1-aminocyclopropyl-glycine synthase (ACCS) and V-Gly is an alternative substrate and mechanism-based inhibitor of ACCS.[19,20] CqsA does not act on d-CoA in the absence of SAM.

The finding that CqsA produces V-Gly/Cy-Gly directly from SAM suggests a two-step reaction process in which V-Gly/Cy-Gly is formed in the first step, and this intermediate either remains bound or is released and then rebound by CqsA along with d-CoA in the subsequent acyltransferase reaction. We used kinetic studies, spectrometry studies, and HRMS analyses to determine that the intermediate is indeed V-Gly and, furthermore, that it is not released (Supplementary Figure 2). Thus, we propose a mechanism in which elimination of MTA from SAM yields a V-Gly-PLP quinonoid intermediate. This intermediate captures the electrophilic carbonyl of d-CoA and subsequently undergoes a decarboxylation reaction to produce Ea-CAI-1 (Scheme 1).

SAM Is the *in Vivo* Substrate for CAI-1 Synthesis. We wondered if SAM is used *in vivo* to synthesize CAI-1. To investigate this, isotope labeling experiments were performed. We introduced a plasmid carrying the *V. cholerae cqsA* gene cloned under the P*tac* promoter into an *E. coli metE* methionine auxotroph [21,22] (see Methods). In this mutant, exogenous L-methionine is required for SAM biosynthesis, and importantly, L-methionine is not a substrate for CqsA (Figure 2a). If SAM is the substrate for CAI-1 synthesis, by growing the *metE* strain in minimal medium with glucose as the sole carbon source and by providing labeled L-methionine, we should observe labeling of CAI-1.

Growth of the *metE* strain in the presence of 1 mM L-methionine resulted in efficient CqsA-dependent production of CAI-1 (molecular ion 215.2005) (Figure 4a). Supplying deuterium-labeled L-methionine (d_8-L-methionine) rather than unlabeled

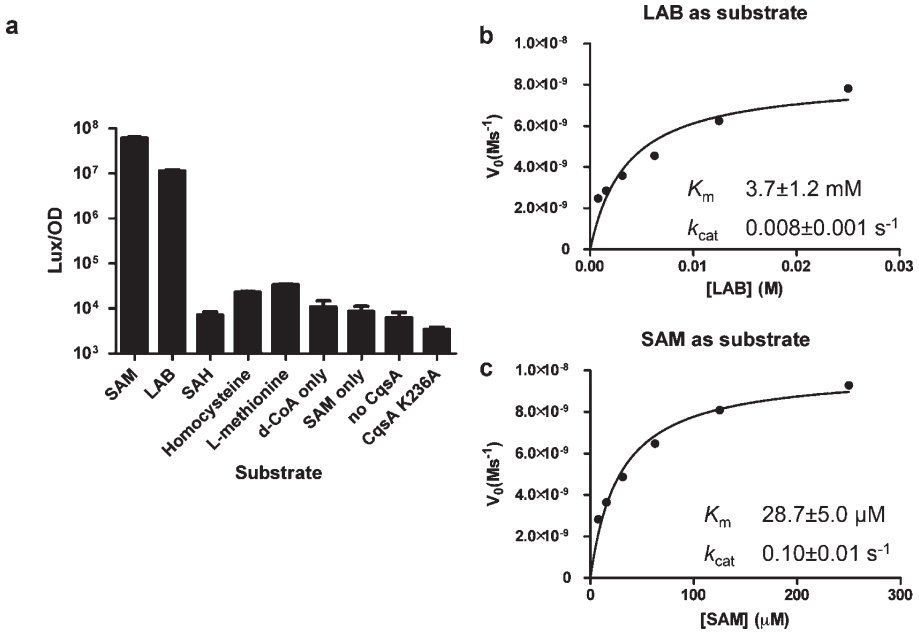


Figure 2. Potential substrates for CqsA and kinetic studies of SAM and LAB as CqsA substrates. (a) Different compounds were examined for activity as CqsA substrates. Reactions were carried out with each potential substrate at 1 mM and d-CoA at 100 μM. Control reactions with SAM only (no d-CoA), without CqsA, and with an inactive CqsA mutant CqsA K236A[13] are also shown. (b, c) Coenzyme A release was measured by a coupled enzyme assay. Kinetic constants for LAB (b) and SAM (c) were estimated by fitting initial velocities to the Michaelis−Menten equation using GraphPad Software.

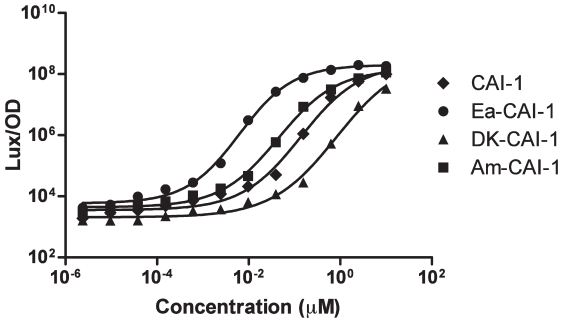


Figure 3. Dose responses of the CqsS receptor for CAI-1 type molecules. *V. cholerae* CqsS dose responses are shown for CAI-1 (◆), Ea-CAI-1 (●), DK-CAI-1 (▲), and Am-CAI-1 (■). Light production is shown at particular concentrations of compounds. Data were fit with a variable-slope sigmoidal dose−response curve.

