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

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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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1 **The di-iron RIC (YtfE) protein of *Escherichia coli* interacts with the DNA-binding**

2 **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

3

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

84   exhibits a broad band at *ca.* 350 nm and Electron Paramagnetic Resonance (EPR)

85   spectroscopy indicates that the principal g-values are below 2 (g=1.96, 1.92 and 1.88), which

86   is indicative of a S=½ spin state in a mixed valence and anti-ferromagnetically coupled

87   Fe(III)-Fe(II) binuclear iron centre. Mössbauer spectroscopy showed that the mixed-valence

88   Fe(III)–Fe(II) di-iron centre of the RIC protein is more labile than that of the μ(oxo)-diferric

89   form (6).

90   RIC proteins possess several highly-conserved amino acid residues of which some have been

91   shown to influence the properties of the di-iron centre and/or function of the protein. In

92   particular, substitution of residues His129, Glu133 or Glu208 of the *E. coli* RIC protein

93   abrogated its ability to protect the Fe-S cluster of aconitase. Moreover, two μ-carboxylate

94   bridges contributed by Glu133 and Glu208, linking the two di-iron site atoms, were shown

95   to be required for the assembly of a stable di-iron centre (9). These studies also demonstrated

96   the important contribution of the conserved His84, His129, His160, His204, Glu133 and

97   Glu208 residues in ligating the di-iron centre within the four-helix bundle fold, and these di-

98   iron coordination roles were recently confirmed by X-ray crystallographic structural studies

99   (10).

100   In the work reported here, we sought to identify proteins that interact with, and support the

101   function of, the RIC protein of *E. coli*. For this purpose, an *E. coli* library was screened for

102   RIC protein interaction partners using the Bacterial Adenylate Cyclase Two Hybrid system

103   (BACTH). Potential interacting gene products were further tested by BACTH, Bimolecular

104   Fluorescence Complementation (BiFC) and pull-down assays. Our protein-protein

105   interaction studies revealed that the RIC protein interacts with the DNA-binding protein from

106 starved cells (Dps). Dps is a symmetrical dodecameric iron-storage protein of the ferritin

107 superfamily that contains a di-iron ferroxidation centre located at the interface between

108 subunits (11–13). Dps sequesters ferrous iron, which is oxidized preferentially by hydrogen

109 peroxide at its di-iron centre and then deposited for storage as Fe(III) oxyhydroxide in the

110 central cavity as an iron core; the sequestered iron can subsequently be released by reduction

111 (13, 14). The ferroxidase activity, DNA-binding and iron-sequestration properties of Dps

112 confer cells with protection from oxidative stress and nutrient deprivation, as judged by the

113 reduced survival of *dps* mutants under stress conditions including starvation, oxidative stress,

114 metal toxicity, and thermal stress (15).   The physiological relevance of the interaction

115 between the RIC protein and Dps was examined and the results revealed that Dps modulates

116 the function of RIC.

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125 **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-*Sau*3A-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).

150 The other four plasmids D to G also contained a ~2 kb insert located upstream of the T18

151 Cya domain, but in these cases the inserts carried the entire *rhtA* gene, encoding an inner-

152 membrane transporter involved in resistance to homoserine/threonine (21), and the *dps* gene,

153 encoding the DNA-binding and iron-storage protein from *s*tarved cells (11). Like RIC, *E. coli*

154 Dps has been implicated in oxidative-stress protection, which raises the possibility of a

155 functional association between these two proteins that might be dependent on their direct

156 interaction.   For this reason, the potential interaction between the two proteins was

157 investigated further in order to establish its validity and determine its physiological purpose.

158

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

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161 To determine whether the interaction between the RIC protein and Dps, as identified through

162 the screening of the pUT18 library, is indeed genuine, further BACTH experiments were

163 performed. To enable such experiments, the gene encoding the RIC protein was cloned into

164 pUT18C and pUT18 vectors (to create T18-RIC and RIC-T18 fusions), and the *dps*-coding

165 region was introduced into the pKNT25 vector (to give Dps-T25 fusions), following which

166 the β-galactosidase activities of the corresponding co-transformants were measured. High β-

167 galactosidase activities were recorded for both sets of the RIC-Dps BATCH combinations

168 tested, with activities 4-6 times greater than those of the controls (Figure 2A), indicative of

169 interaction between the RIC protein and Dps within the cytosol of *E. coli*.

170 A second approach was used to test the proposed RIC-Dps interaction, which involved a

171 Bimolecular Fluorescence Complementation (BiFC) assay. In this method, one of the two

172 proteins of interest is fused to the N-terminal half of the green fluorescent protein (GFP), and

173 the other protein of interest is fused to the C-terminal half; the assay depends upon an

8

174   interaction between the two proteins that promotes the reassembly of the two halves of GFP

175   such that emission of fluorescence is restored (22). Thus, GFP fusions (both the N- and C-

176   terminal domains) were generated for both the RIC protein and Dps, and the fluorescence

177   intensity of the corresponding *E. coli* cells containing plasmids co-expressing the RIC and

178   Dps fusions was measured (Figure 2BC). The data showed that cells expressing RIC[C-GFP]

179   and Dps[N-GFP] exhibit an approximately six-fold higher fluorescence relative to the control,

180   although transformants expressing RIC[N-GFP] and Dps[C-GFP] presented fluorescence levels

181   similar to that of the control samples.

