

YeeO from *Escherichia coli* exports flavins

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Abbreviations: FMN, Flavin mononucleotide; FAD, Flavin adenine dinucleotide; MATE, multidrug and toxic efflux; LB, Lysogeny Broth; M9-glc, M9 minimal media supplemented with 0.4% glucose; M9-glc-caa, M9 minimal media supplemented with 0.4% glucose and 0.4% casamino acids; IPTG, Isopropyl β -D-1-thiogalactopyranoside; PVDF, Polyvinylidene fluoride

Multidrug and toxic compound extrusion (MATE) proteins help maintain cellular homeostasis by secreting metabolic wastes. Flavins may occur as cellular waste products, with their production and secretion providing potential benefit for industrial applications related to biofuel cells. Here we find that MATE protein YeeO from *Escherichia coli* exports both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Significant amounts of flavins were trapped intracellularly when YeeO was produced indicating transport limits secretion of flavins. Wild-type *E. coli* secreted 3 flavins (riboflavin, FMN, and FAD), so *E. coli* likely produces additional flavin transporters.

Introduction

Riboflavin has many uses as a nutritional supplement, a pharmaceutical product, a food dye, and, in a less pure form, as an additive in animal feed.¹ Production of riboflavin in industry has seen a dramatic shift in dependence from expensive chemical production processes to more sustainable biological production methods particularly using 3 different microbes: *Ashbya gossypii*, *Candida famata*, and engineered strains of *Bacillus subtilis*.¹ Riboflavin, however, is less water-soluble than its phosphorylated derivatives that are used as co-factors, such as flavin mononucleotide (FMN)² and flavin adenine dinucleotide (FAD) (Fig. 1A-C); riboflavin is thus not as well-suited to use as a supplement or food dye. These phosphorylated flavins have other purposes as well, with FAD used to treat Vitamin B₂ deficiency.³ High concentrations of FMN may also increase the solubility of riboflavin and decrease susceptibility of certain riboflavin derivatives (i.e., FMN) toward photodegradation.² These phosphorylated flavins are currently synthesized chemically in industry by means of inefficient methods requiring expensive purification steps, including what is needed for separation from unreacted riboflavin.^{3,4} Therefore, it is beneficial to employ metabolic engineering for the production and secretion of specifically FAD and FMN. Certain flavins also play a role in the generation of electricity in microbial fuel cells by acting as electron shuttles,^{5–8} and can potentially be used as additives to increase the electrical current output in microbial fuel cells.

Fermentation processes for the production of riboflavin typically rely on native riboflavin-overproducing organisms. However, metabolic engineering has been employed in a few instances to produce flavins. For *B. subtilis*, riboflavin synthesis genes responsible for the formation of riboflavin from GTP and

ribulose 5-phosphate are encoded on one operon, *ribGBAHTD*.⁹ Increasing copy number and switching promoters of *ribGBAH*, as well as mutating *ribC* (encoding a regulator of the *rib* operon) on the chromosome leads to higher riboflavin production,¹⁰ and expression of *ribA* in particular is the bottleneck in riboflavin synthesis.¹¹ The yeast *Candida famata* was genetically modified by overexpressing the *FMN1* gene (encoding a riboflavin kinase for converting riboflavin to FMN), resulting in a 400-fold increase in FMN production, but still high concentrations of riboflavin were also secreted (with FMN representing less than 70% of flavins secreted).¹² Production of a passive riboflavin transporter, RibM from *Streptomyces davawensis*, in the riboflavin overproducer *B. subtilis* BSHP results in a smaller increase in riboflavin production.¹³

Little work has been performed on the metabolic engineering of flavin production/secretion in the well-known host *E. coli*, besides expressing a genetic fragment from the *B. subtilis* chromosomal DNA (including at least part of the *rib* operon) in *E. coli* to yield a 3.6-fold increase in riboflavin production.¹⁴ Overexpression of flavoproteins in *E. coli* can trap intracellular free FMN as cofactors to result in a more easily purified fraction of FMN, but still at low yields (at most 11% FMN could be recovered according to the maximum amount that can bind to the amount of flavoprotein produced).⁴ Having cells produce and secrete only specific flavins could help in the purification processes in a similar manner, as these flavins could then be collected from culture supernatants. For instance, production of the flavin transporter Bfe from *Shewanella oneidensis* MR-1 increases secretion of FAD, FMN, and riboflavin when produced not only in its natural host, but also in an *E. coli* Δ *ushA* strain (by roughly 21-fold, 3.3-fold, and 2.1-fold, for FAD,

