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A Riboflavin Transporter in *Bdellovibrio exovorous* JSS

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Abstract

The ImpX transporters of the Drug/Metabolite Transporter (DMT) superfamily were first proposed to transport riboflavin (vitamin B2) based on findings of a *cis*-regulatory RNA element responding to flavin mononucleotide (an FMN riboswitch). *BdeHovibrio exovorous* JSS has a homolog belonging to this superfamily. It has 10 TMSs and shows 30% identity to the previously characterized ImpX transporter from *Fusobacterium nucleatum.* However, the ImpX homolog is not regulated by an FMN-riboswitch. In order to test the putative function of the ImpX homolog from *B. exovorous* (BexImpX), we cloned and heterologously expressed its gene. We used functional complementation, growth inhibition experiments, direct uptake experiments and inhibition studies, suggesting a high degree of specificity for riboflavin uptake. The EC50 for growth with riboflavin was estimated to be in the range 0.5–1 μM, estimated from the half-maximal riboflavin concentration supporting the growth of a riboflavin auxotrophic *E. coli* strain, but the K_{half} for riboflavin uptake was 20 μM. Transport experiments suggested that the energy source is the proton motive force, but that NaCl stimulates uptake. Thus, members of the ImpX family members are capable of riboflavin uptake, not only in riboflavin prototrophic species such as *F. nucleatum,* but also in the B2 auxotrophic species, *B. exovorous*.

Keywords

Riboflavin; ImpX transporter; Roseoflavin; FMN biosynthesis

Introduction

Bdellovibrios are predatory bacteria isolated from soil that can lyse and utilize the cytoplasmic constituents of other Gram-negative bacteria as nutrients, potentially providing an alternative approach to the biocontrol of human, animal and plant pathogens [Cao et al.,

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2012; Koval et al., 2013; McNeely et al., 2017]. *Bdellovibrio* is a genus of Gram-negative bacteria that belongs to the Oligoflexia class of Proteobacteria. Two known members of this genus, *B. bacteriovorus* and *B. exovorus,* are obligate predators of other Gram-negative bacteria. While the former species grows in the periplasmic space of the prey cell, the latter grows outside of the prey cell. These predators are ubiquitous in the environment and have been isolated from soil, compost, sewage, activated sludge and marine and terrestrial waters [Davidov et al., 2006a; Davidov et al., 2006b; Jurkevitch, 2006]. The complete genome sequences of both *Bdellovibrio* species are available, and this has allowed comparitive analyses of transport systems in these organisms [Heidari Tajabadi et al., 2017].

The water-soluble vitamin, riboflavin (RF; vitamin B2), is a precursor for two essential metabolic cofactors, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Flavoproteins are universally important for biological processes, including energy metabolism, redox reactions, light emission, biosynthesis, and DNA repair [Garcia Costas et al., 2017; Liu et al., 2017; Plegaria et al., 2017; Stietz et al., 2017]. Riboflavin synthesis has been demonstrated in a wide range of microorganisms through a pathway that creates one riboflavin molecule from one molecule of GTP and two molecules of ribulose 5-phosphate [Fassbinder et al., 2000; Fischer and Bacher, 2010]. The enzymes involved in riboflavin synthesis (Fig. 1) are GTP cyclohydrolase I (RibA), 3,4-dihydroxy-2-butanone 4-phosphate synthase (RibB), pyrimidine deaminase/reductase (RibD), 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase, RibH) and riboflavin synthase (RibC) [Fischer and Bacher, 2010].

The expression of riboflavin biosynthesis and transport genes in many bacteria is regulated by conserved *cis*-regulatory RNAs known as riboflavin nucleotide *(RFN)* elements that were first discovered *in silico* [Vitreschak et al., 2002] and subsequently identified as a class of FMN-sensing riboswitches that regulate gene expression by adopting alternative secondary structures after binding an FMN molecule [Mironov et al., 2002]. *ribB* expression is negatively regulated by an FMN-riboswitch in *E. coli* in the presence of FMN or the FMN-analogue, roseoflavin-mononucleotide [Pedrolli et al., 2015]. Roseoflavin, produced by *Streptomyces davawensis,* is supposed to suppress the activities of flavoproteins in *E. coli* [Langer et al., 2013] and the FMN riboswitch in *B. subtilis* [Jankowitsch et al., 2012; Ott et al., 2009].

The production of vitamins and exchange between microbes and hosts is important; exchange is mediated by efflux and uptake systems [Garcia-Angulo, 2017]. For example, the *E. coli* flavin efflux transporter YeeO has been characterized [McAnulty and Wood, 2014]. Additionally, riboflavin can be used as an electron shuttle for extracellular respiration. Previous analyses of bacterial genomes for genes potentially regulated by FMN riboswitches allowed prediction of multiple families of putative riboflavin uptake transporters [Sun and Rodionov, 2014; Vitreschak et al., 2002]. The functions of representative members of these predicted riboflavin transporter families have been confirmed experimentally [Gutierrez-Preciado et al., 2015; Rodionova et al., 2015]. The RibU transporters belong to the most broadly distributed class of ABC-type ECF-family riboflavin transporters in Gram-positive bacteria [Rodionov et al., 2009] and in *T. maritima* [Karpowich et al., 2016]. Two different types of ABC-type riboflavin transporters, RibXY (TCDB family 3.A.1.17.14) in

