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Functional investigation of *Bacillus subtilis* YrkF's involvement in sulfur transfer reactions

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Abstract: Sulfur incorporation into the molybdenum cofactor (Moco) in the Gram-negative bacterium *Escherichia coli* involves six enzymes. The initial reaction includes the cysteine desulfurase IscS, the sulfurtransferase TusA, and the rhodanese domain-containing protein YnjE. The Gram-positive bacterium *Bacillus subtilis* contains no direct homologs for IscS, but rather four distinct cysteine desulfurases (YrvO, NifS, NifZ, SufS) and YrkF, a two-domain rhodanese protein with an N-terminal domain similar to TusA. Bioinformatic analysis was used to identify potential enzymes involved in the *B. subtilis* Moco thiolation pathway and *in vitro* reactions demonstrated that YrkF can accept sulfur from and enhance the activity of YrvO.

Keywords: cysteine desulfurase, YrvO, YrkF, Moco, Bacillus subtilis, persulfide, sulfurtransferase

1 Introduction

Molybdenum is an essential trace element for approximately two-thirds of organisms, including plants, humans, and bacteria [1]. With the exception of Mo-nitrogenase, which utilizes an iron-sulfur cluster-based cofactor, all studied Mo-containing enzymes contain Mo coordinated by two thiolate ligands of an organic pterin molecule to give the molybdenum cofactor (Moco) and its derivatives [1,2]. Moco-containing proteins constitute a wide range of enzymes, including members of the DMSO reductase, xanthine oxidase, and sulfite oxidase families [3]. As Moco cannot be acquired as a nutrient, species producing Moco-enzymes express biosynthetic enzymes involved in the synthesis of the pterin organic moiety, the insertion of sulfur and Mo to form Moco, and the activation of their respective enzymatic partners [4]. While components in its biosynthetic pathway vary between species, the structure and functionality of the active cofactor is widely conserved [5].

The synthesis of Moco in the Gram-negative bacterium *Escherichia coli* is well understood [4]. The organic scaffolding is synthesized from guanosine triphosphate (GTP) via the intermediates cyclic pyranopterin monophosphate (cPMP) and molybdopterin (MPT) [6], which is subsequently adenylated prior to coordinating Mo [7,8]. The conversion of cPMP to MPT requires the insertion of two sulfur atoms [9], constituting the thiolation branch of Moco biosynthesis (Figure 1). In *E. coli*, sulfur is mobilized from the free amino acid cysteine by the cysteine desulfurase IscS and is passed to MoaD in a reaction involving the sulfurtransferase TusA [10,11]. The rhodanese protein YnjE has also been recognized to participate in the sulfur transfer reaction from IscS to MoaD [12]. Prior to its thiolation, MoaD must be activated via adenylation on its C-terminal glycine residue in a reaction promoted by MoeB [13]. The thiocarboxylated intermediate MoaD then forms a heterotetrameric complex with MoaE, which together comprises the active MPT synthase complex [14]. The substrate cPMP is bound by MoaE and undergoes two rounds of sulfur insertion to form MPT [15].

Despite the presence of active Moco-containing enzymes in Gram-positive bacteria, the biosynthesis of this cofactor remains unexplored in this group of microbes. In this study, we conducted an investigation of biosynthetic components involved in the thiolation branch of the Moco pathway in the Gram-positive bacterium *Bacillus subtilis*. Genomic analysis revealed that *B. subtilis* contains homologs of MoeB, MoaE, and MoaD, but no direct homologs of IscS, TusA, or YnjE (Table 1). Instead, *B. subtilis* contains an array of sulfur transfer proteins and four distinct and biologically active cysteine desulfurases (YrvO, NifZ, NifS, and SufS) known to perform diverse roles in sulfur metabolism [16,17,18].

