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To link to this article DOI: http://dx.doi.org/10.1128/JB.00527-18

Publisher: American Society for Microbiology

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The di-iron RIC (YtfE) protein of *Escherichia coli* interacts with the DNA-binding protein from starved cells (Dps) to diminish RIC-protein-mediated redox stress

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Abstract

The RIC (Repair of Iron Clusters) protein of *Escherichia coli* is a di-iron hemerythrin-like protein that has a proposed function in repairing stress-damaged iron-sulphur clusters. In this work, we performed a Bacterial Two Hybrid screening to search for RIC-protein interaction partners in *E. coli*. As a result, the DNA-binding protein from starved cells (Dps) was identified and its potential interaction with RIC was tested by BACTH, Bimolecular-Fluorescence-Complementation and pull-down assays. Using the activity of two Fe-S-containing enzyme as indicators of cellular Fe-S cluster damage, we observed that strains with single deletions of *ric* or *dps* have significantly lower aconitase and fumarase activities. In contrast, the double *ric dps* mutant strain displayed no loss of aconitase and fumarase activity with respect to the wild type. Additionally, while complementation of the *ric dps* double mutant with *ric* led to a severe loss of aconitase activity, this effect was no longer observed when a gene encoding a di-iron site variant of the RIC protein was employed. The *dps* mutant exhibited a large increase in ROS levels, but this increase was eliminated when *ric* was also inactivated. Absence of other iron-storage proteins, or of peroxidase and catalases, had no impact on RIC-mediated redox stress induction. Hence, we show that RIC interacts with Dps in a manner that serves to protect *E. coli* from RIC-protein-induced ROS.
Importance

The mammalian immune system produces reactive oxygen and nitrogen species that kill bacterial pathogens by damaging key cellular components such as lipids, DNA and proteins. However, bacteria possess detoxifying and repair systems that mitigate these deleterious effects. The *E. coli* RIC (Repair of Iron Clusters) protein is a di-iron hemerythrin-like protein that repairs stress-damaged iron-sulphur clusters. *E. coli* Dps is an iron-storage protein of the ferritin superfamily with DNA-binding capacity that protects cells from oxidative stress. This work shows that the *E. coli* RIC and Dps proteins interact in a fashion that counters RIC-protein-induced ROS. Altogether, we provide evidence for the formation of a new bacterial protein complex and reveal a novel contribution for Dps in bacterial redox-stress protection.

Keywords

*E. coli*, di-iron RIC protein, YtfE, Dps, oxidative stress, nitrosative stress

Running Title

Di-iron RIC protein interacts with Dps
Introduction

During the infection process, bacterial pathogens are able to survive aggressive environments through the activation of specific stress-resistance genes. One such example of a stress-induced gene is $ric$. This gene encodes the ‘Repair of Iron Centre’ (RIC) protein that contains a di-iron centre and contributes to the protection of bacterial pathogens such as *Escherichia coli*, *Haemophilus influenzae*, *Salmonella* spp., *Yersinia* spp. and *Clostridium* spp. during exposure to nitrosative and/or oxidative stress (1). The $ric$ gene is induced upon exposure to either oxidative or nitrosative stress, and in *E. coli*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *H. influenza* and *Cryptococcus neoformans* the RIC protein is thought to confer stress resistance through maintenance of the activity of various Fe-S containing enzymes (1–3). Such an effect is well demonstrated for *E. coli* and *S. aureus* where RIC proteins restore the activity of oxidatively and nitrosatively-damaged Fe-S clusters in the TCA cycle enzymes, aconitase and fumarase (1, 4, paper Jeff cole). In *E. coli*, the RIC protein also acts under non-stress conditions to maintain aconitase and fumarase activities (5). Further, the *E. coli* RIC protein delivers iron (most likely in the ferrous state) for the assembly of Fe-S clusters in spinach apo-ferredoxin and in the *E. coli* Fe-S cluster-assembly scaffold protein, IscU (6). The RIC protein also contributes to the survival of *S. aureus* and *H. influenzae* in activated macrophages, and is required for full virulence in *S. aureus* when infecting the wax moth larva infection-model, *Galleria mellonella* (3, 7). Thus, the RIC protein has an apparent role in bacterial pathogenicity through mediation of Fe-S cluster stability during exposure to redox- and/or nitrosative-stress.

The RIC proteins of *E. coli* and *S. aureus* contain di-iron centres of the histidine/carboxylate type within a four-helix-bundle fold (8). The UV-visible spectrum of oxidized RIC protein
exhibits a broad band at ca. 350 nm and Electron Paramagnetic Resonance (EPR) spectroscopy indicates that the principal g-values are below 2 (g=1.96, 1.92 and 1.88), which is indicative of a S=½ spin state in a mixed valence and anti-ferromagnetically coupled Fe(III)-Fe(II) binuclear iron centre. Mössbauer spectroscopy showed that the mixed-valence Fe(III)–Fe(II) di-iron centre of the RIC protein is more labile than that of the μ(oxo)-diferric form (6).

