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CysB-dependent upregulation of the *Salmonella* Typhimurium *cysJIH* operon in response to antimicrobial compounds that induce oxidative stress

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ABSTRACT

It has been proposed that some antibiotics exert additional damage through reactive oxygen species (ROS) production. Since H_2S protects neurons and cardiac muscle from oxidative stress, it has been hypothesized that bacterial H_2S might, similarly, be a cellular protector against antibiotics. In *Enterobacteriaceae*, H_2S can be produced by the *cysJIH* pathway, which uses sulfate as the sulfur source. CysB, in turn, is a positive regulator of *cysJIH*. At present, the role of *S.* Typhimurium *cysJIH* operon in the protection to reactive oxygen species (ROS) induced by antimicrobial compounds remains to be elucidated. In this work, we evaluated the role of *cysJIH* and *cysB* in ROS accumulation, superoxide dismutase (SOD) activity, reduced thiol accumulation, and H_2S accumulation in *S.* Typhimurium, cultured in either sulfate or cysteine as the sole sulfur source. Furthermore, we assessed the effects of the addition of ceftriaxone (CEF) and menadione (MEN) in these same parameters. In sulfate as the sole sulfur source, we found that the *cysJIH* operon and the *cysB* gene were required to full growth in minimal media, independently on the addition of CEF or MEN. Most importantly, both *cysJIH* and *cysB* contributed to diminish ROS levels, increase the SOD activity, increase the reduced thiols, and increase the H_2S levels in presence of CEF or MEN. Moreover, the *cysJIH* operon exhibited a CysB-dependent upregulation in presence of these two antimicrobials compounds. On the other hand, when cysteine was used as the sole sulfur source, we found that *cysJIH* operon was completely negligible, were only *cysB* exhibited similar phenotypes than the described for sulfate as sulfur source. Unexpectedly, CysB downregulated *cysJIH* operon when cysteine was used instead of sulfate, suggesting a complex regulation of this system.

1. Introduction

Sulfur is an essential element required for the biosynthesis of important biomolecules. In prokaryotes, this element can be assimilated into sulfur-containing amino acids through enzymatic fixation from inorganic sources such as sulfate and/or thiosulfate [1,2], from organic sources such as cysteine [3–6] in aerobiosis or through thiosulfate in anaerobiosis [7]. Considering some functional similarities between mammalian gasotransmitters [8–10], we hypothesized that bacterial H_2S may, similarly, be a cellular protector. Accordingly, non-sulfur bacteria with mutations that suppress H_2S production are highly sensitive to several antimicrobial compounds that exert their bactericidal effect via oxidative stress, including several β-lactam antibiotics [11,12]. The converse is

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also true since an *Escherichia coli* (*E. coli*) strain harboring a plasmid coding for proteins involved in H_2S production, presented increased resistance to several antibiotics [4].

In *E. coli* and other non-sulfur bacteria, the main mechanism to produce H_2S is through β-mercaptopyruvate sulfurtransferase (*sseA*) using cysteine or thiosulfate as a sulfur source [6]. An additional sulfate assimilation pathway that might be involved in H_2S production, includes *cysN*, *cysD*, *cysC* and *cysJIH* operon which shown to be expressed in a CysB-dependent manner [13–16]. The genes *cysJ*, *cysI* and *cysH*, encode for a sulfite reductase (beta subunit), a NADPH dependent sulfite reductase (alpha subunit) and a 3′-phosphoadenosine 5′-phosphosulfate reductase, respectively, which participate in the last step of H_2S synthesis in the sulfate assimilation pathway, suggesting that they might be involved in H_2S production in *Salmonella*.