182   The RIC protein consists of two domains: a short N-terminal 'ScdA_N' domain of ~60

183   residues of unclear function with a highly-conserved pair of Cys residues (10); and a larger

184   C-terminal 'hemerythrin' domain of ~140 residues that forms a di-iron centre. We tested the

185   BiFC interaction between Dps and a truncated form of RIC that lacks the so-called first

186   Scd_N domain to determine which of the two RIC protein domains is responsible for the

187   observed interaction with Dps. The results showed that the degree of interaction between the

188   truncated RIC protein and Dps is similar to that observed when using the full-length protein

189   (Figure 2C). Thus, the interaction observed here between the RIC protein and Dps appears

190   to be mediated through the C-terminal hemerythrin domain of the RIC protein.

191   The interaction between RIC and Dps was also investigated by a pull-down assay. To this

192   end, cells containing plasmids that express non-labelled Dps and N-terminally His-tagged-

193   RIC were treated with formaldehyde, as described in Methods, to promote *in vivo* cross-

194   linking. The cell extract was loaded into a Ni-chelating column and the His-Tag RIC was

195   eluted at 100 mM of imidazole buffer. The fraction was analysed by SDS-PAGE, and

196   Western blotting in which the *E. coli* Dps antibody was used. Also, cells expressing only the

197   non-labelled Dps were treated and analysed similarly to serve as control. The results depicted

198   in Figure 2D show that elution of His-Tag RIC occurred together with a band that has a

199   molecular mass correspondent to that of Dps. This band was proved by Western-blotting to

200   be the *E. coli* Dps (Figure 2D). Therefore, the pull-down assays support the interaction

201   between RIC and Dps.

202

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

204   The RIC protein has been linked to the resistance of *E. coli* to oxidative and nitrosative

205   stresses as its inactivation decreases the survival of *E. coli* upon exposure to hydrogen

206   peroxide or nitric oxide donors (4). Due to the interaction of the RIC and Dps proteins shown

207   above, we questioned whether Dps could contribute to the stress protection afforded by the

208   RIC protein. To test this possibility, a Δ*dps* Δ*ric* double mutant was constructed and the

209   growth of *E. coli* wild type*,* Δ*ric,* Δ*dps*, Δ*dps* Δ*ric* mutants under oxidative and nitrosative

210   stress conditions was tested (Figure 3). The growth experiments showed that inactivation of

211   *ric* resulted in impaired growth under stress conditions imposed by 4 mM H_2O_2 or 250 μM

212   spermine NONOate (Figure 3), which is consistent with previous reports (4). However, the

213   *dps* mutation had little impact on growth under these conditions. Combining the Δ*dps*

214   mutation with the Δ*ric* mutation did not result in any further growth reduction under the same

215   stress conditions, i.e. the Δ*dps* Δ*ric* strain grew similarly to the Δ*ric* strain under the oxidative

216   and nitrosative stress conditions employed (Figure 3). Thus, Dps does not notably

217   compensate for the lack of the RIC protein under peroxide or NO-induced stress.

218   Another characteristic of the *E. coli ric* mutant is the reduced endogenous activity of Fe-S

219   cluster-containing proteins, such as aconitase and fumarase, that contain solvent-exposed Fe-

220   S clusters with a marked sensitivity to redox and nitrosative stress (4). Therefore, the possible

221   contribution of Dps to this phenotype was explored by comparing the aconitase activity of

222   the Δ*dps* and Δ*dps* Δ*ric* strains to that of the wild type and Δ*ric* mutant. The results showed

223   that the Δ*dps* mutation caused a 50% reduction in aconitase activity in log phase (Figure 4A),

224   consistent with a role for Dps in maintaining Fe-S cluster status. As expected, a similar effect

225   was observed for the Δ*ric* mutant, although the reduction in activity (30%) was only

226   approximately half as great as that observed for the Δ*dps* mutant (Figure 4A). Surprisingly,

227   the Δ*dps*Δ*ric* mutant exhibited aconitase activity that was higher than that of the

228   corresponding single mutants and similar to that of the wild type (Figure 4A). These

229   aconitase-activity effects were apparent in both the early-log and the post-exponential phase

230   (OD$_{600}$ 0.6 and 2, respectively; Figure 4A and B), suggesting that the phenotype is

231   independent of growth stage (note that *dps* is stationary-phase induced).

232   Similar effects were observed when testing the activity of another Fe-S enzyme, namely

233   fumarase. The data showed a reduction of 70% in fumarase activity in the Δ*dps* mutant when

234   compared to wild type during the early-log phase (OD$_{600}$=0.6). Accordingly, in the Δ*ric*

235   mutant there was a reduction in fumarase activity of about 40% while the double mutant

236   Δ*dps*Δ*ric* displayed a fumarase activity similar to that of the wild type (Figure 4C).