RIC proteins possess several highly-conserved amino acid residues of which some have been shown to influence the properties of the di-iron centre and/or function of the protein. In particular, substitution of residues His129, Glu133 or Glu208 of the *E. coli* RIC protein abrogated its ability to protect the Fe-S cluster of aconitase. Moreover, two μ-carboxylate bridges contributed by Glu133 and Glu208, linking the two di-iron site atoms, were shown to be required for the assembly of a stable di-iron centre (9). These studies also demonstrated the important contribution of the conserved His84, His129, His160, His204, Glu133 and Glu208 residues in ligating the di-iron centre within the four-helix bundle fold, and these di-iron coordination roles were recently confirmed by X-ray crystallographic structural studies (10).

In the work reported here, we sought to identify proteins that interact with, and support the function of, the RIC protein of *E. coli*. For this purpose, an *E. coli* library was screened for RIC protein interaction partners using the Bacterial Adenylate Cyclase Two Hybrid system (BACTH). Potential interacting gene products were further tested by BACTH, Bimolecular Fluorescence Complementation (BiFC) and pull-down assays. Our protein-protein interaction studies revealed that the RIC protein interacts with the DNA-binding protein from
starved cells (Dps). Dps is a symmetrical dodecameric iron-storage protein of the ferritin superfamily that contains a di-iron ferroxidation centre located at the interface between subunits (11–13). Dps sequesters ferrous iron, which is oxidized preferentially by hydrogen peroxide at its di-iron centre and then deposited for storage as Fe(III) oxyhydroxide in the central cavity as an iron core; the sequestered iron can subsequently be released by reduction (13, 14). The ferroxidase activity, DNA-binding and iron-sequestration properties of Dps confer cells with protection from oxidative stress and nutrient deprivation, as judged by the reduced survival of *dps* mutants under stress conditions including starvation, oxidative stress, metal toxicity, and thermal stress (15). The physiological relevance of the interaction between the RIC protein and Dps was examined and the results revealed that Dps modulates the function of RIC.

**Results**
Identification of novel potential RIC-protein-interaction partners by screening a bacterial two-hybrid E. coli library

We used a genetic approach to further assess the physiological role of RIC in E. coli, by employing the bacterial two-hybrid (BACTH) system (16) to screen the E. coli genome for gene products that could interact with RIC. For this purpose, RIC was fused to the C-terminus of the B. pertussis adenylate cyclase T25 fragment and used as ‘bait’ to screen previously constructed partial-Sau3A-digested E. coli DNA random libraries that express fusions to the N-terminus of the B. pertussis adenylate cyclase T18 fragment (17). We isolated 22 positive recombinant Lac+ colonies, from which plasmids were purified and then transformed into E. coli DHM1 harbouring pKT25-RIC, or the empty vector pKT25 (negative control), or pKT25-TorD (false positive control), followed by the determination of the β-galactosidase specific activities (Figure 1). Seven pKT25-RIC transformants, harbouring plasmids A to G, exhibited significant β-galactosidase activity indicative of a specific interaction (Figure 1). Nucleotide sequencing followed by BLAST analysis was used to identify the genes within the inserts of these plasmids. Sequencing data revealed that plasmids A to C contain an ~2 kb E. coli DNA fragment upstream of the T18 Cya domain, and that all cases included the complete efp and ecnA genes, and part of the ecnB gene. The efp gene encodes the elongation factor EF-P, a translation factor that facilitates the in vitro the formation of the first peptide bond during translation (18, 19). The gene cluster ecnAB expresses two small cell-membrane associated entericidin lipoproteins, forming EcnAB a toxin-antitoxin module that regulates a programmed bacterial cell death under high osmolarity conditions, with EcnA acting as the antidote for the bacteriolytic entericidin, EcnB (20).
The other four plasmids D to G also contained a ~2 kb insert located upstream of the T18 Cya domain, but in these cases the inserts carried the entire rhtA gene, encoding an inner-membrane transporter involved in resistance to homoserine/threonine (21), and the dps gene, encoding the DNA-binding and iron-storage protein from starved cells (11). Like RIC, *E. coli* Dps has been implicated in oxidative-stress protection, which raises the possibility of a functional association between these two proteins that might be dependent on their direct interaction. For this reason, the potential interaction between the two proteins was investigated further in order to establish its validity and determine its physiological purpose.

