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Development of a Biosensor for Crotonobetaine-CoA Ligase Screening Based on the Elucidation of *Escherichia coli* Carnitine Metabolism

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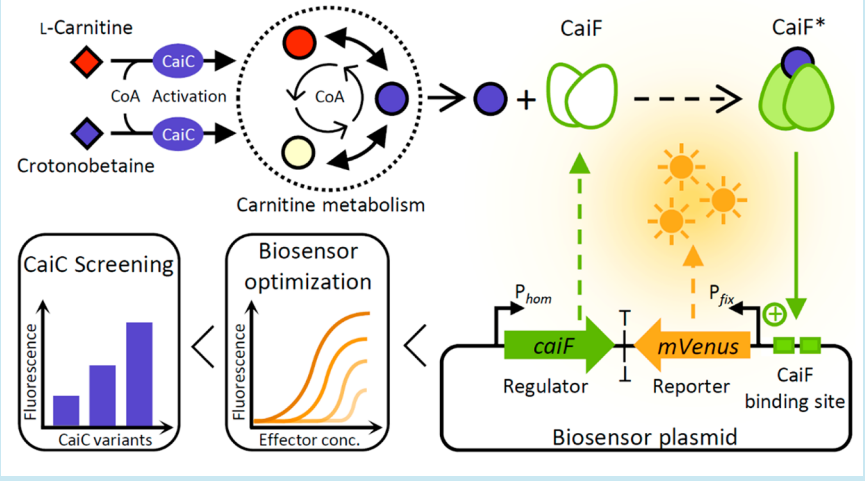

ABSTRACT: L-Carnitine is essential in the intermediary metabolism of eukaryotes and is involved in the β-oxidation of medium- and long-chain fatty acids; thus, it has applications for medicinal purposes and as a dietary supplement. In addition, L-carnitine plays roles in bacterial physiology and metabolism, which have been exploited by the industry to develop biotechnological carnitine production processes. Here, on the basis of studies of L-carnitine metabolism in *Escherichia coli* and its activation by the transcriptional activator CaiF, a biosensor was developed. It expresses a fluorescent reporter gene that responds in a dose-dependent manner to crotonobetainyl-CoA, which is an intermediate of L-carnitine metabolism in *E. coli* and is proposed to be a coactivator of CaiF. Moreover, a dual-input biosensor for L-carnitine and crotonobetaine was developed. As an application of the biosensor, potential homologues of the betaine:CoA ligase CaiC from *Citrobacter freundii*, *Proteus mirabilis*, and *Arcobacter marinus* were screened and shown to be functionally active CaiC variants. These variants and the developed biosensor may be valuable for improving L-carnitine production processes.

KEYWORDS: *carnitine metabolism, biosensor, CaiF, CaiC, metabolite sensing, betaine:CoA ligase*

L-Carnitine ((*R*)-3-hydroxy-4-trimethylaminobutyrate) is a quaternary amine that is essential for the intermediary metabolism of eukaryotes by playing a vital role in energy production and fatty acid metabolism. It is a regulator of the levels of fats in the serum and is involved in the β-oxidation of medium- and long-chain fatty acids as well as in the exchange of acyl and acetyl groups with coenzyme A (CoA) in the mitochondria.[1] Thus, applications for L-carnitine and its derivatives can be found in numerous medical areas, for example, in the treatment of cardiovascular diseases and as a nutritional supplement to improve weight management and training performance.[1]

Carnitine is not only present in eukaryotes but is also often found and is sometimes abundant in soil and natural water bodies;[2,3] thus, it is widely available to microorganisms. Indeed, carnitine plays roles in bacterial physiology and metabolism, *e.g.*, as a final electron acceptor, a compatible solute, or a sole source of carbon, nitrogen, and energy.[4] The carnitine biotransformation pathway of *Escherichia coli* has been employed for the production of L-carnitine in several studies and in industry.[1,5−10]

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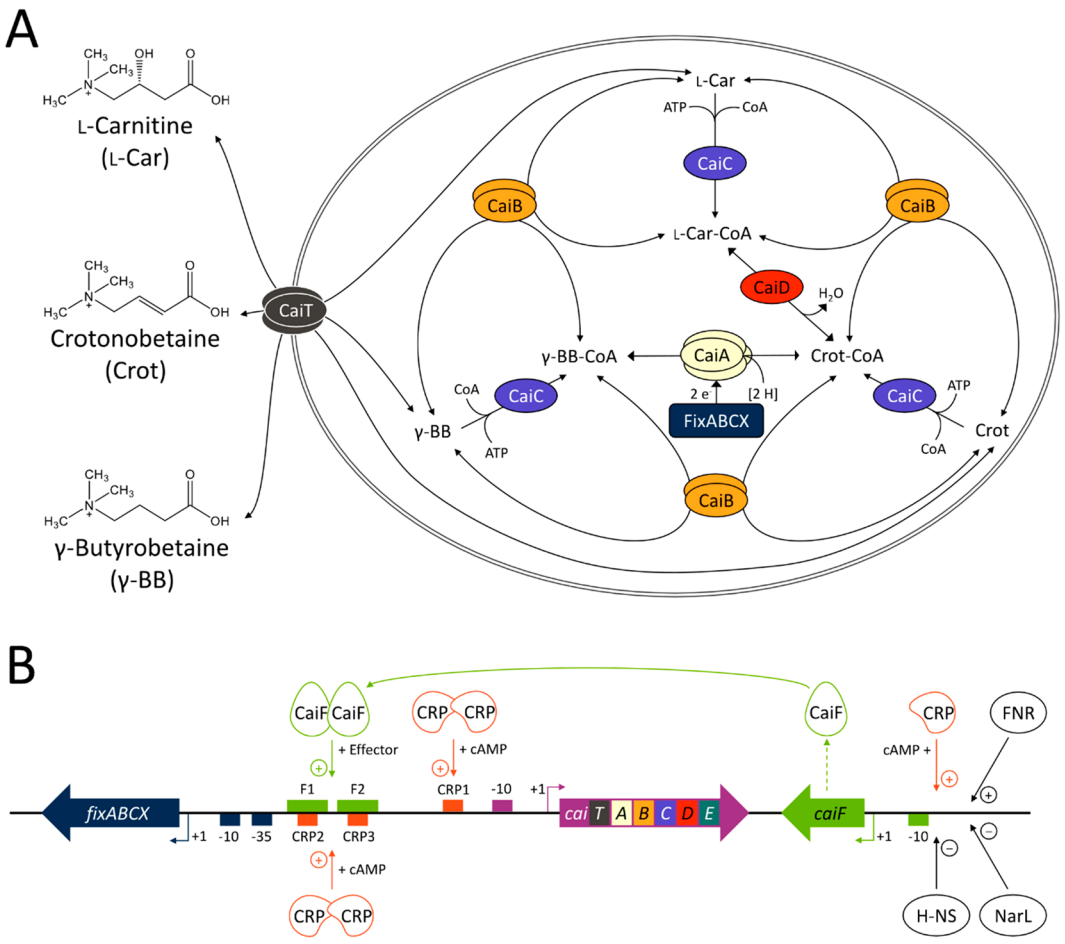

Figure 1. Schematic illustration of carnitine metabolism and its regulation in *E. coli*. (A) *E. coli* carnitine metabolism based on Bernal *et al.*[19] and Meadows *et al.*[4] L-Carnitine (L-Car) is reduced *via* crotonobetaine (Crot) to γ-butyrobetaine (γ-BB) *via* their CoA esters, which is eventually antiported with the substrate carnitine. CaiT, the carnitine:γ-butyrobetaine antiporter, additionally accepts the intermediate crotonobetaine for transport. CaiA is crotonobetainyl-CoA reductase. CaiB (carnitine CoA-transferase) exchanges CoA moieties between all shown TMA compounds. CaiC (betaine:CoA ligase) accepts all three TMA compounds for CoA ligation. CaiD is carnitinyl-CoA dehydratase and FixABCX are the electron transfer flavoproteins. Electron movement is denoted by e^-. (B) Regulation of the carnitine metabolism genes in *E. coli* based on Eichler *et al.*[24] and Buchet *et al.*[25] The *cai* operon (*caiTABCDE*; depicted as single purple arrow) and *fix* operon (*fixABCX*; depicted as single dark blue arrow) are positively regulated by cAMP-CRP with the binding sites CRP1−3 and by CaiF with the binding sites F1 and F2 in the presence of an unknown effector of carnitine metabolism. The expression of *caiF* is positively regulated by cAMP-CRP and the fumarate and nitrate reduction regulatory protein FNR and is negatively regulated by the nitrate/nitrite response regulator protein NarL and the DNA-binding protein H-NS (the regulation type is indicated by the circled + or −). The promoter elements are displayed in corresponding colors and indicate the transcription start sites (+1), −10, and −35 regions.