237   The restoration of aconitase and fumarase activity to wildtype levels in the double *dps ric*

238   mutant (with respect to the corresponding single mutants) suggests that the negative impact

239   of the lack of the RIC protein on such activity is dependent on the presence of Dps (and vice-

240   versa), and this in turn indicates a hitherto unrecognised functional interdependence for these

241   two proteins.

242   The association of the above aconitase-activity effects with the RIC protein was confirmed

243   by complementation using a multicopy plasmid bearing the wild type *ric* gene under control

244   of its natural promoter. Complementation of the single Δ*ric* mutant led to the recovery of

245   aconitase activity to levels similar to those of the wild type (Figure 4D). More importantly,

246   provision of a wild type version of *ric* (in multicopy) caused a large (60%) and significant

247   reduction in the aconitase activity of the Δ*dps*Δ*ric* double mutant (Figure 4D). Thus, as

248   anticipated, the *ric*-complemented double mutant exhibited the same phenotype as the *dps*

249   mutant. This confirms that the RIC protein is responsible for decreasing aconitase activity in

250   a *dps⁻* background.

251   To investigate whether the role of the RIC protein in lowering aconitase activity in the *dps*

252   mutant is dependent on a biochemically-functional version of the RIC protein, the ability of

253   a RIC protein variant (lacking a complete di-iron site due to an E133L substitution; (9)), was

254   used in the complementation experiments (Figure 4D). The resulting activity data clearly

255   show that he non-functional E133L-RIC variant does not enable a notable decrease in

256   aconitase activity when expressed in the Δ*dps*Δ*ric* strain (Figure 4D).

257   In summary, the above data suggest that in the absence of Dps, the RIC protein has a

258   deleterious effect on aconitase and fumarase activities, but that such an effect is not exhibited

259   when Dps is present. This would imply that the interaction between Dps and the RIC protein,

260   as revealed here, acts to ensure that neither of these two proteins can participate in processes

261   that negatively impact the activity of these Fe-S enzymes.

262

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, Δ*ric,* Δ*bfr,* Δ*ftnA,* Δ*bfr*Δ*ric* and Δ*ftnA*Δ*ric* strains, grown to the exponential phase (OD_{600} of 0.6), was determined. Similarly to the Δ*dps* strain, the Δ*bfr* and Δ*ftnA* strains both displayed ~50% lower aconitase activity levels (Figure 5B). But contrary to the effect of combining the Δ*dps* and Δ*ric* 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 Δ*ric* mutation is not additive with respect to lower activity of resulting from the Δ*bfr* or Δ*ftnA* 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.

283

284 **The RIC protein increases intracellular ROS levels when Dps is absent**

285 Dps protects cells from oxidative stress due to its ability to couple the reduction of hydrogen

286 peroxide to water with the oxidation of free-ferrous iron to sequestered-ferric iron. In

287 addition, its association with DNA helps to prevent ROS-induced DNA damage (27). This

288 suggests that the role of Dps in preventing RIC-protein induced inhibition of aconitase

289 activity may arise from the ability of Dps to detoxify ROS that might be produced by the di-

290 iron centre of the RIC protein (e.g. through binding and reduction of oxygen). Therefore, the

291 ROS content of Δ*ric*, Δ*dps* and Δ*dps* Δ*ric* strains were compared with those found in the wild

292 type to determine whether the presence of the RIC protein, in the absence of Dps, results in

293 raised levels of ROS (Figure 6A). Data show that the wild type and Δ*ric* mutant contain

294 similar amounts of ROS while the Δ*dps* strain had significantly higher (~2-fold) levels

295 (Figure 6A). This is as expected given the known role of Dps in redox-stress resistance (27).

296 However, introduction of the *ric* mutation into the *dps* mutant eliminated the increased

297 intracellular ROS levels of the single Δ*dps* mutant (Figure 6A). This suggests that the raised

298 ROS levels of the *dps* single mutant are a consequence of an increase in RIC-protein-

299 dependent ROS production which thus supports a role for Dps in interacting with the RIC

300 protein to restrict its release of ROS species.

301 To discover whether other elements of the redox-stress resistance response might also act to

302 lessen RIC-protein induced ROS production, the Δ*ric* mutation was introduced into a strain

303 (Δ*hpx*) lacking capacity to degrade hydrogen peroxide due to inactivation of both catalase

304 genes as well as the alkyl-hydroperoxide reductase genes (Table 1; (28, 29)). Assay of the

305 resulting aconitase activity levels showed that the Δ*hpx*Δ*ric* quadruple mutant has activity

306 levels similar to those determined for the Δ*ric* and Δ*hpx* mutants (Figure 6B). Therefore, we

14

307   concluded that the three major peroxidases (KatE, KatG, AhpCF) of *E. coli* are not involved

308   in countering any RIC-protein mediated ROS production, at least under conditions where

309   Dps is active.

310

311   **Discussion**

312   Aconitase and fumarase are enzymes of the TCA cycle that are prone to oxidative stress

313   damage. We previously showed that the di-iron RIC protein repairs these enzymes and is

314   able to transfer iron to Fe-S containing proteins (4–6). In the work described here, we

315   screened an *E. coli* BACTH library in order to identify proteins that interact with the RIC

316   protein and thus might be required to assist its function. As a consequence of our screening,

317   Dps emerged as a RIC protein interaction candidate. This suggested interaction was

318   supported by generation and analysis of additional Dps and RIC protein BACTH constructs

319   and by GFP complementation and pull-down assays. Dps belongs to the ferritin superfamily

320   which led us to investigate the possible interaction of RIC with the two other ferritins present

321   in *E. coli*, namely ferritin and bacterioferritin. However, neither of these proteins were found

322   to interact with the RIC protein or to influence its activity *in vivo*.