**E. coli** RIC protein interacts with Dps

To determine whether the interaction between the RIC protein and Dps, as identified through the screening of the pUT18 library, is indeed genuine, further BACTH experiments were performed. To enable such experiments, the gene encoding the RIC protein was cloned into pUT18C and pUT18 vectors (to create T18-RIC and RIC-T18 fusions), and the dps-coding region was introduced into the pKNT25 vector (to give Dps-T25 fusions), following which the β-galactosidase activities of the corresponding co-transformants were measured. High β-galactosidase activities were recorded for both sets of the RIC-Dps BATCH combinations tested, with activities 4-6 times greater than those of the controls (Figure 2A), indicative of interaction between the RIC protein and Dps within the cytosol of *E. coli*.

A second approach was used to test the proposed RIC-Dps interaction, which involved a Bimolecular Fluorescence Complementation (BiFC) assay. In this method, one of the two proteins of interest is fused to the N-terminal half of the green fluorescent protein (GFP), and the other protein of interest is fused to the C-terminal half; the assay depends upon an
interaction between the two proteins that promotes the reassembly of the two halves of GFP such that emission of fluorescence is restored (22). Thus, GFP fusions (both the N- and C-terminal domains) were generated for both the RIC protein and Dps, and the fluorescence intensity of the corresponding E. coli cells containing plasmids co-expressing the RIC and Dps fusions was measured (Figure 2BC). The data showed that cells expressing RIC<sup>C-GFP</sup> and Dps<sup>N-GFP</sup> exhibit an approximately six-fold higher fluorescence relative to the control, although transformants expressing RIC<sup>N-GFP</sup> and Dps<sup>C-GFP</sup> presented fluorescence levels similar to that of the control samples.

The RIC protein consists of two domains: a short N-terminal ‘ScdA_N’ domain of ~60 residues of unclear function with a highly-conserved pair of Cys residues (10); and a larger C-terminal ‘hemerythrin’ domain of ~140 residues that forms a di-iron centre. We tested the BiFC interaction between Dps and a truncated form of RIC that lacks the so-called first Scd_N domain to determine which of the two RIC protein domains is responsible for the observed interaction with Dps. The results showed that the degree of interaction between the truncated RIC protein and Dps is similar to that observed when using the full-length protein (Figure 2C). Thus, the interaction observed here between the RIC protein and Dps appears to be mediated through the C-terminal hemerythrin domain of the RIC protein.

The interaction between RIC and Dps was also investigated by a pull-down assay. To this end, cells containing plasmids that express non-labelled Dps and N-terminally His-tagged-RIC were treated with formaldehyde, as described in Methods, to promote in vivo cross-linking. The cell extract was loaded into a Ni-chelating column and the His-Tag RIC was eluted at 100 mM of imidazole buffer. The fraction was analysed by SDS-PAGE, and Western blotting in which the E. coli Dps antibody was used. Also, cells expressing only the
non-labelled Dps were treated and analysed similarly to serve as control. The results depicted in Figure 2D show that elution of His-Tag RIC occurred together with a band that has a molecular mass correspondent to that of Dps. This band was proved by Western-blotting to be the *E. coli* Dps (Figure 2D). Therefore, the pull-down assays support the interaction between RIC and Dps.

**Dps modulates the function of the RIC protein in maintaining Fe-S cluster status**

The RIC protein has been linked to the resistance of *E. coli* to oxidative and nitrosative stresses as its inactivation decreases the survival of *E. coli* upon exposure to hydrogen peroxide or nitric oxide donors (4). Due to the interaction of the RIC and Dps proteins shown above, we questioned whether Dps could contribute to the stress protection afforded by the RIC protein. To test this possibility, a Δ*dps* Δ*ric* double mutant was constructed and the growth of *E. coli* wild type, Δ*ric*, Δ*dps*, Δ*dps* Δ*ric* mutants under oxidative and nitrosative stress conditions was tested (Figure 3). The growth experiments showed that inactivation of *ric* resulted in impaired growth under stress conditions imposed by 4 mM H₂O₂ or 250 μM spermine NONOate (Figure 3), which is consistent with previous reports (4). However, the *dps* mutation had little impact on growth under these conditions. Combining the Δ*dps* mutation with the Δ*ric* mutation did not result in any further growth reduction under the same stress conditions, i.e. the Δ*dps* Δ*ric* strain grew similarly to the Δ*ric* strain under the oxidative and nitrosative stress conditions employed (Figure 3). Thus, Dps does not notably compensate for the lack of the RIC protein under peroxide or NO-induced stress.
Another characteristic of the *E. coli* ric mutant is the reduced endogenous activity of Fe-S cluster-containing proteins, such as aconitase and fumarase, that contain solvent-exposed Fe-S clusters with a marked sensitivity to redox and nitrosative stress (4). Therefore, the possible contribution of Dps to this phenotype was explored by comparing the aconitase activity of the Δdps and Δdps Δric strains to that of the wild type and Δric mutant. The results showed that the Δdps mutation caused a 50% reduction in aconitase activity in log phase (Figure 4A), consistent with a role for Dps in maintaining Fe-S cluster status. As expected, a similar effect was observed for the Δric mutant, although the reduction in activity (30%) was only approximately half as great as that observed for the Δdps mutant (Figure 4A). Surprisingly, the ΔdpsΔric mutant exhibited aconitase activity that was higher than that of the corresponding single mutants and similar to that of the wild type (Figure 4A). These aconitase-activity effects were apparent in both the early-log and the post-exponential phase (OD_{600} 0.6 and 2, respectively; Figure 4A and B), suggesting that the phenotype is independent of growth stage (note that *dps* is stationary-phase induced).