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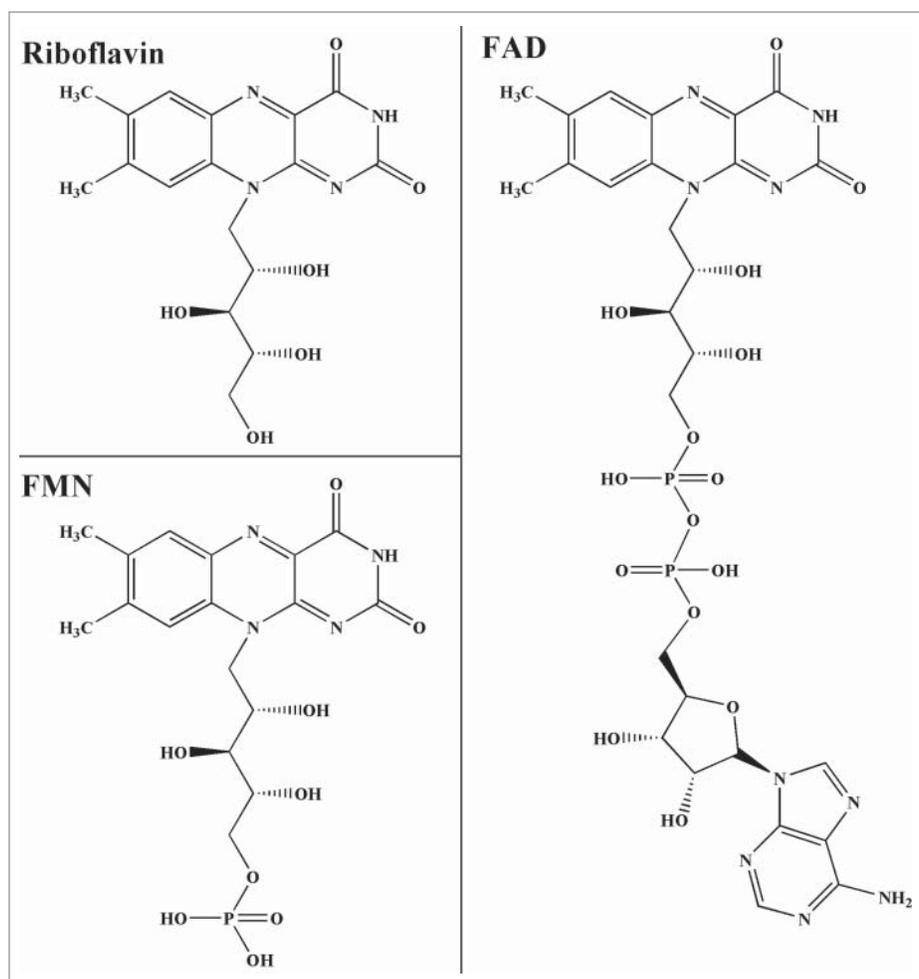


Figure 1. Chemical structures of the 3 flavins in study. The chemical structures of riboflavin, FMN, and FAD are shown.

FMN, and riboflavin in the *E. coli* mutant, respectively).¹⁵ In any case, metabolic engineering goals for riboflavin production should include increases in activity of both riboflavin synthesis and in export.