In this study, we found that treatment of wild type *S.* Typhimurium strain with the bactericidal antibiotic ceftriaxone or the reactive oxygen species (ROS) elicitor menadione revealed an up-regulation of several *cys* genes implicated in the sulfur metabolism, contrasting with the main *sseA*-dependent H_2S production pathway described in *E. coli* [6,11]. Therefore, we evaluate whether H_2S plays a role in the resistance of *Salmonella* to antibiotic-derived ROS. An increase in ROS can lead the bacteria to change expression of genes related to detoxifying machinery, as antioxidant enzymes and genes related with cysteine metabolism to maintain cytoplasmic thiol pools [17]. Taken together, our results suggests that CysB regulates expression of *cysJIH* operon to produce H_2S, which could acts as a cellular protector during oxidative stress in *S.* Typhimurium.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. Typhimurium ATCC 14028s and derivatives (Δ*cysB* and Δ*cysJIH*, constructed in this work as described by Datsenko and Wanner [18], using primers *cysbwannerF* 5′ GCTAATCTGGATGATGTATTATGAAATTGCAGCAGCTTCGTGTAGGCTGGAGCTGCTTCG 3′; *cysBwannerR* 5′ GACACAAACCGACGGTGATATTACTTTTCAG GCAGCTTTACATATGAATATCCTCCTTAG 3′; *cysJIHwannerF* 5′ TTACTGGAACATAACGACGCATGACGACACCGGCTCCACTTGTAGGCT GGAGCTGCTTCG 3′; *cysJIHwannerR* 5′ ATCATACCGCGTAAGGACAATTACCCTTCATGCAGCCCGCCATATGAATATCCTCCTTAG 3′) used in this study were grown routinely at 37 °C in Luria Bertani broth (LB) with shaking to OD_{600} 0.45 and changed to M9-medium supplemented with 0.4% glucose and sulfate source (2 mM $MgSO_4$) or cysteine (0.5 mM). Over expressing strains were constructed cloning the *cysJIH* operon or *cysB* in the pBAD TOPO TA vector according to manufacturer's instructions (Invitrogen) using the primers; pBAD*cysJIHF*: 5′ ATGACGACACCGGCTCC ACTGACTG 3′ and pBAD*cysJIHR*: 5′ CCCTTCATGCAGCCCGCAC TCGCGC 3′ or pBAD*cysBF*: 5′ ATGAAATTGCAGCAGCTTCG 3′ pBAD*cysBR*: 5′ CTTTTCAGGCAGCTTTATAT 3′ and transformed into S. Typhimurium ATCC 14028s. For overexpressing strains, 0.8% L-arabinose was added to the culture. When necessary, growth media were supplemented or not (control) with sub-lethal concentrations of CEF (190 nM) or MEN (300 μM) according to MIC determination for all strains in all culture medium used in this work.

2.2. Determination of intracellular ROS concentration

Bacterial cultures were grown as specified above, using either sulfate or cysteine as the sole sulfur source. When necessary, bacterial cultures were exposed to CEF or MEN for 20 min. Bacteria were centrifuged at 13,000 × *g*, washed in 10 mM potassium phosphate buffer (pH 7.0), and suspended in 500 μl of the same buffer containing 2′, 7′-dichlorofluorescein diacetate (H_2DCFA) for total ROS detection as described by Calderón et al. [19].

2.3. Determination of superoxide dismutase (SOD) activity

Bacterial cultures were grown as specified above, using either sulfate or cysteine as the sole sulfur source. Nevertheless, the washing buffer was 10 mM potassium phosphate (pH 8.5) in this case. When necessary, bacterial cultures were exposed to CEF or MEN for 20 min. SOD activity was assessed by measuring the inhibition of the photochemical reduction of nitro blue tretrazolium (NBT) from crude extracts as previously described [20,21]. A SOD unit was defined as the amount of enzyme causing 50% inhibition of NBT reduction.

2.4. Determination of reduced thiols

Bacterial cultures were grown as specified above, using either sulfate or cysteine as the sole sulfur source. Nevertheless, the washing buffer was 100 mM Tris–HCl (pH 8.0) in this case. When necessary, bacterial cultures were exposed to CEF or MEN for 20 min. Reduced thiols were quantified using Ellman's reagent (DTNB) according to protocol described in Ref. [22] with some modifications. Briefly, after treatment cells were suspended in 500 μl of 50 mM Tris–HCl buffer (pH 8.0) and disrupted by sonication in ice (30s, 3 pulses). Then, 240 μl of crude extract were added in a microplate containing 10 μl of 10 mM DTNB and 40 μl of 1 M Tris–HCl buffer (pH 8.0). Absorbance at 420 nm was measured and the result was normalized by μg of protein.

