RclA is a thermostable copper (II) reductase required for reactive chlorine resistance and host colonization in *Escherichia coli*.

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ABSTRACT

Inflammatory bowel diseases (IBDs) are a growing health concern and have been linked to changes in gut microbiome composition. Enterobacteria, including *Escherichia coli*, bloom to high levels in the gut during inflammation and strongly contribute to the pathology of IBDs. To survive in the inflamed gut, *E. coli* must tolerate high levels of antimicrobial compounds produced by the immune system, including toxic metals like copper and reactive chlorine oxidants such as hypochlorous acid (HOCl). The mechanisms by which both copper and HOCl kill bacteria are complex and poorly characterized. In this work, we show that the widely-conserved bacterial HOCl resistance enzyme RclA catalyzes the reduction of copper (II) to copper (I), and that this activity specifically protects *E. coli* against toxicity resulting from the combination of HOCl and intracellular copper, probably by preventing Cu(III) accumulation. Mutant *E. coli* lacking RclA were highly sensitive to killing by HOCl and were defective in colonizing an animal host. Consistent with the need for RclA to maintain activity under proteotoxic stress conditions, we found that RclA is remarkably thermostable and resistant to inactivation by HOCl. Our results indicate new complexity in the interactions between antimicrobial toxins produced by innate immune cells, and highlight that understanding copper redox reactions both inside and outside of cells is critical to our understanding of how bacteria evade innate immune factors during inflammation.

Keywords: RclA, bacterial stress response, HOCl, copper, inflammation, *E. coli*

46 SIGNIFICANCE STATEMENT

47 During infection and inflammation, the innate immune system uses antimicrobial
48 compounds to control bacterial populations. These include toxic metals like copper and
49 reactive oxidants, including reactive chlorine species like hypochlorous acid (HOCl). We
50 have now found that RclA, an enzyme strongly induced by HOCl in pro-inflammatory
51 *Escherichia coli* and found in many bacteria inhabiting epithelial surfaces, reduces
52 copper (II) to copper (I), and that this activity is required to resist killing by HOCl and for
53 host colonization. This finding indicates that copper redox chemistry plays a critical and
54 previously unappreciated role in bacterial interactions with the innate immune system.
55

56 INTRODUCTION

57 Inflammatory bowel diseases (IBDs), like Crohn's disease and ulcerative colitis,
58 are a growing health problem (1), and are associated with dramatic changes in the
59 composition of the gut microbiome (2-6). Patients with IBDs have increased proportions
60 of proteobacteria, especially *Escherichia coli* and other *Enterobacteriaceae*, in their gut
61 microbiomes, which is thought to contribute to the progression of disease (7-10). The
62 bloom of enterobacteria in the inflamed gut is driven by increased availability of
63 respiratory terminal electron acceptors (*e.g.* oxygen, nitrate, TMAO) and carbon sources
64 (*e.g.* ethanolamine, mucin), which *E. coli* and other facultative anaerobes can use to
65 outcompete the obligate anaerobes (*Bacteroides* and *Clostridia*) that dominate a healthy
66 gut microbiome (2, 3) In addition to these nutritional changes in the gut environment,
67 inflammation leads to increased production of antimicrobial compounds by the innate
68 immune system (9-11), which also impact the bacterial community in the gut. These

69 include antimicrobial peptides, toxic metals (*e.g.* copper), reactive oxygen species
70 (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS) (3, 12,
71 13). Since bacteria living in an inflamed gut are exposed to substantially increased
72 levels of these toxins, the differential survival of proteobacteria during long-term
73 inflammation suggests that *E. coli* may have evolved better mechanisms to resist these
74 stresses than other types of commensal bacteria.

75 RCS are highly reactive oxidants produced by neutrophils and are potent
76 antibacterial compounds (14, 15) that have been reported to play a role in controlling
77 intestinal bacterial populations (16-18). The RCS response of *E. coli* is complex and
78 incompletely understood (15, 19-22), but characteristically involves repair of damaged
79 cellular components, often proteins (15). Protein-stabilizing chaperones are upregulated
80 during RCS stress, including Hsp33 (23, 24) and inorganic polyphosphate (22, 25, 26),
81 and enzymes are expressed that repair oxidized proteins, including periplasmic
82 methionine sulfoxide reductase (MsrPQ) (27) and the chaperedoxin CnoX (28). *E.* coli
83 has multiple HOCl-sensing regulators, including YedVW (which regulates MsrPQ) (27),
84 NemR (regulator of NemA and GloA, which detoxify reactive aldehydes) (19), HypT
85 (which regulates cysteine, methionine, and iron metabolism) (21), and RclR, the RCS-
86 specific activator of the *rclABC* operon (20).

