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Di-iron proteins of the Ric family are involved in iron–sulfur cluster repair

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Abstract A key element in eukaryotic immune defenses against invading microbes is the production of reactive oxygen and nitrogen species. One of the main targets of these species are the iron–sulfur clusters, which are essential prosthetic groups that confer to proteins the ability to perform crucial roles in biological processes. Microbes have developed sophisticated systems to eliminate nitrosative and oxidative species and promote the repair of the damages inflicted. The Ric (Repair of Iron Centers) proteins constitute a novel family of microbial di-iron proteins with a widespread distribution among microbes, including Gram-positive and Gram-negative bacteria, protozoa and fungi. The Ric proteins are encoded by genes that are up-regulated by nitric oxide and hydrogen peroxide. Recent studies have shown that the active di-iron center is involved in the restoration of Fe–S clusters damaged by exposure to nitric oxide and hydrogen peroxide.

Keywords Iron–sulfur · Di-iron · Stress · Bacteria

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Abbreviations

DNIC	Dinitrosyl iron complexes
E.	*Escherichia*
EXAFS	Extended X-ray absorption fine structure
Fe–S	Iron–sulfur
N.	*Neisseria*
NO	Nitric oxide
P.	*Pseudomonas*
R.	*Ralstonia*
Ric	Repair of iron centers proteins
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.	*Staphylococcus*
sp.	Species
XAS	X-ray absorption spectroscopy

Introduction

Iron–sulfur (Fe–S) clusters are considered one of the more ancient catalytic structures which are associated to the origin of life. Found in all life forms, Fe–S containing proteins participate in key cellular processes, e.g., amino acid synthesis, cellular respiration and transcription regulation. In addition to their role in a large spectrum of oxidation–reduction reactions, iron–sulfur proteins are also essential in other physiological functions such as catalysis and sensing of iron and oxygen. Fe–S clusters occur in a large variety of modular structures, being the most

common forms the [2Fe–2S] and [4Fe–4S] centers (Beinert et al. 1997; Kiley and Beinert 2003; Johnson et al. 2005). Although the spontaneous chemical assembly of Fe–S clusters and incorporation into an apoprotein can occur in vitro, by incubation with high concentrations of ferrous iron and sulfide salts under an anaerobic reducing environment, the non-physiological nature of the conditions required makes this process unlikely to occur in vivo. In fact, the biological maturation of Fe–S proteins involves specialized machineries that promote the assembly and insertion of the clusters into proteins and are operative in all life kingdoms (Johnson et al. 2005; Ayala-Castro et al. 2008; Fontecave and Ollagnier-de-Choudens 2008; Lill and Muhlenhoff 2008).

In bacteria, three main Fe–S cluster assembly systems were, so far, identified: the NIF (Nitrogen Fixation), the ISC (Iron Sulfur Cluster) and the SUF (Sulfur assimilation) systems (Fontecave et al. 2005; Johnson et al. 2005; Ayala-Castro et al. 2008; Fontecave and Ollagnier-de-Choudens 2008). Interestingly, it is proposed that ISC and SUF have been inherited by eukaryotic cells, being found in mitochondria and plastids, respectively (Johnson et al. 2005; Pilon et al. 2006; Lill and Muhlenhoff 2008). NIF is associated with the specific maturation of more complex Fe–S clusters, such as those of nitrogenase from *Azotobacter vinelandii*, and ISC and SUF are responsible for the housekeeping Fe–S cluster assembly (Johnson et al. 2005; Ayala-Castro et al. 2008). Despite the differences regarding the function of the proteins that compose each system, two biochemical functions are found in all ISC and SUF systems: (1) a cysteine desulfurase activity, that acts as the sulfur donor for the cluster generation and (2) Fe–S scaffold proteins that provide provisory *loci* where nascent clusters are assembled prior to being transferred to the target Fe–S proteins (Fontecave et al. 2005; Johnson et al. 2005; Ayala-Castro et al. 2008; Fontecave and Ollagnier-de-Choudens 2008). In some organisms (e.g., *Escherichia* (*E.*) *coli*), both ISC and SUF systems are present and the biochemical and genetic analyses performed so far suggest that ISC acts as the main housekeeping Fe–S cluster assembly system, while SUF is a system that becomes active under stress conditions such as iron starvation and/or oxidative stress (Outten et al. 2004; Fontecave et al. 2005; Ayala-Castro et al. 2008). Nevertheless, the simultaneous suppression of the gene clusters coding for the two systems has a lethal effect (Takahashi and Tokumoto 2002).