The metabolic pathways for riboflavin synthesis (and synthesis of its FMN and FAD derivatives) in both prokaryotes and eukaryotes are intricately mapped, but very little is known about flavin secretion.¹⁶ Only one protein, Bfe from *S. oneidensis* MR-1, has been shown to actively secrete flavin (particularly FAD),¹⁵ while RibM from *Streptomyces davawensis* can passively secrete riboflavin.¹³ The process of flavin secretion can play a variety of roles. For *Shewanella* species, flavins play an important role in anoxic cellular respiration by acting as electron-carrying shuttles to reduce insoluble electron acceptors. Such electron acceptors include Fe(III),⁵ potentially Mn(IV), and even certain electrodes to allow for the organism to conduct electrical current in microbial fuel cells.¹⁵ In other organisms such as *Helicobacter pylori*, flavin secretion is involved in chelation of metals, including Fe(III).¹⁷ The recently characterized Bfe is categorized into the multidrug and toxic compound extrusion (MATE) family of transporters.¹⁵ This family of proteins plays roles in maintaining cellular homeostasis by

secreting both xenobiotics and metabolic waste products,¹⁸ so it could be possible that some flavin secretion is simply a result of natural over-accumulation to the point at which they become waste products.

Another MATE transporter, YeeO, secretes dipeptides,¹⁹ and a phenotypic screen revealed that YeeO may play a role in reducing its susceptibility of the host to some antibiotics.²⁰ Here, we show that YeeO is a transporter of flavins FMN and FAD and that it has potential for use in metabolic engineering for the production/secretion of these flavins, as production of YeeO alone causes an extraordinary shift in the flavin secretion profile toward FMN and FAD.

Results

E. coli strains C43(DE3) and BW25113 naturally secrete flavins

Before determining the role of YeeO on flavin secretion, we first determined the amount of flavins secreted by 2 *E. coli* strains using HPLC. Flavin secretions from the wild-type strains C43(DE3) (a B derivative) and BW25113 (a K-12 derivative) were tested and found capable of secreting flavins, but different flavin secretion profiles (Fig. 2). BW25113 secreted almost equimolar

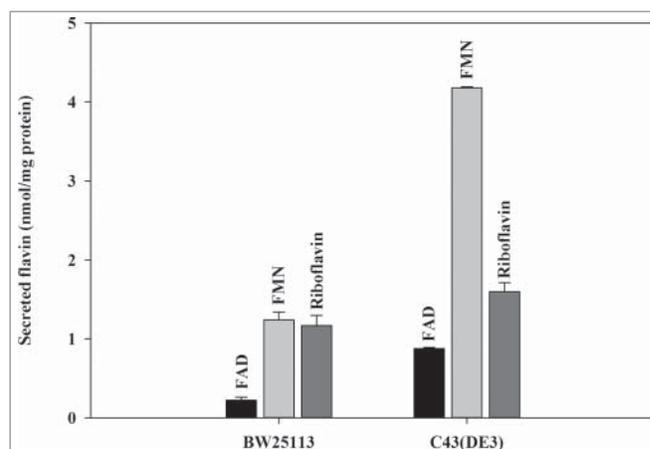


Figure 2. Flavin secretion by *E. coli* BW25113 and C43(DE3). Cultures were grown in M9-glc for 20 hours before flavins in culture supernatants were analyzed by HPLC. Two independent cultures were tested for each strain, and error bars represent one standard deviation.

FMN and riboflavin, while C43(DE3) secreted more FMN than riboflavin. Both strains secreted less FAD than the other 2 flavins. However, BW25113 secreted flavins at a roughly 2.5-fold lower (when normalized according to OD₆₀₀) amount than C43 (DE3). Therefore, the 2 strains likely use varying types of flavin exporters, and the flavin exporters have different specificities toward each of the flavins as substrates.

YeeO exports flavins

Since we found that wild-type strains of *E. coli* are capable of secreting flavins, a search was conducted for proteins responsible for the secretion. We reasoned that since *E. coli* is incapable of uptaking flavins,²¹ any flavin transporter is not passive. A BLASTx search of a protein that actively secretes flavins, Bfe from *S. oneidensis*, against the genomes of BL21(DE3) and MG1655 (C43(DE3) and BW25113 are derived from BL21 (DE3) and MG1655, respectively) revealed the greatest sequence homology between Bfe and YeeO (E value of 3 E-8). However, the overall sequence identity between the 2 proteins is 20% so the proteins are not related.