2.5. RNA isolation and mRNA detection

Total RNA extraction and qRT-PCR analyzes were performed as previously described [19]. Briefly, RNA was obtained with the acid-phenol method from bacterial cultures grown as specified above, using either sulfate or cysteine as the sole sulfur source. When necessary, bacterial cultures were exposed to CEF or MEN for 20 min. Specific primers used for these analyzes were: For *cysJ*; *cysJRTF*: 5′AAGGAGCTGGTTGAGTTGCT 3′ and *cysJRTR*: 5′TTTACCGT CAACCGTCACC 3′. For *cysI*; *cysIRTF*: 5′GCGATTGCCGAGAACG GCAAGCTGG 3′ and *cysIRTR*: 5′CAGGGTGTGCTCCAGCGGCAGATAG 3′. For *cysH*; *cysHRTF*: 5′GCATTGAATGAATTGCCAAA 3′ and 5′CAGT TGGGCGTTGGTTTC 3′. For 16S rRNA gene; *16SFw*: 5′GTAGAA TTCCAGGTGTAGCG 3′and *16SRv*: 5′TTATCACTGGCAGTCT CCTT 3′.

2.6. H_2S production

To monitor H_2S production in *S.* Typhimurium WT and mutant strains, we used a lead acetate detection method [11]. Paper strips saturated by 2% of lead acetate [$Pb(Ac)_2$] were affixed to the inner wall of a cultural tube, above the level of the liquid culture of WT or mutant bacteria. Bacterial cultures were grown as specified above, using either sulfate or cysteine as the sole sulfur source. When necessary, bacterial cultures were exposed to CEF or MEN for 20 min. Stained paper strips were quantified with ImageJ software. The results were normalized per OD.

2.7. Statistics

p values were calculated according the ANOVA test using Bonferroni post-hoc. Values $P < 0.05$ were considered statistically significant.

3. Results

3.1. cysJIH and cysB are required for growth of S. Typhimurium in sulfate medium

To assess whether *cysB* and/or *cysJIH* are required for growth of *S.* Typhimurium, strains containing single gene deletions were generated (Δ*cysB* and Δ*cysJIH*) and growth curves were determined in LB medium. Growth in LB (rich media) shows few differences between all strains tested. Only Δ*cysB* strain suffers a slight delay in lag phase of growth (data not shown). In order to determine whether *cysB* and/or *cysJIH* are required for growth in sulfate (inorganic sulfur source) or cysteine (organic sulfur source), strains were grown to an OD_{600} of 0.45 in LB medium and subsequently changed to M9-sulfate or M9-cysteine medium. Deletion mutant strains of these genes grew defectively in sulfate (Fig. 1A), effect that was opposite in a WT strain overexpressing *cysB* or *cysJIH*. In this sense, when these strains were treated with CEF or MEN lower growth were observed for Δ*cysJIH* strain and similar growth for the Δ*cysB* strain (Fig. 1BC), as compared to the same strains with no treatment (Fig. 1A). In cysteine medium, only Δ*cysB* strain showed a defective growth in absence or presence of CEF and MEN treatment (Fig. 1DEF). For complemented strains, wild type phenotype was restored (data not shown). These results suggest that *cysJIH* and *cysB* are required for bacterial growth, and that differences observed for inorganic and organic sulfur source suggest different pathways for sulfur scavenging.