Other bacterial proteins have also been found to be required for the maturation of Fe–S proteins. *E. coli* was shown to contain another Fe–S cluster assembly system, the CSD system, which is composed of only two proteins that are similar to the SufSE cysteine desulfurase complex of the SUF system. The physiological conditions under which the CSD system is operative remain unknown (Fontecave et al. 2005).

The NfuA family of proteins was recently proposed to be involved in the maturation of the [4Fe–4S] clusters of aconitase-type dehydratases. NfuA from *E. coli* and from *Azotobacter vinelandii* were both shown to bind a $[4Fe–4S]^{2+/1+}$ cluster, and to be more efficient in the transfer of this cluster to an apo-aconitase when compared to the IscU scaffold protein of the ISC system. Currently, these proteins are considered to represent a class of intermediary Fe–S carriers that possibly receive an assembled cluster from the ISC system and transfer it to apo-aconitase and other hypothetical targets. NfuA proteins are also proposed to be important under oxidative stress and iron starvation conditions, by promoting the maturation of damaged Fe–S proteins, like the oxygen sensitive aconitase (Angelini et al. 2008; Bandyopadhyay et al. 2008).

In eukaryotes, frataxin is considered the iron donor for Fe–S cluster assembly and its homologue, the CyaY protein, is currently proposed to perform a similar function in bacteria. In fact, in vitro experiments showed that CyaY is capable of binding and deliver iron for the assembly of Fe–S centers in IscU by a process similar to the one described for IscA. However, while in eukaryotes mutation of the frataxin gene leads to defects in the metabolism of Fe–S proteins, no similar phenotype was observed in bacterial strains with inactivated *cyaY* genes (reviewed in (Ayala-Castro et al. 2008)).

Iron–sulfur clusters are by their nature oxygen-sensitive being rapidly destroyed by reactive oxygen species (ROS) and reactive nitrogen species (RNS). The degree of oxidative/nitrosative damage of the Fe–S clusters is greatly influenced by the surrounding polypeptide matrix (Beinert et al. 1997; Meyer 2008). In general, the more sensitive Fe–S proteins are those that contain solvent exposed clusters, such as the $[4Fe–4S]^{2+/1+}$ family of dehydratases, that include key enzymes of the tricarboxylic acid cycle (e.g.,

aconitase and fumarase A and B) or the branched chain amino acid biosynthesis pathway (e.g., dihydroxyacid dehydratase). The reaction of Fe–S clusters with nitric oxide leads to the conversion of the cluster into stable dinitrosyl–iron complexes (DNIC) that remain bound to the protein, while other RNS and ROS can cause oxidation of the clusters to unstable forms that lead to partial or total disintegration of the centers (Drapier 1997; Imlay 2006). Interestingly, bacteria have evolved mechanisms that profit from the sensitivity of the clusters to oxidative and nitrosative stress, utilizing Fe–S centers as sensory modules of transcriptional regulators that are responsible for activation of genes involved in response to superoxide-induced oxidative stress (SoxR) or to nitric oxide (NsrR), modulating the transition from aerobic to anaerobic metabolism (FNR), or regulating the expression of Fe–S cluster synthesis (IscR) (Kiley and Beinert 2003; Nakano et al. 2006).

A great number of studies have been devoted to the mechanisms of de novo synthesis of Fe–S clusters. Nevertheless, and in spite of being recognized that bacteria can rapidly repair the damage to Fe–S clusters without the need for protein synthesis, the underlying mechanisms remain less understood (Yang et al. 2002; Rogers et al. 2003; Djaman et al. 2004; Justino et al. 2007). This review reports the present knowledge regarding a recently discovered family of microbial proteins that are required for the efficient repair of damaged Fe–S clusters, the Ric family.

The Ric protein family

The Repair of Iron Centers proteins or Ric proteins form a widely spread family found in, at least, five bacterial phyla, in the protozoan pathogen *Trichomonas vaginalis*, and also in some fungi including the pathogen *Cryptococcus neoformans* (Fig. 1). The amino acid sequence comparison of over 100 proteins suggests a subdivision into two groups: one that contains mainly the fungal proteins and the other that includes the majority of the bacterial proteins. In spite of the low amino acid sequence identity and similarity between the two subgroups (ranging from 3 to 11% and from 9 to 22%, respectively), all proteins exhibit a high degree of conservation of residues that are proposed to constitute the ligand sphere of the active di-iron center. The occurrence of Ric paralogs is almost restricted to eukaryotes and it remains to be established if these genes have similar functions.