We tested whether YeeO could secrete flavins by analyzing culture supernatants by HPLC and found that YeeO dramatically increases FMN secretion by 36-fold and FAD secretion by 17-fold (Fig. 3A). YeeO thus displays substrate specificity to both FMN and FAD. One other possibility may be that YeeO is only an inner membrane protein, and that *E. coli* can hydrolyze some of the FAD secreted by YeeO in the periplasm into FMN. In a similar manner, UshA of *S. oneidensis*, has a considerable effect on the flavin secretion profile,²² so we also tested YeeO production in an *ushA*⁻ host to check if UshA of *E. coli* has an impact on the flavin secretion profile. In comparison to the empty vector host, the YeeO producing strain encountered roughly equal decreases in the fold changes of FAD and FMN transport when it carried the *ushA*⁻ mutation (by and -2.7 and -1.8-fold, respectively). UshA thus had a negligible effect here, indicating that if *E. coli* makes any FAD hydrolyzing proteins that localize to the periplasm, it is not UshA. This coincides with the previous finding that the FAD hydrolysis activity of UshA in *E. coli* is weak.²² In any case, secretion of both FAD and FMN occurred at the expense of riboflavin secretion, as riboflavin export decreased 1.8-fold with YeeO production. This provides evidence that YeeO is specific toward certain flavins, and that the increases in secretion of FAD and FMN were not due to cell lysis instead of flavin export.

To test the effect of the expression rate of YeeO on flavin secretion, we also produced YeeO from pCA24N-*yeeO* with varying amounts of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 and 1 mM IPTG) (Fig. 3A, B). As expected, lower production of YeeO (with 0.1 mM IPTG) decreased flavin secretion compared to the empty vector host: 18, 12, and -4.9-fold for FMN, FAD, and riboflavin, respectively at 0.1 mM IPTG compared to 36, 17, and -1.8 with 1 mM IPTG induction.

Production of YeeO impacts the intracellular flavin profile

After seeing that YeeO increases flavin secretion (either directly or indirectly), sonicated cell pellet samples were analyzed

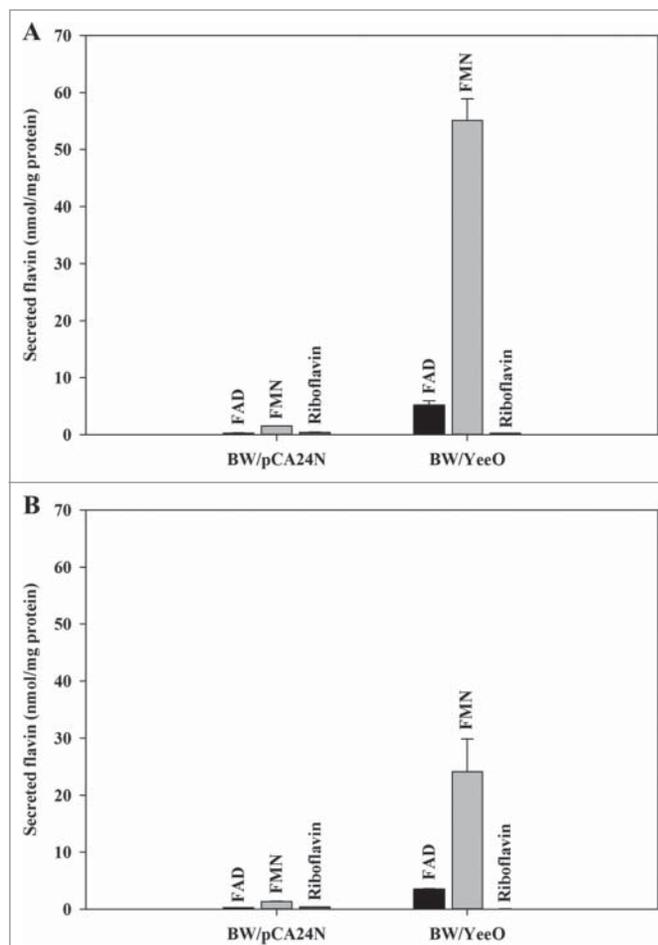


Figure 3. Enhanced flavin secretion upon YeeO production. Cultures were grown in M9-glc-caa and induced with (A) 1 mM IPTG, or (B) 0.1 mM IPTG for 6 hours before flavins in culture supernatants were analyzed by HPLC. BW/pCA24N represents BW25113/pCA24N and BW/YeeO represents BW25113/pCA24N-*yeeO*. Two independent cultures were tested for each strain, and error bars represent one standard deviation.