Similar effects were observed when testing the activity of another Fe-S enzyme, namely fumarase. The data showed a reduction of 70% in fumarase activity in the Δdps mutant when compared to wild type during the early-log phase (OD_{600}=0.6). Accordingly, in the Δric mutant there was a reduction in fumarase activity of about 40% while the double mutant ΔdpsΔric displayed a fumarase activity similar to that of the wild type (Figure 4C).

The restoration of aconitase and fumarase activity to wildtype levels in the double *dps ric* mutant (with respect to the corresponding single mutants) suggests that the negative impact of the lack of the RIC protein on such activity is dependent on the presence of Dps (and vice-
versa), and this in turn indicates a hitherto unrecognised functional interdependence for these two proteins.

The association of the above aconitase-activity effects with the RIC protein was confirmed by complementation using a multicopy plasmid bearing the wild type ric gene under control of its natural promoter. Complementation of the single Δric mutant led to the recovery of aconitase activity to levels similar to those of the wild type (Figure 4D). More importantly, provision of a wild type version of ric (in multicopy) caused a large (60%) and significant reduction in the aconitase activity of the ΔdpsΔric double mutant (Figure 4D). Thus, as anticipated, the ric-complemented double mutant exhibited the same phenotype as the dps mutant. This confirms that the RIC protein is responsible for decreasing aconitase activity in a dps background.

To investigate whether the role of the RIC protein in lowering aconitase activity in the dps mutant is dependent on a biochemically-functional version of the RIC protein, the ability of a RIC protein variant (lacking a complete di-iron site due to an E133L substitution; (9)), was used in the complementation experiments (Figure 4D). The resulting activity data clearly show that the non-functional E133L-RIC variant does not enable a notable decrease in aconitase activity when expressed in the ΔdpsΔric strain (Figure 4D).

In summary, the above data suggest that in the absence of Dps, the RIC protein has a deleterious effect on aconitase and fumarase activities, but that such an effect is not exhibited when Dps is present. This would imply that the interaction between Dps and the RIC protein, as revealed here, acts to ensure that neither of these two proteins can participate in processes that negatively impact the activity of these Fe-S enzymes.
RIC does not interact with other *E. coli* iron-storage proteins

*Escherichia coli* Dps is an iron-sequestering protein composed of 12 identical subunits forming a shell surrounding a central cavity where up to ~500 ferric iron atoms can be sequestered. As *E. coli* encodes two other iron-storage proteins, namely bacterioferritin (Bfr) and ferritin (FtnA), the possibility that the RIC protein might interact with these other iron-storage proteins was also investigated. Thus, corresponding BiFC experiments were performed in cells carrying recombinant plasmids that express the RIC protein with either Bfr or FtnA, as N- or C-terminal fusions to GFP domains. The resulting fluorescence intensity data failed to support any protein-protein interaction between the RIC protein and Bfr or FtnA (Figure 5A).

In a second set of experiments, the aconitase activity of wild type, Δric, Δbfr, ΔftnA, ΔbfrΔric and ΔftnAΔric strains, grown to the exponential phase (OD₆₀₀ of 0.6), was determined. Similarly to the Δdps strain, the Δbfr and ΔftnA strains both displayed ~50% lower aconitase activity levels (Figure 5B). But contrary to the effect of combining the Δdps and Δric mutations, the combined absence of the RIC protein and the Bfr or FtnA proteins resulted in aconitase activities similar to those present in the correspondent single mutant strains (Figure 5B). Thus, the lower aconitase activity caused by the Δric mutation is not additive with respect to lower activity of resulting from the Δbfr or ΔftnA mutations. Further, it can be concluded that (unlike Dps) Bfr and FtnA do not interact with the RIC protein, and that their absence does not result in a RIC-protein dependent decrease in aconitase activity.
The RIC protein increases intracellular ROS levels when Dps is absent

