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Application of an Acyl-CoA Ligase from *Streptomyces aizunensis* for Lactam Biosynthesis

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Supporting Information

**ABSTRACT:** ε-Caprolactam and δ-valerolactam are important commodity chemicals used in the manufacture of nylons, with millions of tons produced annually. Biological production of these highly valued chemicals has been limited due to a lack of enzymes that cyclize ω-amino fatty acid precursors to corresponding lactams under ambient conditions. In this study, we demonstrated production of these chemicals using ORF26, an acyl-CoA ligase involved in the biosynthesis of ECO-02301 in *Streptomyces aizunensis*. This enzyme has a broad substrate spectrum and can cyclize 4-aminobutyric acid into γ-butyrolactam, 5-aminovaleric acid into δ-valerolactam and 6-aminocaproic acid into ε-caprolactam. Recombinant *E. coli* expressing ORF26 produced valerolactam and caprolactam when 5-aminovaleric acid and 6-aminocaproic acid were added to the culture medium. Upon coexpressing ORF26 with a metabolic pathway that produced 5-aminovaleric acid from lysine, we were able to demonstrate production of δ-valerolactam from lysine.

Lactams are important compounds used in the manufacture of commercial polymers. ε-caprolactam (caprolactam) is used in the production of nylon-6 found in fabrics, coatings, and other plastic products. δ-valerolactam (valerolactam) has been proposed as a monomer for nylon 5 and nylon-6,5 synthesis, addition of which tunes the properties of the resulting polymers.1–3 Currently, both caprolactam and valerolactam are synthesized from petrochemical sources. For example, caprolactam production starts from cyclohexanone, which is first converted to its oxime. Treatment of this oxime with acid induces the Beckmann rearrangement to give caprolactam.4 Such production involves energy intensive processes and harsh acidic reaction conditions and produces large amount of waste salts. Unlike chemical dehydration, enzymatic or whole-cell-catalyzed reactions can be performed at lower temperatures and pressure. Although several ω-amino fatty acids have been biosynthesized,2,5,6 full biosynthetic pathways to produce lactams are largely unknown. This is due to a lack of enzymes capable of performing the last ring-closing step. To date, only one enzyme, *Candida antarctica* lipase B (CALB, commercially available as N435), was reported to conduct a reversible aminolysis reaction that can be utilized for valerolactam and caprolactam synthesis.8 However, the reported enzyme requires anhydrous conditions, high temperatures and long reaction times. In addition, the intermolecular aminolysis reaction results in multiple side products, including macrocyclic dimer and trimer lactams, which are hard to eliminate during product purification. As such, there is no suitable enzyme capable of synthesizing industrially important lactams under microbial fermentation conditions.

Previously, ORF26 from *Streptomyces aizunensis*, was hypothesized to be either a 4-aminobutyryl-CoA ligase or a 4-guanidinobutyryl-CoA ligase.9–12 Previous work demonstrated that expression of ORF26 in *in vivo* resulted in the formation of γ-butyrolactam from 4-aminobutyric acid (4-ABA).13 In this study, we overexpressed and purified recombinant ORF26 in *Escherichia coli*, enabling us to explore the catalytic properties of this enzyme. ORF26 has broad substrate specificity, acting on linear or branched acid substrates with positively charged or neutral functional groups on the ω carbon. Yet the enzyme is
selective against negatively charged groups on the ω carbon. ORF26’s ability to activate ω-amino fatty acids led to its application as a general lactam synthase, enabling biosynthesis of butyrolactam, valerolactam and caprolactam (Figure 1). Comparative study of ORF26’s reaction products for different ω-amino fatty acid precursors was performed to better understand this enzyme’s activity as a lactam synthase.