Studies on the *ytfE* gene, encoding the *E. coli* Ric, raised the hypothesis for a role of this protein in the repair of damaged Fe–S clusters. At the genesis of the proposal were observations of increased sensitivity to nitrosative and oxidative stress and lower enzymatic activity of Fe–S proteins of the *E. coli ric* mutant strain (Justino et al. 2005, 2006).

To access the involvement of *E. coli* Ric in the biosynthesis of Fe–S clusters, cells overexpressing two *E. coli* dehydratases, aconitase B and fumarase A, were submitted to oxidative/nitrosative stress and the fate of the $[4Fe–4S]^{2+/1+}$ center was followed by whole cell EPR. The results showed that the formation of oxidatively damaged Fe–S species (i.e., $[3Fe–4S]^{+}$) and DNICs occurred at a higher rate and to a larger extent in cells lacking an active Ric protein. In agreement, the loss of the enzymatic activities was also more pronounced in the *E. coli ric* mutant (Justino et al. 2007). Furthermore, while in the *E. coli ric* mutant no repair is detected (Fig. 2a), addition of the Ric protein promotes a regain of activity similar to the one observed in the *E. coli* wild type strain (Fig. 2b), clearly showing that the repair is dependent on Ric (Justino et al. 2007). Studies performed with *Staphylococcus* (*S.*) *aureus* ScdA, a Ric ortholog (25% identity to the *E. coli* protein), further confirmed the role of the protein (Fig. 2b) (Overton et al. 2008). In an earlier work, *S. aureus* Ric was proposed to have a role in cell wall metabolism affecting cell division and morphology, since the *S. aureus ric* mutant strain showed aberrant morphological defects (Brunskill et al. 1997). This observation led, in several genomes, to the annotation of *ric* genes as "predicted regulator of cell morphogenesis and cell wall metabolism". However, no morphological differences were detected in our *E. coli* and *S. aureus ric* mutant strains (Justino et al. 2005; Overton et al. 2008), which suggests that the previously observed phenotype is most probably related to polar effects exerted on the gene cluster *lytSR*, which codes for a two-component regulatory system involved in cell wall metabolism, and that in *S. aureus* is located downstream of the *ric* gene.

The study of *Neisseria* (*N.*) *gonorrhoeae* Ric homologue (DnrN, 31% amino acid identity to the *E. coli* protein) corroborated and extended the proposed role of these proteins beyond the repair of

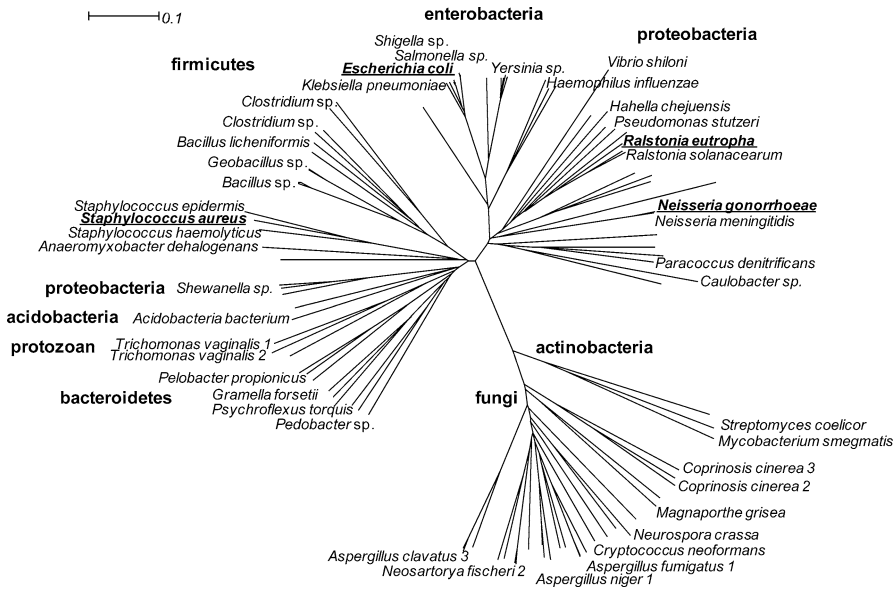

Fig. 1 Distribution of the Ric proteins. Unrooted dendrogram constructed with 102 Ric protein sequences. Kingdom and bacterial phyla are highlighted in *bold* and organisms for which data on Ric proteins is available are *underlined*