Dps protects cells from oxidative stress due to its ability to couple the reduction of hydrogen peroxide to water with the oxidation of free-ferrous iron to sequestered-ferric iron. In addition, its association with DNA helps to prevent ROS-induced DNA damage (27). This suggests that the role of Dps in preventing RIC-protein induced inhibition of aconitase activity may arise from the ability of Dps to detoxify ROS that might be produced by the di-iron centre of the RIC protein (e.g. through binding and reduction of oxygen). Therefore, the ROS content of Δric, Δdps and Δdps Δric strains were compared with those found in the wild type to determine whether the presence of the RIC protein, in the absence of Dps, results in raised levels of ROS (Figure 6A). Data show that the wild type and Δric mutant contain similar amounts of ROS while the Δdps strain had significantly higher (~2-fold) levels (Figure 6A). This is as expected given the known role of Dps in redox-stress resistance (27). However, introduction of the ric mutation into the dps mutant eliminated the increased intracellular ROS levels of the single Δdps mutant (Figure 6A). This suggests that the raised ROS levels of the dps single mutant are a consequence of an increase in RIC-protein-dependent ROS production which thus supports a role for Dps in interacting with the RIC protein to restrict its release of ROS species.

To discover whether other elements of the redox-stress resistance response might also act to lessen RIC-protein induced ROS production, the Δric mutation was introduced into a strain (Δhpx) lacking capacity to degrade hydrogen peroxide due to inactivation of both catalase genes as well as the alkyl-hydroperoxide reductase genes (Table 1; (28, 29)). Assay of the resulting aconitase activity levels showed that the ΔhpxΔric quadruple mutant has activity levels similar to those determined for the Δric and Δhpx mutants (Figure 6B). Therefore, we
concluded that the three major peroxidases (KatE, KatG, AhpCF) of *E. coli* are not involved in countering any RIC-protein mediated ROS production, at least under conditions where Dps is active.

**Discussion**

Aconitase and fumarase are enzymes of the TCA cycle that are prone to oxidative stress damage. We previously showed that the di-iron RIC protein repairs these enzymes and is able to transfer iron to Fe-S containing proteins (4-6). In the work described here, we screened an *E. coli* BACTH library in order to identify proteins that interact with the RIC protein and thus might be required to assist its function. As a consequence of our screening, Dps emerged as a RIC protein interaction candidate. This suggested interaction was supported by generation and analysis of additional Dps and RIC protein BACTH constructs and by GFP complementation and pull-down assays. Dps belongs to the ferritin superfamily which led us to investigate the possible interaction of RIC with the two other ferritins present in *E. coli*, namely ferritin and bacterioferritin. However, neither of these proteins were found to interact with the RIC protein or to influence its activity *in vivo*.

We also observed that inactivation of the RIC protein resulted in lower aconitase and fumarase activity, which is consistent with previous findings indicating that this protein contributes to the protection of solvent accessible Fe-S clusters from ROS damage under aerobic growth conditions (5). Similar results were herein obtained for the single mutant strains of *dps*, *ftnA* and *bfr*, indicating that lack of any of these gene products results in lower endogenous aconitase activity. The role of FtnA and Bfr in aconitase protection was
previously demonstrated as the two ferritins promote the reactivation of aconitase activity following stress damage in *Salmonella enterica* serovar Typhimurium (30). In contrast with our findings with *E. coli*, no loss of aconitase activity was observed for *S. enterica* *ftnA* or *bfr* single mutants in the absence of stress; this discrepancy may be related to different physiological roles and expression control of ferritins in *Salmonella* and *E. coli* species (30, 31).

A surprising result was the finding that the defective aconitase activity of the Δ*dps* and Δ*ric* single mutant strains was reversed when these two mutations were combined in the Δ*dpsΔric* double mutant, such that activity was restored to that measured in the wild type. This result, together with the lower amounts of ROS observed in the Δ*dpsΔric* mutant compared to the Δ*dps* mutant, suggests that the RIC protein is responsible for the generation of ROS, but only in the absence of Dps and, thus, that the interaction of Dps and the RIC protein serves to enable Dps to restrict ROS release (which is presumed to damage the Fe-S cluster of aconitase and fumarase, and hence lower the observed activity of these enzymes in a *dps* mutant) by the RIC protein. Interestingly, other redox-stress resistance components (KatE, KatG and AhpCF) failed to impact the RIC-protein-mediated inhibition of aconitase activity (at least in the presence of Dps). These results suggest that the effect of Dps on the ROS-generation activity of the RIC protein is one that is highly specific and not replicated by the other peroxide-consuming cytosolic factors examined. Indeed, the findings relayed here indicate that a direct interaction is required to enable Dps to quench the ROS-generating activity of the RIC protein. The exact mechanism involved in the apparent quenching of RIC-protein-mediated ROS production by Dps is unclear; such understanding will require *in vitro* reaction studies combining the Dps and RIC proteins. However, two possible processes by which Dps could exert a ROS-quenching action upon the RIC protein can be considered: Dps
might sequester iron released from the di-iron site of the RIC protein and thus restrict Fe-
driven Fenton chemistry; or Dps could consume hydrogen peroxide (or hydroxyl radicals;
(14, 32)) generated by the RIC protein through reaction at its di-iron site with molecular
oxygen.