Chloroflexi and *Thermobaculum terrenum* [Rodionova et al., 2015] and RfuABCD in spirochetes [Deka et al., 2013], are so far restricted to these taxonomic lineages. The PnuC-family riboflavin permease RibM (TC# 4.B.1.1.5) is found only in some Actinobacteria. However the phylogenetic distributions of two other riboflavin permeases, RibN (TC# 2.A.7.3.54) and RfnT (TC# 2.A.1.81.5), are restricted to several classes of Proteobacteria [Garcia Angulo et al., 2013]. A characterized RibZ permease TC# 2.A.1.3.72) has been identified in Firmicutes, but homologs are also present in *Actinobacteria* [Sun and Rodionov, 2014; Vitreschak et al., 2002]. Although ImpX (Integral Membrane Protein X) was originally identified in *Fusobacterium nucleatum* (a commensal-turned pathogen from the fusobacterial phylum) and *Desulfitobacterium halfniense* [Mironov et al., 2002], ImpX transporters regulated by FMN riboswitches were bioinformatically identified across the phyla of Firmicutes (e.g., in *Bacillus clausii, Clostridium beijerinckii, Geobacillus* sp. and *Paenibacillus* sp.) and Fusobacteria (in *Ilyobacterpolytropus* and *Sebaldella termitidis),* as well as in a single species from the proteobacterial phylum, *Marinomonas* sp. [Rodionova et al., 2015]. The involvement of ImpX from *F. nucleatum* in riboflavin uptake was confirmed by functional complementation [Gutierrez-Preciado et al., 2015].

In summary, ImpX transporters had been found previously only in bacteria that are capable of synthesizing riboflavin (B2 prototrophs). In this work, we analyze the distribution of ImpX homologs in an expanded set of bacterial genomes and report their identification in a few bacteria that do not synthesize riboflavin (B2 auxotrophs) including *B. exovorus.* We experimentally characterize the putative ImpX transporter from this organism.

Results

Bioinformatic analysis of ImpX transporters and FMN riboswitch regulons

Similarity searches identified orthologs of ImpX in bacterial genomes representing diverse taxonomic phyla of bacteria. These include Proteobacteria (including *B. exovorous and Bacteriovorax marinus,* another predatory bacterium from the Oligoflexia class, and 4 species of γ-proteobacteria), Fusobacteria (10 species), Actinobacteria (4 species), Firmicutes (9 species) and a single bacterium of the Chloroflexi phylum, *Thermomicrobium roseum* (Fig. 2). The presence of an FMN riboswitch upstream of the *impX* genes from Firmicutes, γ-proteobacteria and some Fusobacteria suggests that transporters with vitamin B2 specificity are conserved across the ImpX transporter family. Interestingly, the majority of bacterial genomes possessing an ImpX homolog also contain a complete set of riboflavin biosynthetic genes, suggesting that ImpX has a tendency to be found in B2 prototrophic microorganisms, possibly serving a scavenging functions or allowing riboflavin export. The only two B2 auxotrophic bacteria possessing ImpX transporters of those examined are *B. exovorous* and *Streptobacillus moniliformis.* The observed positive correlation between ImpX transporters and the presence of the B2 biosynthetic pathway suggests potential involvement of ImpX in vitamin B2 exchange between species in microbial communities, e.g., crossfeeding in biofilms.

B2 auxotrophic species must use a B2 transporter for uptake of this essential vitamin into the cell. However, B2 prototrophs can potentially use the ImpX transporter, not only for the uptake of exogenous B2 (when its concentration inside the cell is low), but also for the

efflux of the vitamin when its intracellular concentration is high, due to the actvity of the biosynthetic pathway. In support of this hypothesis, many characterized members of the DME family are involved in multidrug or metabolite efflux such as the threonine/homoserine transporter, YbiF (TC# 2.A.7.3.6) [Livshits et al., 2003], and the cysteine exporter, YijE (TC# 2.A.7.3.26) [Yamamoto et al., 2015] of *E. coli.* Reconstruction of the riboflavin biosynthetic pathways in the two *Bdellovibrio* species with available genomes suggests that only *B. bacteriovorus* contains the complete set of B2 biosynthetic enzymes. It is a B2 prototroph without a riboflavin transporter. In contrast, the *ribD, ribH* and *ribE* genes are missing in *B. exovorous* JSS, which contains only homologs of *ribBA* and *impX* (gene locus tags, A11Q_1932 and A11Q_1837, respectively). Thus, the putative riboflavin transporter ImpX replaces the *de novo* biosynthetic pathway in this species (Fig.1).

Overexpression of ImpX in a *ribC E. coli* mutant complements the growth defect.