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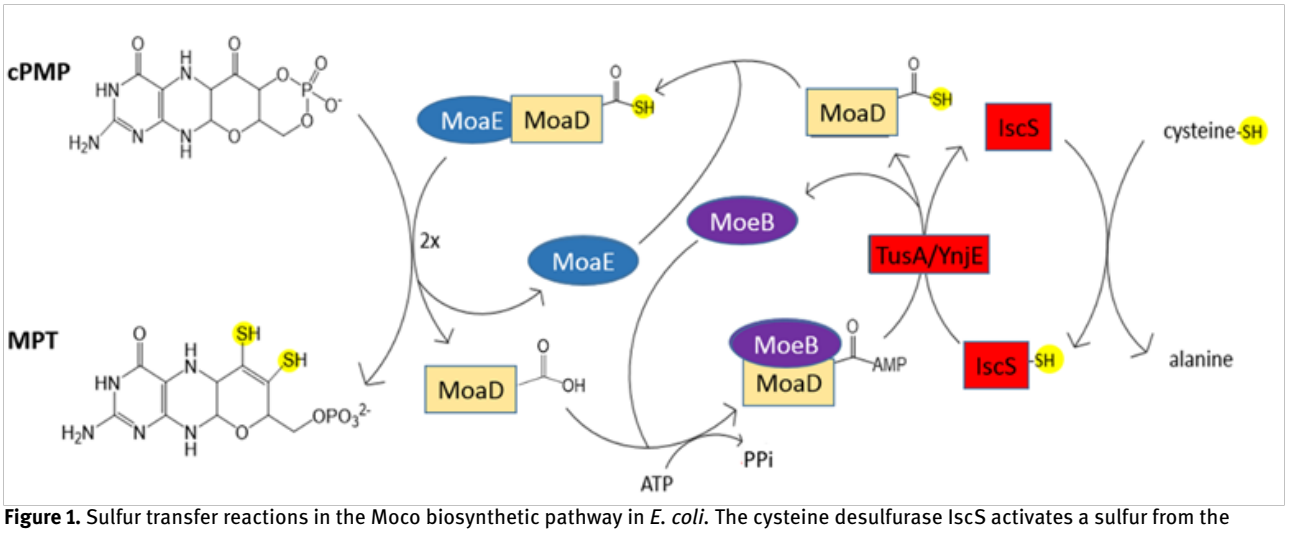

Figure 1. Sulfur transfer reactions in the Moco biosynthetic pathway in *E. coli*. The cysteine desulfurase IscS activates a sulfur from the amino acid cysteine and transfers it to cPMP by a series of sulfur transfer reactions involving enzymes TusA, YnjE, MoeB, MoaE, and MoaD. cPMP is thiolated at two positions to form MPT [4, 9, 10, 12, 14, 15]. Proteins shown in red encode biosynthetic components absent in the *B. subtilis*.

Table 1. Proteins involved in the thiolation branch of Moco biosynthesis in *E. coli* and their orthologs in *B. subtilis*

Escherichia coli protein (locus tag)	Proposed Function	Functional Homolog in Bacillus subtilis	% identity (% similarity)
IscS (b2530)	Cysteine desulfurase	BSU27510 cysteine desulfurase involved in tRNA thiolation (YrvO)	44 (64)
		BSU29590 cysteine desulfurase involved in tRNA thiolation (NifZ)	39 (60)
		BSU27880 cysteine desulfurase (NifS)	35 (54)
		BSU32690 cysteine desulfurase involved in Fe-S cluster formation (SufS)	25 (44)
		BSU02660 putative cysteine desulfurase (YcbU)	32 (55)
YnjE (b1757)	thiosulfate sulfurtransferase	None found	
TusA (b3470)	sulfurtransferase	BSU26530 putative rhodanese-related sulfurtransferase	37 (59)
		BSU26500 hypothetical protein	29 (52)
MoeB (b0826)	Molybdopterin synthase sulfurylase	BSU14270 thiamine/molybdopterin biosynthesis MoeB-like protein	36 (51)
		BSU11700 thiamine/molybdopterin biosynthesis ThiF/MoeB-like protein	29 (48)
		BSU27540 putative enzyme of sulfur-containing coenzyme synthesis	34 (54)
MoaD (b0784)	Molybdopterin synthase small subunit	BSU14310 molydopterin synthase (small subunit)	35 (58)
MoaE (b0785)	Molybdopterin synthase subunit MoaE	BSU14300 molydopterin synthase (large subunit)	35 (54)