E. coli can use carnitine as an osmolyte and as an electron acceptor. The uptake of carnitine is mediated by three transport systems. ProP is a proton-proline symporter for the uptake of proline, glycine betaine, stachydrine, pipecolic acid, ectoine, and taurine belonging to the metabolite:H+ symporter (MHS) family of the major facilitator superfamily (MFS) of transporters.[4,11] ProU is an ATP-dependent ABC transport system encoded by *proVWX* for the uptake of proline and glycine betaine. The genes of both uptake systems are induced under hyperosmotic stress conditions and their gene products accept carnitine as a substrate. CaiT belongs to the substrate:product antiporter of the betaine/carnitine/choline transporter (BCCT) family of secondary transporters.[11] Thus, CaiT only mediates the uptake of carnitine in exchange for a metabolic product of carnitine catabolism.[14−16] Antiport by CaiT is part of *E. coli*'s ability to use carnitine as electron acceptor, a function that has been demonstrated for some pseudomonads and enterobacteria.[12] *E. coli* can use carnitine as a final electron acceptor under anaerobic conditions by reducing it to γ-butyrobetaine (γ-BB) *via* crotonobetaine (Figure 1A). This biotransformation process is facilitated by the enzymes encoded by the divergently transcribed but adjacent operons *caiTABCDE* and *fixABCX*.[13] Inside the cell, the acids are activated in a CoA-dependent manner by CaiC (EC: 6.2.1.48). This ATP-dependent betaine:CoA ligase accepts carnitine as well as the other two trimethylammonium (TMA) compounds, crotonobetaine and γ-BB.[4] Next, the carnitine CoA-transferase CaiB (EC: 2.8.3.21) catalyzes the reversible transfer of the CoA moiety between all of the TMA compounds of carnitine metabolism.[17,18] This allows the energy-efficient recycling of the CoA moiety between the final reduction product, γ-BB-CoA, and the substrate carnitine.[19] Carnitinyl-CoA dehydratase CaiD (EC: 4.2.1.149) catalyzes the reversible dehydration of carnitinyl-CoA to crotonobetainyl-CoA,[17] which is reduced to γ-BB-CoA by crotonobetainyl-CoA reductase CaiA (EC:1.3.8.13). The latter is postulated to form a complex with CaiB for its function.[20] The

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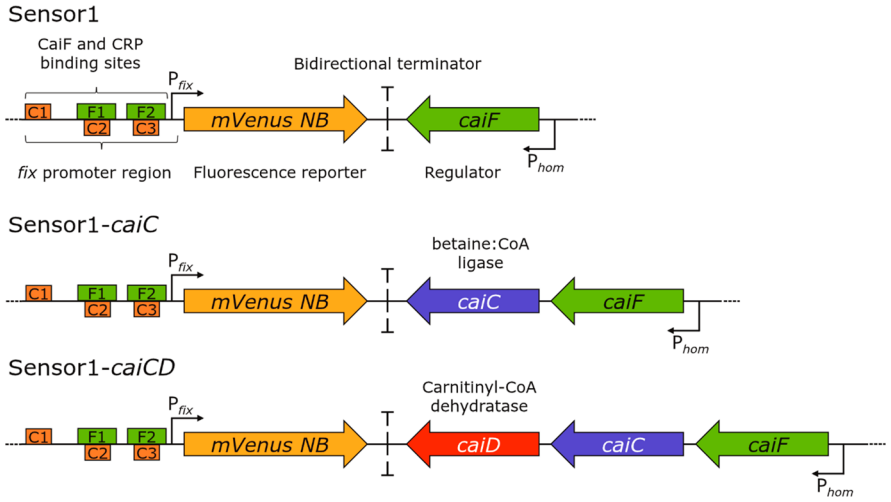

Figure 2. Schematic representation of the biosensor components and designs. Sensor1: The *caiF* gene for the specific transcriptional regulator CaiF is constitutively expressed by the *hom* promoter (P_{hom}). CaiF can bind cooperatively with CRP to its binding sites in the *fix* promoter region F1 and F2, which also includes the cAMP-CRP binding sites C2 and C3, and activate expression of the fluorescence reporter gene *mVenus NB* by P_{fix} after recognition of the effector and in absence of glucose. The first CRP binding site of the *fix* promoter region (C1) is also included upstream in this construct. The *caiF* and *mVenus NB* genes are separated by a bidirectional terminator (T). Plasmid pGP2 was used as the vector backbone. Sensor1-*caiC*: Extension of the previous biosensor design by the *caiC* gene for betaine:CoA ligase using an artificial RBS (*caiC* 5′ UTR (last 35 bp) to start codon: GCGTCCTGGAAGCGTAAGGCACAGGAAGGAGCGATATG, TIR: 400) placed under control of P_{hom}. Sensor1-*caiCD*: Extension of Sensor1 by *caiC* and the *caiD* gene for carnitinyl-CoA dehydratase using the respective native RBS placed under control of P_{hom}.

reductant for this reaction is provided by the enzymes encoded in the *fix* operon.[21,22] The function of CaiE, the last enzyme encoded in the *cai* operon, is still unclear, but it was postulated that it exerts a stimulating influence on the carnitine dehydratase activity.[23]

The *cai* and *fix* operons are transcribed in the presence of either carnitine or crotonobetaine and in the absence of oxygen, glucose, and nitrate.[21,24] This regulation depends on two transcriptional regulators, the cyclic adenosine monophosphate (cAMP) receptor protein (CRP) and the specific transcriptional activator of carnitine metabolism in *E. coli*, CaiF (Figure 1B). The gene *caiF* is located downstream and in opposite orientation to the *cai* operon.[24] CRP requires cAMP as the coactivator to bind to the cAMP-CRP binding sites in the promoter-operator region, while CaiF requires an effector of the carnitine metabolism for activation.[13] On the basis of DNase I footprinting and gel retardation assays that showed no effects of the apparent inducers L-carnitine and crotonobetaine, it is hypothesized that the true effector of carnitine metabolism is a CoA-derivative of these apparent inducers.[25] The exact nature of the effector for gene expression, however, remains elusive.

A better understanding of the regulation of carnitine metabolism could prove helpful for optimizing biotechnological carnitine production, *e.g.*, by enabling dynamic pathway regulation and optimized metabolic flux control, as shown for other processes.[26,27] Moreover, as an alternative to traditional analytical methods such as enzymatic assays, chromatography, or mass spectrometry, which are slow and labor-intensive, genetically encoded biosensors offer an attractive low-cost tool for the quantification of intracellular metabolites.[28] This may be achieved by using nature's diverse repertoire of possibilities for metabolite sensing, such as riboswitches[29,30] or transcription factors, to convert the intracellular metabolite concentration into an easily readable signal such as the expression of a fluorescent protein.[31−35]

We hypothesized that a biosensor based on CaiF may be used as screening tool, studied the activation mechanism of carnitine metabolism, and provide proof that crotonobetainyl-CoA is an essential compound for this process. The development of a biosensor system based on these findings using the transcriptional activator CaiF and the *fix* promoter is described. The biosensor output was optimized by adapting the culture medium and the host strain, and in combination with selected *cai* genes, thus improving the utility value of the biosensor, a metric that considers the biosensor parameters relative input range and output fold induction.[36] In addition, the biosensor was adapted to respond to all TMA compounds of carnitine metabolism. A single biosensor plasmid was designed, allowing *in vivo* detection of exogenous carnitine with high utility in *E. coli*. As a test case, the biosensor was used to identify betaine:CoA ligases homologous to *E. coli* CaiC to further increase the understanding of the physiology of the donor bacteria and to improve carnitine production.