To apply ORF26 for renewable chemical production, ORF26 was overexpressed in E. coli, and both valerolactam and caprolactam were formed in vivo by feeding their respective precursors, 5-aminovaleric acid (5-AVA) and 6-aminocaproic acid (6-ACA). To achieve renewable production of valerolactam, we introduced a two-enzyme pathway into E. coli that converts lysine to 5-AVA. The pathway contains an L-lysine monooxygenase and a 5-aminovaleramide amidohydrolase (davB and davA, respectively, from Pseudomonas putida KT2440).1,14 Introducing the genes encoding the 5-AVA biosynthetic pathway and a gene encoding a fusion of ORF26 with maltose binding protein, MBP-ORF26, enabled the production of valerolactam in E. coli from lysine or directly from glucose.

■ RESULTS

ORF26 Enzymology. Previous efforts to express the gene encoding ORF26 in vivo resulted in the formation of ω-butyrolactam from 4-ABA.13 In order to further explore the catalytic properties of ORF26, we overexpressed and purified recombinant ORF26 in E. coli (Table 1, Figure S1) and tested it against a range of acid substrates (Table 2) using an ATP-PPi release assay. The ATP-PPi release assay allowed us to measure ORF26’s ability to activate various acid substrates with ATP. Following the ATP-PPi release assay, the samples were analyzed via LC–MS for product formation. Various apparent kinetic parameters were estimated for a total of 13 different substrates while assessing acyl-CoA or lactam formation (Table 2, Figure S2). The substrates included (1) short hydroxy- or amino-butyric acids (3-hydroxybutyric, 3-aminobutyric and 2-aminobutyric acid), (2) medium-chain acids (valeric and 4-methyl-hexanoic acid), (3) the ω-amino fatty acids (4-ABA, 5-AVA and 6-ACA), (4) ω-guanidino fatty acids (4-guanidinobutyric and 6-guanidinohexanoic acid), and (5) dicarboxylic acids (glutaric, glutamic and adipic acid). In lactam formation, ORF26 demonstrated broad cyclase activity toward all three ω-amino fatty acids substrates. 4-ABA, 5-AVA and 6-ACA were cyclized into butyrolactam, valerolactam and caprolactam, respectively. As an acyl-CoA ligase, ORF26 demonstrated ligase activity toward the previously hypothesized 4-guanidinobutyric acid, the longer 6-guanidinohexanoic acid and medium chain valeric and 4-methyl-hexanoic acids. ORF26 had optimal activity at pH 8.0 (Figure S3), and therefore all in vitro reactions were conducted at pH 8.0. The enzyme precipitated and became inactive when the pH dropped below 6.0 (Figure S3).

Since ORF26 was hypothesized to be an acyl-CoA ligase,9–12 we anticipated that the ORF26-catalyzed reactions would require ATP or ADP hydrolysis to form the acyl-CoA from the acid and free CoA. As such, we monitored the in vitro reactions for both nucleotide products, in addition to the desired lactams. LC–MS analysis of nucleotides (ATP, ADP, AMP) showed that ORF26 catalyzed a series of reactions (Figure S5). In the absence of ORF26, ATP remained stable throughout the reaction (Figure S6A). In the presence of ORF26 and absence of ω-amino fatty acid substrates, ATP was hydrolyzed to ADP and Pi and small amounts of AMP and PPi (Figure S6B). When ω-amino fatty acids were added into the reaction mixture, the enzyme hydrolyzed ATP to predominantly AMP and Pi at a significantly higher rate with less formation of ADP and Pi. These results suggest that the observed signal increase in the ATP-PPi assay in the presence of ω-amino fatty acid substrate is due to ATP hydrolysis mainly from acid activation and a small fraction from nonspecific ATP hydrolysis by ORF26.