Previous work from our group showed that *B. subtilis* cysteine desulfurases control specificity by partnering with specific sulfur acceptor proteins involved in sulfur trafficking reactions in the biosynthesis of thio-cofactors [19]. The cysteine desulfurase NifZ and the thiouridylase ThiI are dedicated partners in mobilization and insertion of sulfur into the C4 position of uridine 8 of tRNA [18]. YrvO directly interacts with the final sulfur transfer protein MnmA to incorporate sulfur into C2 of uridine 34 of tRNA [16]. This abbreviated pathway dispenses the requirement of the TUS sulfur relay system as observed in *E. coli* [20]. Furthermore *B. subtilis* lacks IscS and other ISC components which participate in formation of Fe-S clusters and other thio-cofactors in Gram-negative bacteria [21,22]. Instead, the assembly of Fe-S clusters is catalyzed by the SUF system, and uses SufS as the primary cysteine desulfurase and the zinc-containing sulfur acceptor SufU [17,23]. The presence of a dedicated enzyme for the synthesis of metalloclusters suggests that the biosynthesis of thio-cofactors is partially uncoupled from iron-sulfur cluster biogenesis [19,24]. Here, we sought to conduct an initial investigation to uncover the identity of the cysteine desulfurase and corresponding sulfur acceptor involved in the Moco thiolation branch in *B. subtilis*.

Inspection of the *B. subtilis* genome sequence pointed to the *yrkF* gene, coding a two-domain rhodanese protein similar to TusA [25]. Proteins containing a rhodanese domain are often involved in sulfur transfer for various cellular processes, particularly in pathways which utilize persulfide chemistry [26]. The similarity between *B. subtilis* YrkF and *E. coli* TusA, along with the presence of a rhodanese domain within YrkF and its lack of involvement in 2-thiouridine formation, prompted us to propose that YrkF functions in sulfur transfer to Moco. Here, we demonstrate that *in vitro* YrkF can serve as a sulfur acceptor of both YrvO and NifZ while only enhancing the catalytic turnover rate of YrvO.

2 Methods

Bioinformatics. All bioinformatic work was performed using the National Center for Biotechnology Information (NCBI) and Integrated Microbial Genomes (IMG) databases and was analyzed using Biology Workbench from the San Diego Supercomputing Center.

Protein expression and isolation. *B. subtilis* cysteine desulfurases were isolated as described previously [16,17,18]. YrkF obtained from *E. coli* cultures expressing *yrkF* under a pT7-7 vector was isolated as described by Hunt [25].

^{35}S-sulfur transfer assays. Reactions (60 µL) contained YrvO (4.8 µM), NifS (4.5 µM), NifZ (4.8 µM), or SufS (4.8 µM) in the presence or absence of 48.4 µM or 96.8 µM YrkF in 50 mM Tris-HCl pH 8. Reactions were initiated by the addition of 200 µM L-cysteine and 10 µCi ^{35}S-cysteine (Perkin-Elmer), incubated for 30 minutes at room temperature, and quenched by the addition of 4 mM N-ethylmaleimide. Reactions were then evaluated in non-reducing SDS-PAGE. Coomassie staining enabled detection of protein bands and migration, and ^{35}S-labeled proteins were visualized using a PhosphorImager (Bio-Rad).

Cysteine desulfurase activity. Assays (800 µL) were performed as previously published [22] in reactions containing 0.75 µM YrvO, NifS, or NifZ, or 1.5 mM SufS with 5 molar equivalents of YrkF in the presence of 2 mM DTT at pH 8. Reactions were initiated by the addition of 0.5 mM L-cysteine. Sulfide production was calculated from a freshly prepared standard curve generated with Na_2S. The specific activity was calculated from the rate of sulfide accumulation over time, from at least three time points.

3 Results

YrkF is the nearest homolog to TusA in *B. subtilis*. Initial bioinformatic analysis of *B. subtilis* genome failed to return orthologs of *E. coli* TusA and YnjE, but revealed the presence of the *yrkF* gene, coding for a two-domain protein. The N-terminus domain of YrkF displays similarity to SirA/TusA proteins, having 37% sequence identity and 59% sequence similarity to the *E. coli* orthologous enzyme (Table 1 and Figure 2), while the C-terminal domain shows sequence similarity to a rhodanese domain protein, PspE [25]. In *E. coli*, TusA participates in sulfur transfer reactions in two biosynthetic pathways: Moco and tRNA 2-thiouridine [10]. In both pathways, TusA first interacts with IscS, promoting the sulfur transfer reaction from the enzyme-persulfide intermediate to the next sulfur carrier protein, either MoaD or TusBCD [20]. In 2-thiouridine biosynthesis, TusA interacts with the TusBCD sulfur-relay complex, which subsequently transfers the sulfur to TusE and ultimately to the MnmA thiouridylase [27]. In *B. subtilis*, orthologs of TusBCD and TusE are also absent, and recent work from our group revealed that the cysteine desulfurase YrvO and the thiouridylase MnmA are sufficient to complete 2-thiouridine synthesis [16]. In Moco biosynthesis, *E. coli* TusA also facilitates the sulfur transfer reaction from IscS to MoaD [10].