for flavins to check how production of the transporter affects the intracellular flavin profile. Unexpectedly, YeeO production increased, rather than decreased, total intracellular flavin concentrations by 3.2-fold (Fig. 4). YeeO caused an increase in intracellular concentrations of both FMN and riboflavin (by 5.9 or 3.3-fold, respectively), but did not affect the intracellular FAD concentration. Cells must have made up for the increased efflux of FMN and FAD caused by YeeO by increasing the concentrations of predecessor flavins. Nonetheless, the concentration of total secreted flavins was still very low compared to intracellular concentrations with or without YeeO production. Intracellular flavin concentrations for the wild-type and the YeeO producing strain were 21 and 136 fold higher than those found in their surrounding media, respectively.

Production of YeeO impacts cellular physiology

To investigate how YeeO impacts cellular physiology, we imaged cells producing YeeO under light microscopy, compared

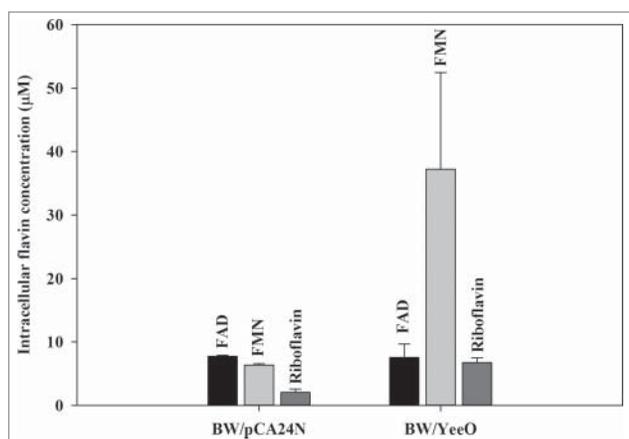


Figure 4. Intracellular flavin profile resulting from YeeO production. Cultures were grown in M9-glc-*caa* and induced with 1 mM IPTG for 6 hours before flavins in sonicated cell pellets were analyzed by HPLC. BW/pCA24N represents BW25113/pCA24N and BW/YeeO represents BW25113/pCA24N-*yeeO*. Two independent cultures were tested for each strain, and error bars represent one standard deviation.

to cells hosting an empty plasmid vector. As seen in Fig. 5, abnormal cell elongation occurs during YeeO production. Measuring the cell lengths of at least 30 cells indicated that they increased by 1.54-fold.

To investigate if YeeO is in fact a membrane protein, we used fluorescence microscopy to localize a GFP-tagged version of YeeO. YeeO, along with the membrane protein positive control, OppA, were seen localizing to cell poles by fluorescence microscopy (Fig. 6A, B). This provides further evidence that YeeO is a membrane protein.

Discussion

Here we show YeeO is a transporter of primarily FMN and also FAD. Production of YeeO shifts flavin secretion

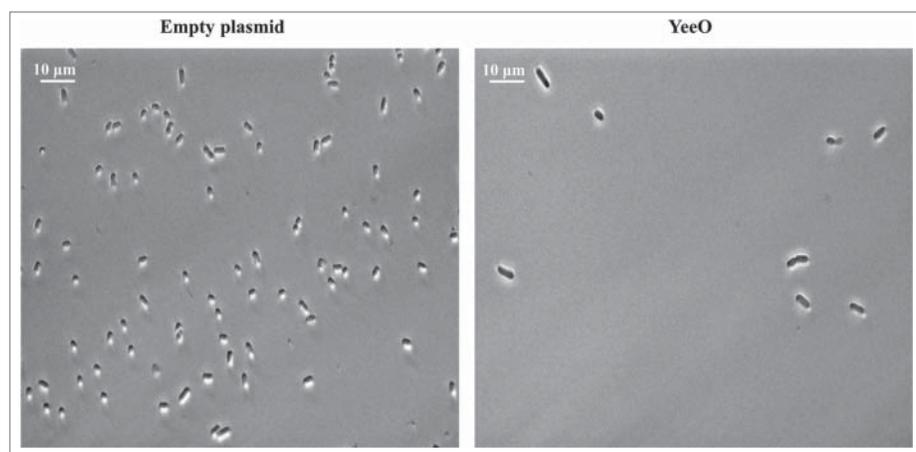


Figure 5. YeeO elongates cells. Cultures were grown in M9-glc-*caa* and induced with 1 mM IPTG for 6 hours before visualizing by phase contrast microscopy. Empty plasmid represents BW25113/pCA24N as control, and YeeO as BW25113/pCA24N-*yeeO*. Scale bar indicates 10 µm.