For lactam production, ORF26 demonstrated a higher substrate affinity for 5-AVA ($K_a = 0.08 \text{ mM}$), followed by 6-ACA ($K_a = 0.16 \text{ mM}$) and 4-aminobutyric acid ($K_a = 4.96 \text{ mM}$), using a constant concentration of ATP and CoA. Moreover, ORF26 demonstrated higher catalytic activity on 5-AVA ($k_{cat}/K_m = 9.19 \text{ min}^{-1} \text{mM}^{-1}$), followed by 6-ACA ($k_{cat}/K_m = 35.85 \text{ min}^{-1} \text{mM}^{-1}$) and the lowest on 4-aminobutyric acid ($k_{cat}/K_m = 1.83 \text{ min}^{-1} \text{mM}^{-1}$). For acyl-CoA production, ORF26 demonstrated catalytic activity for both the 6-guanidinohexanoic acid ($k_{cat}/K_m = 77.83 \text{ min}^{-1} \text{mM}^{-1}$) and 4-guanidinobutyric acid ($k_{cat}/K_m = 18.25 \text{ min}^{-1} \text{mM}^{-1}$) (Table 2, Figure S2, Figure S4). In addition to acting on guanidino-containing substrates, ORF26 also demonstrated activity toward valeric acid ($k_{cat}/K_m = 3.74 \text{ min}^{-1} \text{mM}^{-1}$) and 4-

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**Table 1. E. coli Strains, Plasmids Used**

<table>
<thead>
<tr>
<th>engineered strains</th>
<th>plasmids</th>
<th>host</th>
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<tbody>
<tr>
<td>JZ-171</td>
<td>pBbE2C-ORF26</td>
<td>JW2637–4</td>
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<tr>
<td>JZ-172</td>
<td>pBbE2C-RFP</td>
<td>JW2637–4</td>
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<tr>
<td>JZ-440</td>
<td>pBbA7a-DavB-DavA</td>
<td>BL21(DE3) star</td>
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<td>JZ-441</td>
<td>pBbA7a-DavB-DavA + pET28a-MBP-ORF26</td>
<td>BL21(DE3) star</td>
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Table 2. Summary of ORF26 Kinetic Parameters

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (mM)</th>
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</thead>
<tbody>
<tr>
<td>5-AVA</td>
<td>35.85 (mM)</td>
</tr>
<tr>
<td>6-ACA</td>
<td>3.74 (mM)</td>
</tr>
<tr>
<td>4-ABA</td>
<td>1.83 (mM)</td>
</tr>
</tbody>
</table>

...
methyl-hexanoic acid ($k_{cat}/K_m = 11.68 \text{ min}^{-1} \text{ mM}^{-1}$). No activity was detected for the remaining substrates.

Although ORF26 was predicted to be an acyl-CoA ligase, CoA was not required for lactam formation (Figure S6C, E, and G). The minimal lactam formation system consisted of ORF26, $\omega$-amino fatty acids, ATP and Mg$^{2+}$ (Figure S7). Valerolactam formation was most rapid, followed by caprolactam and butyrolactam in the absence of CoA. In addition to using ATP, ORF26 could also utilize ADP to activate $\omega$-amino fatty acids and catalyze lactam formation. The ADP reaction occurred at $\sim 20$–$50\%$ of the rate compared to the ATP reaction (Figure S6I–K).

**Valerolactam and Caprolactam Production In Vivo.** To demonstrate valerolactam or caprolactam production using whole-cell catalysts, we exogenously added the respective precursors, 2 g/L (20 mM) 5-AVA or 2 g/L (17.7 mM) 6-ACA, to E. coli BL21 (DE3) star expressing pET28-ORF26 (Figure 2A,B). Both valerolactam and caprolactam were observed in the medium upon ORF26 expression (Figure 2A,B). An empty pET28 vector was utilized as a control. After 72 h, exogenous feeding of 5-AVA and 6-ACA to the pET28-ORF26 strain produced a total of 152.7 mg/L (1.5 mM) of valerolactam and 2.02 mg/L (17.9 $\mu$M) of caprolactam, respectively. The empty pET28 vector control displayed valerolactam production of 48.6 mg/L (0.5 mM) and no detectable levels of caprolactam. Valerolactam production in the empty pET28 strain suggests either a spontaneous ring closure of the linear molecule, or enzyme catalysis from endogenous host acyl-CoA enzymes. The native E. coli CaiC, a known crotonobetaine CoA ligase, was previously shown to accept 4-aminobutyric acid as substrate and catalyze the formation of butyrolactam, and its activity for 5-AVA and 6-ACA was also determined and compared to ORF26 (Figure 2A,B). After 72 h, the E. coli strain expressing pET28-CaiC produced 188.8 mg/L (1.9 mM) of valerolactam. No detectable level of caprolactam was observed. These results suggest a broader substrate promiscuity of ORF26 as a lactam synthase in the presence of 2 g/L lactam precursors 5-AVA and 6-ACA.