Taking into account the importance of vitamin B2 salvage for *B. exovorus*, the ImpX transporter from this organism (named *Bex*ImpX) was selected for experimental testing *in vivo*. To determine whether the identified candidate BexImpX protein is a riboflavin transporter, we cloned the *impX* gene from *B. exovorus,* inserted it into the chromosome of *E. coli* and evaluated its ability to complement the growth of a Δ*ribC E. coli* strain. A low concentration of riboflavin in the medium did not support the growth of the Δ*ribC* strain in M9 medium, reflecting the lack of a riboflavin transporter in *E. coli* (data not shown). This strain requires high concentrations of exogenous riboflavin to support growth. However, provision of *Bex*ImpX in this Δ*ribC* strain allowed growth with 3 μM of riboflavin (Fig.3). These results indicate that ImpX facilitates the uptake of riboflavin. Some growth inhibition for ImpX overproducing cells appeared at 0.7mM of riboflavin in *E. coli.*

Inhibition of *E. coli* by roseoflavin in a *Bex*ImpX overproduction strain.

To support the inferred function of the riboflavin transporter, we tested the inhibition of *E. coli* growth by the riboflavin analog, roseoflavin, which targets flavin-dependent enzymes and FMN-riboswiches [Pedrolli et al., 2015]. The growth of *E. coli* wild type and the derivative strain expressing *Bex*ImpX was measured in 96-well plates using the plate-reader, Biotek Elx808 (Fig. 4). Growth of both strains in the presence of 20 μM roseoflavin was not affected, but at a higher concentration (240 μM), inhibition by roseoflavin was observed only for the *Bex*ImpX-producing strain. We conclude that roseoflavin was taken up by the *Bex*ImpX transporter and inhibited the growth of the *impX*$^{+}$ strain.

Riboflavin uptake by ImpX in *E. coli* depends on the proton motive force.

The *E. coli* wild type strain and the derivative strain producing *Bex*ImpX were assayed for riboflavin uptake, measured in M9 media containing 20 mM mannose in the presence and absence of Na^{+}. The *Bex*ImpX-dependent uptake of riboflavin was observed during a 2–15 minute incubation, but no uptake was observed for the wild type *E. coli* strain. To determine the sodium or proton dependency, we measured riboflavin uptake in the *Bex*ImpX strain of *E. coli* in the presence and absence of 0–150 mM NaCl and 0–100 μM cyanide carbonyl *m*-chlorophenyl hydrazone (CCCP) (Fig.5A and 5B). Vitamin B2 uptake was most efficient in an M9 salts solution or in 0.1 X PBS buffer supplemented with 10 mM D-mannose. Under these conditions, an inhibitory effect in the presence of 25 μM CCCP was observed. The

inhibitory effect on riboflavin uptake (35 μM) by nicotinamide or thiamine pyrophosphate at 0.9mM was less then 5%. The data obtained for the uptake using RF titration (Fig. 5C) was analyzed by Graph Pad software (Prizm 6).

Conclusion

In this communication, we provide evidence that an ImpX homologue from *B. exovorous* (*Bex*ImpX), which is distantly related to the previously characterized ImpX from *F. nucleatum* (Fig. 3), is probably a riboflavin-specific transporter. This suggests that close *Bex*ImpX homologues and probably all other ImpX family proteins shown in Fig. 2, have the same specificity.

To support the inferred functional relevance of the vitamin B2 transporter, we experimentally demonstrated the riboflavin transport activity of *Bex*ImpX heterologously expressed in *E. coli,* by showing that this transporter allows the growth of an *E. coli* Δ*ribC* mutant auxotroph at low riboflavin concentrations. The overproduction of the ImpX transporter in *E. coli* showed a mild toxic effect at high concentrations of riboflavin. ImpX also mediates roseoflavin-dependent inhibition of *E. coli* growth. The transport assays revealed an activating effect of sodium at high concentrations. The transporter is probably a proton and/or sodium ion dependent pmf-driven system, as it was shown to be strongly inhibited by CCCP at a concentration of 25 μM (Figs. 5B and 5C). The dependency of uptake on RF concentrations of 0–60 μM using the fluorescence method yielded the 20 μM K_{half} value for ImpX-mediated uptake (Fig. 5C).

Materials and Methods

Bacterial strains, plasmids, media and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1.

Initial studies on the growth of *B. exovorous* strain JSS (ATCC BAA-2330) were conducted in co-cultures in 1/10-strength yeast extract-peptone medium supplemented with calcium [Gordon et al., 1993]. For maintenance, co-cultures contained 1 ml of a stationary phase culture of *B. exovorous* strain JSS and 4 ml of a 24 h peptone-yeast extract (PYE) broth culture of *Caulobacter crescentus* CB2A [Koval et al., 2013]. This mixture was incubated in 125 ml flasks with 20 ml of defined HM buffer. The co-culture was incubated at 30°C with shaking at 150 rpm for 24–48 h. Cultures were transferred to sterile screw-capped tubes and kept at 4°C for up to 1 month. For long-term storage, a 24–48 h culture of strain *B. exovorous* JSS cells was frozen in the presence of 25 % (w/v) glycerol at −80°C [Koval et al., 2013].

The *C. crescentus* strain used in this study, listed in Table 1, was obtained from Dr. L. Shapiro (Stanford University, Stanford, California, USA). *C. crescentus* was maintained on PYE medium. The *E. coli* Δ*ribC* strain was obtained from the CGSC collection.