3.2. Deletion of cysJIH or cysB results in increased ROS, lower SOD activity and reduced thiols

To gain further insight into the roles of *cysJIH* and *cysB* in the oxidative stress response, we measured oxidative stress markers in the different genetic backgrounds. When total ROS levels were evaluated, Δ*cysB* and Δ*cysJIH* strains showed increased level of total ROS in sulfate medium in presence of CEF or MEN as compared to the wild type (Fig. 2A). The SOD activity levels are normally inversely related to the levels of the corresponding ROS. In fact, when ROS levels are higher, lower levels of SOD activity are observed. For the Δ*cysB* and Δ*cysJIH* strains, SOD activity was lower as compared to the wild type strain (Fig. 2B). These results suggest that *cysB* and the *cysJIH* operon participate in ROS degradation when bacteria are grown with a sulfate as a sole sulfur source. Moreover, the reduced thiol content was lower in Δ*cysB* and Δ*cysJIH* strains and higher in overexpressing strains as compared to the wild type strain, only when CEF or MEN was added (Fig. 2C), correlating with ROS level and SOD activity. In M9 + cysteine, total ROS levels were higher only in Δ*cysB* strain (Fig. 2D), but lower than those observed in sulfate medium (Fig. 2A). Few differences were observed for SOD activity in this medium only for Δ*cysB* strain (Fig 2E). No differences in reduced thiol content was observed among strains or treatments; except for the overexpressing *cysB* strain, were we detected an increase amount of reduced thiols (Fig. 2F). These results show that: i) in presence of sulfate as the sole sulfur source, both the *cysJIH* operon and *cysB* play a role in detoxification against ROS; ii) in presence of cysteine as an organic source of sulfur, only CysB seems to be required.

3.3. cysJIH expression is upregulated by CysB in response to ceftriaxone or menadione

In order to correlate a possible regulation of *cysJIH* by CysB in ROS treatment, we evaluated the transcript levels of *cysJ*, *cysI* and *cysH* genes in wild type and Δ*cysB* strains in sulfate medium. The results show that in wild type strain, *cysJ* and *cysH* expression was induced in CEF treatment (2.06 ± 0.44 and 5.3 ± 0.49 fold, respectively) and only *cysH* in MEN treatment (3.8 ± 0.1 fold) and was statistically significant (Fig. 3A). In a Δ*cysB* strain the expression of *cysJ*, *cysI* was decreased (0.47 ± 0.06, 0.57 ± 0.14) and increase for *cysH* in CEF treatment (1.72 ± 0.11) (Fig. 3B) but lower than the wild type strain (Fig. 3A). In MEN treatment a decrease in the expression of the *cysJ* and *cysH* was observed (0.43 ± 0.05 fold