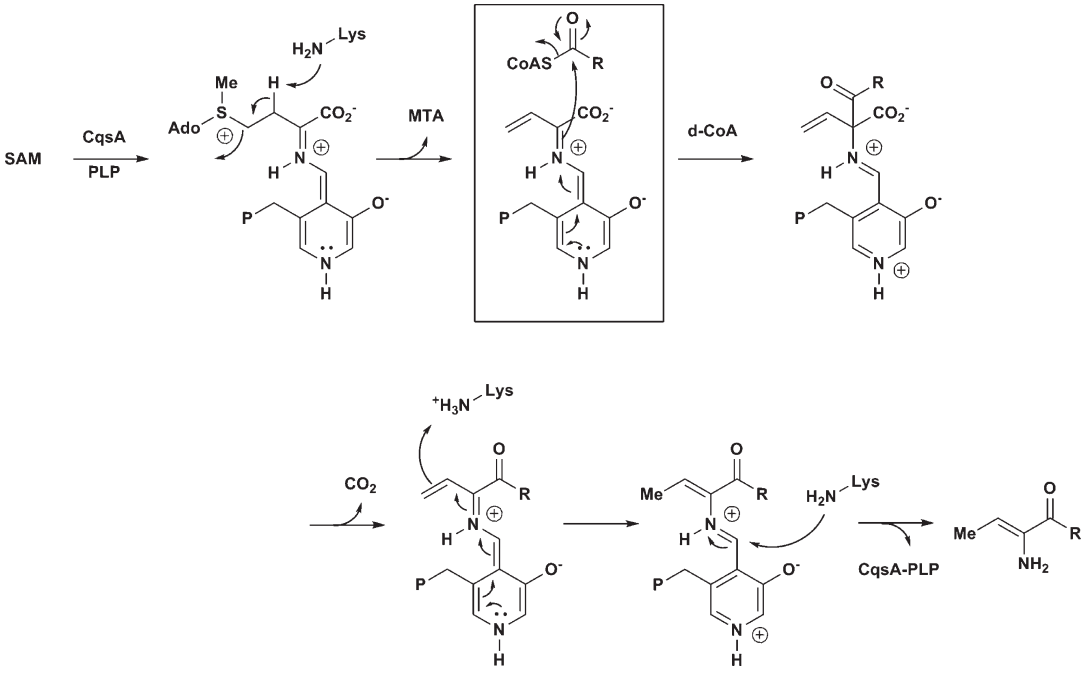
L-methionine resulted in production of a molecular ion of 218.2192 (d_3-CAI-1), and the 215.2005 molecular ion was not produced (Figure 4b), indicating that deuterium-labeled L-methionine, presumably through its incorporation into SAM, is used in the biosynthesis of CAI-1. Importantly, the incorporation of three deuterium atoms rather than four confirms that unsaturated Ea-CAI-1 is formed in the first step in the synthesis of CAI-1, illustrated in Supplementary Figure 3.

To identify the source of the atoms incorporated into the acyl tail of CAI-1, we performed labeling experiments with ^{13}C-glucose. The carbon backbone of the 10-carbon tail of CAI-1 must come from fatty acid biosynthesis and thus should be composed of the isotope present in the sole carbon source, glucose. Indeed, when cells are grown in unlabeled L-methionine and ^{13}C-glucose, the CAI-1 produced has an m/z of 225.2341, an increase indicating the incorporation of 10 carbons from ^{13}C-glucose (Figure 4c). Double labeling with d_8-L-methionine and ^{13}C-glucose produced CAI-1 with an m/z of 228.2526, an increase consistent with the incorporation of three deuterium atoms from d_8-L-methionine into the headgroup and 10 carbons from ^{13}C-glucose into the acyl tail (Figure 4d).

Ea-CAI-1 Is an Intermediate in CAI-1 Synthesis. To examine whether Ea-CAI-1 is an intermediate in *in vivo* CAI-1 synthesis, we used conversion experiments with synthetic Ea-CAI-1 and *V. cholerae* cell lysates. We monitored CAI-1 production using HRMS and the bioluminescence bioassay. Critical for this strategy is that we previously identified *V. cholerae* CqsS receptor mutants exhibiting altered preferences for CAI-1, Am-CAI-1, and other CAI-1 analogues.[23] One mutant, CqsS (W104AS107A) (denoted CqsS*), greatly prefers CAI-1 to Am-CAI-1. The wild-type CqsS receptor has the opposite preference (Am-CAI-1 > CAI-1) (Figure 3).[23] The CqsS* receptor also displays a preference for CAI-1 over Ea-CAI-1 (Supplementary Figure 4). We reasoned that we could use the CqsS* receptor mutant to determine if Ea-CAI-1 is converted to CAI-1 by *V. cholerae* lysates. Specifically, if CAI-1 was produced from Ea-CAI-1, we could measure an increase in CqsS* response using bioluminescence as the readout. We combined Ea-CAI-1 with a *V. cholerae cqsA* lysate or with lysis buffer alone and supplied the reaction mixtures with excess NADPH. CAI-1 production was observed by HRMS and by an increase in CqsS* response only when both the lysate and Ea-CAI-1 were included in the mixture (Figure 5a−c). Inclusion of synthetic deuterium-labeled CAI-1 (d_2-CAI-1) as an internal standard allowed us to quantify the amount of CAI-1 produced from Ea-CAI-1 by *V. cholerae* lysates. In this experimental setup, 22% of the Ea-CAI-1 was converted to CAI-1. Conversion of Ea-CAI-1 to CAI-1 required NADPH, consistent with the fact that the conversion of Ea-CAI-1 to CAI-1 must include one oxidation state change. Thus, Ea-CAI-1 is likely an intermediate in CAI-1 synthesis.