323   We also observed that inactivation of the RIC protein resulted in lower aconitase and

324   fumarase activity, which is consistent with previous findings indicating that this protein

325   contributes to the protection of solvent accessible Fe-S clusters from ROS damage under

326   aerobic growth conditions (5). Similar results were herein obtained for the single mutant

327   strains of *dps*, *ftnA* and *bfr*, indicating that lack of any of these gene products results in lower

328   endogenous aconitase activity. The role of FtnA and Bfr in aconitase protection was

329   previously demonstrated as the two ferritins promote the reactivation of aconitase activity

330   following stress damage in *Salmonella enterica* serovar Typhimurium (30). In contrast with

331   our findings with *E. coli*, no loss of aconitase activity was observed for *S. enterica ftnA* or

332   *bfr* single mutants in the absence of stress; this discrepancy may be related to different

333   physiological roles and expression control of ferritins in *Salmonella* and *E. coli* species (30,

334   31).

335   A surprising result was the finding that the defective aconitase activity of the Δ*dps* and Δ*ric*

336   single mutant strains was reversed when these two mutations were combined in the Δ*dps*Δ*ric*

337   double mutant, such that activity was restored to that measured in the wild type. This result,

338   together with the lower amounts of ROS observed in the Δ*dps*Δ*ric* mutant compared to the

339   Δ*dps* mutant, suggests that the RIC protein is responsible for the generation of ROS, but only

340   in the absence of Dps and, thus, that the interaction of Dps and the RIC protein serves to

341   enable Dps to restrict ROS release (which is presumed to damage the Fe-S cluster of

342   aconitase and fumarase, and hence lower the observed activity of these enzymes in a *dps*

343   mutant) by the RIC protein. Interestingly, other redox-stress resistance components (KatE,

344   KatG and AhpCF) failed to impact the RIC-protein-mediated inhibition of aconitase activity

345   (at least in the presence of Dps). These results suggest that the effect of Dps on the ROS-

346   generation activity of the RIC protein is one that is highly specific and not replicated by the

347   other peroxide-consuming cytosolic factors examined. Indeed, the findings relayed here

348   indicate that a direct interaction is required to enable Dps to quench the ROS-generating

349   activity of the RIC protein. The exact mechanism involved in the apparent quenching of RIC-

350   protein-mediated ROS production by Dps is unclear; such understanding will require *in vitro*

351   reaction studies combining the Dps and RIC proteins. However, two possible processes by

352   which Dps could exert a ROS-quenching action upon the RIC protein can be considered: Dps

353 might sequester iron released from the di-iron site of the RIC protein and thus restrict Fe-

354 driven Fenton chemistry; or Dps could consume hydrogen peroxide (or hydroxyl radicals;

355 (14, 32)) generated by the RIC protein through reaction at its di-iron site with molecular

356 oxygen.

357 Although the absence of the RIC protein in the presence of Dps resulted in reduced aconitase

358 and fumarase activity, lack of RIC protein had no impact on ROS levels when Dps was

359 present. The reason for this effect is unclear but may indicate a role for the RIC protein in

360 supply of iron from Dps for Fe-S cluster repair and/or synthesis.

361 The proposed role of the RIC protein (4) is to repair damaged Fe-S clusters of [Fe-S]-

362 proteins, such as aconitase and fumarase, by donating iron from its di-iron centre leading to

363 the formation of an intermediate mononuclear iron centre that is prone to react with oxygen

364 to generate ROS such as hydrogen peroxide. In this process, the interaction with Dps would

365 fulfil two roles, namely by trapping ROS released by the RIC protein and providing a sink

366 for iron liberated from the di-iron centre of RIC.

367 In conclusion, we report an interaction between the Dps and RIC proteins of *E. coli* which

368 represents the first example of a protein that interacts with the ferritin-like Dps protein. In

369 addition, our results indicate that the Dps-RIC protein interaction contributes to the function

370 of RIC, which is one of the few known bacterial proteins involved in repair.