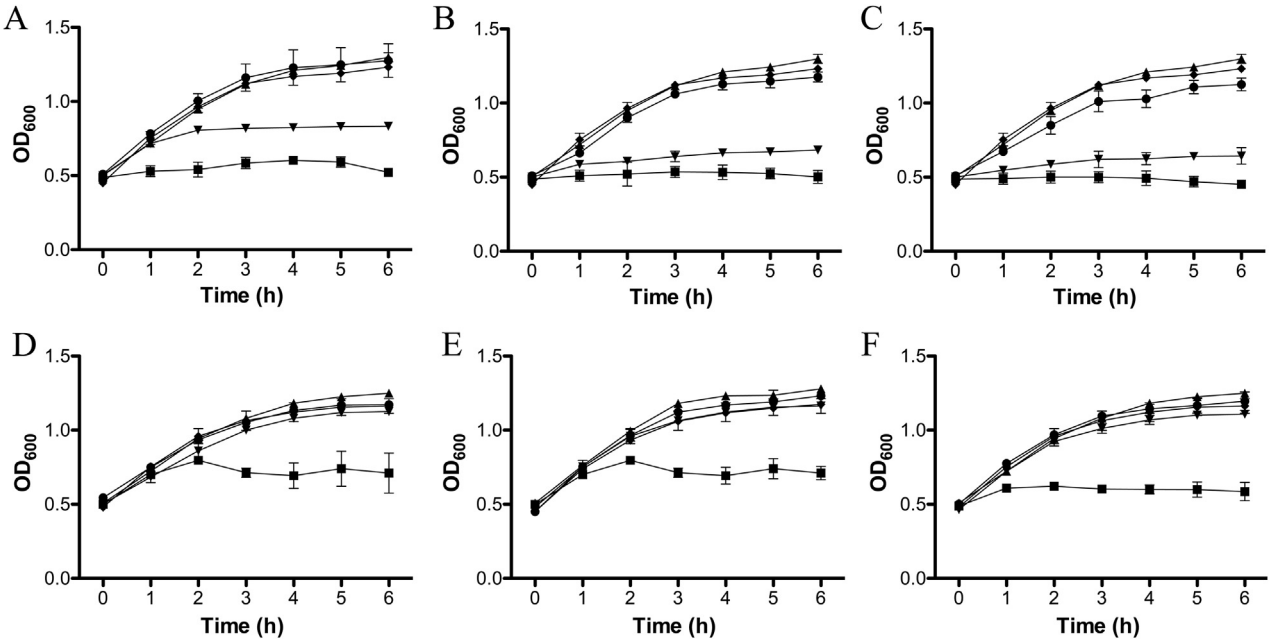

Fig. 1. Growth curves of *S.* Typhimurium wild type, Δ*cysB*, Δ*cysJIH* strains and overexpressing strains. For growth curves, wild type (WT) (circles) Δ*cysB* (squares), Δ*cysJIH* (inverted triangle), WT/pBAD*cysB* (triangle) and WT/pBAD*cysJIH* (rhombus) strains were grown in LB medium up to OD_{600} 0.45 and change to M9-sulfate (A) or cysteine medium (D), LB medium up to OD_{600} 0.45 and change to M9-sulfate or cysteine medium and treated with CEF (B and E, respectively) or LB medium up to OD_{600} 0.45 and change to M9-sulfate or M9-cysteine medium and treated with MEN (C and F, respectively). Error bars indicate SD (n = 6).