87 Innate immune cells also use copper as an antimicrobial agent and copper levels
88 rise in inflamed tissues, although the exact mechanism(s) by which copper kills bacteria
89 are not yet fully understood (29-31). HOCl-stressed *E. coli* down-regulate genes
90 encoding iron import systems (*e.g. fepABCD, fhuACDF*) and upregulate genes for zinc

91 and copper resistance (*e.g. copA, cueO, cusC, zntA, zupT*) (19), suggesting metals may
92 play some role in RCS resistance.
93 The predicted flavin-dependent oxidoreductase RclA of *E. coli* is strongly
94 upregulated in the presence of RCS (20, 32), protects against killing by HOCl via a
95 previously unknown mechanism (20), and is widely conserved among bacteria found on
96 epithelial surfaces. In this work, we have now determined that RclA is a thermostable,
97 HOCl-resistant copper (II) reductase that is required for efficient colonization of an
98 animal host and protects *E. coli* specifically against the combination of HOCl and
99 intracellular copper, probably by preventing the formation of highly reactive Cu(III). We
100 also found that, surprisingly, extracellular copper effectively protects bacteria against
101 killing by HOCl in cell culture, most likely by catalyzing the breakdown of HOCl in the
102 media before it reaches the bacterial cells.
103

104 RESULTS

105 **RclA is conserved among bacterial inhabitants of epithelial surfaces.** In *E. coli*, the
106 expression of the *rcl* operon (*rclA*, *rclB*, and *rclC*) is controlled by the RCS specific
107 transcriptional activator RclR (20). RclA is the most phylogenetically conserved protein
108 of the Rcl system (Figure 1) and is found almost exclusively in bacteria known to
109 colonize epithelial surfaces (SI Dataset S1), suggesting that it may play an important
110 role in host-microbe interactions in many species. Bacteria encoding RclA homologs
111 include Gram negative species (*e.g. Salmonella enterica*), Gram positive species (*e.g.*
112 *Streptococcus sanguinis*), obligate anaerobes (*e.g. Clostridium perfringens*), facultative
113 anaerobes (*e.g. Staphylococcus aureus*), pathogens (*e.g. Enterococcus faecalis*),

114 commensals (*e.g. Bacteroides thetaiotaomicron*), and probiotics (*e.g. Lactobacillus*
115 *reuteri*), suggesting that RclA's function is broadly conserved and not specific to a single
116 niche or type of host-microbe interaction. RclR, RclB, and RclC are much less widely
117 conserved, and are found only in certain species of proteobacteria, primarily members
118 of the *Enterobacteriaceae*.
119
120 **RclA contributes to HOCl resistance and host colonization.** An *rclA* mutant of *E.*
121 *coli* is more susceptible to HOCl-mediated killing than the wild-type (20). In this work,
122 we utilized a growth curve-based method to measure sensitivity to sub-lethal HOCl
123 stress by quantifying changes in lag phase extension (LPE) of cultures grown in the
124 presence of HOCl. Using this method, we observed significant increases in LPE for a
125 Δ*rclA* mutant strain compared to the wild type grown in the presence of various
126 concentrations of HOCl (Supplemental Figure 1). This confirms previous results and
127 validates the importance of RclA for resisting HOCl-mediated oxidative stress in *E. coli*.
128 To directly test the role of RclA in interactions with an animal host, we examined the
129 ability of *E. coli* to colonize the intestine of *Drosophila melanogaster*, where the
130 presence of enterobacteria is known to stimulate antimicrobial HOCl production by the
131 dual oxidase Duox (16, 18). Since an *E. coli* K-12 strain did not efficiently colonize *D.*
132 *melanogaster* (Supplemental Figure 2), we used the colonization-proficient *E. coli* strain
133 Nissle 1917 (33, 34) in these experiments (Figure 2). *E. coli* Nissle 1917 Δ*rclA* mutants
134 had a significant defect in their ability to colonize *Drosophila* (Figure 2), which was
135 largely eliminated at the early timepoints of colonization when host Duox expression