The similarities between TusA and YrkF, along with the lack of a homolog for the YnjE rhodanese protein, led

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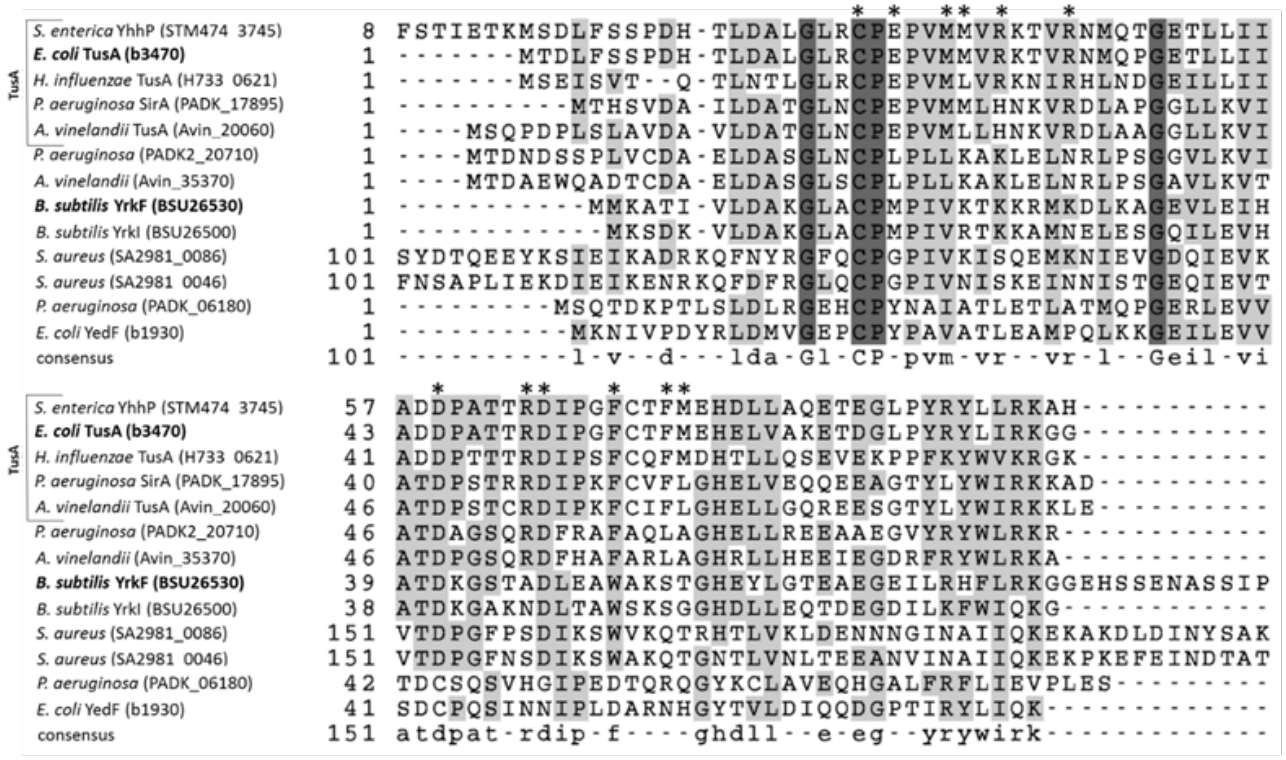