RESULTS AND DISCUSSION

Design and Construction of a Carnitine Metabolism Biosensor. On the basis of the known regulatory mechanism of carnitine metabolism in *E. coli* (Figure 1B), we sought to develop a plasmid-based carnitine biosensor (Figure 2). In *lacZ* fusion experiments, the *fix* promoter has previously been shown to exhibit a higher fold induction compared to the *cai* promoter.[13] Thus, the *fix* promoter was used here to control the expression of the promoterless reporter gene *mVenus NB*. The other important element of the biosensor is the specific transcription factor CaiF, which is required for the transcriptional activation of the *fix* promoter and is responsible for the recognition of the carnitine-related effector. In the biosensor plasmid, *caiF* is constitutively expressed from the *hom* promoter from *Corynebacterium glutamicum*, which is a weak constitutive promoter in *E. coli*,[37] consequentially eliminating regulation of the *caiF* promoter by CRP, FNR, H-NS, and NarL (Figure 1B). CaiF binds cooperatively with cAMP-CRP to the *fix* promoter region to activate expression.[25] Thus, it was hypothesized that the biosensor only depends on the concentration of the effector

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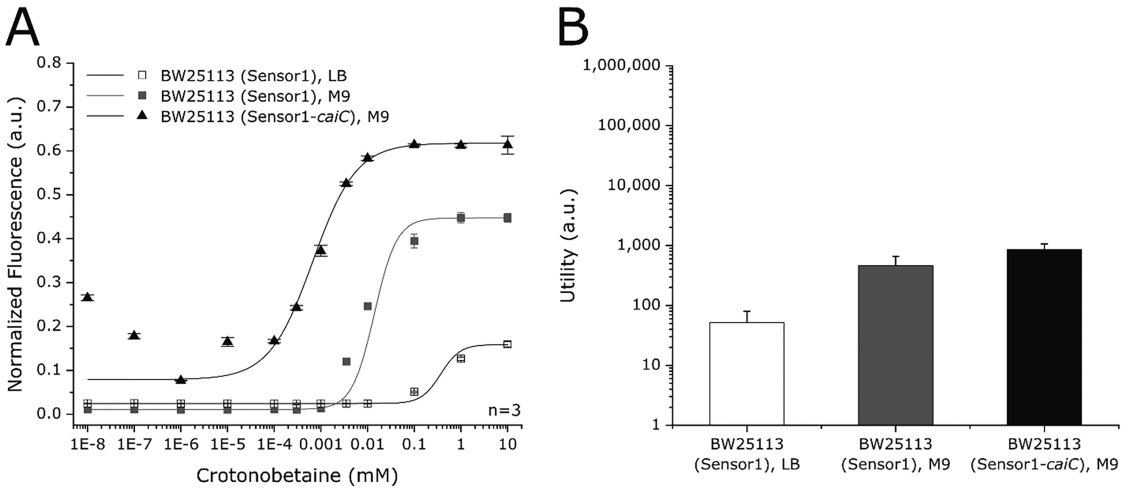

Figure 3. Improving the biosensor utility and sensitivity. (A) Dose−response curves to exogenous crotonobetaine for Sensor1 in *E. coli* BW25113 (wt) cultivated in LB medium or in M9 medium and for Sensor1-*caiC* in *E. coli* BW25113 in M9 medium. The fluorescence was determined at each indicated effector concentration and normalized to the cell density. The curves were fitted to the Hill equation. The maximum normalized fluorescence signal observed during the cultivation process is shown. Error bars indicate the standard deviation of the mean of three replicates. (B) Utility of the three different dose−response curves calculated from the Hill functions in Figure 3A.

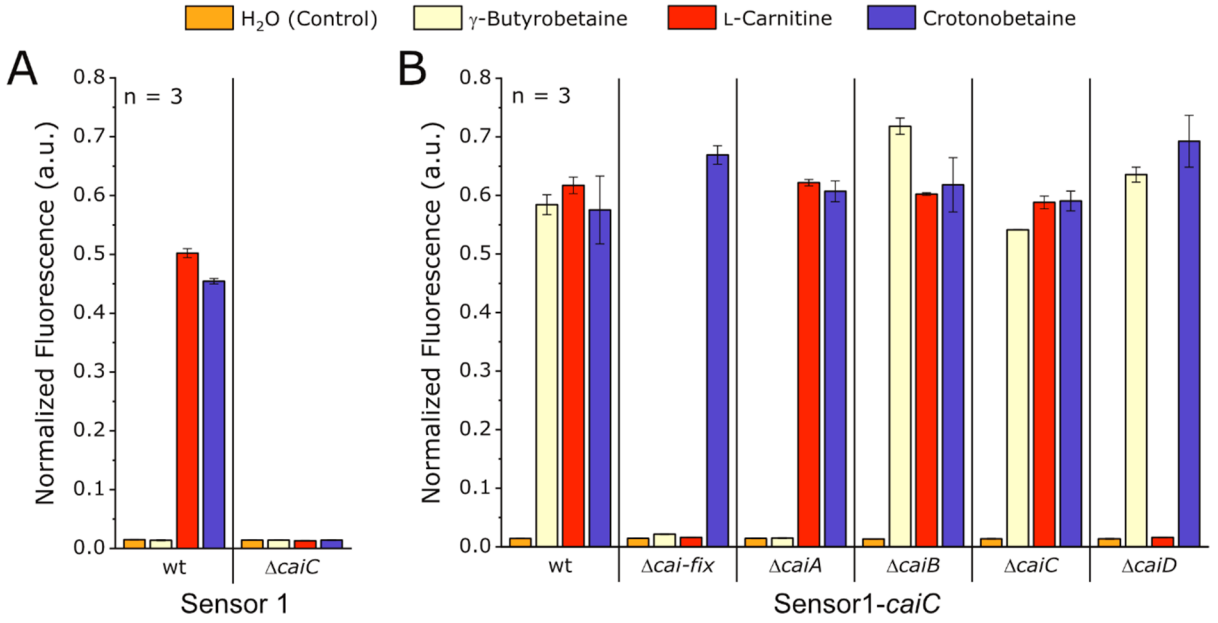

Figure 4. Identification of crotonobetainyl-CoA as a coactivator of CaiF in carnitine metabolism by loss-of-function analysis. The fluorescence response to the three different TMA compounds, L-carnitine, crotonobetaine, and γ-butyrobetaine, at a concentration of 2 mM and to water as the control by different biosensor strains in the M9 medium is depicted. The maximum normalized fluorescence signal observed during the cultivation process is shown. Error bars indicate the standard deviation of the mean of three replicates. (A) Sensor1 in *E. coli* BW25113 (wt) and *E. coli* BW25113 with the deletion of *caiC* (Δ*caiC*). (B) Sensor1-*caiC* in *E. coli* BW25113 (wt) and *E. coli* BW25113 strains with deletions of the *cai-fix* operons (Δ*cai-fix*), and single deletion of *caiA*, *caiB*, *caiC*, and *caiD* (Δ*caiA*, Δ*caiB*, Δ*caiC*, and Δ*caiD*, respectively).

in the absence of glucose, as this results in the high cAMP levels required for CRP binding. The *caiF* and *mVenus NB* genes were separated by a bidirectional terminator to avoid interactions. Plasmid pGP2 was constructed from pVWEx1[38] and used as the backbone for the biosensor plasmid.

Since it was hypothesized that the true effector of carnitine metabolism is a CoA-derivative of one of the apparent inducers, carnitine or crotonobetaine,[25] the biosensor variant Sensor1-*caiC* was constructed. To enable the biosynthesis of the CoA-esters carnitinyl-CoA, crotonobetainyl-CoA and γ-BB-CoA, the betaine:CoA ligase gene *caiC* was expressed in a synthetic operon with *caiF* under control of the *hom* promotor. For the 5′ untranslated region (5′ UTR), an artificial ribosome binding site (RBS) with 10% of the native RBS transcriptional initiation rate (TIR) was designed and placed in front of *caiC* to compensate for the 10-fold increased gene copy number by transferring the gene from the chromosome to the plasmid.

