Oxidative stress enhances the expression of sulfur assimilation genes: preliminary insights on the *Enterococcus faecalis* iron-sulfur cluster machinery regulation

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The Firmicutes bacteria participate extensively in virulence and pathological processes. Enterococcus faecalis *is a commensal microorganism; however, it is also a pathogenic bacterium mainly associated with nosocomial infections in immunocompromised patients. Iron-sulfur [Fe-S] clusters are inorganic prosthetic groups involved in diverse biological processes, whose in vivo formation requires several specific protein machineries.* Escherichia coli *is one of the most frequently studied microorganisms regarding [Fe-S] cluster biogenesis and encodes the iron-sulfur cluster and sulfur assimilation systems. In Firmicutes* species*, a unique operon composed of the* sufCDSUB *genes is responsible for [Fe-S] cluster biogenesis. The aim of this study was to investigate the potential of the* E. faecalis sufCDSUB *system in the [Fe-S] cluster assembly using oxidative stress and iron depletion as adverse growth conditions. Quantitative real-time polymerase chain reaction demonstrated, for the first time, that Gram-positive bacteria possess an OxyR component responsive to oxidative stress conditions, as fully described for* E. coli *models. Likewise, strong expression of the* sufCDSUB *genes was observed in low concentrations of hydrogen peroxide, indicating that the lowest concentration of oxygen free radicals inside cells, known to be highly damaging to [Fe-S] clusters, is sufficient to trigger the transcriptional machinery for prompt replacement of [Fe-S] clusters.*

Key words: *suf* operon - [Fe-S] cluster assembly - Firmicutes - oxidative stress - iron depletion

Iron-sulfur ([Fe-S]) clusters are ubiquitous inorganic prosthetic groups involved in biological processes as diverse as electron transfer, redox and non-redox catalysis and gene regulation (Frazzon & Dean 2003, Lill 2009). Although [Fe-S] clusters are easily assembled in vitro from elementary $Fe^{2+/3+}$ and S^{2-} in a reductive environment (Malkin & Rabinowitz 1966, Kiley & Beinert 2003), this process in vivo requires several specific protein machineries.

[Fe-S] cluster assembly and delivery are most frequently studied in *Proteobacteria*, for which three types of biosynthetic machineries have been described: nitrogen fixation (*NIF*), iron-sulfur cluster (*ISC*) and sulfur assimilation (*SUF*). The *NIF* system was first described in *Azotobacter vinelandii* and comprises structural and regulatory genes specialised in maturation of nitrogenase and *NIF* (Jacobson et al. 1989a, b, Rubio & Ludden 2008). The *ISC* system, highly conserved in *Proteobacteria*, is encoded by the *iscRSUA-hscBA-fdx* operon and represents the housekeeping genes for Fe/S protein maturation (Zheng et al. 1998). Lastly, the *sufABCDSE* operon encoded by the *SUF* system plays a key role in growth under stressful conditions, such as oxidative and nitric oxide stresses and iron starvation (Takahashi & Tokumoto 2002, Fontecave et al. 2005). Four well-characterised *SUF* regulators have already been described, namely, Fur and the oxidant-responsive (ORE) activators I, II and III, which correspond to the proteins OxyR, IHF and IscR, respectively (Py & Barras 2010). Likewise, c*is*-interacting elements for these regulators have been characterised in *Escherichia coli* (Lee et al. 2004) (Fig. 1). The transcriptional repressor and global regulator of cellular iron, Fur and the activator complex IscR-OxyR-IHF compete for the same promoter region of the *sufABCDSE* operon (Guerinot 1994, Hantke 2001, Schwartz et al. 2001, Outten et al. 2004), thus tightly controlling the expression of *SUF* genes.

The Firmicutes phylum corresponds to a group of bacteria that extensively participates in human virulence and pathological processes. *Enterococcus faecalis* is a commensal microorganism that colonises the mammalian gastrointestinal tract; however, it is also a pathogenic bacterium, mainly associated with nosocomial infections in immunocompromised patients (Fisher & Phillips 2009), such as urinary tract, wound, bloodstream and endocardium infections (Sava et al. 2010). *E. faecalis* virulence is mainly due to factors related to adherence and invasion of host tissues, immunomodulation, toxin-mediated damage and resistance to a wide range of antimicrobial agents (Arias et al. 2010).