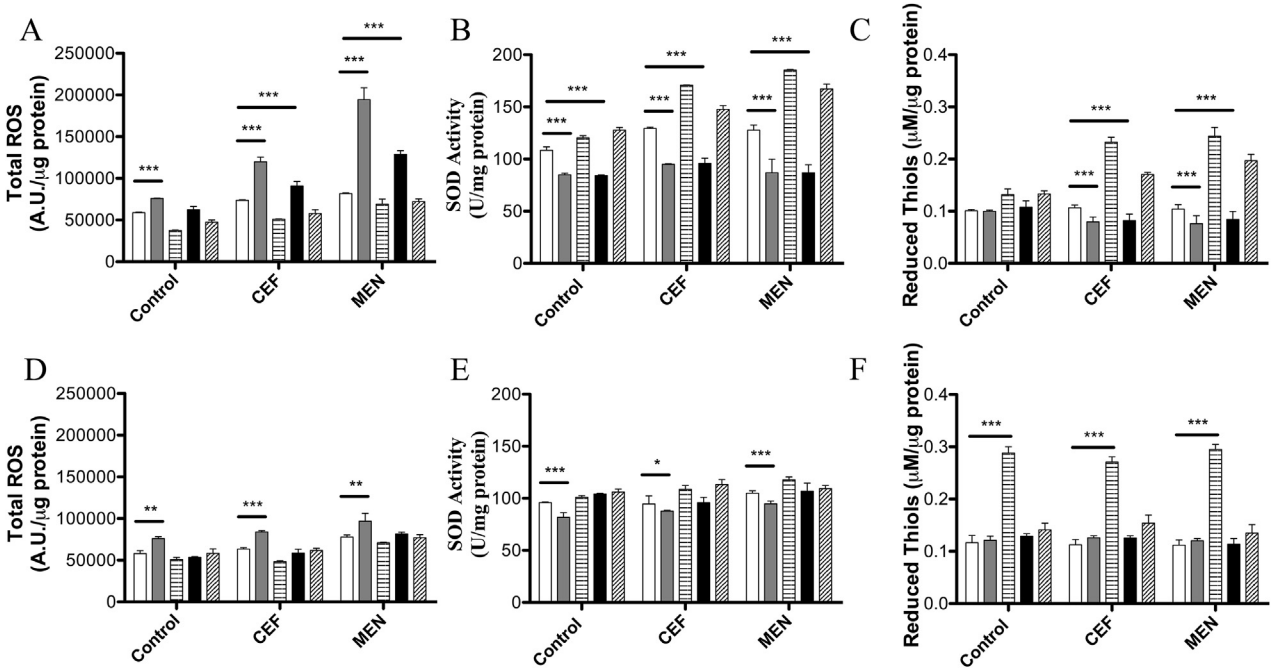

Fig. 2. Total ROS, SOD activity and reduced thiols of S. Typhimurium wild type and mutant strains. For total ROS, SOD activity and reduced thiol content the strains were grown in LB up to OD_{600} 0.45, change to M9-sulfate or cysteine medium and treated with CEF or MEN for 20 min (A) total ROS, (B) SOD activity and (C) reduced thiols in M9-sulfate medium. (D) total ROS, (E) SOD activity and (F) reduced thiols in M9-cysteine medium. For all graphics, Wild type (WT) (white bars) Δ*cysB* (grey bars), WT/pBAD*cysB* (horizontal lines bars), Δ*cysJIH* (black bars) and WT/pBAD*cysJIH* (diagonal lines bars) strains. Experiments were repeated three times and asterisks represent statistically significant differences as compared with untreated cells from each strain (*P < 0.05; **P < 0.01; ***P < 0.001). Error bars indicate SD (n = 6).

and 0.44 ± 0.007 fold, respectively) and increase for *cysH* (1.91 ± 0.13 fold) but lower than the wild type strain (Fig. 3A). In presence of cysteine, the contrary effect was observed. In a wild type strain, expression of *cysJ*, *cysI* and *cysH* remained unchanged or decreased upon treatment with CEF treatment or MEN (Fig. 3C), respectively. In contrast, in a Δ*cysB* genetic background (Fig. 3D) an increased expression of *cysJ* and *cysH* was observed in presence of CEF (1.5 ± 0.03 fold; 1.95 ± 0.08 fold, respectively) or MEN

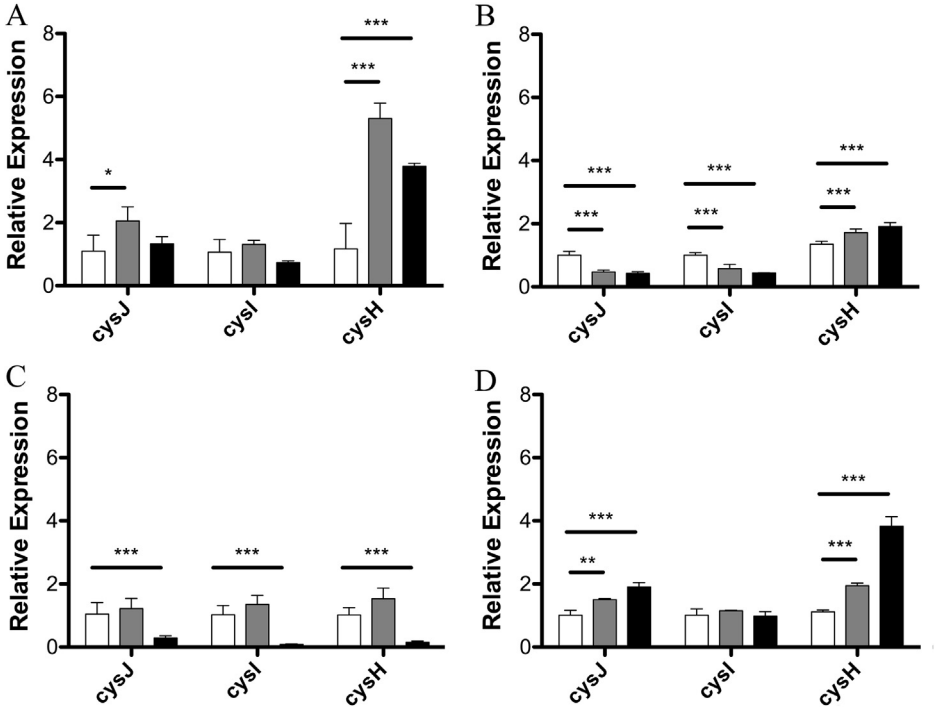

Fig. 3. *cysJIH* expression in a Δ*cysB* against ROS. Wild type and Δ*cysB* strains were grown in LB medium up to OD_{600} 0.45 and change to M9-sulfate (A and B) or cysteine (C and D) medium and treated with CEF or MEN for 20 min. The transcript levels of *cysJ*, *cysI* and *cysH* were detected by qRT-PCR in WT (A and C) and Δ*cysB* (B and D) strains. White bars indicate control treatment; grey bars indicate CEF treatment and black bars indicate MEN treatment. Experiments were repeated three times and asterisks represent statistically significant differences as compared with untreated cells from each strain (*P < 0.05; **P < 0.01; ***P < 0.001). Error bars indicate SD (n = 6).

treatment (1.9 ± 0.14 fold and 3.83 ± 0.3 fold, respectively). These results suggest that *cysB* positively regulates *cysJ* and *cysI* expression in a medium with sulfate and negatively the expression of *cysJ* and *cysH* in medium with cysteine.