profiles of *E. coli* toward FMN and FAD at the expense of riboflavin secretion. YeeO can thus be utilized in a metabolic engineering strategy for production and secretion of flavins. The transporter also shifts flavin profiles intracellularly with increases in riboflavin and FMN concentrations. While this may indicate that the production/secretion of flavins by *E. coli* is limited primarily by riboflavin, FAD and FMN synthesis rather than transport, flavins still seem to be trapped intracellularly, even with YeeO production. This may be due to the fact that most flavins are postulated to exist as bound co-factors,²¹ and are thus unavailable as substrates for YeeO. Another possible explanation for the phenomenon is that YeeO acts to secrete FMN and FAD only across the inner membrane, and transport across the outer membrane is a bottleneck. The latter hypothesis would explain why intracellular concentrations (including both cytoplasmic and periplasmic fractions) of FMN are increased with YeeO production, with the extra secreted FMN getting trapped in the periplasm, and cells producing extra FMN in order to make up for the loss of FMN and FAD in the cytoplasm from secretion. Further experiments regarding isolation and analyses of periplasmic and cytoplasmic domains would confirm this. Regardless, production of YeeO profoundly shifts intracellular and extracellular flavin profiles.

While a previous study indicates that *E. coli* is capable of secreting flavins,⁵ we show here the flavin secretion profiles of 2 different *E. coli* strains (one being a B derivative (C43(DE3), and the other being a K-12 derivative (BW25113)). *E. coli* secretes more quantifiable amounts of flavins when incubated at longer periods of time, and with richer media (i.e. M9 minimal media supplemented with 0.4% glucose and 0.4% casamino acids compared to M9 minimal media supplemented with 0.4% glucose). Both strains are capable of secreting all 3 flavins tested for, and they have different flavin secretion profiles. There is thus a possibility that multiple flavin transporters exist in each strain (with each one having different substrate specificity among the flavins), and each strain naturally expresses these different transporters at variable levels.

YeeO was only shown previously to be one of 4 proteins made by *E. coli* allowing the secretion of dipeptides,¹⁹ and a $\Delta yeeO$ mutation was found to increase susceptibility to a variety of antibiotics.²⁰ We show here that YeeO has another unrelated function: the secretion of FMN and FAD. YeeO is thus one of 2 proteins known to secrete at least FAD in bacteria, with the other being Bfe from *S. oneidensis*.¹⁵ Flavins are secreted by *S. oneidensis* to act as electron shuttles for exoelectrogenesis; however, the reason(s) for flavin secretion in *E. coli* is unknown. Since both Bfe and YeeO belong to the MATE family of proteins, there is the possibility that flavin secretion occurs in part

due to over-accumulation of intracellular flavins.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The *E. coli* strains used in this study are listed in Table 1. The Keio²³ and ASKA²⁴ collections were used for isogenic single-gene deletion mutants of BW25113, and for obtaining pCA24N-based plasmids for overexpression, respectively. All experiments were conducted in 250 mL shake flasks incubated at 37°C with 250 rpm shaking in Lysogeny Broth (LB),²⁵ unless otherwise indicated. M9 minimal medium supplemented with 0.4% glucose and 0.4% casamino acids (M9-glc-*caa*)²⁶ was used where indicated. Chloramphenicol was used at 30 µg/mL for the maintenance of pCA24N-based plasmids, and kanamycin was used at 50 µg/mL for selecting mutants having the kanamycin resistance cassette on the chromosome.