All of the constructs were confirmed by DNA sequencing using the primers listed in Table 2. To see if expression of the *B. exovorous impX* gene can restore growth of *E. coil ribC* mutant cells in the presence of riboflavin, a constitutive promoter driven *impX* gene was

moved into the *E. coli* chromosome. First, the *impX* gene was amplified from *B. exovorous* ATCC BAA-2330 DNA using the primers Bex-Bam-R and Bex-Kpn-F (listed in Table 2). The resulting fragment was digested with *Kpn*I and *BamH*I and cloned into the same sites of pKD13-*rrnB*T:Ptet, yielding the plasmid pKDT_Ptet-*impX*, in which *impX* expression is under the control of the *tet* promoter (Ptet). Present in this plasmid, the fragment *"km*^{r}*:rrnB*T:P*tet-impX"* was PCR amplified, gel purified and then electroporated into wild type BW25113 cells expressing the λ-Red recombinase. The cells were incubated with shaking at 37°C for 1 hour and then applied onto LB + Km agar plates. The Km^r colonies were verified for the *"km*^{r}*:rrnB*T:P*tet-impX"* substitution for the 67 bp *intS/yfdG* intergenic region between the 117th and 51st nucleotides relative to the start codon of *yfdG* by colony PCR (using primers kt and intS-ver-R) and subsequently by sequencing. This yielded strain BW25123_impX.

The chromosomal region carrying *"km*^{r}*:rrnB*T:P*tet-impX"* in BW25123_impX was transferred into *E. coli ribC* deletion mutant cells by P1 transduction. The selection medium was LB+Km+riboflavin. A P1 transductant was purified and confirmed by PCR, yielding *E. coli* Δ*ribC*_impX that was used for the growth assays.

Riboflavin growth experiment and roseoflavin inhibition of the *E. coli* ImpX$^+$ strain.

To compare growth of strains *E. coli* Δ*ribC* and *E. coli* Δ*ribC*_impX, minimal medium M9 with a carbon source (0.5% glucose) and a nitrogen source (20 mM NH_4Cl) was used. The cells of *E. coli* Δ*ribC* and Δ*ribC_impX* were grown overnight in LB, supplemented with 0.7 mM riboflavin. The overnight cultures were washed twice using M9 salts. The strains inoculated into M9 for growth without washing are presented in Fig. 3.

The strains of *E. coli*: Δ*ribC* ImpX$^+$ and Δ*ribC* (as control) were inoculated into M9 medium with different concentrations of riboflavin: 0, 0.03 *μM,* 0.27 *μ*M and 26.5 *μ*M. The cultures were started at OD_{600} = 0.02, and growth was measured by following the increase in the absorbance at 600 nm.

For the experiment with the riboflavin analogue inhibitor, roseoflavin, the *E. coli* wild type strains with and without *impX* were grown overnight and inoculated into LB medium at different concentrations of roseoflavin. The starting absorbance was OD_{600} = 0.005.

Bioinformatic analyses

A search for B2 regulatory elements (FMN riboswitches) for the analysis and prediction of candidate transporters was conducted as described [Rodionova et al., 2015; Sun and Rodionov, 2014]. A maximum likelihood phylogenetic tree was constructed with the PhyML program, and an alignment was generated using the Muscle programs [Edgar, 2004; Lefort et al., 2017]. The operon co-localization of *impX* with the riboflavin biosynthetic genes is marked by an arrow; taxonomic groups are indicated in black font. The system analysis of homologues for riboflavin genes was produced using the PubSEED platform as described in [Magnusdottir et al., 2015].

Riboflavin uptake measurements

The *E. coli* wild type and the derivative strain expressing *BeximpX* were grown to OD_{600} equal to 1, and the cells were collected and washed with M9. Riboflavin uptake was measured in M9 salts or 0.1XPBS buffer or 40 mM Tris-HCl buffer supplemented with 20 mM mannose containing 0.04 mM riboflavin and 10 mM $MgSO_4$. The effects of the different concentrations of NaCl or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) on *Bex*ImpX-mediated uptake were tested (Fig 5A–B). Following uptake experiments at 25°C for various periods of time, cells were collected and washed with PBS buffer. For cell extraction of riboflavin, 0.2ml of 5% perchloric acid was added followed by incubation on ice for 10 minutes with periodic vortexing. After centrifugation, 0.05 ml of supernatant was added to 0.2 ml K_2HPO_4 on black plates, and the fluorescence was measured. The total concentration of FMN and riboflavin in the *E. coli* cells were detected by fluorescence as described in [Munro and Noble, 1999].