[4Fe–4S] clusters of aconitase-type dehydratases, to include the [2Fe–2S] cluster of NsrR and the [4Fe–4S] cluster of the regulator FNR (Overton et al. 2008). It was shown that deletion of the gonococcal *ric* gene affected the regulation of *aniA* and *norB* genes (Fig. 2c). The *aniA* gene is up-regulated by the holo [4Fe–4S]-FNR; upon exposure to a transient NO-stress the cluster of FNR is inactivated by nitrosylation, leading to the down regulation of *aniA* expression in both the parental and mutant *N. gonorrhoeae* strains. After the rapid consumption of NO, the expression of *aniA* returns to the initial levels in the parental strain, due to the repair of the Fe–S center of FNR. However, in the absence of *ric*, the repression of *aniA* is maintained, in agreement with the lack of repair of the FNR nitrosylated Fe–S center. Similarly, nitrosylation causes inactivation of the [2Fe–2S] containing NsrR regulator, with consequent de-repression of the *norB* gene, an NsrR regulated gene. While in the wild type strain recovery of the NsrR cluster allows reactivation of its repressor function, in the mutant strain high levels of *norB* are still observed due to the failure to repair the inactive form of NsrR generated by the stress (Overton et al. 2008).

Before the reports on *E. coli ytfE*, Vollack and Zumft published that *Pseudomonas* (*P.*) *stuzeri* DnrN, a Ric homologue (45% identity to the *E. coli* YtfE), controlled the transcriptional pattern and the mRNA stability of the *nirS* operon, having the *P. stutzeri dnrN* mutant a more prolonged induction of the *nirS* operon after a challenge with nitric oxide (Vollack and Zumft 2001). At the time no explanation for this effect was put forward; however, it can now be interpreted in light of what is currently known about the Ric proteins since the behavior is similar to the above mentioned for the *norB* gene in the gonococcal *ric* mutant.

In the denitrifying bacterium *Ralstonia* (*R.*) *eutropha* the deletion of the gene *norA*, encoding a Ric homologue, causes a three-fold increase in the levels of transcription of the *norAB* operon (coding for a nitric oxide reductase), with consequent increase in the intracellular levels of nitric oxide. The protein shares a high degree of identity with the *E. coli* Ric (49% identity) and also contains a di-iron center (Strube et al. 2007). The protein was shown to ligate nitric oxide, a general characteristic of the di-iron proteins, and its high cytoplasmic concentration ($\sim$20 μM) led the authors to propose that NorA is a NO scavenger (Strube et al. 2007). Nevertheless, no attempt to address a role of this protein in Fe–S assembly was yet reported.

Expression and regulation of Ric genes

The current available data shows that the transcriptome of *E. coli* exposed to NO releasers strongly depends on the experimental conditions used, and

