YeeO from *Escherichia coli* exports flavins

Michael J McAnulty and Thomas K Wood

1Department of Chemical Engineering; Pennsylvania State University; University Park, PA USA; 2Department of Biochemistry and Molecular Biology; Pennsylvania State University; University Park, PA USA

**Keywords:** flavin, flavin adenine dinucleotide, flavin mononucleotide, transporter

**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. 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*. Riboflavin, however, is less water-soluble than its phosphorylated derivatives that are used as co-factors, such as flavin mononucleotide (FMN) 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. High concentrations of FMN may also increase the solubility of riboflavin and decrease susceptibility of certain riboflavin derivatives (i.e., FMN) toward photodegradation. 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. 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, 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*. 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, and expression of *ribA* in particular is the bottleneck in riboflavin synthesis. 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). Production of a passive riboflavin transporter, RibM from *Streptomyces dassauensis*, in the riboflavin overproducer *B. subtilis* BSHP results in a smaller increase in riboflavin production.

Little work has been performed on the metabolic engineering of flavin production/synthesis 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. Over-expression 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). 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* ΔushA strain (by roughly 21-fold, 3.3-fold, and 2.1-fold, for FAD,
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 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 *Streptomycetes 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,15 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 1. Chemical structures of the 3 flavins in study. The chemical structures of riboflavin, FMN, and FAD are shown.](image)

![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.](image)
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, 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, 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. 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 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
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 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 cofactors, 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, 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, and a ΔyeeO mutation was found to increase susceptibility to a variety of antibiotics. 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*. 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 Keio<sup>23</sup> and ASKA<sup>24</sup> 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),<sup>25</sup> unless otherwise indicated. M9 minimal medium supplemented with 0.4% glucose and 0.4% casamino acids (M9-glc-caa)<sup>26</sup> 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 OD<sub>600</sub> 0.025 in 25 mL M9-glc-caa, and incubated for 20 h after the OD<sub>600</sub> 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/OD<sub>600</sub><sup>27</sup>). For cells with pCA24N-based plasmids, overnight cultures were refreshed to OD<sub>600</sub> 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 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<sup>+</sup> and Km<sup>+</sup> indicate chloramphenicol and ampicillin resistance, respectively.

| Strains         | Genotype                          | Source |
|-----------------|-----------------------------------|--------|
| C43(DE3)        | F-ompT hsdS8 (R- mB-) gal dcm (DE3)| Lucigen 23 |
| BW25113         | rm83 ΔlacZ4787 hsdR514 Δ(arabAD)567 Δ(rhaBAD)568 rph-1 |        |
| BW25113 ushA    | BW25113 ushA ΔΔKm<sup>R</sup> | 23     |
| Plasmids        |                                   |        |
| pCA24N          |                                   | 24     |
| pCA24N-yeeO     |                                   | 24     |
| pCA24N-gfp      |                                   | 24     |
| pCA24N-yeeO-gfp |                                   | 24     |
| pCA24N-oppA-gfp |                                   | 24     |

![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.](image-url)
concentrations, while cell pellets were washed in 1/2 volume, then resuspended in one tenth the volume of the spun down culture in M9-glcl-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 described with some modifications. All samples were filtered through a 0.22 μm polyvinylidene fluoride (PVDF) filter before 10 μL 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 μm ODS2 (24 cm × 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 standard curves made by running each flavin under investigation (riboflavin, FMN, and FAD) was confirmed by retention time, co-elution with standard curves made by running each flavin and absorbance spectra with those from the standards. 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 standard curves made by running each flavin and 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-glcl-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× magnification, with epi-fluorescence (470 nm excitation and 525 nm emission).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University. We are grateful for the Keio and ASKA strains provided by the Genome Analysis Project in Japan. We thank Brian W. Kwan for providing microscopy images of the OppA producer and its empty plasmid control. We also thank Tatiana N. Laremore (Penn State Proteomics and Mass Spectrometry Facility, University Park, PA) for translating an article in Russian.