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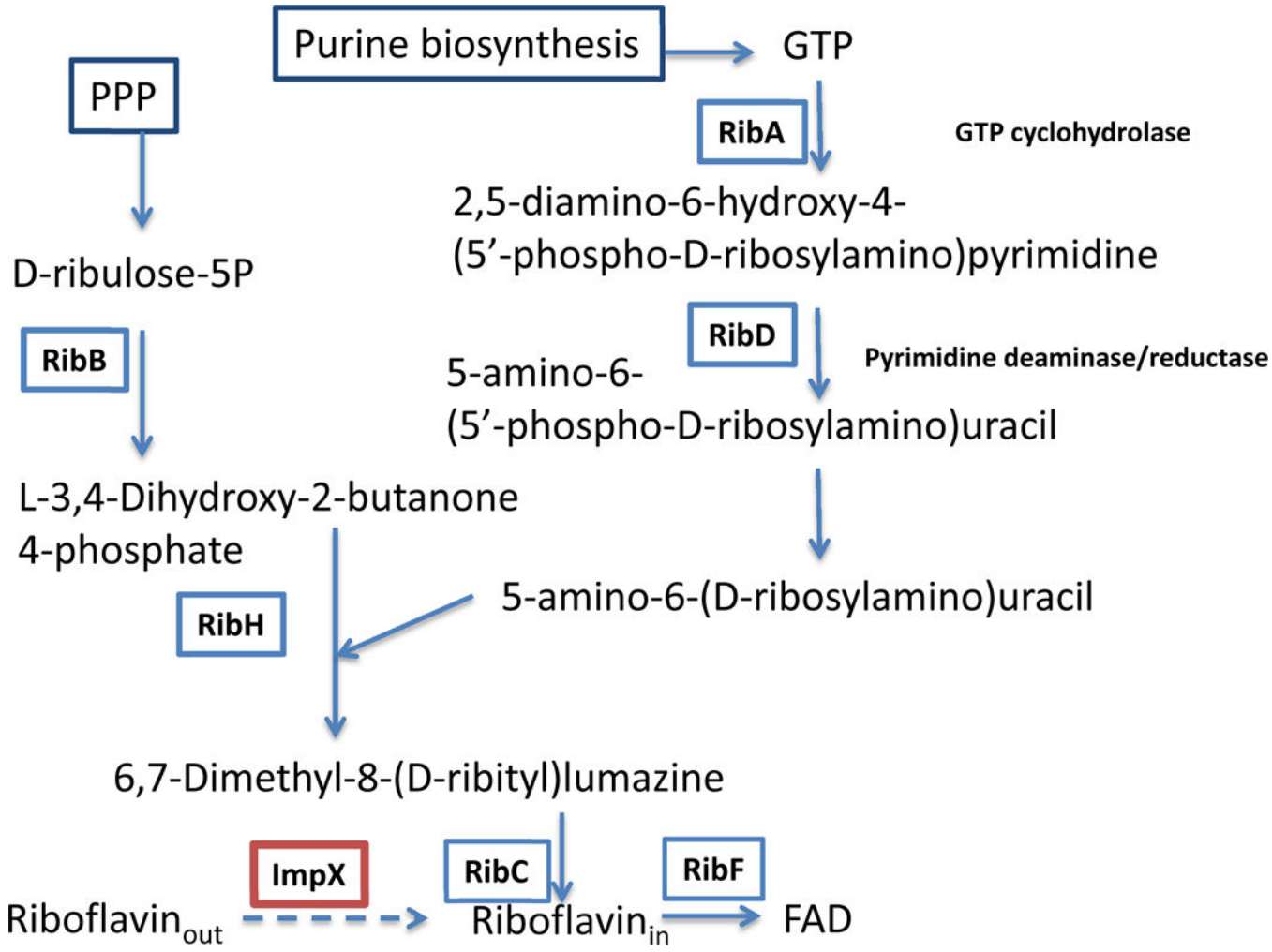

Fig. 1.
The pathway for riboflavin biosynthesis and transport in bacteria. PPP - pentose phosphate pathway.