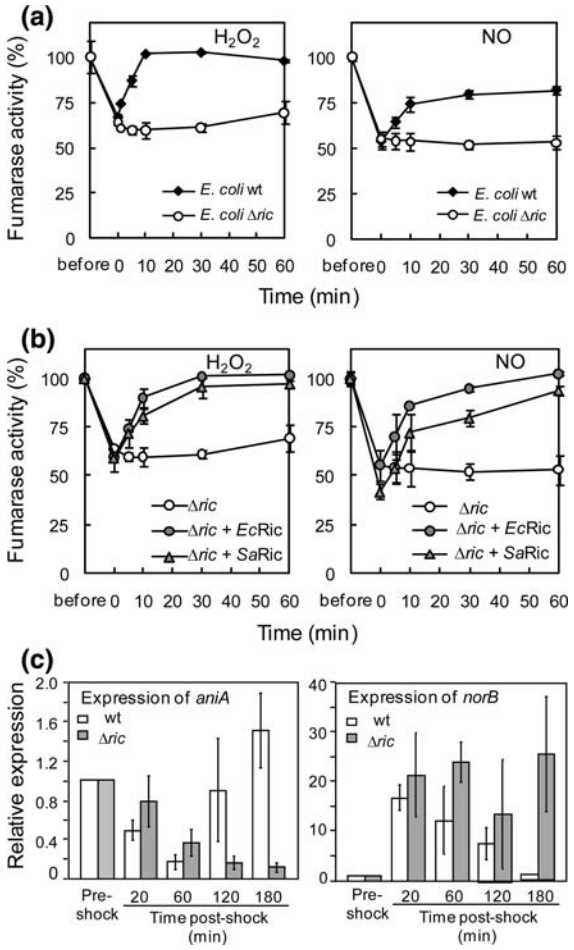

Fig. 2 Repair of Fe–S clusters by *E. coli*, *S. aureus* and *N. gonorrhoeae* Ric proteins. **a** The *E. coli ric* mutant is unable to repair the [4Fe–4S] cluster of the fumarase A enzyme following damage by 4 mM H_2O_2 and 150 μM NO. **b** Addition of *E. coli* and *S. aureus* Ric promotes the repair of the [4Fe–4S] cluster of fumarase A damaged by hydrogen peroxide and nitric oxide. **c** The *N. gonorrhoeae ric* mutant exhibits a slower rate of repair of the NO-damaged [2Fe–2S] cluster of NsrR and of the [4Fe–4S] cluster of FNR, which is reflected in the altered transcription of the FNR-regulated *aniA* gene and the NsrR-regulated *norB* gene. Adapted from Justino et al. (2007) and Overton et al. (2008)

ytfE is included in the short list of genes common to all assays. The high levels of induction exhibited by *ytfE* in all those experiments suggested a role of the gene in nitric oxide metabolism (Mukhopadhyay et al. 2004; Flatley et al. 2005; Justino et al. 2005; Pullan et al. 2007). In organisms other than enterobacteria, namely *S. aureus*, *N. gonorrhoeae*, *N. meningitidis, R. eutropha*, *P. stutzeri* and the pathogenic fungi *Cryptococcus neoformans*, the *ric* gene is also induced by RNS (Pohlmann et al. 2000; Vollack and Zumft 2001; Overton et al. 2006; Chow et al. 2007; Heurlier et al. 2008). The *E. coli ric* gene was shown to be up regulated by nitrate and nitrite, particularly during anaerobic growth, and it was proposed that this regulation was dependent on the levels of NO produced during nitrate/nitrite respiration (Bodenmiller and Spiro 2006; Filenko et al. 2007). The *E. coli ric* gene is also among the group of genes that are induced by heat shock, a condition that causes protein denaturation (Richmond et al. 1999). Furthermore, hydrogen peroxide and iron limitation cause a small induction of *ric* expression (our unpublished results), and in a microarray study of *S. aureus* the *ric* gene was up-regulated by oxidative stress (Chang et al. 2006). Other microarray analyses showed that in *Salmonella enterica* and *Yersinia pestis* the *ric* genes were significantly up-regulated during host-pathogen interaction (Eriksson et al. 2003; Kim et al. 2003; Sebbane et al. 2006).

A bioinformatics study firstly raised the hypothesis that the nitric oxide-sensitive transcriptional regulator NsrR controlled the expression of *E. coli ytfE*, based on the presence of NsrR binding motifs in the *ric* promoter region (Rodionov et al. 2005) and this prediction was then confirmed in *E. coli* (Bodenmiller and Spiro 2006). Like in enterobacteria, the expression of the *ric* genes in *N. gonorrhoeae* and *N. meningitidis* is regulated by the NsrR repressor (Overton et al. 2006; Heurlier et al. 2008).

Interestingly, the regulation of the NO-dependent expression of *ric* seems to be different according to the organism. In *P. stutzeri* the induction of the *ric* gene in response to NO depends on the DnrD regulator (Vollack and Zumft 2001), while in *R. eutropha* the *ric* gene forms an operon with the nitric oxide reductase encoding gene, which is regulated by the nitric oxide sensor NorR, whose encoded gene is upstream and divergently transcribed from the *ric–norB* operon (Pohlmann et al. 2000).

The *E. coli ric* gene was also shown to be de-repressed when FNR and Fur are absent (Justino et al. 2006); nevertheless, further studies have suggested that this is a product of indirect effects, possibly at the level of the regulation of NsrR. In fact, no binding of Fur to the *ric* promoter could be observed (our unpublished results) nor does this promoter contain any canonical FNR binding sites (Constantinidou et al. 2006).