On the basis of these results, and with the aim of further investigating ORF26 in achieving renewable production of

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Product $^b$</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
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<tr>
<td>(S)-3-hydroxybutyric acid</td>
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<td>8.03 ± 0.17</td>
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<td>7.65 ± 0.17</td>
<td>11.68</td>
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<tr>
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<td>4.96 ± 0.56</td>
<td>9.09 ± 0.35</td>
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<td>5.88 ± 0.17</td>
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<td>0.17 ± 0.03</td>
<td>3.12 ± 0.99</td>
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<tr>
<td>6-guanidinohexanoic acid</td>
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<td>6.46 ± 0.18</td>
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</tbody>
</table>

$^a$The kinetic parameters of ORF26 were determined in a 100 mM HEPES, pH 7.5 at 25 °C using the ATP-PPi release assay using 5 $\mu$M ORF26, 1 mM CoA and 1 mM ATP. $^b$Other nonproduct forming pathways could occur such as ATP hydrolysis or acyl-OAMP intermediate hydrolysis. $^c$Further kinetic analysis can be found in Figure S2.
valerolactam, we introduced a two-enzyme pathway, which converts lysine to 5-AVA into *E. coli* BL21 (DE3) star, resulting in strain JZ-441 (Table 1).1−3 Introducing the genes encoding the 5-AVA biosynthetic pathway and a gene encoding a fusion of ORF26 with maltose binding protein, MBP-ORF26, enabled the production of valerolactam in *E. coli* from lysine (Figure 3A). JZ-440, which contains both *davA* and *davB* but lacks MBP-ORF26, was used as a negative control. Cultures of JZ-441 and JZ-440 were supplemented with 0, 1, 5, and 10 g/L of lysine. Samples were collected at 24, 48 and 72-h time points and analyzed via LC−MS. A linear trend was observed in valerolactam production with increasing supplemented lysine (Figure 3B). Cells growing in medium supplemented with 10 g/L of lysine produced a maximum of 705 mg/L of valerolactam after 72 h. At 0 g/L lysine feeding, JZ-441 produced 146 mg/L of valerolactam after 72 h (Figure 3B). JZ-440, which lacks MBP-ORF26, produced measurable levels of valerolactam, producing a maximum of 67 mg/L after 72 h when supplemented with 10 g/L of lysine. This is most likely due to the catalysis from endogenous acyl-CoA ligase host enzymes.13

The three-enzyme JZ-441 strain utilizes lysine, which is converted to 5-AVA by the *DavB-DavA* enzymatic pathway and subsequently converted into valerolactam by MBP-ORF26. In order to identify the bottleneck of this three-enzyme pathway, we measured lysine consumption and 5-AVA production in both JZ-440 and JZ-441 strains when cultured with 0 g/L, 1 g/L (6.84 mM), 5 g/L (34.2 mM) and 10 g/L (68.4 mM) lysine (Figure S9). After 24 h, both JZ-440 and JZ-441 consumed more than half of the endogenously added lysine (Figure S9A,B). By 72 h, the majority of the supplemented lysine was converted into 5-AVA (Figure S9C,D), suggesting high stability and catalytic efficiency of both *DavB* and *DavA* in *E. coli*. Overall, these results suggest cyclization of 5-AVA to valerolactam by ORF26 is the rate-limiting step in this three-enzyme pathway.