Although the absence of the RIC protein in the presence of Dps resulted in reduced aconitase
and fumarase activity, lack of RIC protein had no impact on ROS levels when Dps was
present. The reason for this effect is unclear but may indicate a role for the RIC protein in
supply of iron from Dps for Fe-S cluster repair and/or synthesis.

The proposed role of the RIC protein (4) is to repair damaged Fe-S clusters of [Fe-S]-
proteins, such as aconitase and fumarase, by donating iron from its di-iron centre leading to
the formation of an intermediate mononuclear iron centre that is prone to react with oxygen
to generate ROS such as hydrogen peroxide. In this process, the interaction with Dps would
fulfil two roles, namely by trapping ROS released by the RIC protein and providing a sink
for iron liberated from the di-iron centre of RIC.

In conclusion, we report an interaction between the Dps and RIC proteins of E. coli which
represents the first example of a protein that interacts with the ferritin-like Dps protein. In
addition, our results indicate that the Dps-RIC protein interaction contributes to the function
of RIC, which is one of the few known bacterial proteins involved in repair.
Materials and Methods

Bacterial strains and growth conditions

*Escherichia coli* strains used in this work are listed in Table 1, and were grown at 37 °C. *E. coli* XL2Blue and *E. coli* reporter strain DHM1 non-reverting adenylate cyclase deficient (cya) were used as host strain and for detection of protein-protein interactions, respectively.

Construction of the *E. coli* double mutant strains was performed by bacteriophage P1-mediated transduction (33), and the corrected mutations were confirmed by PCR using primers listed in Table 2.

*E. coli* cells were grown in LB medium under aerobic conditions in flasks containing a 1/5 volume of culture or under anaerobic conditions in rubber seal-capped flasks filled with medium and extensively bubbled with nitrogen prior to growth. For the stress assays, cells were grown, at 37 °C and 150 rpm, in M9B minimal medium (60 mM K₂HPO₄, 33 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 1.7 mM sodium citrate, 1 mM MgSO₄ and 10 μM MnCl₂, pH 7) supplemented with 10 μg/mL thiamine and 40 μg/mL L-arginine, L-leucine, L-proline, L-threonine and 40 mM glucose. Cultures at an OD₆₀₀ of 0.3 were either left untreated or exposed to 4 mM H₂O₂ for 6 h or to 250 μM spermine-NONOate for 9 h.

BACTH experiments

The Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) system assay (16) was used to identify RIC-interacting proteins. *E. coli* RIC protein was fused to the C-terminal of
**Bordetella pertussis** Cya (adenylate cyclase) T25 domain (pKT25-RIC) and used to screen an *E. coli* MC4100 gene library containing chromosomal fragments fused to the N-terminal of *B. pertussis* Cya T18 domain. The DNA fragments were obtained by partial digestion with Sau3AI and cloning into the BamHI site of pUT18 plasmids (17). About 1 µg of pUT18BamHI DNA library was transformed together with pKT25-RIC into *E. coli* DHM1 cells by electroporation. Blue colonies present in AmpR CmR selective plates (L-agar with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)) were identified after incubation at 30 °C for 36 h, and cells with the highest β-galactosidase were considered to contain recombinant plasmids harbouring genes encoding polypeptides that interact with the *E. coli* RIC protein. Twenty two colonies were obtained and the corresponding plasmids were isolated, co-transformed with pKT25-RIC plasmid in *E. coli* DHM1, and the strength of the protein-protein interactions observed was again estimated by quantification of the β-galactosidase activity. Seven isolates considered positive were named ‘A’ to ‘G’ (Figure 1), and subject to nucleotide sequencing using primer T18Fw (Table 2). To identify the encoded genes, the sequences were screened against the *E. coli* K-12MG1655 genome using BLAST. Genes coding for the RIC protein and Dps were PCR amplified from *E. coli* K-12 genomic DNA using the oligonucleotides described in Table 2, and cloned into pKT25 (fused to Cya C-terminal T25 domain), pKNT25 (fused to Cya N-terminal T25 domain), pUT18 (fused to Cya N-terminal T18 domain) and pUT18C (fused to Cya C-terminal T18 domain) plasmids, and the enzyme *Pfu* DNA polymerase (Thermo Scientific). The resulting recombinant plasmids encoded Dps or RIC with either a C- or N-terminally linked T25 or T18 domain from the *B. pertussis* Cya protein. Two complementary plasmids, one carrying a T25 fragment and the other a T18 fragment, were co-transformed into the *E. coli* DHM1 strain.
E. coli DHM1 cells containing the ric-encoding pUT18 or pUT18C plasmids were co-transformed with complementary pKTN25 empty plasmid that served as negative controls.

In all cases, false positives were tested by co-transformation of E. coli DHM1 with plasmids containing each gene and pKT25-TorD, which expresses E. coli TorD that binds non-specifically to a wide variety of polypeptides (34).