Improving the Utility and Sensitivity of the Biosensor. *E. coli* BW25113 was transformed with Sensor1, and cells were grown in LB medium supplemented with crotonobetaine or not. The cells responded to the effector at a concentration range of 0.1−10 mM by a maximum 6.5-fold increase in fluorescence (Figure 3A).

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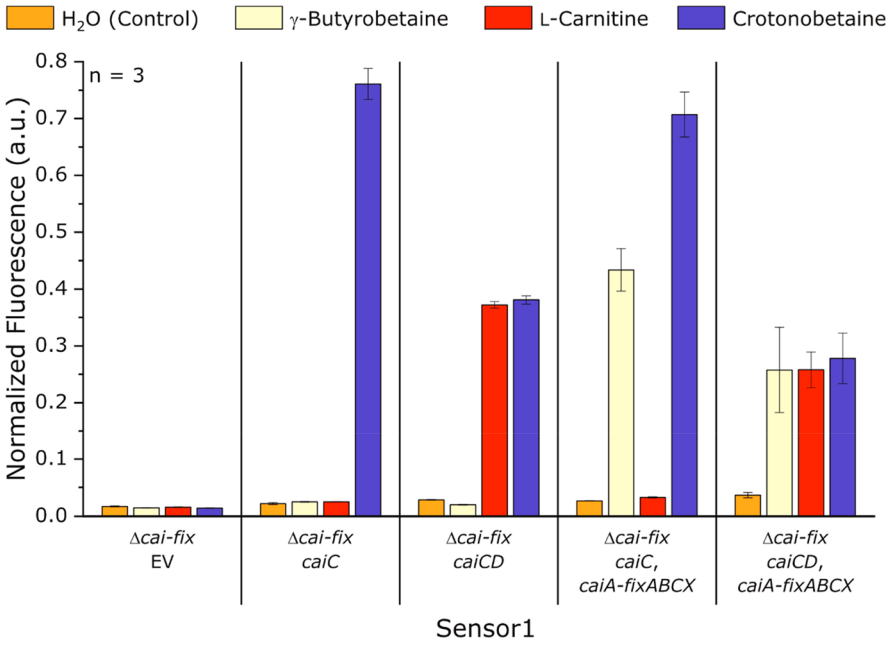

Figure 5. Gain-of-function biosensor experiment to identify key enzymes for the biosensor system. The fluorescence response to the three different TMA compounds L-carnitine, crotonobetaine and γ-butyrobetaine, at a concentration of 2 mM and to water as a control by strains with deleted carnitine metabolism (Δ*cai-fix*), in which parts of the metabolism were reconstructed by the plasmid-based expression of selected *cai* and *fix* genes are shown. *E. coli* BW25113 Δ*cai-fix* (Sensor1) was used as the basis and transformed with pEKEx3 (empty vector, EV), pEKEx3-*caiC* (*caiC*), pEKEx3-*caiCD* (*caiCD*), pEKEx3-*caiC* + pPLib2-*caiA-fixABCX* (*caiC*, *caiA-fixABCX*), and pEKEx3-*caiCD* + pPLib2-caiA-*fixABCX* (*caiCD*, *caiA-fixABCX*). The maximum normalized fluorescence signal observed during the cultivation process is shown. Error bars indicate the standard deviation of the mean of three replicates.

The uptake of exogenously applied TMA compounds such as crotonobetaine occurs *via* the importers ProP and ProU, while CaiT as an antiporter requires a cosubstrate. It was hypothesized that the cultivation conditions, particularly the nutrients or other osmolytes in the complex LB medium that are transported by ProP/ProU (*e.g.*, L-proline, ectoine, and glycine betaine), inhibit the uptake of TMA compounds into the cell,[11] and, thus, reduce the biosensor output as observed for other biosensors.[39] Therefore, the sensor strain *E. coli* BW25113 (Sensor1) was used to determine the dose−response behavior to crotonobetaine over a concentration range from 0.01 nM to 10 mM in the LB complex medium and in the glycerol minimal M9 medium (Figure 3A). Dose−response curves were fitted to these data using the Hill equation, and the utility factor was calculated by multiplying the relative input range by the output fold induction of the biosensor[36] (Figure 3B). In the glycerol M9 medium, *E. coli* BW25113 (Sensor1) showed 8.9-fold higher utility compared to the LB medium. Under these conditions, the biosensor was more sensitive and responded to lower effector concentrations as indicated by a shift in the half maximal effective concentration (EC_{50}) from 370 μM in the LB medium to 14 μM in the M9 medium. Therefore, the glycerol M9 minimal medium was used for all subsequent experiments.

On the basis of the hypothesis that betaine:CoA ligase CaiC may limit the sensitivity of the sensor system, *caiC* was added to the biosensor plasmid and expressed constitutively (Sensor1-*caiC*, Figure 2). As expected, this biosensor already showed fluorescence at lower effector concentrations, which improved its concentration range and shifted the EC_{50} to 0.7 ± 0.3 μM. In addition, the maximum fluorescence level was increased. At effector concentrations ≤0.01 μM, Sensor1-*caiC* showed higher than expected background fluorescence. The utility values of both Sensor1 and Sensor1-*caiC* were comparable (~1.9-fold), as the fold induction of Sensor1-*caiC* was reduced, but its concentration range was increased by a higher sensitivity for lower effector concentrations (Table S1).

Biosynthesis of Crotonobetainyl-CoA Is Required for CaiF Function. To test if biosynthesis of a CoA ester by betaine:CoA ligase CaiC is required for activation of the *fix* promoter in Sensor1, a loss-of-function experiment regarding CaiC was performed. Sensor1 was used to transform *E. coli* BW25113 (called wt) and the isogenic mutant BW25113 Δ*caiC*. These strains were cultivated in the presence of one of the TMA compounds, L-carnitine, crotonobetaine or γ-butyrobetaine, and the mVenus fluorescence was measured (Figure 4A). In the wt parental strain, carnitine and crotonobetaine acted as effectors, while the addition of neither carnitine nor crotonobetaine resulted in Sensor1 fluorescence in BW25113 Δ*caiC* (Figure 4A).

Furthermore, the biosensor function in the presence of a constitutive *caiC* expression, *i.e.*, using Sensor1-*caiC*, was assayed in various *cai* deletion strains (Figure 4B) and a strain with deletions of both operons (Δ*cai-fix*). As expected, the constitutive expression of *caiC* from Sensor1-*caiC* compensated for the deletion of *caiC* from the chromosome. In the parental wt strain, Sensor1-*caiC* sensed the known effectors carnitine and crotonobetaine and, surprisingly, also sensed γ-butyrobetaine, which was thought to be an inhibitor of carnitine metabolism.[24] The finding that the sensing of γ-butyrobetaine only occurred when betaine:CoA ligase was synthesized constitutively (as in Sensor1-*caiC*), but not when *caiC* was under native control (as when using Sensor1 in the wt) (Figure 4A), indicated that uninduced *caiC* levels are insufficient to induce native *cai fix* operons. Thus, γ-butyrobetainyl-CoA is not the natural inducer of the *cai fix* operons. To confirm this finding, *caiC* expression was analyzed by qRT-PCR for strains wt (Sensor1) and wt

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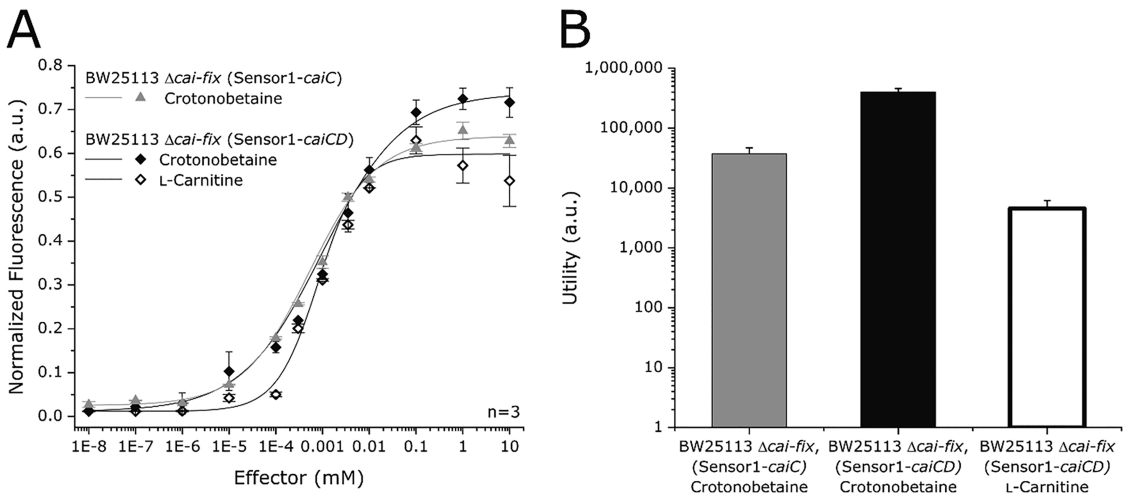