References

1. Stahmann KP, Revuelta JL, Seulberger H. Three biotechnological processes using Alkaneus gypsii, Candida famata, or Bacillus subtilis compete with chemical riboflavin production. Appl Microbiol Biotechnol 2000;53:509-16. PMID:10855708; http://dx.doi.org/10.1007/s002530051649
2. Grobin A, Hird G, Lambot B, Onai K, Pullen S, inventors; Eisai Co. Ltd., assignee. Pharmaceutical composition comprising 5′ monophosphate ester of riboflavin and solubilized riboflavin. United States patent US 20030162751 A1. 2001 Aug 28.
3. Grimmer J, Kiefer H, inventors; BASF Aktiengesellschaft, assignees. Preparation of riboflavin 5′-phosphate (fmm) and its sodium salt in a lactone. United States patent US 5095115 A. 1992 March 10.
4. Kraus U, Svensson V, Wirtz A, Knips-Grundhagen E, Jünger K-E. Colfactor trapping, a new method to produce flavin mononucleotide. Appl Environ Microbiol 2011;77:1097-100. PMID:21131527; http://dx.doi.org/10.1128/AEM.01541-10
5. von Canstein H, Ogawa J, Shimizu S, Lloyd JR. Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Appl Environ Microbiol 2008;74:615-23. PMID:18065612; http://dx.doi.org/10.1128/AEM.01387-07
6. Velasquez-Orra SB, Head IM, Curtis TP, Scott K, Lloyd JR, von Canstein H. The effect of flavin electron shuttles in microbial fuel cells current production. Appl Microbiol Biotechnol 2010;85:1373-81; PMID:19697021; http://dx.doi.org/10.1007/s00253-009-2172-8
7. Masuda M, Fregua S, Wang Y-F, Tsujimura S, Kano K. Flavins contained in yeast extract are exploited for anodic electron transfer by Lactococcus lactis. Bioelectrochemistry 2010;78:173-5. PMID:19717350; http://dx.doi.org/10.1016/j.bioelechem.2009.08.011
8. Khan MT, Browne WR, van Dijl JM, Harmsen HMJ. How can Fusobacterium prunaeiizii employ riboflavin for extracellular electron transfer? Antioxid Redox Sign 2012;17:1433-40; http://dx.doi.org/10.1089/ars.2012.4701
9. Mitonov VN, Kraev AS, Chikidjas ML, Chernov BK, Stepanov AI, Skryabin KG. Functional organization of the riboflavin biosynthesis operon from Bacillus subtilis 168. Mol Gen Genet 1994;242:201-8. PMID:8159171; http://dx.doi.org/10.1007/BF00930140
10. Perkins JB, Sloma A, Hermann T, Theriault K, Zachgo E, Erdenberger T, Hannert N, Chatterjee NP, Williams V, Rufo GA, et al. Genetic organization of Bacillus subtilis for the commercial production of riboflavin. J Ind Microbiol Biotechnol 1999;22:8-18; http://dx.doi.org/10.1007/s10292.9900587
11. Hümbelin M, Grieser V, Keller T, Schurrer W, Haiker M, Holmman H-P, Ritz H, Richter G, Bacher A, van Loon APGM. GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are rate-limiting enzymes in riboflavin synthesis of an industrial Bacillus subtilis strain used for riboflavin production. J Ind Microbiol Biotechnol 1999;22:1-7; http://dx.doi.org/10.1007/s10292.9900590
12. Yatsyshyn YV, Ishchuk OP, Voronovskyi AV, Fedorovych DV, Sibiryak AA. Production of flavin mononucleotide by metabolically engineered yeast Candida famata. Metab Eng 2009;11:163-7; PMID:19558965; http://dx.doi.org/10.1016/j.ymben.2009.01.004
13. Hemberger S, Pedrolli DB, Stols J, Vogl C, Lehmann M, Mack M. RibM from Streptomyces davawensis is a riboflavin/roseoflavin transporter and may be useful for the optimization of riboflavin production strains. BMC Biotechnol 2011;11:119. PMID:22136195
14. Rahimovich PM, Beburov M Iu, Linarch VZ, Bandrin SV, Stepansov AI. [Expression of Bacillus subtilis riboflavin operon in Escherichia coli cells in the form of hybrid plasmids pPR1 and pPR2]. Dokl Akad Nauk SSSR 1978;238:1459-61; PMID:415855
15. Kozłowski NJ, Gralnick JA. Flavin electron shuttles dominate extracellular electron transfer by Shewanella oneidensis. mBio 2013;4 pii: e00553-12. PMID:23322638; http://dx.doi.org/10.1128/mBio.00553-12
16. Abbas CA, Sibiryak AA. Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev 2011;75:321-60. PMID:21646432; http://dx.doi.org/10.1128/MMBR.00030-10
17. Woorr DJ, Grertt MM, Vandenbroucke-Grauls CMJE, Kusters JG. Helicobacter pylori ribA-mediated riboflavin production is involved in iron acquisition. J Bacteriol 1998;180:1473-9. PMID:9515332
18. Moriyama Y, Hiasa M, Matsumoto T, Omoto H. Multiple drug and toxic compound extrusion (MATE)-type proteins as anchor transporters for the excretion of Russian.