136 was reduced with RNAi. This shows that RclA's contribution to *E. coli* HOCl resistance
137 is important for efficient host colonization.
138
139 **RclA reduces copper (II) to copper (I).** Although the role of RclA in protection from
140 RCS has been appreciated, the physiological function of RclA was unknown. Based on
141 its homology to other flavin-dependent disulfide oxidoreductases (35), we hypothesized
142 that RclA catalyzed the reduction of an unknown cellular component oxidized by RCS.
143 RclA is homologous to mercuric reductase (MerA), an enzyme that reduces Hg(II) to
144 Hg(0) (36) (Figure 3 and Supplemental Figure 3). HOCl is capable of oxidizing transition
145 metals, including iron (37, 38) and manganese (39, 40). Although RclA and MerA share
146 a conserved CXXXXC active site (Figure 3), MerA has an extra N-terminal domain and
147 two additional conserved cysteine pairs for metal binding. RclA only has one conserved
148 cysteine pair (Figure 3 and Supplemental Figure 3), indicating that, if its substrate is a
149 metal, it must have a different metal-binding mechanism than MerA. We measured the
150 specific activity (SA) of RclA in the presence of a panel of biologically relevant metals.
151 We did not test mercury, since under normal conditions we do not expect *E. coli* to
152 encounter this metal in its environment. In the absence of any metal, RclA slowly
153 oxidized NADH (0.0303 μmole NAD^+ min^{-1} mg^{-1} RclA), consistent with the background
154 activity of other flavin-dependent oxidoreductases in the absence of their specific
155 substrates (41, 42). Two of the metals we tested significantly affected RclA SA, as
156 measured by NADH oxidation. Copper greatly increased the SA of RclA, while zinc
157 caused a decrease in SA (Figure 4A). These results suggested that copper was a
158 substrate of RclA, and that zinc may inhibit RclA activity.

159 Since RclA is an NADH oxidase, the results shown in Figure 4A strongly
160 suggested that this enzyme was concurrently reducing copper. Copper exists in four
161 possible oxidation states, Cu(I), Cu(II), and the less common and highly reactive Cu(III)
162 and Cu(IV) states (43). The copper salt used in our RclA SA determinations was $CuCl_2$,
163 suggesting that RclA was reducing this Cu(II) species to Cu(I). This was very surprising,
164 since Cu(I) is generally thought of as a toxic species that causes oxidative stress (44,
165 45), so it was not obvious either why *E. coli* would produce Cu(I) during RCS stress or
166 why production of Cu(I) might be protective. We therefore first sought to validate that
167 RclA was in fact reducing Cu(II) to Cu(I) while oxidizing NADH to NAD^+. We measured
168 Cu(I) accumulation in RclA reactions directly using the Cu(I)-specific chelator
169 bathocuproinedisulfonic acid (BCS) (46). NADH spontaneously reduces Cu(II) (47) at
170 rates too slow to impact the measurements made here, but BCS increases the rate of
171 this non-enzymatic copper reduction by shifting the equilibrium of the reaction towards
172 Cu(I) (48). Stopping RclA reactions with a mixture of BCS and EDTA, to chelate any
173 remaining Cu(II), allowed us to observe RclA-dependent Cu(I) accumulation
174 (Supplemental Figure 4). We observed a significant increase in BCS/Cu(I) complex
175 formation only in reactions containing RclA, NADH, and Cu(II), and not in reactions
176 lacking any single component (Figure 4B, Supplemental Figure 4). Taken together,
177 these findings show that RclA has Cu(II) reductase activity and generates Cu(I) as a
178 product.
179
180 **How does Cu(II) reductase protect *E. coli* against HOCl?** Both HOCl and copper
181 cause oxidative stress in bacteria and Cu(I) is generally considered more toxic than

182 Cu(II) (15, 29, 49-52), so we were surprised that a Cu(II) reductase protected *E. coli*
183 against HOCl. Copper reacts with the ROS hydrogen peroxide (H_2O_2) to form highly
184 reactive hydroxyl radicals (31, 45, 53, 54), but how the presence of copper influences
185 bacterial sensitivity to RCS has not been investigated before this study. The chemistry
186 of reactions between HOCl and copper is complicated. HOCl can oxidize Cu(II) to highly
187 reactive Cu(III) (55-59) and both Cu(I) and Cu(II) are known to catalyze the breakdown
188 of HOCl (55-58). At near-neutral pH, similar to that in the large intestine or bacterial
189 cytoplasm, Cu(I) accelerates the decomposition of HOCl to O_2 and chloride ions by as
190 much as 10^8-fold (56).
191 We envisioned three possible mechanisms that might explain how Cu(II)
192 reduction to Cu(I) by RclA could protect bacteria against HOCl. First, since the copper
193 exporters of *E. coli* (CopA and CusCFBA) are upregulated by HOCl treatment (19) and
194 only transport Cu(I) (44, 60-62), it is possible that RclA facilitates the rapid export of
195 cytoplasmic copper, allowing it to react with and eliminate HOCl outside the cell.
196 Secondly, RclA might facilitate an HOCl-degrading Cu(I) / Cu(II) redox cycle in the
197 cytoplasm. Thirdly, RclA-catalyzed reduction of Cu(II) to Cu(I) might limit the production
198 of Cu(III) in the cytoplasm. Cu(III) is a known product of the reaction between HOCl and
199 Cu(II) (55-58), and is a highly reactive oxidant (59), with the potential to react with and
200 damage almost any cellular component. Uncontrolled production of Cu(III) could greatly
201 potentiate the ability of HOCl to kill bacterial cells.
202
203 **Extracellular $CuCl_2$ protects both wild-type and Δ*rclA E. coli* against HOCl.** To
204 distinguish between the above models, we first used growth curves in the presence of