Figure 6. Characterization of Sensor1-*caiC* for crotonobetaine and Sensor1-*caiCD* as a dual-input biosensor for L-carnitine and crotonobetaine in a Δ*cai-fix* background. (A) Dose−response curves to exogenous crotonobetaine for Sensor1-*caiC* and to crotonobetaine and L-carnitine for Sensor1-*caiCD* in *E. coli* BW25113 Δ*cai-fix* in M9 medium. The fluorescence was determined at each indicated effector concentration and normalized to the cell density. The curves were fitted to the Hill equation. The maximum normalized fluorescence signal observed during the cultivation process is shown. Error bars indicate the standard deviation of the mean of three replicates. (B) Utility of the three different dose−response curves calculated from the Hill functions in Figure 6A.

(Sensor1-*caiC*), with *cysG* as the reference gene encoding siroheme synthase. The resulting Cq values for *caiC* for these strains were 31.7 ± 0.9 and 21.7 ± 0.3, respectively, and the Cq values for the reference gene *cysG* did not differ significantly in the two strains (29.7 ± 1.4 and 28.6 ± 0.4, respectively) (Figure S1). This confirmed that *caiC* expression is higher in the wt (Sensor1-*caiC*) strain compared to the wt (Sensor1) strain.

CoA transferase CaiB was not required for sensing by Sensor1-*caiC*, since the CoA esters can be synthesized from the three betaines (carnitine, γ-butyrobetaine, and crotonobetaine) (Figure 4B). In the absence of crotonobetainyl-CoA reductase CaiA, *i.e.*, when γ-butyrobetainyl-CoA cannot be converted to crotonobetainyl-CoA, Sensor1-*caiC* only sensed carnitine and crotonobetaine, but not γ-butyrobetaine (Figure 4B). This finding is commensurate with the view that carnitinyl-CoA and/or crotonobetainyl-CoA are natural inducers of the *cai fix* operons. In the absence of carnitinyl-CoA dehydratase (CaiD), Sensor1-*caiC* did not sense carnitine but did sense crotonobetaine and γ-butyrobetaine (Figure 4B). Finally, when only CaiC was present in addition to CaiF (as in the Δ*cai-fix* strain carrying Sensor1-*caiC*), only crotonobetaine was sensed (Figure 4B). Taken together, these loss-of-function experiments revealed that crotonobetainyl-CoA is required for CaiF activity in carnitine metabolism and, thus, crotonobetainyl-CoA is proposed as a coactivator of CaiF.

To obtain independent evidence of the formation of the CaiF coactivator, gain-of-function experiments were performed in the Δ*cai-fix* strain carrying Sensor1. For reconstruction, various carnitine metabolism genes were expressed from a second plasmid (Figure 5). As expected from the loss-of-function analysis, the expression of *caiC* in Δ*cai-fix* led to the detection of crotonobetaine only, whereas *caiCD* expression resulted in the detection of both carnitine and crotonobetaine (Figure 5). Upon the additional expression of *caiA* and *fixABCX*, γ-butyrobetaine was also detected by the biosensor system.

One of two interesting findings was that the addition of all three betaines resulted in biosensor output in the absence of CoA transferase CaiB (see strain Δ*caiB* in Figure 4B and strain Δ*cai-fix* carrying plasmids pEKEx3-*caiCD* and pPLib2-*caiA-fixABCX* in Figure 5). The carnitine CoA-transferase CaiB (EC: 2.8.3.21) catalyzes the reversible transfer of the CoA moiety between all of the TMA compounds of carnitine metabolism (carnitine, crotonobetaine, γ-BB). CaiB is not essential, as each TMA compound can also be activated with CoA by the ATP-dependent betaine:CoA ligase CaiC (EC: 6.2.1.48). It is not necessary to invoke other CaiB-like enzyme activities, although they may exist, *e.g.*, acetate is activated by acetyl-CoA synthetase (a CoA ligase similar to CaiC) and acyl-CoA transferases transfer the CoA moiety from acetyl-CoA to other acids such as propionate or succinate (a CoA transferase similar to CaiB). Thus, the finding that the addition of all three betaines resulted in biosensor output in the absence of CoA transferase CaiB can be explained by crotonobetainyl-CoA being generated from crotonobetaine *via* CaiC, from carnitine *via* CaiC and CaiD and from γ-butyrobetaine *via* CaiC and CaiA. The latter conversion of γ-butyrobetaine *via* CaiC to γ-butyrobetainyl-CoA and then to crotonobetainyl-CoA by CaiA is the second unexpected finding. This was not anticipated, since CaiB was thought to play a role in this interconversion based on enzymatic assays and cross-linking experiments with glutaraldehyde analyzed by SDS-PAGE and immunostaining.[20] Moreover, the reduction of crotonobetainyl-CoA to γ-butyrobetainyl-CoA was described as irreversible in *E. coli* and *Proteus* sp.[17,40] By contrast, the results shown here are consistent with a report on the oxidation of γ-butyrobetainyl-CoA to crotonobetainyl-CoA occurring in HK4, a carnitine-producing strain isolated from soil, which is phylogenetically related to *Agrobacterium* and *Rhizobium*.[41] However, the involvement of a different enzyme catalyzing the oxidation of γ-butyrobetainyl-CoA to crotonobetainyl-CoA cannot be excluded.

The gain-of-function experiments support the finding from the loss-of-function experiments that crotonobetainyl-CoA is required for CaiF activity in carnitine metabolism. The result is also plausible when considering the biological function of carnitine metabolism in *E. coli*. It is assumed that under anaerobic conditions, carnitine is converted into crotonobetaine

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via their CoA esters so that it can serve as an alternative terminal electron acceptor when common electron acceptors (*e.g.*, nitrates or fumarate) are not present.[4] The decisive step for this growth advantage is not the conversion of carnitinyl-CoA into crotonobetainyl-CoA, but the subsequent electron transfer to crotonobetainyl-CoA forming γ-butyrobetainyl-CoA. Therefore, it is reasonable that crotonobetainyl-CoA is detected by CaiF and, for example, carnitine or carnitinyl-CoA are not detected, as in this way the detection mechanism is closely linked to the decisive metabolic reaction. In addition, environmental crotonobetaine, if available, can also be utilized in the absence of carnitine, offering more options for survival in unfavorable environmental conditions.

Optimization and Characterization of a Dual-Input Biosensor for L-Carnitine and Crotonobetaine. To optimize the biosensor regarding its response to the dual input of L-carnitine and crotonobetaine, two strategies were followed. First, the response of Sensor1-*caiC* to lower effector concentrations (Figure 3) led to the hypothesis that these effects were based on interference of the *cai fix* gene products with the intracellular effector level in this low concentration range. Therefore, the dose−response behavior of Sensor1-*caiC* to crotonobetaine was observed in the absence of the *cai fix* gene products using strain *E. coli* BW25113 Δ*cai-fix* (Figure 6A). The response behavior for effector concentrations >0.01 μM and the EC_{50} of 0.5 ± 0.3 μM remained almost unchanged compared to *E. coli* BW25113 (Sensor1-*caiC*) (Figure 3A). By contrast, the background noise at very low effector concentrations was abolished, which reduced the minimum fluorescence level and increased the concentration range. Thus, the resulting utility of the biosensor Sensor1-*caiC* used in BW25113 Δ*cai-fix* (Figure 6B) was improved compared to its use in BW25113 (Figure 3B).