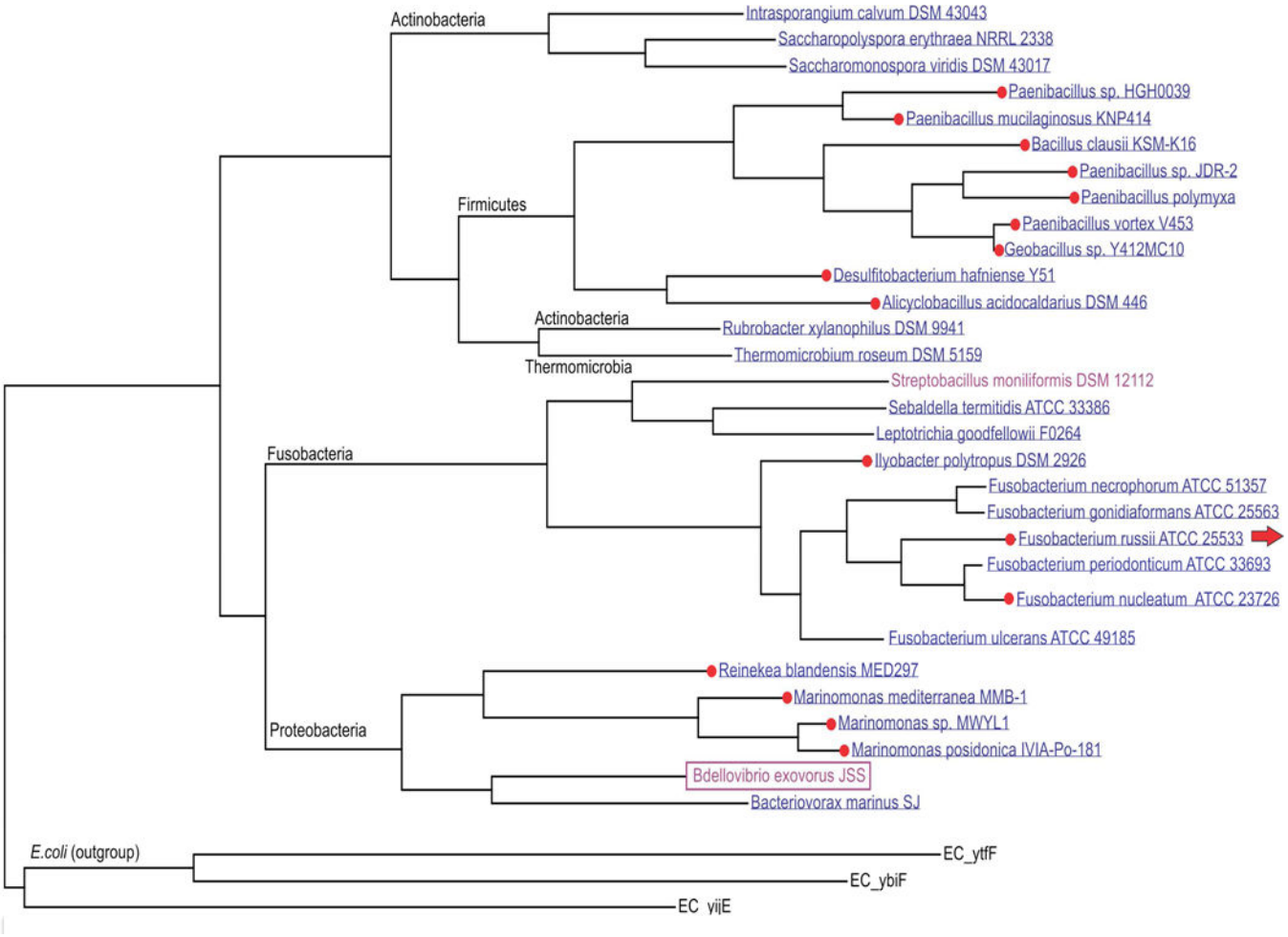

Fig. 2.

The maximum likelihood phylogenetic tree of ImpX proteins from *Bdellovibrio exovorous* and other bacterial species. Red dots show species that have an *impX* transporter gene controlled by an FMN riboswitch. The presence of a complete set of riboflavin biosynthetic genes in bacterial genomes is shown by blue (B2 prototrophs), while B2 auxotrophic species are shown in red. Three other members of the 10 TMS Drug/Metabolite Exporter (DME) family, YtfF, YbiF and YijE from *E. coli,* were used as an outgroup.