3.4. H_2S production by cysJIH correlates with increased resistance to oxidative stress

In order to correlate the oxidative stress resistance with H_2S production, WT, Δ*cysB* and Δ*cysJIH* strains were grown independently in LB to OD_{600} 0.45 and transferred to M9-sulfate or M9-cysteine medium and treated with CEF or MEN. Then, H_2S production was determined by $Pb(AC)_2$ analysis as described in methods. The results show that in sulfate media (Fig. 4A), H_2S levels were increased in the wild type strain in presence of CEF or MEN, and that treatment of Δ*cysB* and Δ*cysJIH* strains with CEF or MEN led to a lower increase in H_2S levels than wild type strain. With cysteine as an organic source, lower levels of H_2S were observed only in strain Δ*cysB* as well as when treated with CEF or MEN (Fig. 4B). These results suggest that the differences observed in resistance to ROS could be related to H_2S production by *cysJIH* operon in presence of sulfate.

4. Discussion

Current antimicrobial therapies, which cover a wide array of bacterial targets [23], fall into two general categories: bactericidal drugs, which kill bacteria with an efficiency of >99.9%, and bacteriostatic drugs, which merely inhibit growth [24]. The bactericidal mechanism(s) of antibiotics is currently attributed to the class of specific drug-target interaction; however our understanding of many bacterial responses that occur as a consequence of this interaction remains incomplete [25–28]. Kohanski et al. [29], proposed a model in which for a bactericidal antibiotic the primary target of interaction stimulates oxidation of NADH via the electron transport chain dependent on the tricarboxylic acid cycle, increasing ROS production. In this sense, several bacterial species could be able to use H_2S as a cellular protector to increase resistance to ROS triggered by bactericidal antibiotics [11]. *Enterobacteriaceae*, including *Salmonella* genus, generates H_2S in their natural environments mainly from thiosulfate [1,4]; however, the biochemistry and physiological roles of this gas in these species remains poorly described [11]. In this work, we propose a model in which H_2S production against ROS depends on the sulfur source and the presence of the transcriptional regulator of the cysteine regulon, CysB, and the products of the *cysJIH* operon. Our results show that the sulfur source is important for *Salmonella* growth. *cysB* was required for growth in all the sulfur sources tested in this work, probably due to the global role of this gene in cysteine regulon expression. Regarding to *cysJIH*, this was required only when the sulfur source was sulfate, probably because this operon acts in the last step of the sulfate assimilation pathway [13]. Our results also strongly suggest that in a Δ*cysJIH* strain, ROS response is diminished probably due to low SOD activity and reduced thiol content in sulfate medium. This effect, as suggested by Shatalin et al. [11], is probably due to a deficient H_2S production and hence a less protector effect. The results showed in Fig. 4A confirm that asseveration. In a Δ*cysB* strain the production of H_2S under CEF and MEN treatment was less than that observed in wild type strain, and in a Δ*cysJIH* strain this effect was similar to the observed for Δ*cysB*, which could suggest the participation of *cysJIH* in sulfate assimilation.