For β-galactosidase activity determination (35), at least 3 representative colonies of each transformation plate were inoculated, in duplicate, in LB medium, and following an overnight growth at 37 °C, transformant cultures were re-inoculated (at a 0.01 dilution) into LB with ampicillin (100 μg/mL), kanamycin (50 μg/mL) and IPTG (0.5 mM). When cultures reached an OD<sub>600</sub>=0.5 (approximately after 16 h of growth, at 30 °C), 1 mL of each culture was collected by centrifugation (5000 g, 5 min at 4 °C). The pellets were lysed by incubation with 100 μL BugBuster HT 1x (Novagen) at 37 °C, for 30 min. Cellular debris was then removed by centrifugation and the β-galactosidase activities were assayed in 20 μL suspensions in a microplate reader. The assays were initiated by addition of a reaction mixture comprising: 0.27% β-mercaptoethanol (v/v) and 0.9 mg/mL ONPG (o-nitrophenyl-β-D-galactopyranoside) in buffer A (60 mM Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 40 mM NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 1 mM MgSO<sub>4</sub>.7H<sub>2</sub>O, 10 mM KCl). Reactions were incubated at 28 °C, and the absorbance was recorded at 420 nm at 2 min intervals, for 90 min. The β-galactosidase specific activity was defined as ONP/min/milligram of protein. Interactions were considered positive for those reactions where β-galactosidase activity was at least four times higher than the negative control.

**Bimolecular fluorescence complementation (BiFC) assays**
BiFC assay was performed essentially as described previously (36). For this purpose, the genes encoding RIC protein, a truncated version of the RIC protein (lacking the first 57 amino acid residues in N-terminal (9)), Dps, Bfr and FtnA were PCR amplified from genomic DNA of *E. coli* K-12 using the oligonucleotides described in Table 2. The DNA fragments were cloned into vectors (pET11a-link-N-GFP and pMRBAD-link-C-GFP (36)) that express the green fluorescence protein, GFP, to allow formation of corresponding N- or C-terminal GFP fusions, respectively. Cloning was achieved using *Xho*I and *Bam*HI sites (for cloning into pET11a-link-N-GFP) or *Nco*I and *Aat*II sites (for cloning into pMRBAD-link-C-GFP) sites, except for Dps for which *Sph*I replaced *Nco*I. All recombinant plasmids were sequenced confirming the integrity of the genes and the absence of undesired mismatches. Cells harboring pET11a-link-N-GFP and pMRBAD-link-C-GFP served as negative control.

*E. coli* BL21(DE3)Gold (Agilent) was co-transformed with the resulting recombinant pET11a-link-N-GFP and pMRBAD-link-C-GFP vectors, in various combinations (RIC/Dps, truncated-RIC/Dps, RIC/Bfr and RIC/FtnA), and plated on selective LB-agar. Colonies were inoculated in LB medium, grown overnight, at 37 °C and 150 rpm, and plated onto inducing LB agar medium containing 20 µM IPTG and 0.2% of arabinose. After an overnight incubation at 30 °C followed by two days incubation at room temperature, colonies were suspended in PBS and spread onto 1.7% agarose slides. Cells were examined for green fluorescence in a Leica DM6000 B upright microscope coupled to an Andor iXon+ camera, using a 1000x amplification and a FITC filter. The images were analysed using the MetaMorph Microscopy Automation and Image Analysis Software.

*Pull-down and Western Blot assays*
The genes encoding RIC and Dps were amplified from *E. coli* K-12 genomic DNA by PCR, using the oligonucleotides listed in Table 2, cloned into pET28a and pACYCDuet-1 vectors, respectively, and sequenced which confirmed their integrity and the absence of undesired mutations. *E. coli* BL21(DE3)Gold was transformed with the following pair of plasmids: i) pET28a-RIC (expressing the RIC protein fused to a N-terminal His-Tag-RIC) and pACYCDuet-1-Dps (expressing a non-labelled Dps); and ii) pET28a (empty vector) together with pACYCDuet-1-Dps. Cells harboring the later pair of recombinant plasmids served as control samples. Cells were grown in LB medium, supplemented with 10 μM of Fe and the appropriate antibiotics, at 30 °C to an OD₆₀₀ of 0.3. At this time, 0.3 mM IPTG was added to induce the expression of the His-tagged-RIC and Dps proteins, and after 4 h the cross-linking agent formaldehyde (1% final concentration) was added to the cells. The cross-linking reaction (25) was carried at 37 °C for 20 min, and the reaction was stopped by incubation with glycine (final concentration of 0.5 M) at room temperature for 5 min. Bacterial cells were harvested by centrifugation, washed twice with PBS and resuspended in PBS. Cells were disrupted in a French Press (Thermo) and cell debris were removed by centrifugation. The total protein concentration of the supernatants was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific). For the pull-down experiments, these supernatants were loaded into Ni-Chelating Sepharose Fast Flow columns (GE Healthcare), which were first washed with 10 mM Tris-HCl (pH 7.5), and the proteins were eluted with imidazole containing buffers. The protein fractions were analysed by 12.5% SDS-PAGE and Western blotting. For Western Blot analysis, samples that were first resolved by SDS-PAGE were transferred to a nitrocellulose blotting membrane (GE Healthcare) in a Trans-blot semi-dry cell apparatus (Bio-Rad). The membrane was blocked by addition of TBS (20 mM Tris-HCl pH 7.5, 500
mM NaCl) containing 5% of dried skimmed milk and incubation at room temperature for 1 h. Then, the membrane was incubated with the primary antibody against *E. coli* K-12 Dps (1:1000 dilution in TBS-T (TBS + 0.05% Tween-20) plus 5% of dried skimmed milk). Following an overnight incubation at 4 °C, the membrane was washed with TBS-T and incubated with the secondary antibody (anti-rabbit IgG-alkaline phosphatase from Sigma) diluted 1:10000 in TBS-T + 5% of dried skimmed milk). The reaction proceeded for 1 h at room temperature, and the color was developed by addition of 10 μL of NBT-BCIP (Sigma) in 10 mL buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl2).