**DISCUSSION**

Natural product biosynthesis continues to be a rich source of enzyme candidates with novel biochemical activities. ORF26, an enzyme in the *Streptomyces aizunensis* ECO-02301 biosynthetic cluster, was identified to be a 4-guanidinobutyryl-CoA ligase, which has interesting implications for the ECO-02301 loading mechanism.12,15,16 ORF26 activated a wide range of medium chain fatty acid substrates and their functionalized analogs. Both linear and branched fatty acids were accepted as substrates. However, the enzyme seems to have little tolerance of polar groups at C3 position. ORF26 displays substrate promiscuity toward positively charged substrates, such as the ω-amino fatty acids or the ω-guanidino fatty acids, despite the fact that some substrates such as 6-guanidinohexanoic acid have two more carbons in their backbone than the putative native substrate. However, enzyme activity on substrates that have a negatively charged group on the ω-terminal end, such as a carboxylic acid group, was not observed, even though glutaric acid and adipic acid have similar steric hindrance as 6-guanidinohexanoic acid. This suggests that ORF26 has strict substrate charge specificity and preferentially favors substrates with a positively charged group on the ω-terminal end.
The ability of ORF26 to accept various ω-amino fatty acids was explored for lactam biosynthesis. For lactam formation, ORF26 does not require CoA as substrate. The activation of ω-amino fatty acids by ATP facilitates cyclization. This enables five-membered, six-membered and even seven-membered ring formation at mild temperatures, resulting in the production of important industrial lactams such as valerolactam and caprolactam via fermentation. Unlike the reversible aminolysis enzyme CALB that was previously described, ORF26 performs the reaction under ambient conditions. The lactam product observed is exclusively cyclized monomer, without dimer or trimer contaminants. These novel features make ORF26 an ideal candidate for lactam biosynthesis. However, the enzyme catalyzed significant ATP and ADP hydrolysis during the reaction. Directed evolution of ORF26 toward less futile ATP and ADP hydrolysis could potentially improve ORF26 as a lactam synthase.

By introducing davB and davA into E. coli, we demonstrated valerolactam biosynthesis from lysine. Valerolactam production in autoinduction medium was lysine dependent. At concentrations of lysine below 1 g/L, valerolactam production correlated with the lysine concentration from the ZYM-5052 medium. High concentrations of lysine (e.g., 10 g/L) resulted in ~5 fold increase in valerolactam production compared to nonsupplemented cultures. Even without lysine feeding, direct lysine production from endogenous lysine and glucose was sufficient to produce ~50% of the valerolactam produced when 5 g/L lysine was fed to the culture. Interestingly, in the absence of ORF26 a small amount of valerolactam was observed (Figure 3B, Figure S9). The observed valerolactam could result from catalysis by innate E. coli enzymes, most likely CaiC. Caprolactam was also biosynthesized by feeding its precursor 6-aminocaproic acid. With an already established 6-aminocaproic acid biosynthetic pathway in E. coli, ORF26 holds promise to enable full caprolactam biosynthesis in a microbial host.6,7

**CONCLUSION**

ORF26 is an enzyme in the ECO-02301 biosynthetic cluster previously identified to catalyze 4-guanidinobutyryl-CoA formation and was able to activate a wide range of substrates. While acting on ω-amino fatty acids, ORF26 can catalyze the ring closing reaction to produce butyrolactam, valerolactam and caprolactam. Biosynthesis of polymer precursors such as caprolactam, valerolactam and butyrolactam were demonstrated from their respective ω-amino fatty acids. E. coli harboring a
pathway to convert lysine to 5-AVA and ORF26 were able to produce valerolactam from lysine or glucose.

**MATERIALS AND METHODS**

**Strains and Plasmids.** All the strains and plasmids utilized in this study are listed in Table 1. The link to the plasmids sequence information is listed in Supporting Information (Table S1).