Our results also demonstrated that, in *S.* Typhimurium, sulfate acts as a source of H_2S, depending on CysB and some members of the *cysJIH* operon. *cysJ* and *cysH* genes were overexpressed in presence of CEF and *cysH* in presence of MEN in sulfate media and depend on CysB. However, in the presence of cysteine, only *cysJ* and *cysH* genes had an increased expression in a Δ*cysB* genetic background. This could be explained by previous described experiments [15,30], in which they showed the presence of two binding site for CysB in the *cysJIH* promoter region. One of them (described as CBS-J1) located upstream of −35 box and is described as an activation site, and the other one (CBS-J2) located downstream of −35 box. In presence of high concentrations of CysB, due to an increase in cysteine, CysB might be able to bind to the CBS-J2 site, repressing the expression of the operon. It is noteworthy that the expression levels of each cistron of the *cysJIH* operon were not equivalent, which is in agreement with observations described [31], that analyzed the *E. coli*'s growth phase transcriptome in glucose minimal medium. Using high-resolution transcriptome maps they showed that one-third of *E. coli*'s operons are complex, with internal promoters and terminators generating multiple transcription units and allowing differential gene expression within these operons. They observed differential expression profiles in polycistronic genes, for 43% of the encoded operons, despite being in the same operons. In this sense, in a large proportion (36%), the operons possess a complex architecture with internal promoters or terminators that generate multiple transcription units. These observations indicate that the operon's architecture allows fine-tuning of gene expression warranting further work to fully

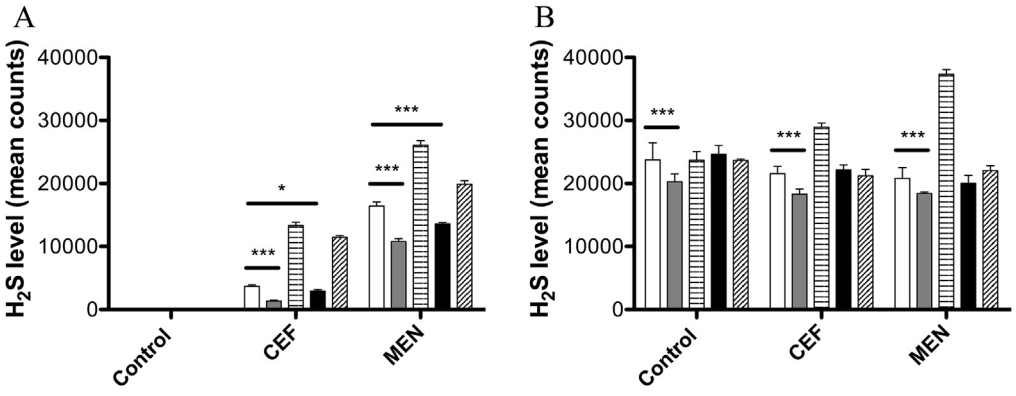

Fig. 4. H_2S production in Δ*cysB* and Δ*cysJIH* strains against ROS. Wild type, Δ*cysB* and Δ*cysJIH* strain were grown in LB medium up to OD_{600} 0.45 and change to M9-sulfate (A) or M9-cysteine (B) medium and treated with CEF or MEN. For all graphics, Wild type (WT) (white bars) Δ*cysB* (grey bars), WT/pBAD*cysB* (horizontal lines bars), Δ*cysJIH* (black bars), and WT/pBAD*cysJIH* (diagonal lines bars) strains. Data were normalized to the control in which no treatment was amended. Experiments were repeated three times and asterisks represent statistically significant differences as compared with untreated cells from each strain (*P < 0.05; **P < 0.01; ***P < 0.001). Error bars indicate SD (n = 6).

elucidate the differential expression of *cysJIH* under antibiotic induced stress.

Our results also relate, for the first time, *cysJIH* with ROS and antibiotic resistance. In this sense, in strains overexpressing *cysJIH* an increased concentration of reduced thiols became evident, which could be compensating a possible fail in removal of ROS by the antioxidant enzyme activity as suggest by Turnbull et al. [17]. This observation is supported by the fact that a Δ*cysK* strain, which should accumulate H_2S, is 3-fold more resistant to ciprofloxacin in comparison to an *S.* Typhimurium wild type strain [32]. Moreover, a transient depletion of free cysteine that acts as a reducing agent that fuels the Fenton reaction [33], would allow bacteria to resist the oxidative stress. The treatment of bacterial infections is becoming more difficult due to a rapid decline of the current antibiotic arsenal, the development of antibiotic resistance and the slow rate of new drug development [34]. Considering that endogenous H_2S might diminish the effectiveness of many clinically used antibiotics, further work could provide new tools and useful information for the development of novel potential therapies against pathogenic bacteria.

Conflict of interest

None.

Acknowledgments

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