205 copper and HOCl to identify how combinations of HOCl and copper influenced LPE of
206 wild type and Δ*rclA* mutant *E. coli* (Figure 5). Consistent with the ability of Cu to
207 decompose HOCl to non-toxic O_2 and Cl^- (55-58), addition of copper to HOCl-containing
208 media greatly decreased LPE for both the wild type and the *rclA* null mutant (Figure 5),
209 indicating that the presence of exogenous copper strongly protected *E. coli* against
210 HOCl. Copper being uniformly protective between the two strains makes it likely that
211 extracellular copper had reacted with and detoxified the HOCl before cells were
212 inoculated into the media.
213
214 **RclA does not protect against HOCl by facilitating copper export.** To test whether
215 the Cu(II) reductase activity of RclA is important for exporting copper during HOCl
216 stress, we measured intracellular copper concentrations before and after HOCl stress.
217 We found that the *rclA* mutant contained, on average, more intracellular copper before
218 HOCl stress than the wild-type but that both strains contained similar amounts of copper
219 after HOCl stress (Supplemental Figure 5). This indicates that although RclA may affect
220 basal copper homeostasis, it does not facilitate copper export during HOCl stress.
221
222 **RclA protects *E. coli* against the combination of HOCl and intracellular copper.**
223 Next, we aimed to investigate how intracellular copper affects the HOCl resistance of *E*
224 *coli*. To address this, we grew wild type and Δ*rclA* mutant *E. coli* overnight in minimal
225 media with and without copper before inoculating the strains into copper-free media to
226 perform HOCl-stress growth curves. If RclA's primary function was to drive cytoplasmic
227 HOCl decomposition, then the presence of intracellular copper would be expected to

228 protect wild-type *E. coli* against HOCl, and growth under copper-free conditions would
229 be expected to increase the sensitivity of the wild-type to match that of the Δ*rclA* mutant.
230 However, we actually observed that LPE extension after HOCl stress of the Δ*rclA*
231 mutant was decreased to that of wild-type when grown and stressed in copper-free
232 media (Figure 5). The decreased sensitivity of the Δ*rclA* mutant when intracellular
233 copper is limited suggests that the physiological role of RclA is to resist the stress
234 resulting from the combination of HOCl and copper in the cytoplasm. The combination
235 of copper and HOCl is known to generate strong oxidizing intermediates, most notably
236 Cu(III) (55-59). Our data demonstrate that intracellular copper potentiates killing by
237 HOCl, and that RclA's Cu(II) reductase activity counteracts this effect.
238
239 **RclA is thermostable and resistant to denaturation by HOCl and urea.** We
240 hypothesized that the copper reductase activity of RclA was likely to be relatively stable
241 under denaturing conditions because it must remain active during exposure to HOCl
242 stress, which is known to cause extensive protein misfolding and aggregation *in vivo*
243 (15, 21, 22, 26-28). To test this hypothesis, we first measured RclA activity after
244 treatment with protein denaturing agents (HOCl and urea) *in vitro*. HOCl treatment (with
245 0, 5, 10, and 20-fold molar ratios of HOCl to RclA) was done on ice for 30 minutes and
246 urea treatment (0, 2, 4, and 6 M) was carried out at room temperature for 24 hours.
247 RclA retained full copper reductase activity at all HOCl levels tested, indicating that it is
248 highly resistant to treatment with HOCl (Figure 6A). By comparison, lactate
249 dehydrogenase had significantly decreased activity after treatment with a 5-fold excess
250 of HOCl (Figure 6B). RclA also retained 35.8% of full activity after being equilibrated in 6

251 M urea (Figure 6C). Finally, we used circular dichroism (CD) spectroscopy to measure
252 the melting temperature (T_m) of RclA, which was 65 °C (Figure 6D, Supplemental Figure
253 6), indicating that RclA is thermostable relative to the rest of the *E. coli* proteome, which
254 has an average T_m of approximately 55 °C (56, 63).
255