Identification of the Enzymes Responsible for the Conversion of Ea-CAI-1 to CAI-1. On the basis of the preceding

Scheme 1. Proposed CqsA Reaction Scheme[a]



[a] In the CqsA reaction using SAM and d-CoA as substrates, we propose a reaction sequence that combines a β,γ-elimination of SAM with the release of MTA and an acyltransferase reaction with the release of CoA. V-Gly is likely an enzyme-bound intermediate (boxed). This scheme is based on this study and inspired by the schemes for ACCS and CqsA.[13,14,20] The lysine amino moiety in the figure depicts the active site Lys236 residue on CqsA.[13].

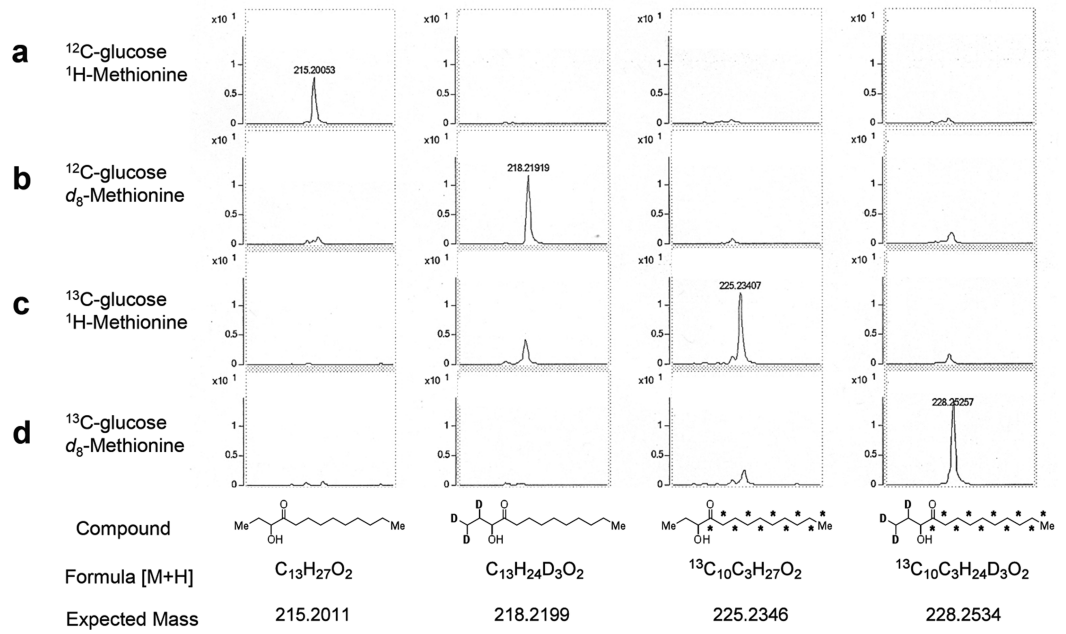


Figure 4. *In vivo* CAI-1 isotope labeling experiments. *In vivo* CAI-1 labeling experiments were performed by supplementing L-methionine or d_8-L-methionine in combination with glucose or ^{13}C-glucose in M9 minimal medium. HRMS spectra are enlarged to show specific regions around molecular weights 215, 218, 225, and 228. Corresponding chemical structures, compound formulas, and expected molecular weights are indicated at the bottom of each column. In the structures, predicted deuterium atoms and ^{13}C carbon atoms are denoted with "D" and "∗", respectively.

results, we hypothesized that two chemical transformations are required for conversion of Ea-CAI-1 to CAI-1 and one conversion must involve a reduction that uses NADPH. Enamines are often unstable in aqueous solution as a result of rapid hydration to produce the corresponding ketones.[24,25] Therefore, we proposed that an initial hydrolysis of Ea-CAI-1 would produce tridecane-3,4-dione (DK-CAI-1) (Figure 1), which could be reduced in an NADPH-dependent manner to CAI-1. We divided