**ORF26 Protein Expression and Purification.** The gene encoding ORF26 (GenBank: AAX98201.1) was purchased from DNA 2.0 (Menlo Park, CA). The synthetic gene was optimized with *E. coli* codon usage and delivered in pDNA2.0-ORF26. For expression and purification of ORF26, the pDNA2.0-ORF26 plasmid was digested with Ndel and Xhol and cloned into pET28b in order to produce ORF26 with an N-terminal 6xHis tag. The resulting plasmid, pET28b-ORF26, was transformed into *E. coli* BL21 Star (DE3) for ORF26 overexpression. pET28-CaiC was cloned using the same Ndel and Xhol restriction site cloning methodology as pET28-ORF26.

For the N-terminal 6xHis ORF26 expression, the overnight culture was inoculated (1:100 v/v) into 1 L of Luria–Bertani (LB) media containing 50 μg/mL kanamycin. The culture was grown at 37 °C until the OD600 nm (optical density measured at a wavelength of 600 nm) reached 0.6 and cooled on ice for 20 min. 0.5 mM IPTG was added to induce pET28-ORF26 overexpression for 16 h at 18 °C. The cells were harvested by centrifugation (8000g, 10 min, 4 °C), resuspended in 40 mL of lysis buffer (50 mM HEPES, pH 8.0, 0.3 M NaCl, 10% glycerol (v/v) and 10 mM imidazole), and lysed by sonication on ice. Cellular debris was removed by centrifugation (20000g, 60 min, 4 °C). The N-terminal 6xHis ORF26 protein was purified by ThermoFisher HisPur cobalt resin, dialyzed against 50 mM HEPES, pH 8.0, 1 mM DTT and 8% glycerol (v/v). The dialyzed proteins were concentrated to 18 mg/mL using 10 kDa MWCO Amicon Ultra filters (Millipore). Concentrated 6xHis ORF26 protein was aliquoted, flash frozen in liquid nitrogen and stored at −80 °C. Both DNA and protein concentrations were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific). The purified ORF26 was checked on SDS-PAGE gel for purity (Figure S1).

**ATP-PPi Release Assays for ORF26.** The substrate range of ORF26 was determined by ATP-PPi release assays as previously described. Without prior knowledge about the reaction pathways, the ATP-PPi assay served a semiquantitative method to compare substrate-induced acceleration of ATP consumption. For kinetic investigation of ORF26 activity with different substrates, the inorganic pyrophosphate released by enzymatic reaction was measured continuously using the EnzChek Pyrophosphate Assay Kit (Invitrogen). A typical assay contained in a total volume of 150 μL: 5 μM of ORF26, 0–60 mM substrates, 1 mM ATP, 1 mM CoA and 1 mM MgCl2 in 100 mM HEPES, pH 7.5. 2-aminocapro-7-methylpurine ribonucleoside (MESG), p-nitrophenylphosphate and inorganic pyrophosphatase were added according to the protocol. Reactions were initiated by the addition of ATP and monitored at 360 nm with SpectraMax M2 ( Molecular Devices, Sunnyvale, CA). Initial velocities were calculated using the standard curve for inorganic pyrophosphate. For each substrate, control reactions were carried out without enzyme or without ATP. The rates of PPi release were converted to observed rates, and Michaelis–Menten kinetic parameters were estimated using GraphPad prism software (GraphPad Prism version 7.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com) (Figure S2). The activities assayed are listed in Table 2 (Sigma-Aldrich). During preparation of substrate stock solution, 6-guanidinohexanoic acid displayed low solubility under neutral pH, and HCl was added to obtain 100 mM stock solution in pH ~ 2.0.