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372

373

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 $^{\circ}$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 K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 1 mM $MgSO_4$ and 10 µM $MnCl_2$, 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 OD_{600} of 0.3 were either left untreated or exposed to 4 mM H_2O_2 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

18

394   *Bordetella pertussis* Cya (adenylate cyclase) T25 domain (pKT25-RIC) and used to screen

395   an *E. coli* MC4100 gene library containing chromosomal fragments fused to the N-terminal

396   of *B. pertussis* Cya T18 domain. The DNA fragments were obtained by partial digestion with

397   *Sau*3AI and cloning into the *Bam*HI site of pUT18 plasmids (17). About 1 µg of

398   pUT18*Bam*HI DNA library was transformed together with pKT25-RIC into *E. coli* DHM1

399   cells by electroporation. Blue colonies present in Amp^R Cm^R selective plates (L-agar with 5-

400   bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)) were identified after incubation

401   at 30 °C for 36 h, and cells with the highest β-galactosidade were considered to contain

402   recombinant plasmids harbouring genes encoding polypeptides that interact with the *E. coli*

403   RIC protein. Twenty two colonies were obtained and the corresponding plasmids were

404   isolated, co-transformed with pKT25-RIC plasmid in *E. coli* DHM1, and the strength of the

405   protein-protein interactions observed was again estimated by quantification of the β-

406   galactosidase activity. Seven isolates considered positive were named 'A' to 'G' (Figure 1),

407   and subject to nucleotide sequencing using primer T18$_{Fw}$ (Table 2). To identify the encoded

408   genes, the sequences were screened against the *E. coli* K-12MG1655 genome using BLAST.

409   Genes coding for the RIC protein and Dps were PCR amplified from *E. coli* K-12 genomic

410   DNA using the oligonucleotides described in Table 2, and cloned into pKT25 (fused to Cya

411   C-terminal T25 domain), pKNT25 (fused to Cya N-terminal T25 domain), pUT18 (fused to

412   Cya N-terminal T18 domain) and pUT18C (fused to Cya C-terminal T18 domain) plasmids,

413   and the enzyme *Pfu* DNA polymerase (Thermo Scientific). The resulting recombinant

414   plasmids encoded Dps or RIC with either a C- or N-terminally linked T25 or T18 domain

415   from the *B. pertussis* Cya protein. Two complementary plasmids, one carrying a T25

416   fragment and the other a T18 fragment, were co-transformed into the *E. coli* DHM1 strain

19

417   (*cya⁻*). *E. coli* DHM1 cells containing the *ric*-encoding pUT18 or pUT18C plasmids were co-

418   transformed with complementary pKTN25 empty plasmid that served as negative controls.

419   In all cases, false positives were tested by co-transformation of *E. coli* DHM1 with plasmids

420   containing each gene and pKT25-TorD, which expresses *E. coli* TorD that binds non-

421   specifically to a wide variety of polypeptides (34).

422   For β-galactosidase activity determination (35), at least 3 representative colonies of each

423   transformation plate were inoculated, in duplicate, in LB medium, and following an overnight

424   growth at 37 °C, transformant cultures were re-inoculated (at a 0.01 dilution) into LB with

425   ampicillin (100 μg/mL), kanamycin (50 μg/mL) and IPTG (0.5 mM). When cultures reached

426   an OD_{600}=0.5 (approximately after 16 h of growth, at 30 °C), 1 mL of each culture was

427   collected by centrifugation (5000 *g*, 5 min at 4 °C). The pellets were lysed by incubation with

428   100 μL BugBuster HT 1x (Novagen) at 37 °C, for 30 min. Cellular debris was then removed

429   by centrifugation and the β-galactosidase activities were assayed in 20 μL suspensions in a

430   microplate reader. The assays were initiated by addition of a reaction mixture comprising:

431   0.27% β-mercaptoethanol (v/v) and 0.9 mg/mL ONPG (o-nitrophenyl-β-D-

432   galactopyranoside) in buffer A (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 1 mM

433    $MgSO_4.7H_2O$, 10 mM KCl). Reactions were incubated at 28 °C, and the absorbance was

434   recorded at 420 nm at 2 min intervals, for 90 min. The β-galactosidase specific activity was

435   defined as ONP/min/milligram of protein. Interactions were considered positive for those

436   reactions where β-galactosidase activity was at least four times higher than the negative

437   control.

438   *Bimolecular fluorescence complementation (BiFC) assays*

439 BiFC assay was performed essentially as described previously (36). For this purpose, the

440 genes encoding RIC protein, a truncated version of the RIC protein (lacking the first 57 amino

441 acid residues in N-terminal (9)), Dps, Bfr and FtnA were PCR amplified from genomic DNA

442 of *E. coli* K-12 using the oligonucleotides described in Table 2. Tthe DNA fragments were

443 cloned into vectors (pET11a-link-N-GFP and pMRBAD-link-C-GFP (36)) that express the

444 green fluorescence protein, GFP, to allow formation of corresponding N- or C-terminal GFP

445 fusions, respectively. Cloning was achieved using *Xho*I and *Bam*HI sites (for cloning into

446 pET11a-link-N-GFP) or *Nco*I and *Aat*II sites (for cloning into pMRBAD-link-C-GFP) sites,

447 except for Dps for which *Sph*I replaced *Nco*I. All recombinant plasmids were sequenced

448 confirming the integrity of the genes and the absence of undesired mismatches. Cells

449 harboring pET11a-link-N-GFP and pMRBAD-link-C-GFP served as negative control.