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Oxidative stress and iron depletion are common cellular challenges faced by *E. faecalis* upon host invasion (Caza & Kronstad 2013, Winterbourn & Kettle 2013), which suggests an important role for the *SUF* system in pathogenesis. Firmicutes species encode a genetic cluster for [Fe-S] cluster assembly, which is mostly composed of *E. coli SUF* homologues (*sufC*, *sufD*, *sufS*, *sufB*) and an additional *sufU* gene, as well as an *E. coli iscU* orthologue (Riboldi et al. 2009). Recently, the demonstration of complementation *in trans* of *E. coli sufABCDSE* by the *E. faecalis sufCDSUB* suggested a degree of similarity between these machineries (Riboldi et al. 2011). Additionally, the *E. faecalis SUF* gene cluster seems to be transcribed as an operon and potential regulatory *cis*-elements homologous to *E. coli* Fur, IHF and OxyR-binding sequences were identified within the *E. faecalis SUF* promoter region (Fig. 1, Supplementary data).

Considering the high degree of conservation of the *SUF* system in Firmicutes, the sole presence of the *sufCDSUB* operon in *E. faecalis* and the fact that iron depletion and cellular oxidative stress are linked to the regulation of the SUF machinery in well-characterised organisms, the aim of this study was to investigate the potential of the *E. faecalis sufCDSUB* system in [Fe-S] cluster assembly, using oxidative stress and iron depletion as adverse growing conditions.

MATERIALS AND METHODS

Strains and media - *E. faecalis* strain ATCC 51299 (Swenson et al. 1995), here referred to as strain X1, which corresponds to a vancomycin-resistant and clinically relevant strain isolated from peritoneal fluid during a clinical outbreak, was used in this study. Two other *E. faecalis* strains were used to confirm the results obtained with the X1 strain. These are the widely used and well-characterised laboratory strains FA22 (Jacob et al. 1975) and JH22 (Yagi & Clewell 1980). Cultures were performed with brain heart infusion (BHI) broth and/or media. Vancomycin was used when necessary at a final concentration of 30 µg/mL.

Hydrogen peroxide (H_2O_2) and 2',2'-dipyridyl (DIP) challenge conditions - *E. faecalis* strains were grown to an optical density at 600 nm of 0.5, harvested by centrifugation and suspended in 0.9% (w/v) NaCl in the following concentrations of H_2O_2: 2.0 mM, 5.0 mM, 10.0 mM, 20.0 mM, 30.0 and 40.0 mM. Cultures were incubated at 37ºC in a water bath for 30 min and the RNA was subsequently extracted. Iron starvation was induced by the addition of 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM or 1 mM to BHI-broth prior to the incubation of the cultures at 37ºC.

Quantitative real-time polymerase chain reaction (qPCR) procedures - Total RNA was isolated from exponentially growing control and stressed cells, using a CTAB-modified protocol (Salter & Conlon 2007). Two micrograms of total RNA were used for cDNA synthesis using the M-MLV reverse transcriptase (Promega) and random hexamer and/or oligo-dT primers (Invitrogen). *E. faecalis* V583 reference sequences for primer design for *sufC* (GenBank EF2394), *sufD* (EF2393), *sufS* (EF2392), *sufU* (EF2391), *sufB* (EF2390), *kat* (EF1597), *fur* (EF1525), *oxyR* (EF2958), *23S rRNA* (EF23SD), elongation factor for transporter RNA (*tuf*) (EF0201), RNA polymerase beta chain (*rpoB*) (EF3238) and gyrase beta chain (*gyrB*) (EF0005) were obtained from the Institute for Genomic Research (tigr.org). qPCR was performed in an ABI-7500 (Applied Biosystems) thermal cycler using 96-well plates and SYBR green (Bio-Rad) as a dye. Expression levels of *rpoB*, *gyrB*, *tuf* and 23S ribosomal RNA (*23SrRNA*) were evaluated as possible reference controls for data normalisation. The specific linear behaviour of these candidate genes was analysed carefully (see below). PCR conditions were as follows: an initial denaturation step at 95ºC for 3 min followed by 45 cycles at 95ºC for 15 s and 58ºC for 45 s. Melting curves were estimated by the application of an additional step that increased the temperature of the samples from 70-95ºC (1ºC min^{-1}).