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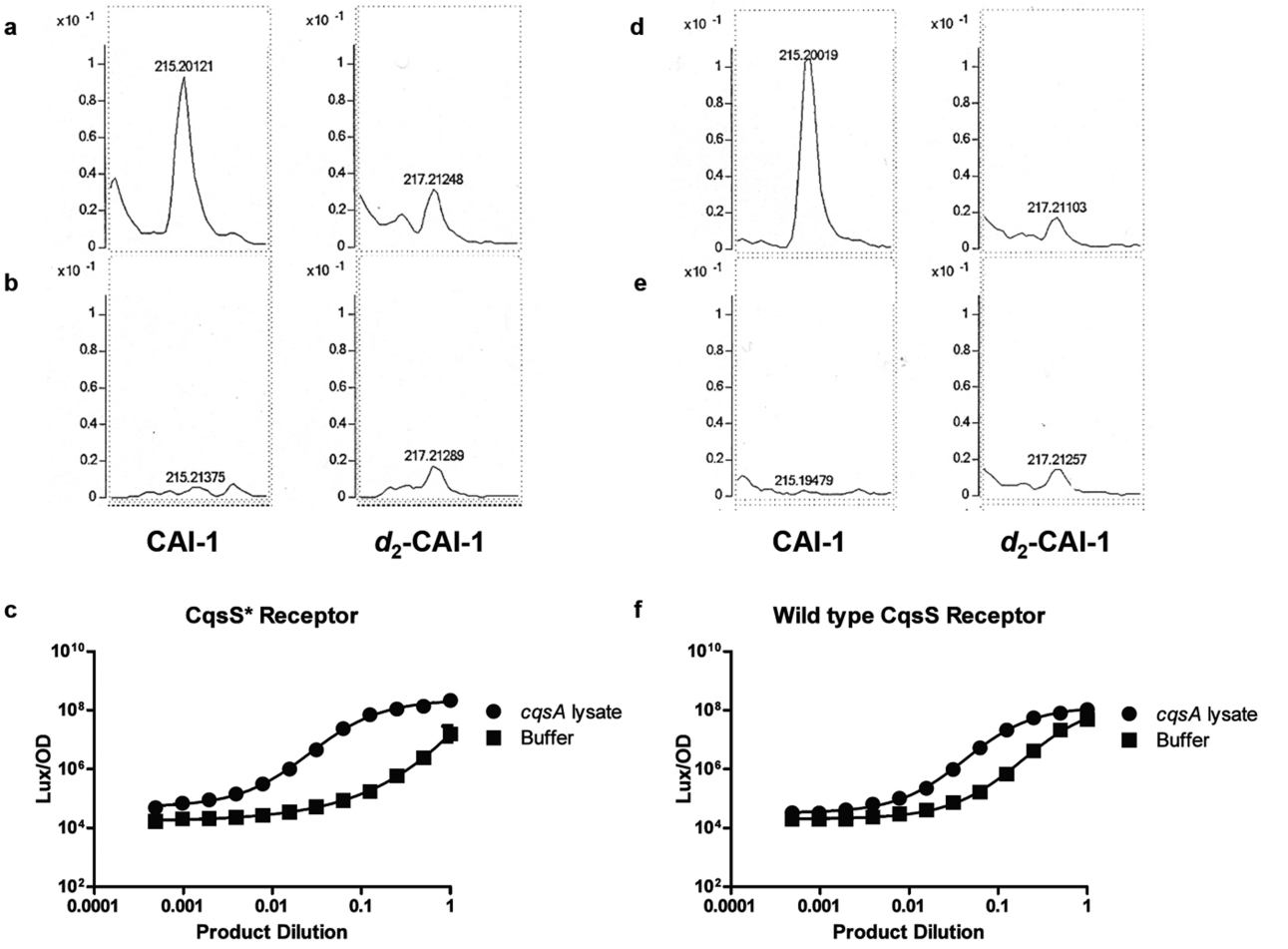

Figure 5. Conversion of Ea-CAI-1/DK-CAI-1 to CAI-1. Conversion experiments were performed with a *V. cholerae cqsA* lysate (a, d) or lysis buffer (b, e) providing Ea-CAI-1 (a−c) or DK-CAI-1 (d−f) and excess NADPH. In a, b, d, and e, HRMS spectra are enlarged to show peaks for CAI-1 and for the internal standard, d_2-CAI-1, as noted. In c and f, dose responses measured by light production are shown for the CqsS* receptor for Ea-CAI-1 to CAI-1 conversion (c) and for the wild-type CqsS receptor for DK-CAI-1 to CAI-1 conversion (f). Products were diluted as noted for reactions with *cqsA* lysate (●) and buffer (■).

our efforts to characterize the steps leading from Ea-CAI-1 to CAI-1 into an analysis of each chemical event: hydrolysis of Ea-CAI-1 to DK-CAI-1 and reduction of DK-CAI-1 to CAI-1.

Ea-CAI-1 to DK-CAI-1 conversion could either require an enzyme(s) or it could occur by spontaneous hydrolysis (see Discussion). To identify possible enzymes involved in the conversion, we fractionated *V. cholerae* lysates (see Methods). To each fraction we added Ea-CAI-1 and screened for DK-CAI-1 formation using the CqsS* receptor (see Methods). The CqsS* receptor prefers DK-CAI-1 over Ea-CAI-1 (Supplementary Figure 4), so we could determine which fractions contained Ea-CAI-1 to DK-CAI-1 converting activity by assaying for an increase in CqsS* receptor response. HRMS is not suitable for measuring the production of DK-CAI-1 because of the low ionization efficiency of DK-CAI-1. Despite multiple attempts, we were unable to identify a fraction containing an activity that increased the CqsS* receptor response. While not conclusive, we suggest that Ea-CAI-1 to DK-CAI-1 conversion occurs spontaneously. We return to this point in the discussion.

To identify enzymes responsible for conversion of DK-CAI-1 to CAI-1, we first tested if DK-CAI-1 could be converted to CAI-1 using *V. cholerae cqsA* lysates. DK-CAI-1 was prepared chemically and was found to have weak activity as a ligand for wild-type CqsS (Figure 3). Thus, if DK-CAI-1 is converted to CAI-1, we could monitor this reaction through an increase in the wild-type CqsS response and by using HRMS. CAI-1 production indeed occurred when we incubated a *V. cholerae cqsA* mutant lysate with DK-CAI-1 and NADPH (Figure 5d,f). No production occurred in the buffer control or in reactions lacking NADPH (Figure 5e). These results indicate the presence of an enzyme(s) capable of converting DK-CAI-1 to CAI-1 in an NADPH-dependent manner.