450 *E. coli* BL21(DE3)Gold (Agilent) was co-transformed with the resulting recombinant

451 pET11a-link-N-GFP and pMRBAD-link-C-GFP vectors, in various combinations (RIC/Dps,

452 truncated-RIC/Dps, RIC/Bfr and RIC/FtnA), and plated on selective LB-agar. Colonies were

453 inoculated in LB medium, grown overnight, at 37 ℃ and 150 rpm, and plated onto inducing

454 LB agar medium containing 20 µM IPTG and 0.2% of arabinose. After an overnight

455 incubation at 30 ˚C followed by two days incubation at room temperature, colonies were

456 suspended in PBS and spread onto 1.7% agarose slides. Cells were examined for green

457 fluorescence in a Leica DM6000 B upright microscope coupled to an Andor iXon+ camera,

458 using a 1000x amplification and a FITC filter. The images were analysed using the

459 MetaMorph Microscopy Automation and Image Analysis Software.

460   *Pull-down and Western Blot assays*

461   The genes encoding RIC and Dps were amplified from *E. coli* K-12 genomic DNA by PCR,

462   using the oligonucleotides listed in Table 2, cloned into pET28a and pACYCDuet-1 vectors,

463   respectively, and sequenced which confirmed their integrity and the absence of undesired

464   mutations. *E. coli* BL21(DE3)Gold was transformed with the following pair of plasmids : i)

465   pET28a-RIC (expressing the RIC protein fused to a N-terminal His-Tag-RIC) and

466   pACYCDuet-1-Dps (expressing a non-labelled Dps); and ii) pET28a (empty vector) together

467   with pACYCDuet-1-Dps. Cells harboring the later pair of recombinant plasmids served as

468   control samples. Cells were grown in LB medium, supplemented with 10 μM of Fe and the

469   appropriate antibiotics, at 30 °C to an OD_{600} of 0.3. At this time, 0.3 mM IPTG was added

470   to induce the expression of the His-tagged-RIC and Dps proteins, and after 4 h the cross-

471   linking agent formaldehyde (1% final concentration) was added to the cells. The cross-

472   linking reaction (25) was carried at 37 °C for 20 min, and the reaction was stopped by

473   incubation with glycine (final concentration of 0.5 M) at room temperature for 5 min.

474   Bacterial cells were harvested by centrifugation, washed twice with PBS and resuspended in

475   PBS. Cells were disrupted in a French Press (Thermo) and cell debris were removed by

476   centrifugation. The total protein concentration of the supernatants was determined by the

477   Pierce BCA Protein Assay Kit (Thermo Scientific). For the pull-down experiments, these

478   supernatants were loaded into Ni-Chelating Sepharose Fast Flow columns (GE Healthcare),

479   which were first washed with 10 mM Tris-HCl (pH 7.5), and the proteins were eluted with

480   imidazole containing buffers. The protein fractions were analysed by 12.5% SDS-PAGE and

481   Western blotting

482   For Western Blot analysis, samples that were first resolved by SDS-PAGE were transferred

483   to a nitrocellulose blotting membrane (GE Healthcare) in a Trans-blot semi-dry cell apparatus

484   (Bio-Rad). The membrane was blocked by addition of TBS (20 mM Tris-HCl pH 7.5, 500

485   mM NaCl) containing 5% of dried skimmed milk and incubation at room temperature for 1

486   h. Then, the membrane was incubated with the primary antibody against *E. coli* K-12 Dps

487   (1:1000 dilution in TBS-T (TBS + 0.05% Tween-20) plus 5% of dried skimmed milk).

488   Following an overnight incubation at 4 ˚C, the membrane was washed with TBS-T and

489   incubated with the secondary antibody (anti-rabbit IgG-alkaline phosphatase from Sigma)

490   diluted 1:10000 in TBS-T + 5% of dried skimmed milk). The reaction proceeded for 1 h at

491   room temperature, and the color was developed by addition of 10 μL of NBT-BCIP (Sigma)

492   in 10 mL buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$)..

493   *Enzyme activity assays and determination of endogenous ROS*

494   *E. coli* wild type, Δ*ric*, Δ*dps* and Δ*dps* Δ*ric* strains that were transformed with either pUC18,

495   pUC18-RIC or pUC18-RIC-E133L (prepared described in (9)) were tested for endogenous

496   aconitase and fumarase activities. To this end, the *E. coli* cells strains were grown in LB

497   medium at 37 ᵒC, under aerobic conditions, to an OD_{600} of 0.6 and 2, as indicated in each

498   case.

499   For the aconitase assays, cells grown to the desired cell density were centrifuged, washed in

500   reaction buffer (50 mM Tris-HCl, 0.6 mM $MnCl_2$, pH 8), and the pellets were frozen in liquid

501   nitrogen. The following experiments were performed under anaerobic conditions. Prior to

502   the activity assay, the cell pellets were resuspended in reaction buffer containing 0.5 mg/mL

503   lysozyme and 0.2 mg/mL DNAse and incubated on ice for 10 min, and then centrifuged at

504   9600 *g* for 10 min, at 4 ᵒC. The aconitase activity was determined in these supernatants (falta

505   qtds usadas) in reaction mixtures that also contained 200 μM $NADP^+$, 1 U isocitrate

506   dehydrogenase and 30 mM sodium citrate (9). and by recording the formation of NADPH at

507   340 nm.