The second strategy aimed at developing a plasmid-based dual-input biosensor system for L-carnitine and crotonobetaine. On the basis of the findings that the biosynthesis of crotonobetainyl-CoA is required for CaiF function and that *caiCD* expression resulted in the detection of both carnitine and crotonobetaine when Sensor1 was used, Sensor1-*caiCD* was constructed. Sensor1-*caiCD* was designed to coexpress *caiF* with *caiC* and *caiD* by adding the gene for betaine:CoA ligase *caiC* and carnitinyl-CoA dehydratase *caiD* with their respective native RBS to Sensor1 (Figure 2). Thus, all of the necessary elements for the detection of L-carnitine and crotonobetaine were combined on a single plasmid. The CaiC activity constitutively induced by this plasmid was increased compared to Sensor1-*caiC*, since the stronger native RBS of *caiC* was used. The dose−response behavior of the biosensor was determined using strain *E. coli* BW25113 Δ*cai-fix* (Sensor1-*caiCD*) and the addition of the betaines to the glycerol minimal M9 medium in a concentration range from 0.01 nM to 10 mM. The dose−response curves were fitted to these data using the Hill equation (Figure 6A), and the utility of the biosensor system was calculated from these functions (Figure 6B). Compared to the data for crotonobetaine with Sensor1-*caiC* in this strain, Sensor1-*caiCD* showed an increased maximum fluorescence level and a similar EC_{50} (0.8 ± 0.5 μM), which improved the utility 10.8-fold. Notably, Sensor1-*caiCD* also functions at crotonobetaine concentrations between 10 μM and 10 mM. In this concentration range, the function of Sensor1-*caiC* was limited by its lower CaiC activity.

The initial sensor system, strain BW25113 (Sensor1) in M9 medium (Figure 3), also responded to crotonobetaine and carnitine (Figure 4A), but in contrast relies on the genomic *cai* genes. Compared to this strain, the fold induction for crotonobetaine was improved by ∼50% and the relative input range increased ∼570-fold, resulting in an 875-fold increase in utility.

The detection of L-carnitine by *E. coli* BW25113 Δ*cai-fix* (Sensor1-*caiCD*) (Figure 6) occurred with ∼90-fold reduced utility compared to detection by the same strain in the presence of crotonobetaine, since the relative input range was reduced ∼70-fold and a 25% lower fold induction was observed. The EC_{50} was not significantly changed (1.0 ± 0.3 μM).

As detection of L-carnitine requires activation with CoA by CaiC and conversion to crotonobetainyl-CoA by CaiD, the response behavior was changed. Nevertheless, although less efficient than with crotonobetaine, this *in vivo* biosensor system was able to report external L-carnitine concentrations in the range of 0.1−100 μM. This detection range is similar to that of a microfluidic chip-based L-carnitine biosensor using the carnitine acetyltransferase reaction, which was reported to be 0.2−100 μM,[42] and the utility was comparable to previously designed biosensor systems for other analytes.[36]

Screening for Homologous Variants of the Betaine:-CoA Ligase CaiC. As a first application, the biosensor developed here was used to screen for betaine:CoA ligases. Three different CaiC homologues were assayed from *Citrobacter freundii*, *Proteus mirabilis*, and *Arcobacter marinus* with 85.3%, 71.6%, and 53.8% amino acid sequence identity to *E. coli* CaiC, respectively (Figure S1). *C. freundii* and *P. mirabilis* were chosen because their ability to metabolize carnitine to γ-butyrobetaine had been demonstrated earlier.[12] For *Proteus* sp., the enzymatic functions of CaiA, CaiB, and CaiD were confirmed, and CaiC was proposed to have betaine:CoA ligase activity on the basis of sequence similarity.[40] *A. marinus*, on the other hand, was isolated from an aquatic marine environment in 2010.[43] Its genome[44] contains a cluster of ORFs with homology to the *cai* operon as well as a second cluster positioned ∼8 kb upstream with homology to the *fix* operon and a second copy of a CaiD homologue, indicating that *A. marinus* may catabolize carnitine, a property known from other *Arcobacter* species.[45] However, the enzymes selected for this screening procedure were not known to be active as betaine:CoA ligase.

The heterologous *caiC* genes were codon-harmonized (see Supporting Information) for *E. coli* and cloned into the pEKEx3 vector in the same way as *caiC* from *E. coli* was used in the experiments above (see Figure 5). While all of the CaiC homologues showed activity, the intensity of the biosensor output differed, and the extent of sequence identity to the *E. coli* variant correlated with the signal intensity of the biosensor output (Figure 7). To confirm that the CaiC homologues are active as crotonobetaine:CoA ligases, enzyme activity assays were performed with crude extracts of these strains and the strain expressing *E. coli* $CaiC^{Ec}$. Indeed, all of the CaiC variants showed crotonobetaine:CoA ligase activity (Figure 8). These results suggest that all three homologues indeed possess betaine:CoA ligase activity. Notably, this indicates that carnitine metabolism is conserved not only in the order *Enterobacterales* but also in Epsilonproteobacteria, to which *A. marinus* belongs. No indications of horizontal gene transfer have been found because the *cai fix* operons are neither part of genomic islands nor are they flanked by transposon-related sequences. Since carnitine concentrations in the habitat of this marine organism can be expected to be lower than those in the habitats of other microbes that colonize the intestines,[2,46] it is tempting to speculate that the $CaiC^{Am}$ variant may show enzymatic

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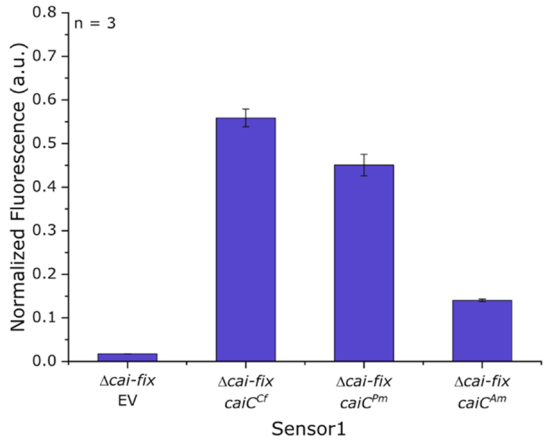

Figure 7. Screening for homologous variants of the betaine:CoA ligase CaiC. Fluorescence response to crotonobetaine at a concentration of 2 mM by *E. coli* BW25113 Δ*cai-fix* (Sensor1) strains transformed with pEKEx3 derivatives expressing homologues CaiC variants, derived from *C. freundii* ($caiC^{Cf}$), *P. mirabilis* ($caiC^{Pm}$), and *A. marinus* ($caiC^{Am}$). pEKEx3 was used as the empty vector control (EV). The maximum normalized fluorescence signal observed during the cultivation process is shown. Error bars indicate the standard deviation of the mean of three replicates. For comparison, the fluorescence response to crotonobetaine by a control strain with *caiC* from *E. coli* can be found in Figure 5.

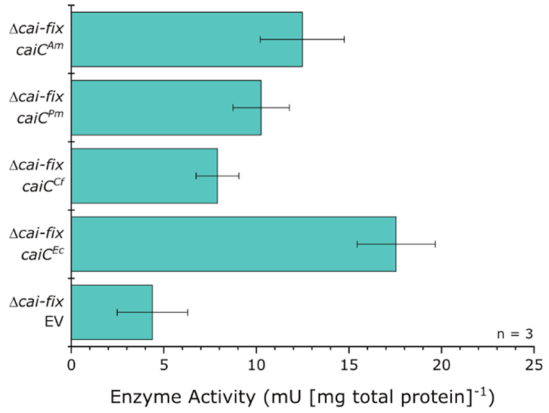

Figure 8. Crotonobetaine CoA ligase activities in crude extracts of *E. coli* BW25113 Δ*cai-fix* (Sensor1) strains transformed with pEKEx3 derivatives expressing homologues CaiC variants, derived from *E. coli* ($caiC^{Ec}$), *C. freundii* ($caiC^{Cf}$), *P. mirabilis* ($caiC^{Pm}$), and *A. marinus* ($caiC^{Am}$). pEKEx3 was used as the empty vector control (EV). Means of three replicates are shown. Error bars were calculated by error analysis from the standard deviations of the enzyme assays and Bradford measurements.

properties that are useful for further applications, such as a lower K_M value.