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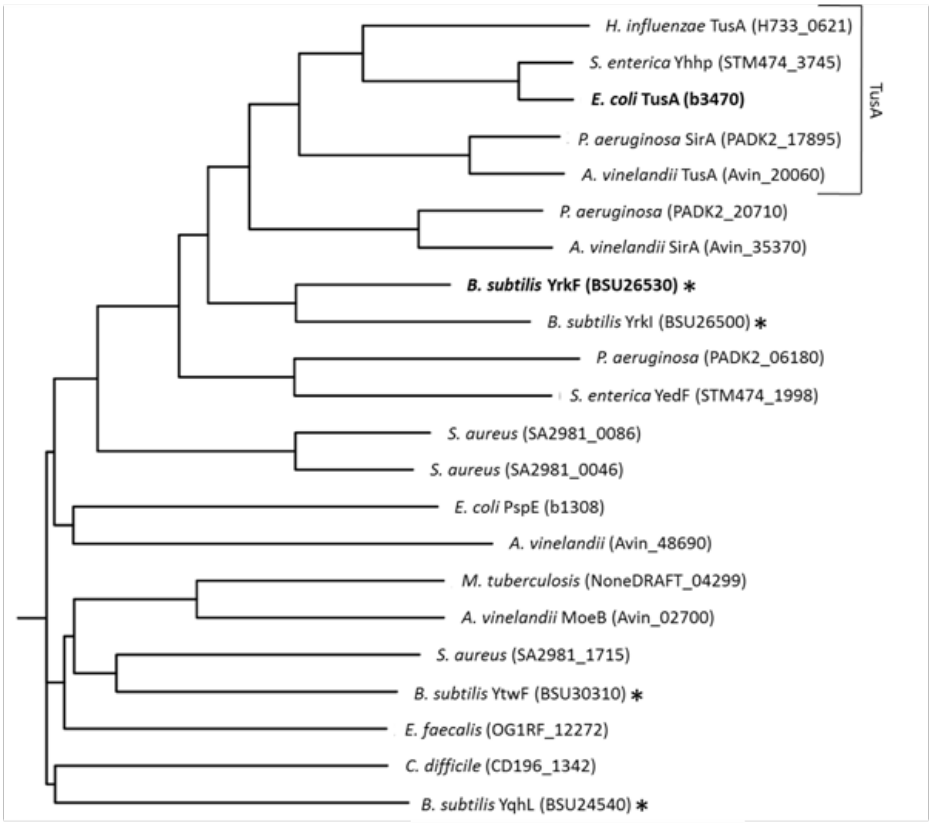

Figure 2. Bioinformatic analysis of TusA homologs. A. Sequence alignment (Gonnet series) generated from a BLAST search of TusA and YrkF against genomes of several species of Gram-positive and Gram-negative bacteria. Starred residues are involved in TusA-IscS interaction [24]. B. Phylogenetic tree showing rhodanese and sulfur transfer proteins with sequence similarity to *E. coli* TusA or *B. subtilis* YrkF. *B. subtilis* rhodanese proteins are starred.

us to investigate whether YrkF could perform analogous functions to those of YnjE and TusA in *B. subtilis*. A phylogenetic analysis shows the relationship between YrkF and TusA homologs in several species (Figure 2B). Sequence alignment of *B. subtilis* YrkF with *E. coli* TusA (Figure 2A) demonstrates conservation of several residues critical for the interaction between TusA and IscS [28]. The active cysteine residue in *E. coli* TusA ($Cys19^{TusA}$) is fully conserved in all species inspected, including *B. subtilis* YrkF ($Cys15^{YrkF}$). The *E. coli* IscS-TusA complex structure shows that $Cys19^{TusA}$ is in close proximity to $Cys328^{IscS}$, supporting the mechanism involving intermolecular persulfide-sulfur transfer between these residues [28]. The partial conservation in YrkF of TusA residues involved in direct interaction with the cysteine desulfurase IscS suggests that YrkF might interact with a cysteine desulfurase similar to IscS in *B. subtilis* during sulfur transfer reactions.