Data analysis - Data analyses were performed using the comparative critical threshold ($^{\Delta\Delta}$Ct) method (Livak & Schmittgen 2001). Relative expression levels of strains

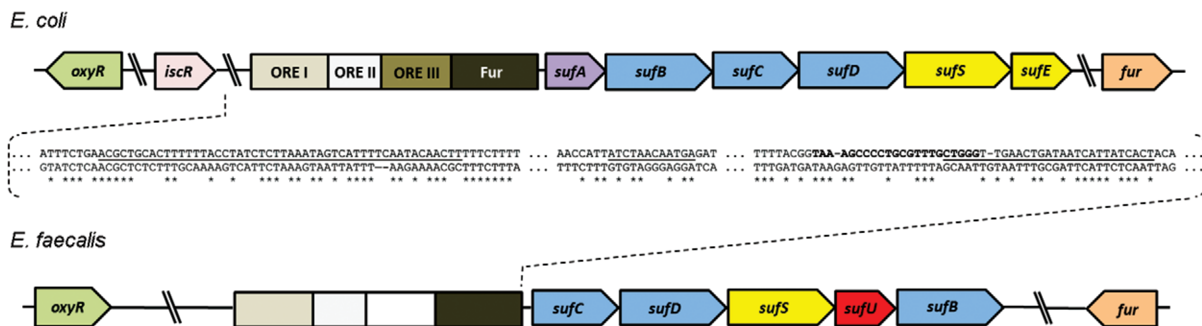

Fig. 1: molecular machinery for the biosynthesis of *SUF* [Fe-S] clusters in *Escherichia coli* and *Enterococcus faecalis* and the genomic localisation of Fur, OxyR and IscR regulators. Genes and *cis*-element regions with similar functions and/or orthologous sequences are depicted by the same colours. The boxes represent promoter-associated *cis*-elements for OxyR [oxidant-responsive (ORE) I], IHF (ORE II), IscR (ORE III) and Fur binding. The inset shows a pair-wise DNA alignment of *E. coli* and *E. faecalis* promoter regions. Underlined sequences, from left to right, correspond to the ORE I, ORE II and Fur elements, respectively, while the DNA region depicted in bold corresponds to the ORE III *cis*-element.

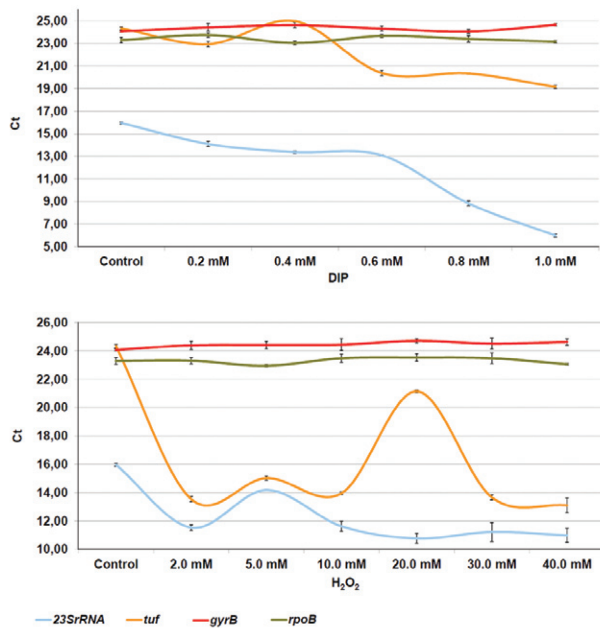

Fig. 2: critical threshold (Ct) values for quantitative polymerase chain reaction of gyrase beta chain (*gyrB*), RNA polymerase beta chain (*rpoB*), elongation factor for transporter RNA (*tuf*) and 23S ribosomal RNA (*23SrRNA*) genes of *Enterococcus faecalis* strain X1 under different simulations of iron depletion (left panel) and oxidative stress (right panel). DIP: 2',2'-dipyridyl; H_2O_2: hydrogen peroxide.

from the control group were calculated in the same way, only discriminating 0 as the value for $^{\Delta}Ct_{stress\ condition}$ during the process of $^{\Delta\Delta}Ct$ determination. The ABI 7500 Real-Time PCR SDS Software v.2.0 was used to analyse thresholds and baselines of individual transcriptional profiles. An internal control was determined using the same method. Experiments were performed in biological triplicate and experimental quadruplicate. The constitutive genes were carefully considered to identify the optimal normalisation gene among the set of candidates by the three following algorithms: NormFinder (Andersen et al. 2004), geNorm (Vandesompele et al. 2002) and BestKeeper (Pfaffl et al. 2004). qPCR results were analysed through one-way ANOVA using the SAS software package and results presenting $p < 0.05$ were considered statistically significant.