www.landesbioscience.com 391Bioengineered
19. Hayashi M, Tabata K, Yagasaki M, Yonetani Y. Effect of multidrug-efflux transporter genes on dipeptide resistance and overproduction in *Escherichia coli*. FEMS Microbiol Lett 2010; 304:12-9; PMID:20067529; http://dx.doi.org/10.1111/j.1574-6968.2009.01879.x

20. Nichols RJ, Sen S, Choo YJ, Beltrao P, Zaretk M, Chaba R, Lee S, Kazmierczak KM, Lee KJ, Wong A, et al. Phenotypic landscape of a bacterial cell. Cell 2011; 144:143-56; PMID:21185072; http://dx.doi.org/10.1016/j.cell.2010.11.052

21. Wilson AC, Pardee AB. Regulation of flavin synthesis by *Escherichia coli*. J Gen Microbiol 1962; 28:283-303; PMID:14007303; http://dx.doi.org/10.1099/00221287-28-2-283

22. Covington ED, Gebmann CB, Kotloski NJ, Gralnick JA. An essential role for UshA in processing of extracellular flavin electron shuttles by *Shewanella oneidensis*. Mol Microbiol 2010; 78:519-32; PMID:20807196; http://dx.doi.org/10.1111/j.1365-2958.2010.07353.x

23. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Danekou KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006; 2:2006-0008; PMID:16738554; http://dx.doi.org/10.1038/msb4100050

24. Kitagawa M, Ara T, Artifazzaman M, Ioka-Nakamichi T, Inamoto E, Tounaga H, Mori H. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. DNA Res 2005; 12:291-9; PMID:16769691; http://dx.doi.org/10.1093/dnares/dsi012

25. Sambrook JF, Maniatis T. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1989.

26. Rodriguez RL, Tait RC. Recombinant DNA techniques: an introduction. Menlo Park, Calif.: The Benjamin/Cummings Publishing Company, Inc., 1985.

27. Vardar G, Wood TK. Protein engineering of toluene-o-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for synthesizing 4-methylresorcin, methylhydroquinone, and pyrogallol. Appl Environ Microbiol 2004; 70:3253-62; PMID:15184119; http://dx.doi.org/10.1128/AEM.70.6.3253-3262.2004

28. Volkmer B, Heinemann M. Condition-dependent cell volume and concentration of *Escherichia coli* to facilitate data conversion for systems biology modeling. PloS One 2011; 6:e23126; PMID:21829590; http://dx.doi.org/10.1371/journal.pone.0023126

29. Pallotta ML. Evidence for the presence of a FAD pyrophosphatase and a FMN phosphohydrolase in yeast mitochondria: a possible role in flavin homeostasis. Yeast 2011; 28:693-705; PMID:21915900; http://dx.doi.org/10.1002/yea.1897

30. Soo VWC, Hanson-Manful P, Patrick WM. Artificial gene amplification reveals an abundance of promiscuous resistance determinants in *Escherichia coli*. Proc Natl Acad Sci U S A 2011; 108:1484-9; PMID:21173244; http://dx.doi.org/10.1073/pnas.1012108108