YrkF is an effective sulfur acceptor. The ability of YrkF to serve as an effective sulfur acceptor in *B. subtilis* was initially assessed in ^{35}S-labeling experiments. YrkF was incubated with each of the four cysteine desulfurases (YrvO, NifZ, NifS, and SufS) in the presence of ^{35}S-cysteine. Radiolabeling of YrkF, indicative of persulfide transfer, was observed only in reactions containing NifZ or YrvO (Figure 3). Reactions performed in the presence of NifS and SufS showed ^{35}S-labeling of both cysteine desulfurases, demonstrating the ability of these enzymes to form a persulfide-enzyme intermediate. However in NifS and SufS containing reactions, ^{35}S-labeling associated with YrkF was comparable to the control reaction containing only YrkF and ^{35}S-cysteine, suggesting that these enzymes are not suitable sulfur donors to YrkF. In contrast, significant YrkF labeling was observed in the presence of NifZ and YrvO, indicating efficient persulfide-sulfur transfer from these cysteine desulfurases to YrkF. It is important to note that, the activity of NifZ (V_{max} 350 ± 26 nmol S^{2-} min^{-1} mg^{-1}) [18] under reducing conditions (2 mM DTT) is over 12-fold higher than that of YrvO (V_{max} 28.8 ± 0.5 nmol S^{2-} min^{-1} mg^{-1}) [16] and thus an assay monitoring single turnover conditions may be necessary to discern whether YrvO or NifZ is the preferred sulfur donating partner for YrkF.

YrkF enhances the activity of YrvO. Sulfur acceptor proteins can enhance the reactivity of their cysteine desulfurases, thus increasing the enzyme turnover rate of the second half of the sulfurtransferase reaction

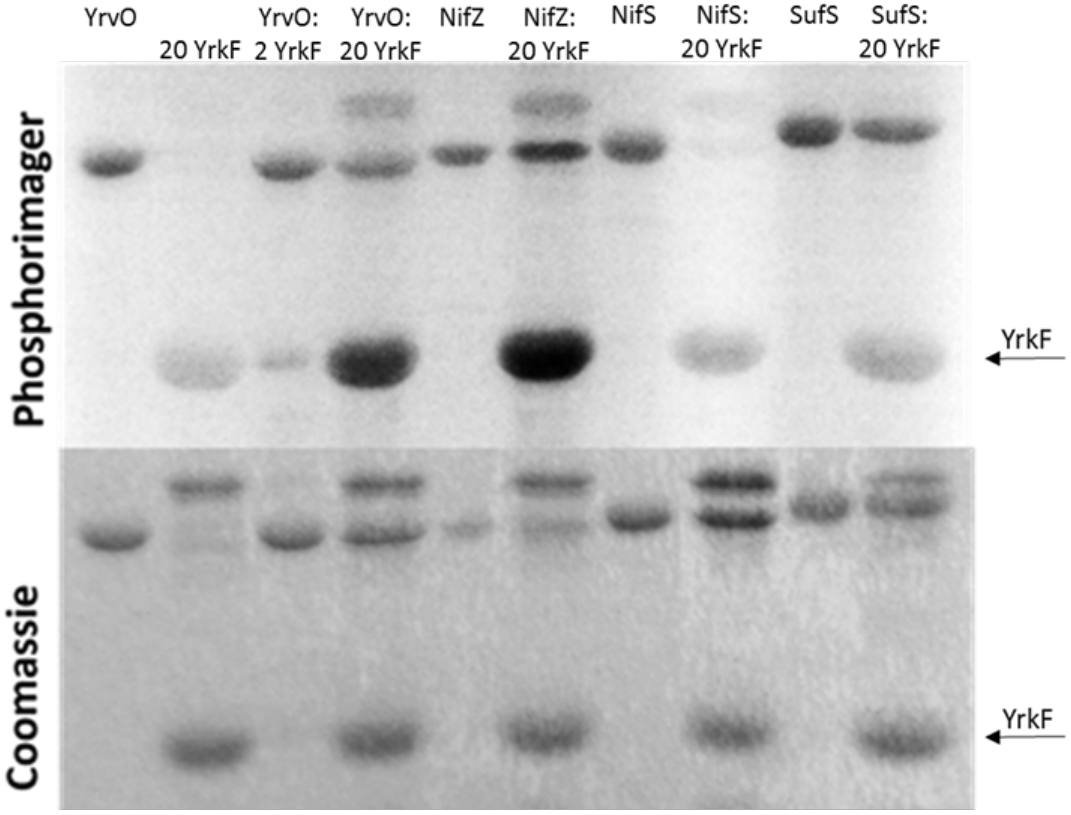

Figure 3: ^{35}S-labeling of YrkF with *B. subtilis* cysteine desulfurases. *B. subtilis* cysteine desulfurases were incubated with ^{35}S-Cys in the presence or absence of 2 or 20 equivalents of YrkF. Following SDS-PAGE (non-reducing) separation, proteins were visualized using a PhosphorImager (top) and a Coomassie stain (bottom).