508 For the fumarase activity assays (37), once the cells reached the desired cell density they

509 were centrifuged, washed with 50 mM sodium phosphate pH 7.3 buffer, and frozen in liquid

510 nitrogen. Cell pellets were resuspended in 2 mL of the same phosphate buffer, lysed by five

511 freeze-thaw cycles that used liquid nitrogen and a water bath at room temperature. The

512 resulting cell extracts were cleared by addition of sodium deoxycholate, to a final

513 concentration of 0.5%. Fumarase activity was determined under anaerobic conditions in

514 reaction mixtures that contained the cell lysates falta qtds usadas , 50 mM L-malate, and xxx

515 of fumarate, and by following the consumption of fumarate at 240 nm.

516

517 Endogenous reactive oxygen species content was determined in *E. coli* wild type, Δ*ric*, Δ*dps,*

518 Δ*dps* Δ*ric*, Δ*bfr,* Δ*bfr* Δ*ric,* Δ*ftnA,* Δ*ftnA* Δ*ric* strains (Table 1). Cells were grown aerobically

519 to an OD_{600} of 0.6, collected by centrifugation, resuspended in PBS, and distributed in 96-

520 well microtitre plates. Following the addition of dichloro-dihydro-fluorescein diacetate (10

521 µM DCFH-DA), the fluorescence was measured in a spectrofluorimeter Varian Cary

522 (Agilent) at λ_{ex} = 485 nm and λ_{em} = 538 nm,, and for 2 h. The Fluorescence Intensity (FI)

523 was normalized in relation to the optical density of each culture at 600 nm.

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542   <mark>Reformatar referencias</mark>

543   **References**

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665   **Table 1 –Strains and plasmids used in this study**

E.coli	Description	Source
Strains		
DHM1	F′, *cya-854, recA1, endA1, gyrA96* (Nal^R), *thi1, hsdR17, spoT1, rfbD1, glnV44(AS)*	Euromedex
Wild type	K-12 ATCC 23716	ATCC
Δ*ric*	K-12 Δ*ric*::*cat*	(38)
Δ*dps*	JS091 Δ*dps*::*kan*	(39)
Δ*bfr*	JW3298 Δ*bfr*::*kan*	(39)
Δ*ftnA*	MC4100 Δ*ftnA*::*spc*	(31)
Δ*dps*Δ*ric*	K-12 Δ*dps*::*kan*, Δ*ric*::*cat*	This study
Δ*bfr*Δ*ric*	K-12 Δ*bfr*::*kan*, Δ*ric*::*cat*	This study
Δ*ftnA*Δ*ric*	K-12 Δ*ftnA*::*spc*, Δ*ric*::*cat*	This study
MG1655	F$^-$ WT	(40)
SJ90	BW25113 Δ*ric*::*cat*	(41)
LC106 (*hpx*)	Δ*ahpCF'kan::'ahpḞ*, Δ(*katG17*::Tn*10*)1, Δ(*katE12*::Tn*10*)1	(29)
Δ*hpx*Δ*ric*	LC106 Δ*ric1*::cat	This study
XL2 Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F' *proAB$^+$, lacIqZ*ΔM15 Tn*10* (Tet^r)]	Agilent
BL21Gold(DE3)	*E. coli* B, F$^-$, *ompT, hsdS* (r$_B^-$ m$_B^-$), *dcm$^+$*, Tet^r, *gal*, *λ*(DE3), *endA*, Hte	Agilent

Plasmids

pUT18/pUT18C	Vector that allows construction of in-frame fusions at the N-terminus/C-terminus of T18 fragment (amino acids 225-399 of CyaA)	(16)
pKT25/pKNT25	Vector that allows construction of in-frame fusions at the N-terminus/C-terminus of T25 fragment (amino acids 1-224 of CyaA)	(16)
pUT18/pUT18C-RIC	RIC fused to T18 fragment in N/C-terminal	This study
pKT25/pKNT25-RIC	RIC fused to T25 fragment in N/C-terminal	This study
pUT18/pUT18C-Dps	Dps fused to T18 fragment in N/C-terminal	This study
pKT25/pKNT25-Dps	Dps fused to T25 fragment in N/C-terminal	This study
pUT18-Zip	Leucine zipper fused to T18 fragment in the N-terminal	(16)
pKT25-Zip	Leucine zipper fused to T25 fragment in the C-terminal	(16)
pUT18-TorD	TorD fused to T18 fragment in N-terminal	(17)
pKT25-TorD	TorD fused to T25 fragment in C-terminal	(17)
*Bam*HI	pUT18 plasmid that contains chromosomal fragments obtained by partial digest of the MC4100 chromosomal DNA with Sau3A1 and cloned into de *Bam*HI site	(17)
pUC18	Expression vector	ATCC