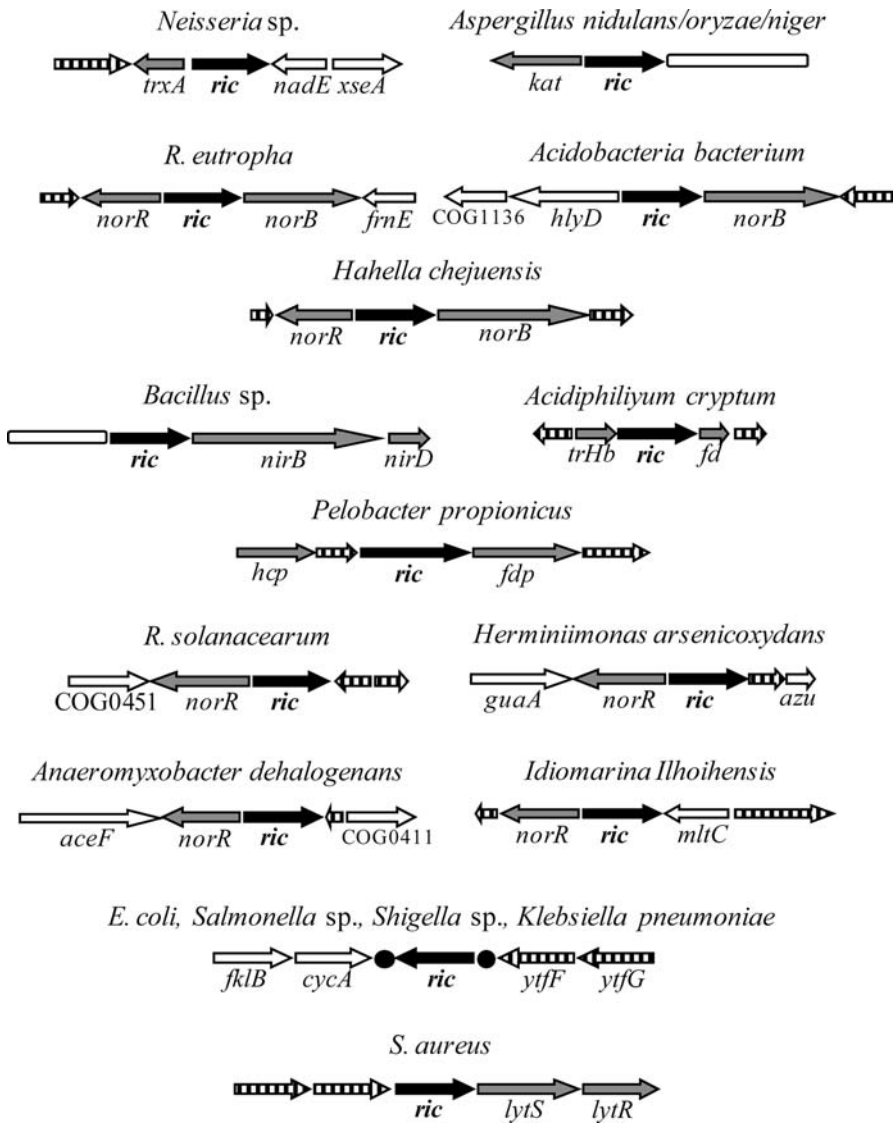

Fig. 3 Genomic organization of the *ric* genes. *Arrows* depict genes, *white blocks* indicate regions not conserved, and *black dots* indicate regions of gene insertion. *Black arrows* indicate the *ric* genes, *striped arrows* represent hypothetical genes, and *grey arrows* highlight genes that encode the following proteins: *fd*, a putative [4Fe–4S] ferredoxin; *fdp*, putative flavodiiron NO reductase; *hcp*, hybrid cluster protein; *kat*, catalase; *lytR* and *lytS*, autolysis regulatory system; *nirB* and *nirD*, nitrite reductase; *norB*, NO reductase; *norR*, NO sensor/regulator; *trHb*, putative truncated globin; and *trxA*, thioredoxin I