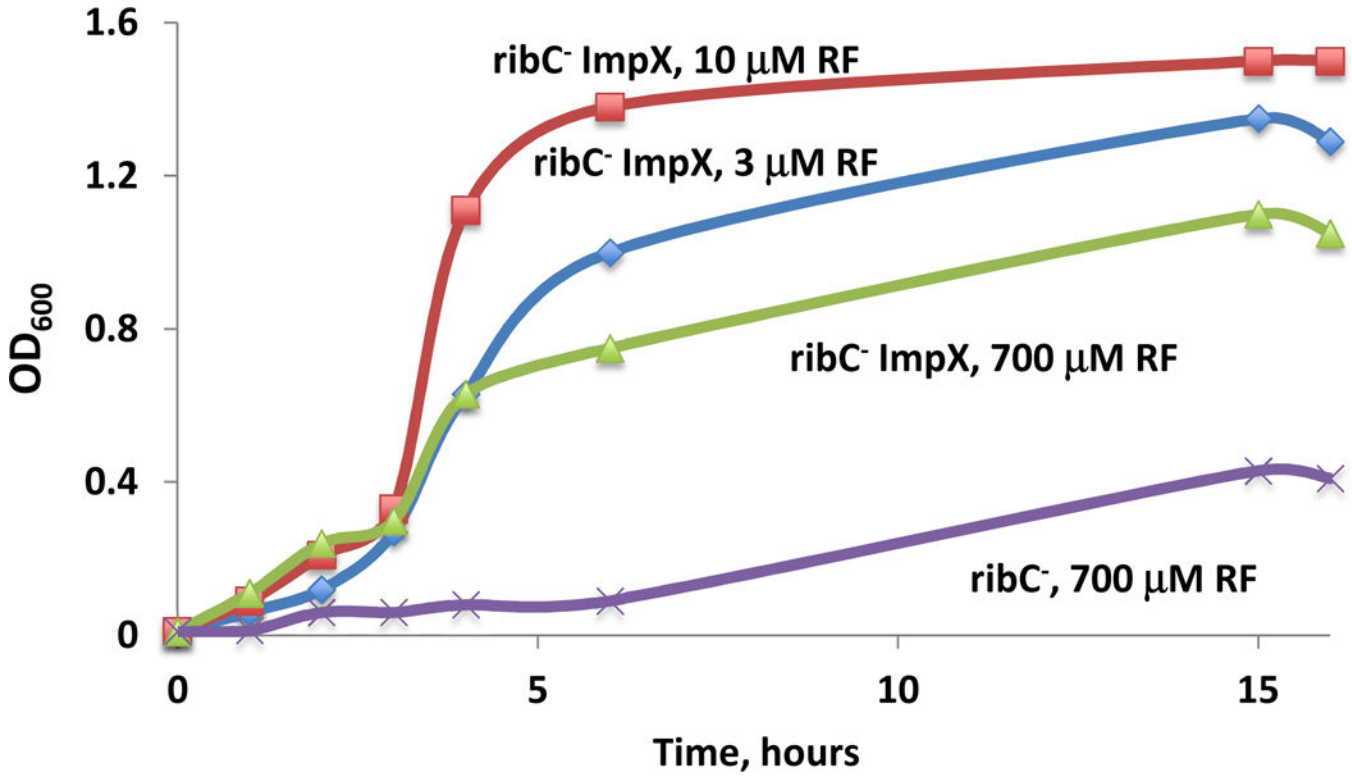

Fig. 3.

Growth of *E. coli* with **varied concentrations of** riboflavin (RF). The growth curves are marked purple for 0.7 mM RF with the Δ*ribC* strain; other lines - Δ*ribC* ImpX$^+$, blue diamonds - 3μM RF; red squares - 10 μM RF; green triangles - 0.7 mM RF.

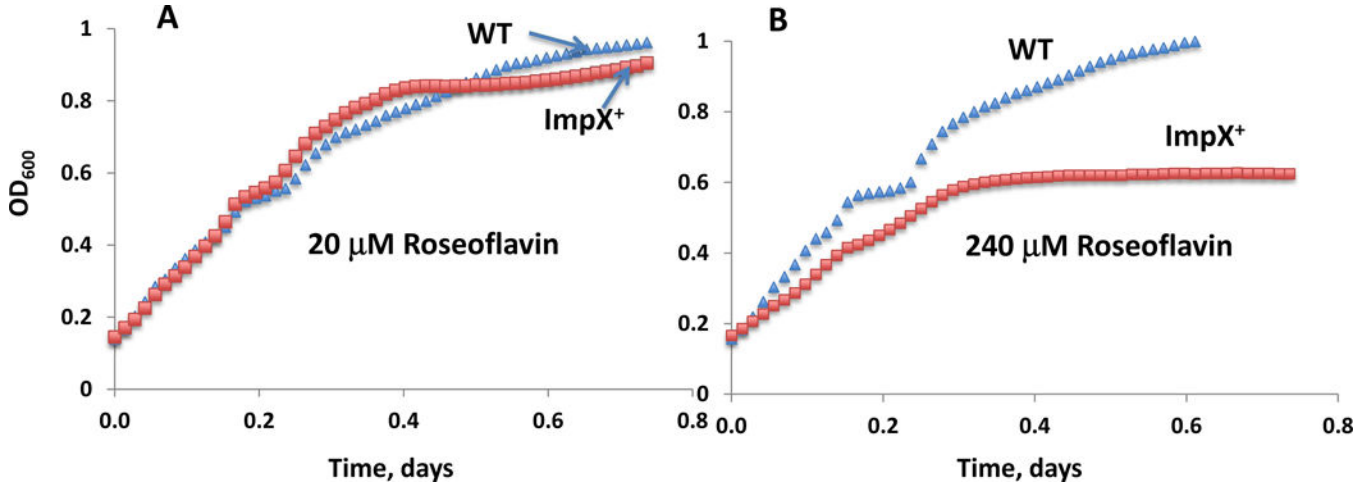

Fig. 4.

Growth inhibition of *E. coli* with roseoflavin at concentrations of 20 μM (**A**) and 240 μM (**B**); WT *E. coli* - blue triangles, isogenic *impX*$^{+}$ strain of *E. coli* - red squares. The BW25113 wild type strain was used with conditions as specified under Materials and Methods.