RESULTS

Relative expression of the SUF operon: finding appropriate housekeeping genes for data normalisation - In an attempt to find good constitutive gene candidates for our qPCR measurements, we determined the expression profile of *rpoB*, *gyrB*, *tuf* and *23SrRNA* under the different growth conditions studied here. As illustrated in Fig. 2, the linearity observed for the *gyrB* and *rpoB* genes was satisfactory, with a minor variation of 1 Ct among the different cellular growth conditions. However, *tuf* and *23SrRNA* did not exhibit a regular expression pattern, as verified by the wide Ct ranges observed under the various conditions. Additionally, *in silico* analysis of these candidate constitutive genes using the algorithms Normfinder, geNorm and BestKeeper predicted *gyrB* and *rpoB* to be the most stably expressed genes for data normalisation. Similar experiments with *E. faecalis* FA22 and JH22 strains corroborated these results (Supplementary data).

Oxidative stress enhances sufCDSUB expression dramatically - To study the expression pattern of the *E. faecalis* SUF operon under challenging conditions, cultures were subjected to oxidative stress by being exposed to increasing quantities of H_2O_2 and were separately grown under iron limitation with increasing concentrations of the iron chelator, DIP. As demonstrated in Fig. 3, qPCR revealed extremely negative $^{\Delta\Delta}Ct$ values for all H_2O_2 concentrations tested. These values were statistically significant when compared to the control group and indicated a substantial transcriptional increase of these genes, as illustrated by relative gene expression ($2^{-\Delta\Delta Ct}$) in the heat-map shown in Fig. 4. *kat*, a gene whose expression pattern is indicative of oxidative stress, was included in the experiment as a positive control to indicate this growth challenge. As expected, *kat* expression levels were up regulated in all conditions tested, which confirmed that the cells were under oxidative stress. The *sufCDSUB* genes showed a significant transcriptional induction in all H_2O_2 concentrations tested, with an up to 10,000-fold increase in gene expression, when compared to control growth conditions (Fig. 4).

The *oxyR* gene was included in this study after identification of a putative *oxyR* regulatory region in the *E. faecalis* genome similar to the characteristic *cis*-acting responsive element for oxidative stress previously verified in *E. coli* (Supplementary data). Thus, to analyse the putative connection between OxyR and the transcription of the *sufCDSUB* operon, the expression pattern of *oxyR* was also quantified. As expected, upon exposure to H_2O_2 the relative expression of *oxyR* was significantly enhanced (Figs 3, 4). Importantly, the expression profiles obtained for the *E. faecalis* X1 strain were similar to those obtained for the FA22 and JH22 strains (Supplementary data), reinforcing the data presented here.

The challenge of iron-limiting environments - Iron limitation resulted in the least conclusive data, because little effect on *SUF* expression was observed in lower concentrations of DIP. However, an increase in the transcriptional activation of *sufCDSUB* could be clearly identified when 0.6 mM DIP was added to the growth medium (Fig. 4). It seems that the *E. faecalis SUF* operon experiences a decrease in its expression pattern when exposed to increasing iron chelator concentrations, as verified upon exposure to 0.8 mM and 1.0 mM DIP; however, an enhancement in *sufCDSUB* expression was still notable. This same DIP-concentration-dependent decrease in the transcriptional activation of *SUF* could be observed when the data were normalised relative to both the constitutive *gyrB* and *rpoB* genes (Figs 3, 4). *kat* and *oxyR* showed the same expression pattern, with the highest activation in 0.6 mM DIP and subsequent transcriptional decay in increasing concentrations of DIP. Transcription of *sufB* behaved in an unusual manner, reaching a 10-fold transcriptional induction under 0.2 mM DIP; notwithstanding, this signal was lost when data were normalised