A comprehensive analysis of all genomes available shows a high variability of the *ric* gene organization. Figure 3 displays the genomes in which the *ric* gene is located near genes encoding proteins related to oxidative and nitrosative stress. For example, in *Neisseria* sp. a thioredoxin gene is found upstream and divergently transcribed from *ric* while in some fungi a *kat* gene precedes *ric*. In *R. eutropha*, *Acidobacteria bacterium* and *Hahella chejuensis* the gene *norB*, encoding an NO reductase, is located downstream of *ric*. A different organization is found in the *Bacillus* genus, where the genes following the *ric* gene encode a nitrite reductase. In *Acidiphilium cryptum* the *ric* gene is located downstream of a gene encoding a truncated globin, a protein that belongs to a family involved in nitric oxide or oxygen detoxification (Angelo et al. 2008). Interestingly, in *Pelobacter propionicus* Ric contains a putative [4Fe–4S] center in an extra domain in the C-terminal region. In this organism, *ric* is included in a gene cluster that contains *hcp* (encoding the hybrid cluster protein that in *E. coli* affords protection against oxidative stress (Almeida et al. 2006)) and *fdp*, which codes for a putative nitric oxide reductase of the flavodiiron protein family (Saraiva et al. 2004; Vicente et al. 2008).

In the genomes of two *Ralstonia* species, *Herminiimonas arsenicoydans*, *Hahella chejuensis*,

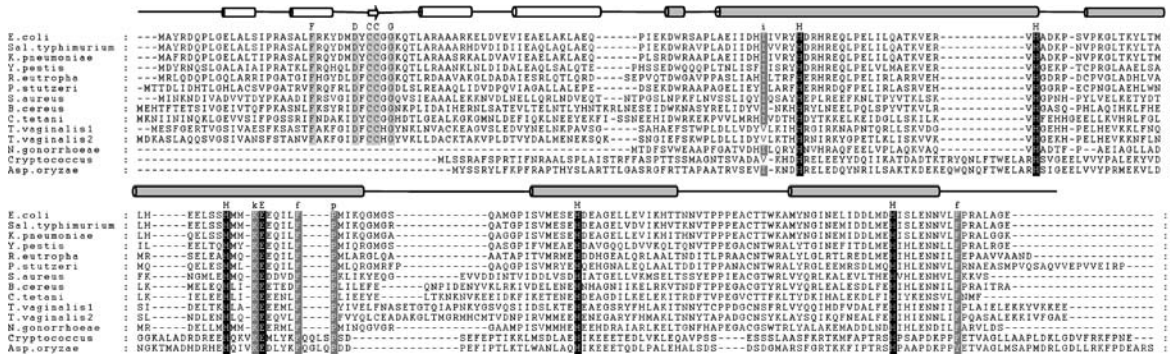

Fig. 4 Amino acid sequence analysis of selected Ric proteins. Organism and protein sequence gi number each corresponding to the abbreviation: E. coli, *E. coli* K-12 MG1655 (16132031); Sal. typhimurium, *Salmonella typhimurium* LT2 (16767645); K. pneumoniae, *Klebsiella pneumoniae* subsp. *Pneumoniae* (150957923); Y. pestis, *Yersinia pestis* CO92 (16123677); S.aureus, *S. aureus* NCTC 8325 (88194036); R. eutropha, *R. eutropha* JMP134 (72122218); P. stutzeri, *P. stutzeri* (11071577); T. vaginalis1 and T. vaginalis2, *Trichomonas vaginalis* G3 (121909109) and (121888849); B.cereus, *Bacillus cereus* ATCC 14579 (30020272); C.tetani, *Clostridium tetani* E88 (28211509); N. gonorrhoeae, *Neisseria gonorrhoeaen* FA 1090 (59717975); Cryptococcus, *Cryptococcus neoformans* var. *neoformans* B-3501A (134106553); Asp. oryzae, *Aspergillus oryzae* (83773074). Residues highlighted in *black boxes* are strictly conserved in all 102 Ric sequences; residues conserved in more than 80% of the sequences are displayed in *dark grey boxes*, and *light grey boxes* indicate residues that are conserved in more than 90% of the sequences. A consensus of the predicted secondary structures is also represented: *Full lines* represent coil regions, *arrows* represent β-sheet and *cylinders* represent α-helical regions. Adapted from Overton et al. (2008)

Anaeromyxobacter dehalogenans and *Idiomarina ihoihensis*, *ric* is downstream and divergently transcribed from the regulator NorR. The same analysis showed that among all presently available genomes, including all enterobacteria, *ric* is never found in the close vicinity of the NsrR regulator.