Isolation of supernatant and intracellular samples for HPLC analysis

For wild-type *E. coli* without plasmids, overnight cultures were refreshed to OD₆₀₀ 0.025 in 25 mL M9-glc-*caa*, and incubated for 20 h after the OD₆₀₀ reached 0.2. The turbidity at 600 nm was then measured and used to normalize samples based on protein content (using 0.22 mg protein/mL/OD₆₀₀²⁷). For cells with pCA24N-based plasmids, overnight cultures were refreshed to OD₆₀₀ 0.025 in 25 mL M9-glc-*caa*, and induced with 1 mM IPTG, unless otherwise indicated, when turbidities reached around 0.2. After induction (6 h), supernatants were collected after measuring the turbidity and

concentrated 5 or 10-fold using a centrifugal evaporator. For determination of intracellular concentrations, turbidity was measured for estimating the cellular volume (3.6 µL cellular volume/mL culture/OD₆₀₀²⁸), and the entire culture was centrifuged at 7,520 g for 10 minutes at 4°C. Supernatants were kept for later analysis and comparison with secreted vs. intracellular

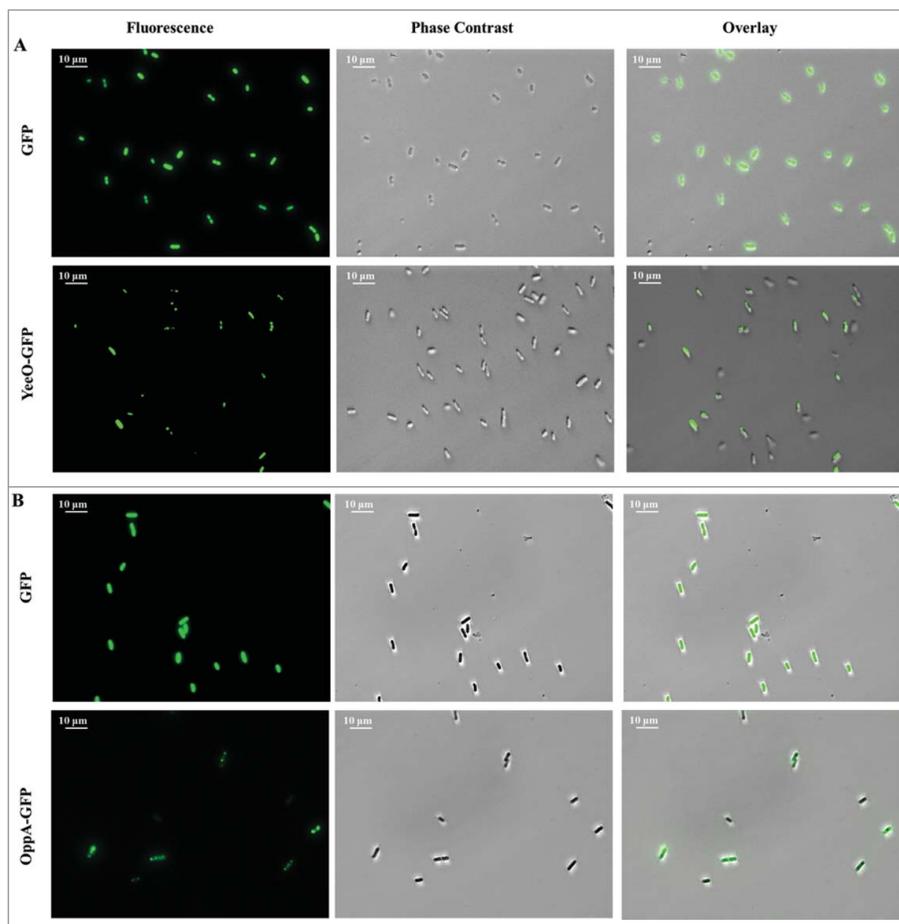


Figure 6. Representative microscopy images of cells producing GFP-tagged YeeO. **(A)** Cultures were grown in M9-glc-*caa* and induced with 1 mM IPTG for 6 hours, and **(B)** cultures were grown in LB and induced with 1 mM IPTG for 2 hours. All samples were visualized by phase contrast and epi-fluorescence, and overlay images are indicated. GFP represents BW25113/pCA24N-*gfp*, YeeO represents BW25113/pCA24N-*yeeO-gfp*, and OppA represents BW25113/pCA24N-*oppA-gfp*. Scale bar indicates 10 µm.