We fractionated cell lysates and screened for enzymes capable of converting DK-CAI-1 to CAI-1. A protein, VC1059, annotated as a short chain dehydrogenase containing an NADPH binding domain, was identified in the active fraction. We overexpressed and purified the *V. cholerae* VC1059 protein from *E. coli*. Recombinant VC1059 protein converts DK-CAI-1 to CAI-1 when excess NADPH is present (Supplementary Figure 5). Within the *V. cholerae* genome, VC1059 has seven highly conserved homologues, and we attempted to test whether any of these other enzymes is also capable of converting DK-CAI-1 to CAI-1. We could successfully overexpress and purify four of the proteins. Of these four proteins, VC1059 has the highest activity, and two of the other four purified proteins, VCA0301 and VC2021, possess detectable activity in converting DK-CAI-1 to CAI-1 (Figure 6a). We engineered deletions of *vc1059* and *vca0301*. VC2021 is the

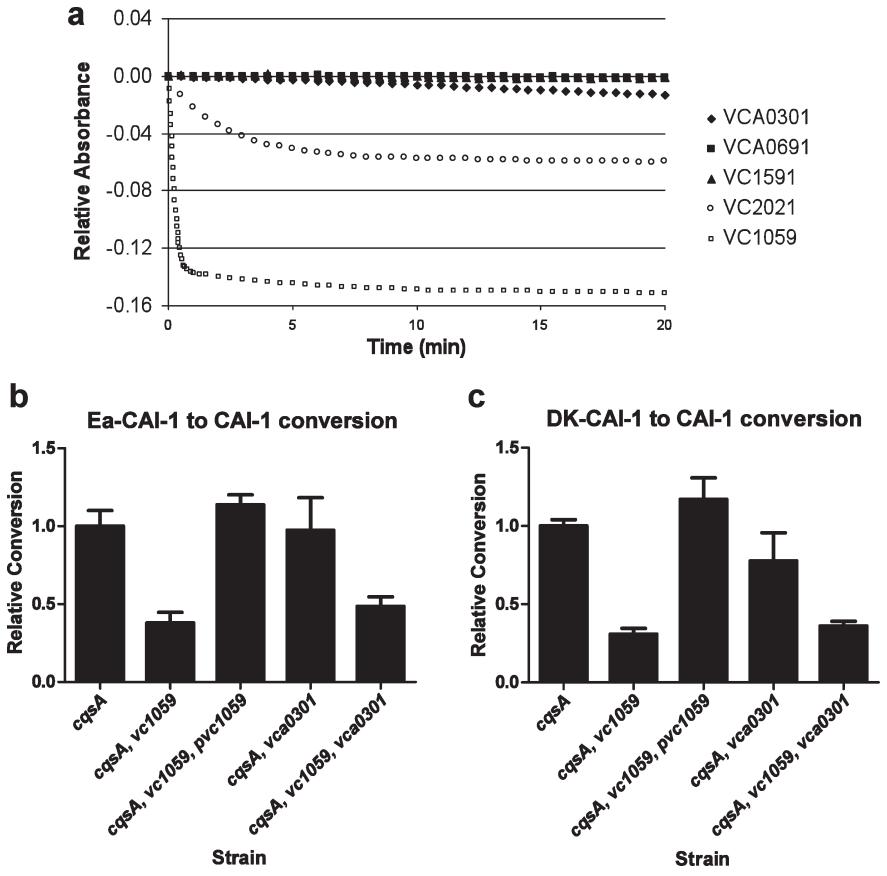

Figure 6. Conversion experiments with VC1059-type enzymes and *V. cholerae* mutant lysates. (a) *In vitro* conversion of DK-CAI-1 to CAI-1 following provision of DK-CAI-1, NADPH, and one of the recombinant proteins: VCA0301 (◆), VCA0691 (■), VC1591 (▲), VC2021 (○), and VC1059 (□). NADPH decreases were monitored. At each time point, relative absorbance at 340 nm was calculated by subtracting the initial absorbance at time zero. The reading interval is shorter for VC1059 because of its rapid reaction speed. Only selected time points for VC1059 are shown after the absorbance reaches a plateau. Conversion of Ea-CAI-1 (b) or DK-CAI-1 (c) to CAI-1 was carried out with *V. cholerae* mutant lysates. Mutations are noted in the figure. Conversion efficiencies are normalized to that of the *cqsA* single mutant.

V. cholerae homologue of the *E. coli* FabG protein, an essential protein in fatty acid biosynthesis,[26] so we could not construct a *V. cholerae vc2021* deletion mutant. Lysate from the *cqsA, vc1059* double mutant converted significantly less (~50%) of the Ea-CAI-1 or DK-CAI-1 to CAI-1 than did lysate from the *cqsA* single mutant (Figure 6b,c). Introduction of a plasmid expressing *vc1059* into the *cqsA, vc1059* mutant strain restored Ea-CAI-1/DK-CAI-1 to CAI-1 conversion (Figure 6b,c). By contrast, deletion of the *vca0301* gene in a *cqsA V. choleare* mutant did not significantly impair conversion of Ea-CAI-1/DK-CAI-1 to CAI-1 (Figure 6b,c). Indeed, the *cqsA, vc1059, vca0301* triple mutant exhibited conversion activity similar to that of the *cqsA, vc1059* double mutant (Figure 6b,c). These results are consistent with our observation that the recombinant VC1059 protein is more efficient than the recombinant VCA0301 protein at converting DK-CAI-1 to CAI-1. Together these results strongly suggest that DK-CAI-1 is an intermediate in CAI-1 synthesis and that VC1059 is the primary enzyme that carries out the final step in CAI-1 synthesis (Scheme 2). However, when cell-free culture fluids from the *V. cholerae vc1059* or *vc0301* single mutants or the *vc1059, vc0301* double mutant were assayed by mass spectrometry, none of the mutants were defective in CAI-1 production. Presumably, this is because residual conversion occurs in the *vc1059* mutant and in the *vc1059, vca0301* double mutant as a result of the redundant activity of VC2021 and/or the other homologues