CONCLUSIONS

The expression analysis of a transcriptional fusion of the *fix* promoter, including its CaiF binding sites, with a fluorescent reporter gene, combined with genetic loss-of-function and gain-of-function experiments, revealed that the biosynthesis of crotonobetainyl-CoA was required for the function of the transcriptional activator CaiF. Therefore, crotonobetainyl-CoA is proposed to be the sought-after coactivator of CaiF. In addition, these experiments suggested that oxidation of γ-butyrobetainyl-CoA to crotonobetainyl-CoA is catalyzed by CaiA. On the basis of these findings, biosensor Sensor1-*caiC* was developed and shown to detect extracellularly added crotonobetaine in a concentration range of 0.01−10 μM and at ∼25-fold induction, corresponding to a utility factor of 37 000. *E. coli* BW25113 Δ*cai-fix* (Sensor1-*caiCD*) was developed a as dual-input biosensor for the detection of L-carnitine in addition to the improved detection of crotonobetaine with ∼50- and ∼60-fold induction and utility factors of ∼4500 and ∼400 000, respectively. As an application, the biosensing *E. coli* BW25113 Δ*cai-fix* (Sensor1) strains were assayed by transformation with a vector expressing homologous CaiC variants from *C. freundii*, *P. mirabilis*, and *A. marinus*. This allowed the CaiC function to be scored and to show that this enzyme is conserved not only in the order *Enterobacterales* but also in Epsilonproteobacteria.

MATERIALS AND METHODS

Bacterial Strains, Strain Construction, Media, and Growth Conditions. All of the bacterial strains and plasmids are listed in Table S2. KanR gene deletions strains were obtained from the Keio collection.[47] The *cai-fix* deletion strain was constructed using the λ Red recombinase method,[47] plasmid pKD4 and the corresponding primers dcaiFix_FRT-Km_fw and dcaiFix_FRT-Km_rv shown in Table S3. The removal of the kanamycin cassette from the *E. coli* BW25113 derivative strains was carried out with plasmid pCP20 as described earlier[48] and confirmed by PCR amplification using gene-specific flanking primers. *E. coli* BW25113 and its derivatives were used for the biosensor experiments. *E. coli* DH5α was used as a cloning host and for plasmid propagation. For cloning, cells were grown at 37 °C in lysogeny broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} sodium chloride). The media were supplemented with antibiotics where appropriate for selection: kanamycin (50 μg mL^{-1}), spectinomycin (100 μg mL^{-1}), and chloramphenicol (25 μg mL^{-1}).

Standard molecular genetic techniques were performed as described elsewhere.[49] The enzymes were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and used following the manufacturer's recommendations. Genes were amplified by PCR using the respective primers delivered from Metabion GmbH (Planegg/Steinkirchen, Germany) and ALLin HiFi DNA Polymerase according to the manufacturer (highQu GmbH, Kraichtal, Germany). Cloning of the amplified PCR products was performed using restriction enzyme digestion and Gibson assembly.[50] All of the oligonucleotides used in this study are listed in Table S3. When indicated, constructs were cloned using an artificial ribosomal binding site (RBS, predicted using the online RBS calculator from the Salis laboratory at Penn State University, https://salislab.net/software/). New vectors were confirmed by insert sequencing.

Construction of pGP2 and pGP2-Based Biosensor Vectors. As a precursor, the plasmid pGP2 was designed on the basis of the expression vector pVWEx1.[38] The pGP2 backbone was constructed from 3 fragments derived from pVWEx1, which were assembled using the Gibson method. The fragments were amplified using the primers pGP2_F1_fw, pGP2_F1_rv, pGP2_F2_fw, pGP2_F2_rv, pGP2_F3_fw, and pGP2_F3_rv. Fragment 1 consisted of pHM519 $oriV_{Cg}$, fragment 2 contained Kan^R and the *E. coli* p15A *ori*, and fragment 3 contained the multiple cloning site and the *rrnB* T1 and *rrnB* T2 terminators. Compared to pVWEx1, the $lacI^q$ and *tac* promoter were removed in the pGP2 plasmid.

The pGP2-Sensor1 plasmid was constructed in two steps. First, pGP2 was cut with *Bam*HI, and the *hom* promoter

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(amplified with the primers CS1_pHom_fw and CS1_pHom_rv from the genome of *C. glutamicum* ATCC 13032) and *caiF* (amplified with the primers CS1_caiF_fw and CS1_caiF_pGP2_rv from the genome of *E. coli* BW25113) were inserted into the restriction site *via* Gibson assembly. The resulting plasmid pGP2-*Phom-caiF* was subsequently opened at the restriction site of Bsp1407I, and the *mVenus NB* fragment (amplified with the primers CS1_mVenusNB_fw and CS1_mVenusNBsc_rv from the plasmid pEB1-*mVenus NB*[51]) and the *cai-fix* promoter region fragment (amplified with primers CS1_pCai-Fix_fw and CS1_pGP2_pCai-Fix_rv from the *E. coli* BW25113 genome) were inserted *via* Gibson assembly. The bidirectional terminator BBa_B1001[52] was introduced between *caiF* and *mVenus NB via* primers CS1_caiF_pGP2_rv and CS1_mVenusNB_fw. The forward primers for *caiF* and *mVenus NB* harbored optimized RBS for their respective genes. pEB1-*mVenus NB* was a gift from Philippe Cluzel (Addgene plasmid # 103986). To construct the pGP2-Sensor1-*caiC* plasmid, pGP2-Sensor1 was opened at the AvrII restriction site, and *caiC* (amplified with the primers CS2_caiC_RBS_fw and CS2_caiC_rv from the genome of *E. coli* BW25113) was inserted *via* Gibson assembly. Plasmid pGP2-Sensor1-*caiCD* was built according to the procedure for pGP2-CarSen1-*caiC*, and the inserted *caiCD* was in this case amplified with the primers CS2_caiC_fw and CS1_caiD_rv.

Construction of pPLib2 and pPLib2-Based Expression Vectors. Plasmid pPLib2 was constructed from four fragments which were assembled using the Gibson method. Fragment 1 consisted of CmR, the *E. coli* CloDF13 *ori* and *tonB* terminator, and it was amplified from the MP4 plasmid using the primers pPLib2_MP4_F1_fw and pPLib2_MP4_F1_rv. For Fragment 2, the primers pPLib2_Plac_F2_fw and pPLib2_Plac_F2_rv were used to amplify the *lac* promoter region from the *E. coli* BW25113 genome. Fragment 3 contained the superfolder green fluorescent protein (sfGFP)[53] and was amplified from the pSB1C3-BBa_I746909 plasmid with the primers pPLib2_sfGFP_F3_fw and pPLib2_sfGFP_F3_rv, which introduced an *Eco*RI restriction site followed by an optimized artificial RBS for sfGFP (forward primer) and a XhoI restriction site (reverse primer). Fragment 4 harbored the *rrnB* T1 terminator and the T7Te terminator, which were amplified by the primers pPLib2_rrnB_F4_fw and pPLib2_rrnB_F4_rv with the pSB1C3-BBa_I746909 plasmid as the template. pPLib2-sfGFP was created with the contribution of Svenja Vinke during planning and cloning.

To construct plasmid pPLib2-*caiA-fixABCX*, *caiA* was amplified from the *E. coli* BW25113 genome with primers caiA_pPLib2_fw and caiA_fixA_rv, and *fixABCX* was amplified from the *E. coli* BW25113 genome with primers fixA_caiA_fw and fixX_pPLib2_rv. Both fragments were introduced into pPLib2-sfGFP digested with *Eco*RI and XhoI *via* Gibson assembly, replacing sfGFP.