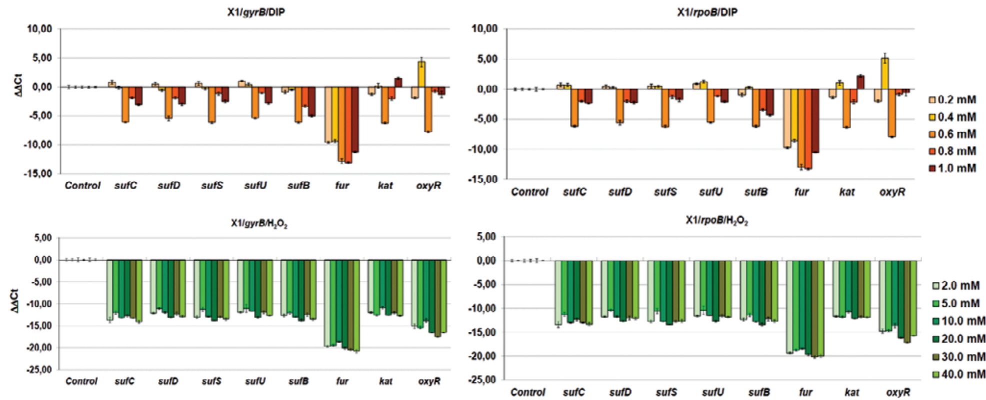

Fig. 3: comparative critical threshold ($^{\Delta\Delta}$Ct) data after normalisation relative to the expression profiles of the constitutive genes gyrase beta chain (*gyrB*) (upper and lower left panels) or RNA polymerase beta chain (*rpoB*) (upper and lower right panels). Graphics represent experiments performed with the *Enterococcus faecalis* X1 strain upon increasing conditions of iron depletion [2',2'-dipyridyl (DIP): 0.2-1.0 mM; upper left and right panels] or oxidative stress [hydrogen peroxide (H_2O_2): 2.0-40 mM; lower left and right panels]. Error bars are also included to indicate data variance.

relative to *gyrB* expression (Fig. 4). Transcription of *fur* showed more than a 1,000-fold increase in gene expression under all concentrations of DIP tested and these results remained highly significant after normalisation to both of the constitutive genes used. However, the pattern of *fur* expression verified for the X1 strain was not reproduced in the FA22 and JH22 strains.

DISCUSSION

This paper describes the transcriptional pattern of genes related to the [Fe-S] cluster assembly in *E. faecalis* cells under oxidative stress and iron depletion conditions. Basic bioinformatics analyses enabled us to identify regions in the *sufCDSUB* promoter region that are similar to the *E. coli SUF* system *cis*-elements involved in the binding of the regulators OxyR (ORE I), IHF (ORE II) and Fur.

In an attempt to further characterise the *E. faecalis SUF* system, the present work demonstrated a concomitant response of *sufCDSUB* and its putative transcriptional modulators, *oxyR* and *fur,* because *sufCDSUB* upregulation always followed an increase in the expression of both regulators. Recently, the viability of *E. coli* double mutants of the *ISC* and *SUF* systems that encoded the *sufCDSUB* gene cluster from *E. faecalis* was described, indicating that the *SUF* systems from both bacteria might act in a similar manner in vivo (Riboldi et al. 2011). The data presented here suggest a similar type of regulation for the *SUF* systems of both *Proteobacteria* and Firmicutes, highlighting an additional degree of homology between both *E. coli* and *E. faecalis SUF* systems. Differences in the expression patterns among the *E. faecalis* strains used here could be due to the genetic diversity of *Enterococcus* isolates obtained from different niches (Vebø et al. 2010), because the X1 strain was originally isolated from a blood infection (most likely presenting a more invasive phenotype), whereas both FA22 and JH22 are laboratory standardised strains. Further experiments are needed to achieve a consistent conclusion about iron depletion and its relation to the *sufCDSUB* [Fe-S] cluster assembly machinery.