Discussion. Quorum sensing controls virulence factor expression and biofilm production in *V. cholerae*. The CAI-1/CqsS signaling pathway is of interest for the development of new therapeutics.[3] Synthetic CAI-1 represses virulence genes *in vitro*,[7,12] and a commensal *E. coli* strain expressing the *cqsA* gene and therefore producing CAI-1 inhibits pathogenesis of *V. cholerae* in an infant mouse model.[3] Despite the critical role that CAI-1 plays in the life cycle and virulence of *V. cholerae*, the mechanism of CAI-1 biosynthesis is not known.

In this study, we identify SAM and d-CoA as the *in vivo* substrates for CqsA. This reaction produces a novel quorum-sensing molecule Ea-CAI-1. Ea-CAI-1 is more potent than CAI-1 or amino-CAI-1 as a CqsS agonist and is readily detected in *V. cholerae* cell-free culture fluids.[18] Nonetheless, we do not believe that Ea-CAI-1 is the major *V. cholerae* autoinducer because of its low abundance. Enamino-carboxylic acids have been proposed as biosynthetic reaction intermediates; however, they typically undergo rapid hydrolysis to the corresponding α-keto acids.[20,27−29] Thus, our finding of Ea-CAI-1 in reaction products and in cell-free culture fluids is surprising. Although there exist examples of free enamino-ketones, these molecules universally require additional stabilizing conjugated π-systems.[30] Thus, a dialkyl substituted free enamino-ketone analogous to Ea-CAI-1 has not been described previously.

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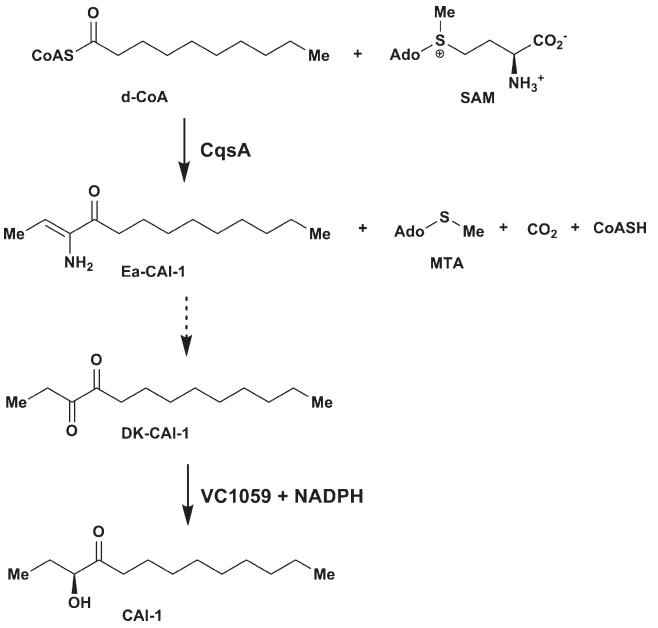
Scheme 2. CAI-1 Biosynthesis Scheme[a]



[a] SAM and d-CoA are the *in vivo* substrates for CqsA, which produces Ea-CAI-1, MTA, and CoASH. Ea-CAI-1 is converted to CAI-1 through the intermediate DK-CAI-1. We suggest that conversion of Ea-CAI-1 to DK-CAI-1 occurs spontaneously. VC1059 is the primary enzyme responsible for converting DK-CAI-1 to CAI-1.

Amino acid sequence analysis shows that CqsA bears homology to PLP-dependent acyl-CoA transferases, and previous work has shown that CqsA mediates an acyltransferase reaction between LAB and d-CoA to produce Am-CAI-1.[7,12,13] We find that CqsA is also capable of catalyzing the fragmentation of SAM. The ability of CqsA to catalyze the elimination reaction of SAM is somewhat surprising because of the low homology between CqsA and ACCS, an enzyme known to catalyze such a reaction. Nonetheless, an absorption peak at ~530 nm is observed when SAM is added to the CqsA protein, indicating the formation of a PLP quinonoid intermediate,[20] which would be activated to undergo either β,γ-elimination of SAM or acyltransfer (Supplementary Figure 6). Inspired by precedent examples of PLP-dependent enzymes[13,14,31,32] and based on our observations, we propose a mechanism for this transformation catalyzed by CqsA. It involves the combination of two PLP-mediated steps, directly facilitating both the elimination of MTA from SAM and carbon−carbon bond formation with the carbonyl group of d-CoA (Scheme 1). We note that the proposed mechanism in Scheme 1 is our preferred model but is not exclusive (*i.e.*, it is possible that MTA elimination happens after the ligation of the CoA thioester onto SAM) (Supplementary Figure 6). Future structural studies of the CqsA enzyme in complex with substrate analogues could clarify how CqsA possesses two distinct PLP-dependent catalytic activities. We note that we did not explore the possibility that decanoyl-acyl carrier protein, rather than d-CoA, is the fatty acid substrate for CqsA. This is a plausible scenario because decanoyl-acyl carrier protein and d-CoA have similar chemical properties and acyl−acyl carrier proteins are the substrates for LuxI type autoinducer synthases.[5]