Construction of pEKEx3-Based Expression Vectors. To construct the expression plasmids, the pEKEx3 vector was digested with *Bam*HI. The PCR products were amplified using the following oligonucleotides: *caiC*: caiC_px3_fw + caiC_px3_rv; *caiCD*: caiC_px3_fw + caiD_px3_rv; $caiC^{Pm}$: caiC_Pm_px3_5k_fw + caiC_Pm_pX3_rv; $caiC^{Cf}$: caiC_Cf_px3_5k_fw + caiC_Cf_pX3_rv; $caiC^{Am}$: caiC_Am_px3_5k_fw + caiC_Am_pX3_rv. *caiC* and *caiCD* were amplified from the *E. coli* BW25113 genome. The genes $caiC^{Am}$, $caiC^{Cf}$, and $caiC^{Pm}$ (RefSeq identifiers: WP_099309900, WP_100271523, and WP_012368455, respectively) were codon-harmonized for *E. coli* using the Codon Adaptation Index profile fitting strategy,[54] and genes were synthesized with an attached artificial RBS at a translation initiation rate similar to that of *E. coli caiC* RBS by Eurofins Genomics Germany GmbH (Ebersberg, Germany).

Biosensor Experiments. M9 minimal medium (6.78 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.1% (v/v) trace element solution, pH 7.0) with 24 g L^{-1} glycerol as the carbon source was used for the biosensor experiments. The trace element solution contained (in mg L^{-1}) $ZnSO_4{\cdot}7H_2O$ (10), $MnSO_4{\cdot}7H_2O$ (100), $FeSO_4{\cdot}7H_2O$ (100), $CuSO_4$ (2), and $NiCl_4{\cdot}6H_2O$ (0.2) and had pH ∼ 1.0. The LB medium was used for biosensor experiments where indicated. The biosensor experiments were carried out at 37 °C in 48-well microtiter FlowerPlates with the BioLector cultivation system (m2p-laboratories GmbH, Baesweiler, Germany).[55,56] The formation of biomass was recorded as backscatter light intensity at a wavelength of 620 nm with signal gain factor of 50. The fluorescence of the yellow fluorescent protein mVenus NB was measured after excitation at a wavelength of 488 nm as fluorescence emission at 520 nm with signal gain factor of 70. The normalized fluorescence was calculated as fluorescence at 520 nm over the backscatter light intensity at 620 nm with Biolection software version 2.4.5.0 (m2p-laboratories GmbH, Baesweiler, Germany). The maximum normalized fluorescence signal observed during the entire cultivation was used. In all cases, the time of the maximum normalized fluorescence coincided with the end of the exponential growth phase of the culture. Precultures for the biosensor experiments were done at 37 °C in M9 medium supplemented with 1% (v/v) LB medium. When a biosensor experiment was done in LB medium, the precultures were also done in the LB medium. Cells were harvested (3200*g*, 7 min) and resuspended in the cultivation medium to an optical density at 600 nm (OD_{600}) of 2. This suspension was used to inoculate the FlowerPlate wells, with 50 μL of the suspension to a working volume of 1 mL and a starting OD_{600} of 0.1. Antibiotics were supplemented in the biolector experiments as stated above. Strains carrying three antibiotic resistances were cultivated at half the antibiotic concentration to reduce the total antibiotic burden. L-Carnitine, crotonobetaine and γ-butyrobetaine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

RNA Isolation and qRT-PCR. *E. coli* strains BW25113 (Sensor1) and BW25113 (Sensor1-*caiC*) were cultivated analogously to the biosensor experiments, with the use of 100 mL baffled shake flasks with 10 mL medium on a shaking incubator (shaking frequency: 180 min^{-1}, eccentricity: 2.5 cm). The medium was supplemented with 2 mM γ-butyrobetaine and the cultivation was carried out in biological triplicates to the exponential phase (OD_{600} ≈ 2.75). The cells were harvested from 1 mL samples by centrifugation (4 °C, 12 000*g*, 1 min). The cell pellets were immediately frozen and stored at −80 °C. RNA isolation and qRT-PCR were performed as previously described[57] with slight modifications.

For RNA isolation, the samples were homogenized in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 5 mg mL^{-1} of lysozyme for 30 min at 37 °C. The total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instruction. Subsequently, DNase digestion was performed using the RNase-free DNase Set and "RNeasy MinElute" kits (Qiagen, Hilden, Germany) to eliminate possible genomic and plasmid DNA

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contamination. A quality control with Taq polymerase (New England Biolabs, Ipswich, MA, USA) was performed to confirm RNA purity. The RNA concentration was measured using a spectrophotometer (NanoDrop, ND-1000). Fifty nanograms of each RNA sample were used in quantitative real-time PCR (qRT-PCR) with the SensiFAST SYBR No-ROX One-Step Kit (Bioline, London, UK) and the CFX96 cycler system (Bio-Rad, Hercules, USA), which were used according to the manufacturers' instructions. The temperature profile employed in all qRT-PCRs was 45 °C for 10 min (reverse transcription), 95 °C for 2 min, followed by 40 cycles of: 95 °C for 5 s, 52 °C for 15 s, and 72 °C for 10 s. A melt curve analysis was done with the measurements between 65 and 95 °C. The primers qCaiC_fw and qCaiC_rv were used for the target gene *caiC*, and the reference gene *cysG* was amplified with primers qCysG_fw and qCysG_rv (Table S3).

Assay of CaiC Betaine:CoA Ligase Activity in *E. coli* Crude Extracts. Cultivation was done in 500 mL baffled flasks containing 50 mL of the LB medium supplemented with antibiotics, as stated above, on a shaking incubator (shaking frequency: 180 min^{-1}, eccentricity: 2.5 cm) at 37 °C. The cells were harvested from 45 mL of the culture by centrifugation (4 °C, 4000*g*, 7 min) sampled during the exponential growth phase at an OD_{600} of 2.5−2.8. The cell pellets were immediately frozen and stored at −80 °C. The cell pellets were resuspended in 1.5 mL of the assay buffer (100 mM Tris-HCl, 1 mM Dithiothreitol (DTT), pH 7.8), lysed by sonication (Ultraschalldesintegrator Sonoplus GM 200, Sonotrode M72, Bandelin electronic GmbH & Co KG, Berlin, Germany) for 2.5 min (cycle 0.5, amplitude 55), and centrifuged for 60 min at 4 °C and 20 000*g* to remove the cell debris. The supernatant was desalted using Vivaspin 6, 10.000 MWCO PES centrifugal concentrators (Sartorius Lab Instruments GmbH & Co. KG), according to the manufacturer's instructions, and the assay buffer to achieve a >99.9% buffer exchange. The resulting crude extracts were used to measure the enzyme activities. The protein concentrations were determined with Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA), using bovine serum albumin as the standard.

The betaine:CoA ligase activity was assayed as described for other CoA ligases[58,59] with slight modifications. In short, the reaction was followed by measuring AMP formation in a coupled assay with myokinase, pyruvate kinase, and lactate dehydrogenase, and the oxidation of 2 mol of NADH per mol of crotonobetaine was monitored spectrophotometrically at 340 nm (ε_{NADH} = 6220 M^{-1} cm^{-1}, d = 1 cm, 37 °C). The 1 mL reaction mixture contained 100 mM Tris-HCl at pH 7.8, 1 mM DTT, 20 mM $MgCl_2$, 2 mM ATP, 0.4 mM coenzyme A, 0.24 mM NADH, 2 mM phosphoenolpyruvate, 5 mM crotonobetaine, 5 U mL^{-1} of myokinase, 1 U mL^{-1} of pyruvate kinase, 1.5 U mL^{-1} of lactate dehydrogenase, and 50 μL of cell extract. The reaction was initialized by the addition of crotonobetaine. Where necessary, the cell extract was diluted with the assay buffer to achieve a rate of absorption change in the range of 0.03−0.15 per min.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00234.

- qRT-PCR C*q* values for *caiC* expression from Sensor1-*caiC* (Figure S1); Sequence alignment of CaiC homologues from *C. freundii*, *P. mirabilis*, and *A. marinus* to *E. coli* CaiC (Figure S2); Codon-harmonized sequences of the *caiC* genes from *C. freundii*, *P. mirabilis*, and *A. marinus*; Overview of the biosensor parameters derived from the Hill functions (Table S1); Strains and plasmids used in this study (Table S2); Oligonucleotides used in this study (Table S3) (PDF)

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Author Contributions

P.K. designed the experiments, P.K. and D.F. conducted the experiments, P.K., D.F and V.F.W analyzed data, and P.K. and V.F.W. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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