**4-Guanidinobutyl CoA Product Identification.** To confirm ORF26’s native activity as a 4-guanidinobutyl CoA ligase, the quenched reaction with 4-guanidinobutyric acid was analyzed for CoA products as described previously. The mass measurements were carried out in the TOF-Scan monitoring mode for the detection of [M − H]− ions (4-guanidinobutyl-CoA, m/z = 893.1825). Negative controls were carried out using no enzyme, no ATP, no substrate or no CoA.

**Product Analysis of In Vitro ORF26 Lactam Formation.** To compare formation of various lactams by ORF26, a reaction mixture containing 57 μM of ORF26, 5 mM ω-amino fatty acids substrates, 1 mM ATP or ADP, 1 mM CoA and 1 mM MgCl2 in 100 mM HEPES (pH = 8) were incubated at 25 °C. The reactions were quenched by addition of methanol to a final concentration of 50% (v/v) at multiple time points (0 min, 15 min, 1 h, 2 h, 4 and 19 h). The resulting quenched reactions were kept at 4 °C and filtered through a 3 kDa MWCO Amicon Ultra-0.5 ml. Centrifugal Filters (Millipore) at 8000g for 30 min. The filtered solutions were analyzed for lactams and nucleotides using HPLC–MS (see Supporting Information).

**In Vivo Lactam Production.** *E. coli* JW2637Δrflc contains a knockout of gabT, which encodes a GABA transaminase. This host was initially used to confirm production of valerolactam and caprolactam in vivo. *E. coli* JZ-171 (ORF26) and JZ-172 (RFP negative control) were inoculated overnight in LB medium, which was prepared from dehydrated powder according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). The overnight culture was diluted to OD600 nm ~ 0.01 in LB medium and induced with 50 ng/mL anhydrotetracycline which OD600 nm reached ~0.6. 5-AVA and 6-ACA were added exogenously into the medium to reach a final concentration of 1 mM. To analyze 24 h lactam production, 600 μL of culture were taken for each sample and cooled in 4 °C room. The samples were spun at 13 000 rpm for 10 min, and 500 μL of supernatant was taken and filter spin at 13 000 rpm for 10 min (0.5 mL 3000 MW cutoff filter, EMD Millipore). 100 μL filtered supernatant was taken and mixed 1:1 volume with MeOH.

For the in vivo lactam production comparison between ORF26 and CaiC, *E. coli* BL21 (DE3) star cells were used as a host containing either pET28-ORF26 or pET28-CaiC. Cultures were grown in 10 mL of autoinduction ZYM-5052 medium according to the published protocol. S-AVA and 6-ACA were added exogenously into the medium to reach a final concentration of 2 g/L. Samples were cultured at 200 rpm and 25 °C. Samples taken at 24, 48 and 72 h were prepared and analyzed as previously described.
In Vivo Valerolactam Biosynthesis. The davA and davB genes were ordered as gBlocks (Integrated DNA Technologies, Coralville, IA) and cloned into the BglII and XhoI sites on pBBaA7a-DavB-DavA. To circumvent ORF26's limited solubility during incubation, MBP-ORF26 was utilized. For high-density shake flask cultures, Studier's autoinduction ZYM-5052 medium was prepared according to the published protocol. Lysine at various concentrations (0, 1, 5, and 10 g/L) were included in the ZYM-5052 medium. Kanamycin (20 μg/mL) and ampicillin (100 μg/mL) were added where desired to provide selective pressure for plasmid maintenance.

E. coli strains (JZ-441) harboring plasmids containing davA, davB and MBP-ORF26 were inoculated into 10 mL of LB overnight. On day 2, the overnight culture was inoculated 1:100 (v/v) into 25 mL Studier's autoinduction ZYM-5052 medium with various concentrations of lysine (0 g/L to 10 g/L) and appropriate antibiotics. The culture was incubated at 37 °C. When the OD600 nm reached around 0.6, the culture was cooled to 25 °C. The culture was then placed at 25 °C incubator and the valerolactam titer was analyzed at 24, 48 and 72 h.

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**REFERENCES**