We were able to reconstitute CAI-1 synthesis using synthetic Ea-CAI-1 and *V. cholerae* cell lysates, and we propose DK-CAI-1 as an intermediate in this conversion. Although we cannot rule out the participation of an enzyme in catalyzing the conversion from Ea-CAI-1 to DK-CAI-1, we could not identify such an enzyme. One possibility is that, in our chromatographic fractions, an Ea-CAI-1 to DK-CAI-1 converting enzyme was inhibited by the product of the reaction. This possibility was eliminated by supplementing lysate fractions with purified VC1059 protein and excess NADPH and finding that Ea-CA-1 was still not converted to DK-CAI-1. We anticipate that Ea-CAI-1 is susceptible to general acid-catalyzed hydrolysis with the rate-limiting step, under our conditions, being protonation of the β-carbon of the enamine to yield an iminium ion that is subsequently hydrolyzed.[24] A large number of general acids are typically present in bacteria, one or some of which could be responsible for mediating the protonation of Ea-CAI-1.

No α-enaminoketones have been implicated in biological processes. The related α-enamino acids, although proposed as biosynthetic products, have never been detected directly; rather, the corresponding α-keto acids are typically observed.[25] We, however, observed exactly the opposite for the enaminoketone Ea-CAI-1. Detection of DK-CAI-1 by HRMS has been challenging because of its low ionization potential. Thus, the high detection limit has hampered our quantification of DK-CAI-1 in both cell-free culture fluids and *in vitro* conversion reactions of Ea-CAI-1 to CAI-1. Nonetheless, we unequivocally showed that VC1059 and its homologues can convert DK-CAI-1 to CAI-1 *in vitro*. Mutation of the *vc1059* gene encoding a short chain dehydrogenase enzyme results in significantly impaired conversion of DK-CAI-1 to CAI-1 and, importantly, also Ea-CAI-1 to CAI-1, supporting the role of DK-CAI-1 as an intermediate in the biosynthesis of CAI-1 (Scheme 2). Although VC1059 appears to be the predominant enzyme responsible for the conversion of DK-CAI-1 to CAI-1, other VC1059 homologues, namely, VC2021 and VCA0301, are also capable of reducing DK-CAI-1 to CAI-1 albeit with lower efficiency *in vitro*.

It is intriguing to us why such a complicated synthetic route exists for CAI-1, especially since the immediate product of CqsA, Ea-CAI-1, potently induces quorum sensing (Figure 3). One possibility is that CAI-1 possesses properties that are optimized for signaling. For example, CAI-1 likely persists in the environment longer than Ea-CAI-1, which is sensitive to hydrolysis to give DK-CAI-1. Second, regulation of the production and/or activity of the conversion enzymes could play crucial roles in producing the precise final blend of molecules required for quorum sensing. For example, induction of enzymes converting DK-CAI-1 to CAI-1 could increase production of the potent agonist CAI-1 from the weak agonist DK-CAI-1 and, as a consequence, could promote a stronger quorum-sensing response. The enzymes that convert Ea-CAI-1 to CAI-1 also exist in *E. coli*, which lacks the CAI-1/CqsS system, since introduction of *cqsA* into *E. coli* is sufficient to confer CAI-1 production. The general existence of the conversion enzymes suggests the possibility that conversion might be an "accidental" consequence of the housekeeping functions of the other enzymes. Curiously, the study of Ng *et al.*[18] shows that *V. harveyi* uses Ea-C8-CAI-1 (the direct product of the *V. harveyi* CqsA reaction) rather than C8-CAI-1 (the final product of the conversion reaction) as its primary quorum-sensing molecule. These data suggest that the conversion from Ea-CAI-1-type molecules to CAI-1-type molecules is not an absolute requirement for CAI-1/CqsS quorum-sensing systems.

SAM is the second most widely used enzyme substrate after ATP.[33] Our work here shows that SAM is the preferred substrate for CqsA *in vivo*. SAM is also the substrate for another major class of autoinducers in Gram-negative bacteria, acryl-homoserine lactones (acyl-HSL), as well as for the AI-2 autoinducer family. It is intriguing to us that acyl-HSL, AI-2, and CAI-1 synthesis all require SAM as a substrate. The use of SAM as the universal substrate in these autoinducer production pathways reveals a possible ancient origin for quorum-sensing signals in Gram-negative bacteria. Linking the production of all of these signals to an essential metabolite, SAM, suggests that perhaps these signals originated as a byproduct of essential metabolism. Alternatively, it could be beneficial to use a widely available substrate and housekeeping-type reactions to produce the earliest communication signals. Consistent with this second idea, because SAM is an abundant metabolite and given its high reactivity in biological systems, it could be the optimal substrate to ensure constant and efficient production of autoinducers and, in turn, to ensure accurate representation of population densities. SAM is required for DNA, RNA, and protein production, and thus information about the growth potential of the environment could also be encoded in the quorum-sensing signals due to a requirement for SAM in their synthesis. We wonder if cells detecting quorum-sensing signals also extract information about their growth conditions from these molecules. Finally, *V. cholerae* apparently does not have an acyl-HSL quorum-sensing signal. Using the CAI-1 system in place of an acyl-HSL system could provide advantages in certain niches as enzymes inactivating HSL molecules have been discovered.[34−36]