Biochemical characterization of Ric proteins

Until now, three Ric proteins have been isolated and studied at the biochemical level. The *E. coli* Ric protein (YtfE), the first Ric to be isolated, is a $\sim$25 kDa protein that forms homodimeric complexes (Justino et al. 2006). Dimer formation has also been observed for the Ric proteins of *S. aureus* and *R. eutropha*. In particular, the study of the *R. eutropha* protein revealed that the dimer formation is dependent on the oxidation of cysteine residues and consequent formation of disulfide bridges, since reduction with dithiothreitol promotes the disappearance of the dimer (Strube et al. 2007); this feature was also observed for the proteins of *E. coli* and *S. aureus* (our unpublished results). The two cysteinyl residues of the highly conserved motif (DfCCgG), located at the N-terminal domain of bacterial Ric proteins (Fig. 4), are likely candidates for the disulfide bridging. Nevertheless, the relevance of Ric dimerization for its physiological activity needs further studies since bacterial Ric proteins lacking the N-terminal domain, like the *N. gonorrhoeae* Ric (Overton et al. 2008), still retain the in vivo role in the repair of Fe–S clusters.

The *E. coli*, *S. aureus* and *R. eutropha* Rics are iron proteins containing 2 metal atoms per polypeptide chain. Their characterization by UV/visible and EPR spectroscopies have established that the iron atoms form a non-heme binuclear iron center of the histidine/carboxylate type (Justino et al. 2006; Strube et al. 2007; Overton et al. 2008). The UV/visible spectra of the *E. coli* Ric exhibits a broad band at *c.* 360 nm in the oxidized form, that is bleached when the protein is reduced (Justino et al. 2006). Similar spectral features were described for the two other Ric proteins and are attributed to the absorbance of the (μ-oxo)-bridged diferric center (Kurtz 1997). The EPR spectra of the as-isolated Ric proteins exhibit a single signal with principal g-values below 2.0, typical of a mixed valence antiferromagnetically coupled binuclear iron center in a $S = ½$ state (Kurtz 1997). The di-iron center of *E. coli* Ric has reduction potentials, assessed by an EPR monitored redox titration, of $E = +260$ and $+110$ mV (Justino et al. 2006).

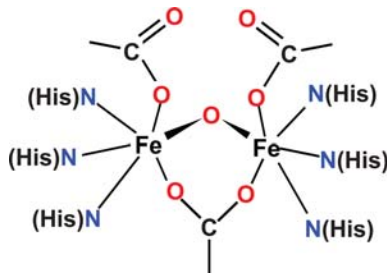

Fig. 5 Model of the *E. coli* Ric di-iron center in the oxidized state. The two upper oxygen ligands of the two iron atoms can be also provided by a single carboxylate group, leading to the formation of a second μ-carboxylate bridge. Adapted from Todorovic et al. (2008)

The *E. coli* Ric was further analyzed by resonance Raman and XAS/EXAFS spectroscopies. These studies led to a model of the center (Fig. 5) in which the di-iron center is bridged by a μ-oxo and one or two μ-carboxo bridges, and coordinated by six histidinyl residues and two or three carboxylate ligands (from aspartate or glutamate residues) (Todorovic et al. 2008).

A comprehensive comparison of the amino acid sequences of all available Ric proteins revealed a total conservation of five histidine residues (His^{84}, His^{105}, His^{129}, His^{160} and His^{204}; numbering refers to the residues of *E. coli* K-12 Ric) and a very strong conservation of Glu^{133} (99 out of 102 sequences), thus constituting good candidates to be involved in the ligand sphere of the di-iron center (Fig. 4). Furthermore, the bacterial Ric proteins have predicted secondary structures consistent with a four helix-bundle fold (Fig. 4), typically present in di-iron proteins (Justino et al. 2005). The binuclear iron center is the active center since the *E. coli* apo-Ric is unable to promote the repair of oxidatively damaged Fe–S cluster (Justino et al. 2007).

In summary, the Ric proteins are the first example of di-iron containing proteins involved in Fe–S biosynthesis and their discovery contributed to increase the knowledge of the mechanisms sustaining the repair of nitrosative/oxidative stress damaged Fe–S clusters. Further studies are required to clarify the molecular basis of the function of Ric proteins.

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