Table 1. *E. coli* strains and plasmids used in this study. Chloramphenicol (30 µg/mL) was used to maintain pCA24N-based plasmids, and kanamycin (50 µg/mL) was used to select for mutants having the kanamycin resistance cassette. Cm^R and Km^R indicate chloramphenicol and ampicillin resistance, respectively

Strains	Genotype	Source
C43(DE3)	F – ompT hsdSB (rB- mB-) gal dcm (DE3)	Lucigen
BW25113	<i>rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	23
BW25113 <i>ushA</i>	BW25113 <i>ushA</i> Ω Km ^R	23
Plasmids		
pCA24N	Cm ^R ; <i>lacI^q</i> , pCA24N	24
pCA24N- <i>yeeO</i>	Cm ^R ; <i>lacI^q</i> , pCA24N P _{T5-lac} :: <i>yeeO</i>	24
pCA24N- <i>gfp</i>	Cm ^R ; <i>lacI^q</i> , pCA24N P _{T5-lac} :: <i>gfp</i>	30
pCA24N- <i>yeeO-gfp</i>	Cm ^R ; <i>lacI^q</i> , pCA24N P _{T5-lac} :: <i>yeeO::gfp</i>	24
pCA24N- <i>oppA-gfp</i>	Cm ^R ; <i>lacI^q</i> , pCA24N P _{T5-lac} :: <i>oppA::gfp</i>	24

concentrations, while cell pellets were washed in $\frac{1}{2}$ volume, then resuspended in one tenth the volume of the spun down culture in M9-glc-*caa*. Resuspended cells were sonicated at 5 pulses of 30 to 60 seconds at 7 W to lyse open the cells. Sonicated samples were spun down at 17,000 g for 1 minute to get rid of cellular debris and supernatants were kept as the intracellular samples.

HPLC analysis of samples

HPLC analysis for the detection and quantification of the 3 flavins under investigation (riboflavin, FMN, and FAD) was conducted as previously described²⁹ with some modifications. All samples were filtered through a 0.22 μ m polyvinylidene fluoride (PVDF) filter before 10 μ L was fractionated by HPLC (Waters 717 autosampler with 2 model 515 pumps, and a 2996 photodiode array detector) with a reversed-phase column (Spherisorb 5 μ m ODS2 (24 cm \times 4.6 mm)). Separations were conducted using a flow rate of 1 mL/min with 5 minutes of flow with buffer (A) 20 mM potassium phosphate buffer (pH 6.0) in 25% methanol, then 10 minutes of flow with buffer (B) 20 mM potassium phosphate buffer (pH 6.0) in 50% methanol. Absorbance (instead of fluorescence) at 268.6 nm was used to detect the flavin compounds. Chemicals used as standards for comparisons include riboflavin from Sigma-Aldrich (cat. no. R4500-25G), FMN sodium salt hydrate from Sigma-Aldrich (cat. no. F8399-1MG), and FAD disodium salt hydrate from Alfa Aesar (cat. no. A14495). Peaks corresponding to those of riboflavin, FMN, and FAD were confirmed by retention time, co-elution with standards, and by comparing absorbance spectra with those from the standards. Total quantities of the flavins were calculated by comparing peak areas with standard curves made by running each flavin standard. Flavin concentrations were normalized where

indicated according to OD₆₀₀ of the sample at the time of collection.

Microscopy

For the YeeO producer and its empty plasmid control, cells were grown overnight, then were refreshed to OD₆₀₀ 0.025 in 25 mL M9-glc-*caa*, and induced with 1 mM IPTG when the turbidity reached approximately 0.2. After induction (6 h), the final turbidity was measured, and a droplet of the culture was placed on a glass slide for visualization by microscopy. For the OppA positive control and its empty plasmid control, cells were grown overnight, then refreshed and induced with 1 mM IPTG when turbidities reached around 1 (2 h induction). Phase contrast images were taken using a microscope (Zeiss, Axio Scope. A1) at 1000 \times magnification, with epi-fluorescence (470 nm excitation and 525 nm emission).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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