pUC18-RIC	Vector for expression of RIC	(4)
pUC18-RIC-Glu133Leu	Vector for expression of RIC-Glu133Leu	(9)
pET11a-link-GFP	Vector for expression of fusions with N- terminal fragment of GFP	(36)
pMRBAD-link-GFP	Vector for expression of fusions with C- terminal fragment of GFP	(36)
pET11a-RIC-GFP	RIC fused to N-terminal GFP fragment	This study
pMRBAD-RIC-GFP	RIC fused to C-terminal GFP fragment	This study
pET11a-Dps-GFP	Dps fused to N-terminal GFP fragment	This study
pMRBAD-Dps-GFP	Dps fused to C-terminal GFP fragment	This study
pET11a-Bfr-GFP	Bfr fused to N-terminal GFP fragment	This study
pMRBAD-Bfr-GFP	Bfr fused to C-terminal GFP fragment	This study
pET11a-FtnA-GFP	FtnA fused to N-terminal GFP fragment	This study
pMRBAD-FtnA-GFP	FtnA fused to C-terminal GFP fragment	This study
pET11a-RICTrunc-GFP	Truncated RIC fused to N-terminal GFP fragment	This study
pMRBAD-RICTrunc-GFP	Truncated RIC fused to C-terminal GFP fragment	This study
pET-28a	Expression vector	Novagen
pET-28a-RIC(HisTag)	Vector for expression of N-terminal Poly-HisTag-RIC	This study
pACYCDuet-1-Dps	Vector for expression of Dps	This study

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668   **Table 2 – Oligonucleotides used in this study**

Primer Name	Sequence
Construction of plasmids used in BACTH	
ric_Fw	GAGGTGTCGACTATGGCTTATC
ric_Rv	CTTTTAGGATCCTCACCCGCC
dps_Fw	GTTAATTACTGGGATCCAACATCAAGAGG
dps_Rv	TCCTGTCAGGTACCCGCTTTTATC
T18_Fw	CATTAGGCACCCCAGGCTTTAC
T18_Rv	GAGCGATTTTCCACAACAAGTC
T18C_Fw	CATACGGCGTGGCGGGGAAAAG
T18C_Rv	AGCGGGTGTTGGCGGGTGTCG
T25_Fw	ATGCCGCCGGTATTCCACTG
T25_Rv	CGGGCCTCTTCGCTATTACG
NT25_Fw	CACCCCAGGCTTTACACTTTATGC
NT25_Rv	CAATGTGGCGTTTTTTTCCTTCG
Construction of plasmids used in BiFC	
ric_xhoFw	GAATGAGGTCTCGAGTATGGCTTATC
ric_bamRv	GCGCAATGGGATCCAGCTTTTAGA
ric_ncoFw	GAGGTATCAGCCATGGCTTATCG
ric_aatRv	CCAGCTTTTAGACGTCTCACCC
dps_xhoFw	CGTTAATTACTCGAGCATAACATCAAG
dps_bamRv	GTACTAAGGATCCGCACCATCAGC
dps_sphFw	CAAGAGGATATGCATGCATGAGTACCGCTA
dps_aatRv	CATCAGCGATGGGACGTCTCGATGTTAG

bfr_xhoFw	GAGTGGAAGCGCTCGAGTCAAAAAATG
bfr_bamRv	GGAGGGTTCTGGATCCCGACACG
bfr_ncoFw	GAAGGAGTCAAACCATGGAAGGTGATAC
bfr_aatRv	CGGACGTCCCTTCTTCGCGGATC
truncric_xhoFw	CTTTAAGAAGGCTCGAGACATATGGCTG
truncric_ncoFw	GGAGATATACCCATGGCTGAACAAC
ftna_xhoFw	CAAATATAACCTTTCTCGAGCACTATC
ftna_bamRv	TGAAACGGATCCAGTAAACCTGC
ftna_ncoFw	GAGCACTACCATGGTGAAACCAGAAAT
ftna_aatRv	CGGAGAGGACGTCTTTTGTGTGTC

Construction of plasmids used for protein expression

pric_ndeFw	AAGAATGAGGTATCACATATGGCTTATCGC
pric_ecoriRv	GGCTGTTTATTGGTAAGAATTCGGCTGCTG
pdps_ndeFw	GAGGATATGAACATATGAGTACCGC
pdps_kpnRV	GTACTAAAGTTCGGTACCATCAGCG

Double mutant construction confirmation

Conf_dps_Fw	CAGAATAGCGGAACACATAGC
Conf_dps_Rv	GATGCACTAAATAAGTGCGTTG
Conf_bfr_Fw	CTCTTCAAAGAGTGGAAGCG
Cof_bfr_Rv	GATCTCTTATTAACCGGGAGG
Conf_ftnA_Fw	CAAATTATAGTGACGCCACAG
Conf_ftnA_Rv	ACCGATCAGAGTAAGATTTGC
Conf_ric_Fw	AAGAATGAGGTATCACATATGGCTTATCGC

Conf_ric_Rv	GGCTGTTTATTGGTAAGAATTCGGCTGCTG

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