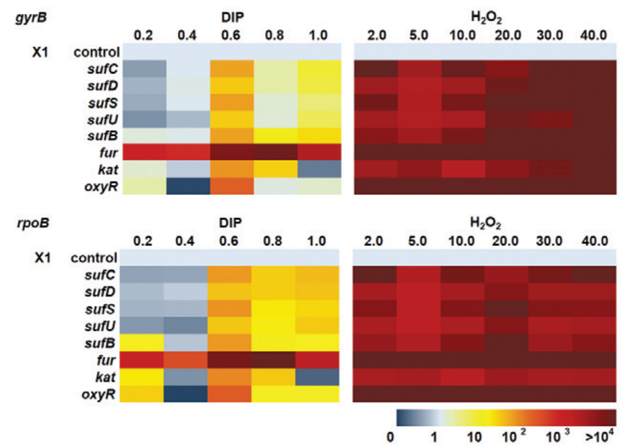

Fig. 4: general relative gene expression patterns ($2^{-\Delta\Delta Ct}$) of *sufCDSUB*, *fur*, *kat* and *oxyR* genes normalised relative to the expression profile of the constitutive genes gyrase beta chain (*gyrB*) (upper left and right panels) or RNA polymerase beta chain (*rpoB*) (lower left and right pannels) for the *E. faecalis* X1 strain. Challenging cellular conditions included increasing conditions of iron depletion [2',2'-dipyridyl (DIP): 0.2-1.0 mM; upper and lower left panels] and oxidative stress [hydrogen peroxide (H_2O_2):2.0-40 mM; upper and lower right panels]. Colours relate to the degree of relative gene expression from absent (blue) to high expression (dark red), as depicted in the label.

Pathogens have evolved complex iron acquisition and oxidative stress response mechanisms to survive and establish infections. Microbial virulence phenotypes have already been associated with the [Fe-S] cluster machinery, as well as with Fur and OxyR regulators and their respective *cis*-elements, which are involved in the regulation of microbial pathogenicity and are important in overcoming host defence mechanisms during both the infection and colonisation processes. Fur is a regulator of the iron uptake and transport systems (Clarke et al. 2001), inhibiting the expression of its targets upon iron binding and thus ensuring the cellular iron homeostasis. For instance, Fur is required for the expression of *Staphylococcus aureus* virulence determinants, such as

cytolysin and immunomodulatory proteins and for protection against host neutrophils (Johnson et al. 2011). Additionally, Fur has been reported to play a role in the virulence of *Bacillus cereus* (Newton et al. 2005), *Listeria monocytogenes* (Harvie et al. 2005) and *Streptococcus suis* (Aranda et al. 2010).

In a similar manner, OxyR has been attributed a role in the virulence processes of *E. coli* (Johnson et al. 2006), *Porphyromonas gingivalis* (Wu et al. 2008) and *Bacteroides fragilis* (Sund et al. 2008). Moreover, OxyR is associated with *Pseudomonas aeruginosa* resistance to human neutrophils (Lau et al. 2005) and the secretion of potent cytotoxic factors (Melstrom Jr et al. 2007). The host colonisation processes in *Klebsiella pneumoniae* (Hennequin & Forestier 2009) and *Xylella fastidiosa* (Toledo et al. 2011) have also been linked to OxyR, which may also be involved in antimicrobial resistance, because its inactivation was shown to increase *Mycobacterium tuberculosis* sensitivity to isonicotinic acid hydrazide (Pagán-Ramos et al. 2006). The well-characterised interaction between OxyR and Fur upon cellular stress could explain the constant association of transcriptional induction between both Fur and OxyR elements verified here.

Because *E. faecalis* is a potentially pathogenic bacterium, the data described here suggest the presence of *SUF* regulators and *cis*-elements similar to those characterised in *E. coli* may contribute to the understanding of the virulence mechanisms employed by this microorganism. Accordingly, our results support involvement of the *sufCDSUB* machinery and its putative regulatory elements Fur and OxyR in *E. faecalis* infectivity, host immune defence evasion and/or colonisation processes, as has been observed for the maintenance of the bacterium redox balance through the activity of lactate dehydrogenase (LDH) (Rana et al. 2013). Those authors demonstrated that LDH is crucial for *E. faecalis* infectivity and resistance to several environmental stresses, such as exposure to H_2O_2. Likewise, *E. faecalis* could potentially be relying on the *SUF* system to maintain an additional level of protection against host defence mechanisms and this hypothesis prompts further investigation.

In summary, the work presented here describes innovative data related to the expression of the *E. faecalis sufCDSUB* biosynthetic machinery involved in [Fe-S] clusters assembly. Moreover, the *SUF* genes are shown to be transcriptionally up-regulated upon exposure to cellular oxidative stress.

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