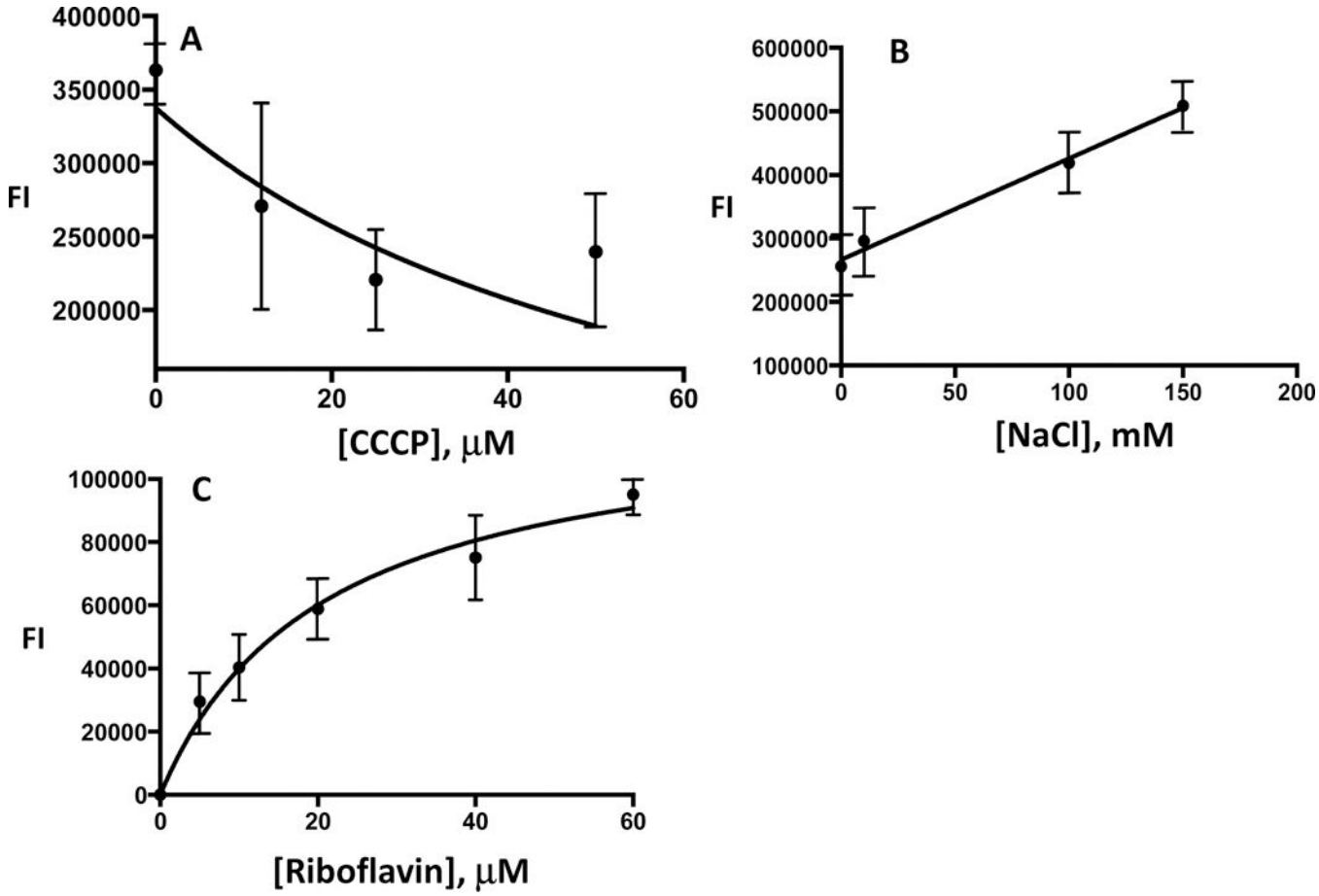

Fig. 5.

Determination of riboflavin (RF) uptake using the fluorescence method. **A.** Dependency on the CCCP concentration. The increase in fluorescence after 4 minutes of uptake was measured using 0–25 μM CCCP. **B.** The dependency of the uptake of RF on the NaCl concentration. The increase in fluorescence after 4 minutes of uptake was measured with 0–150 mM NaCl. **C.** The dependency of uptake on the RF concentration (0–60 μM) giving a calculated K_{half} = 20 μM. The increase in fluorescence after 4 and 8 minutes was measured as described in the text, normalized to a standardized OD_{600}. FI is fluorescence intensity (the FI background for *E. coli* cells was subtracted).

Table 1-

Strains and plasmids used in this study.

Strain or Plasmid	Relevant characteristics	Source
Bdellovibrio exovorous JSS	Wild type	ATCC BAA-2330
Caulobacter crescentus CB15N	Wild type	SDSU, Dr. Shapiro
Escherichia coli BW25113	Wild type	In house
E. coli BSV13	Δ*ribC*	CGSC
Plasmid PKD13	Amp^+ Kan^+ $Temp^-$	In house
Plasmid PCP20	Amp^+ Kan^+ $Temp^-$	In house

Table 2-

Oligonucleotides used in this study

Primer name	Sequence (5’ to 3’)
Bex-Bam-R	ATGGATCCTTAATTTGTATGACGGCGTGAAAGCTG
Bex-Kpn-F	TTAGGTACCATGGGTTTTATCTTTATTATTTTGGG
Bex-ver-R	TCAGGATAAGAAATAAGGAATACG
intS-Bex-P2	GATAGTTGTTAAGGTCGCTCACTCCACCTTCTCATCAAGCCAGTCCGCCCTTAATTTGTATGACGGCGTGAAAGC
intS-ver-R	TCCAAGTCTTAATCGATCGATACTTG
Kt	CGGCCACAGTCGATGAATCC
rib-ver-R	TGATATTCAGCTCTGGCAGGTCGTG
ribC-ver-F	TGATATACTTCTGCACGTGAACAC
ribC1-P1	ATGTTTACGGGGATTGTACAGGGCACCGCAAAACTGGTGTCGATTGACGAGTGTAGGCTGGAGCTGCTTC
ribC2-P2	TCAGGCTTCTGTGCCTGGTTGATTCATGGCATTTTCTCGTGCCGCCAGCACATTCCGGGGATCCGTCGACCTG