[16-18,23,29,30]. The effect of YrkF on the activity of *B. subtilis* cysteine desulfurases was determined using sulfide assays. YrkF was found to specifically enhance the rate of sulfide formation by YrvO, but not of SufS, NifS or NifZ. The fold enhancement of YrvO's cysteine desulfurase activity was 2.18 ± 0.55, while YrkF had negligible impact on the activity of SufS (fold enhancement 1.13 ± 0.42), NifS (1.24 ± 0.18), or NifZ (0.97± 0.12) (Figure 4).

4 Discussion

B. subtilis does not contain a direct homolog of *E. coli* IscS, TusA, or YnjE, and the mechanism of sulfur mobilization for Moco biosynthesis is hypothesized to rely on a novel biosynthetic pathway. Of the four cysteine desulfurase enzymes found in *B. subtilis*, YrvO has the most significant sequence similarity to IscS (64%) (Table 1). YrvO is also involved in tRNA 2-thiouridine formation, a pathway known to be connected to Moco biosynthesis in *E. coli* via the shared sulfur intermediate TusA [10]. While a BLAST search of YnjE sequences against the *B. subtilis* genome yields no homologous proteins, sequence analysis revealed the presence of YrkF, a two-domain rhodanese protein with 59% sequence similarity to TusA. Importantly, TusA's catalytic cysteine residue, along with several residues known to interact with the cysteine desulfurase IscS, are conserved in YrkF (Figure 2A). ^{35}S-labeling experiments established that both YrvO and NifZ can effectively transfer sulfur to YrkF, suggesting that either could serve as the *in vivo* partner for sulfur mobilization reactions. However, cysteine desulfurase assays demonstrated that YrkF could enhance the reactivity of YrvO only, suggesting that these two proteins might work together in sulfur transfer reactions in *B. subtilis*, particularly in sulfur transfer to Moco biosynthesis. Our future work is geared towards the investigation of the involvement of YrkF in the biosynthesis of Moco.

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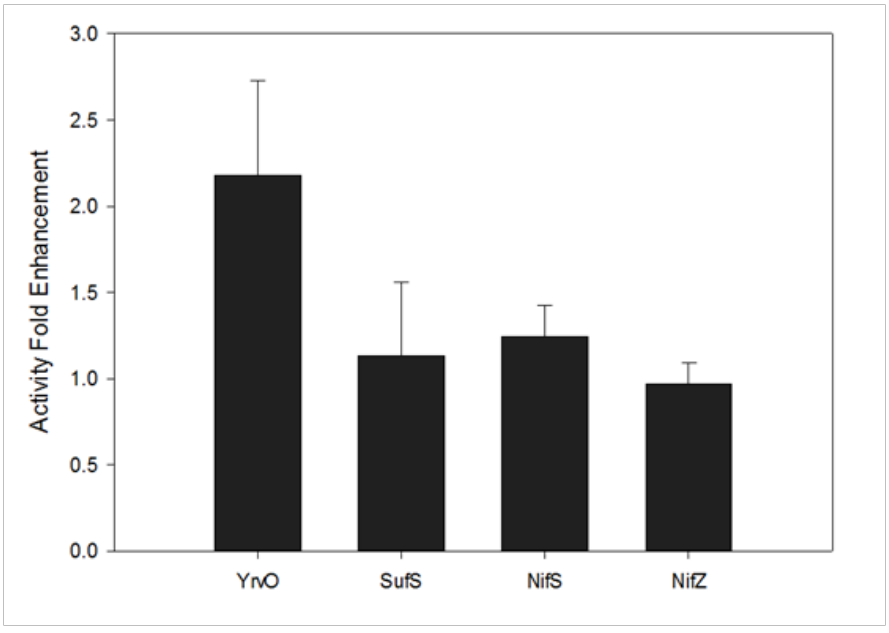

Figure 4: Activity fold enhancement in sulfide assays of *B. subtilis* cysteine desulfurases by YrkF. Cysteine desulfurase activity assays monitoring the production of S^{2-} were performed in the presence or absence of 5 molar equivalents of YrkF. YrkF enhances the activity of YrvO more than two fold, but does not enhance the activity of the other cysteine desulfurases.

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