**Enzyme activity assays and determination of endogenous ROS**

*E. coli* wild type, Δric, Δdps and Δdps Δric strains that were transformed with either pUC18, pUC18-RIC or pUC18-RIC-E133L (prepared described in (9)) were tested for endogenous aconitase and fumarase activities. To this end, the *E. coli* cells strains were grown in LB medium at 37 °C, under aerobic conditions, to an OD$_{600}$ of 0.6 and 2, as indicated in each case.

For the aconitase assays, cells grown to the desired cell density were centrifuged, washed in reaction buffer (50 mM Tris-HCl, 0.6 mM MnCl$_2$, pH 8), and the pellets were frozen in liquid nitrogen. The following experiments were performed under anaerobic conditions. Prior to the activity assay, the cell pellets were resuspended in reaction buffer containing 0.5 mg/mL lysozyme and 0.2 mg/mL DNAse and incubated on ice for 10 min, and then centrifuged at 9600 g for 10 min, at 4 °C. The aconitase activity was determined in these supernatants (falta qtds usadas) in reaction mixtures that also contained 200 μM NADP$^+$, 1 U isocitrate dehydrogenase and 30 mM sodium citrate (9), and by recording the formation of NADPH at 340 nm.
For the fumarase activity assays (37), once the cells reached the desired cell density they were centrifuged, washed with 50 mM sodium phosphate pH 7.3 buffer, and frozen in liquid nitrogen. Cell pellets were resuspended in 2 mL of the same phosphate buffer, lysed by five freeze-thaw cycles that used liquid nitrogen and a water bath at room temperature. The resulting cell extracts were cleared by addition of sodium deoxycholate, to a final concentration of 0.5%. Fumarase activity was determined under anaerobic conditions in reaction mixtures that contained the cell lysates, 50 mM L-malate, and xxx of fumarate, and by following the consumption of fumarate at 240 nm.

Endogenous reactive oxygen species content was determined in E. coli wild type, Δric, Δdps, Δdps Δric, Δbfr, Δbfr Δric, ΔftnA, ΔftnA Δric strains (Table 1). Cells were grown aerobically to an OD₆₀₀ of 0.6, collected by centrifugation, resuspended in PBS, and distributed in 96-well microtitre plates. Following the addition of dichloro-dihydro-fluorescein diacetate (10 µM DCFH-DA), the fluorescence was measured in a spectrofluorimeter Varian Cary (Agilent) at λₑₓ = 485 nm and λₑₘ = 538 nm, and for 2 h. The Fluorescence Intensity (FI) was normalized in relation to the optical density of each culture at 600 nm.

Acknowledgments

We thank Professor Hirotada Mori (Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan) for provision of the mutants from the Keio collection, and Professor James Imlay (Department of Microbiology, B103 CLSL, 601 S Goodwin Ave, Urbana, IL 61801, USA) for the Hpx mutant. We also grateful to Professors
Tracy Palmer and Frank Sargent (Centre for Bacterial Cell Biology, Newcastle University) for providing the BACTH E. coli library.

This work was financially supported by: Project LISBOA-01-0145-FEDER-007660 (Microbiologia Molecular, Estrutural e Celular) funded by FEDER funds through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) and by national funds through FCT - Fundação para a Ciência e a Tecnologia" for grants PTDC/BBB-BQB/5069/2014, and SFRH/BD/118545/2016 (LSOS). This project has also received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement number 810856.

References


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Table 2 – Oligonucleotides used in this study
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**Construction of plasmids used for protein expression**

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