METHODS

Cloning, Overexpression, and Purification of VC1059 and Homologues. DNA manipulations were performed following standard procedures.[37] *V. cholerae* genes *vc1059, vca0301, vca0691, vca1057, vca1108, vc1591, vc0979, and vc2021* were amplified, cloned into pET28b (Novagen) using NdeI/XbaI and *Bam*HI sites, and sequenced. Plasmids were transformed into *E. coli* BL21 (DE3). A complete strain list can be found in Supporting Information. VCA1057 was not expressed, and VCA1108 and VC0979 were insoluble following induction. The other N-His_6-tagged proteins were purified as described in Supplementary Methods.

Strain Construction. Reporter strains are the CqsS wild-type reporter MM920 (*V. cholerae* El Tor C6706str *cqsA*, *luxQ* carrying *luxCDABE* from *V. harveyi* and the CqsS* reporter WYZ682. WYZ682 is derived from MM920 and contains a double mutation in the *cqsS* gene, CqsS (W104A S107A).[23] *V. cholerae* double *cqsA*, *vc1059* and *cqsA*, *vca0301* mutants and the *V. cholerae* triple *cqsA*, *vc1059*, *vc0301* mutant were derived from a *V. cholerae cqsA* parent strain.

***In Vitro* Enzyme Assay with Purified CqsA.** *In vitro* reactions with 1 μM CqsA, 100 μM d-CoA, and 1 mM substrates were performed in 20 mM HEPES, 200 mM NaCl, at pH 8.0 at RT. Reactions were terminated with acetonitrile (ACN) (50% (v/v) final concentration) following 1 h reactions. After centrifugation, 10 μL of the reaction was added in triplicate into 190 μL of a 50-fold diluted overnight culture of MM920 grown in LB medium with 10 μg/L tetracycline. Light production and OD_{600} were measured in 96-well plates following 6 h of incubation using an Envision 2103 Multilabel Reader (Perkin-Elmer). For HRMS with SAM and d-CoA, 100 μM of one or both substrates was added in the above buffer. Following 30 min, reaction products were extracted using DCM (see Supplementary Methods for details).

Kinetic Analyses with SAM and LAB. Coenzyme A release was measured by a coupled enzyme assay.[31] SAM and LAB concentrations were varied while d-CoA was held constant at 100 μM. We used 100 nM CqsA for experiments with SAM and 1 μM CqsA for experiments with LAB. A standard curve was generated to calculate CoA release.

***In Vivo* Labeling Experiment.** A plasmid carrying the *V. cholerae cqsA* gene under the P*tac* promoter[38] was introduced into an *E. coli* Δ*metE::kan* strain from the KEIO collection.[39] Pellets from 1 mL of overnight cultures of WYZ1455 grown in LB medium were washed with M9 minimal medium without glucose or L-methionine and resuspened in 5 mL of M9 medium supplemented with 1 mM L-methionine or d_8-L-methionine and 0.4% (w/v) glucose or ^{13}C-glucose. Following overnight growth, cell-free culture fluids were extracted with DCM and analyzed by HRMS. L-Methionine, glucose, and ^{13}C-glucose were purchased from Sigma-Aldrich, and d_8-L-methionine came from Cambridge Isotope Laboratories.

Conversion Experiments with Lysates. To prepare crude cell lysates, 3 mL of overnight cultures of different strains were pelleted and resuspended in 1 mL of lysate buffer (20 mM HEPES, 100 mM NaCl at pH 8.0). Cells were lysed by sonication, and lysates were harvested by centrifugation. In Ea-CAI-1/DK-CAI-1 to CAI-1 conversion experiments, final concentrations of 100 μM Ea-CAI-1 or DK-CAI-1 were added to 200 μL of lysates containing 500 μM NADPH. Reactions were incubated at RT for 30 min and then divided in half. Twenty microliters of each reaction was terminated with 20 μL of ACN. Following centrifugation, 5 μL of the cleared fluid was added in triplicate into 195 μL of a 50-fold diluted *V. cholerae* reporter strain and assayed. MM920 was used for assaying DK-CAI-1 to CAI-1 conversion and WYZ682 was used for assaying Ea-CAI-1 to CAI-1 conversion. The remaining portions of the reactions were supplied with a final concentration of 5 μM d_2-CAI-1. Each sample was extracted with 200 μL of DCM for HRMS.

***In Vitro* Enzymatic Assays with Purified VC1059 and Homologues.** *In vitro* reactions containing 50 nM purified VC1059 or another homologue, 100 μM DK-CAI-1, and 100 μM NADPH were carried out in 20 mM HEPES, 100 mM NaCl at pH 8.0 at RT. Absorbance at 340 nm was monitored by a DU-800 spectrophotometer (Beckerman Coulter) for 20 min.

Chemical Synthesis. Chemical synthesis of Ea-CAI-1 is described in the study by Ng *et al.*[18] Synthetic procedures for DK-CAI-1 and d_2-CAI-1 are described in an earlier study.[13]

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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