256 DISCUSSION

257 The antimicrobial function of copper in host-microbe interactions is well-
258 established (29, 31, 49, 50), although the exact mechanism(s) by which copper kills
259 bacteria remain incompletely known (52, 53). In this work, we identified a new way in
260 which copper toxicity contributes to host-bacteria interactions via its reactions with RCS.
261 We identified RclA as a highly stable Cu(II) reductase (Figure 4) required for resisting
262 killing by the combination of HOCl and intracellular copper in *E. coli* (Figure 5). In the
263 absence of *rclA*, *E. coli* had a significant defect in host colonization which was partially
264 eliminated when production of HOCl by the host was reduced (Figure 2). Duox
265 activation and HOCl production are rapid host responses that occur at early stages of
266 bacterial colonization of the gut, in agreement with our data showing that RclA is
267 important for initial establishment. As the course of infection progresses, additional
268 antimicrobial effectors, such as antimicrobial peptides regulated by NF-κB signaling,
269 become more prominent (27), which could explain the similar colonization levels of
270 mutant and wild-type strains at later time points. The amount of copper in bacterial cells
271 is low (29, 30), but how much is unbound by protein and its redox state under different
272 conditions are unknown (29). Given the broad conservation of RclA among host-

273 associated microbes, there is likely to be a common and previously unsuspected role for
274 copper redox reactions in interactions between bacteria and the innate immune system.
275 Copper accumulates in host tissues during inflammation (64, 65), as do RCS (66,
276 67). Our discovery that even very low concentrations of extracellular copper can protect
277 bacteria against RCS adds a new and important facet to understanding copper's role in
278 innate immunity. Since a large proportion of host tissue damage during inflammation is
279 due to HOCl (68, 69), the presence of copper in inflamed tissues may play an important
280 role not only in killing bacteria, but potentially in protecting host cells, although this
281 hypothesis will require further testing. Our results also show that media copper
282 concentrations are a key variable in experiments testing the sensitivity of cells to HOCl,
283 and that care must be taken to account for media copper content and use metal-free
284 culture vessels in such experiments.
285 While RclA itself is widely conserved, the *rclABCR* locus as a whole is restricted
286 to certain enteric proteobacteria, including *E. coli*, *Salmonella*, *Citrobacter*, *Raoultella*,
287 *Serratia* and *Shigella*. These genera are notable for their close association with gut
288 inflammation and the ability of pathogenic strains to bloom to very high levels in the gut
289 in disease states (2, 3, 7-10, 70, 71). We hypothesize that the ability to survive
290 increased levels of antimicrobial compounds (including RCS) in the inflamed gut is
291 important for the ability of enterobacteria to exploit this niche, and our *in vivo* results
292 with the Δ*rclA* mutant support this idea (Figure 2). We do not currently know the
293 physiological roles of RclB, which is a small predicted periplasmic protein, or RclC,
294 which is a predicted inner membrane protein, although deletion of either of these genes
295 results in increased HOCl sensitivity in *E. coli* (20). We hypothesize that they may

296 support the copper-dependent activity of RclA, and are currently pursuing experiments
297 to test this idea.
298

299 EXPERIMENTAL PROCEDURES

300 Detailed experimental procedures are described in the *SI Appendix*.
301

302 ***Protein expression and purification.*** Twin-strep tagged RclA was expressed in M9
303 minimal media (72) containing 2 g l^{-1} glucose and 100 µg ml^{-1} ampicillin at 20 ^{o}C.
304 Recombinant RclA was purified using a 1 mL StrepTrap HP column (GE, 28-9136-30
305 AC) according to the manufacturer's instructions.
306

307 ***Colonization of D. melanogaster with E. coli Nissle 1917.*** *Canton-S D. melanogaster*
308 were used as a wild-type line. Duox-RNAi *D. melanogaster* were obtained from crosses
309 of *UAS-dDuox-RNAi* (17) with *NP1-GAL4* (enterocyte - specific driver). Adult female *D.*
310 *melanogaster* were starved for 2h at 29˚C prior to being fed a 1:1 suspension of
311 bacteria (OD_{600} = 200) and 2.5% sucrose applied to a filter paper disk on the surface of
312 normal fly food. *D. melanogaster* were sterilized and rinsed in sterile PBS before being
313 homogenized in screw-top bead tubes with PBS. *E. coli* colonies were identified by
314 morphology and counted.
315

316 ***NADH oxidase activity.*** RclA activity was assayed in 20 mM HEPES, 100 mM NaCl,
317 pH 7 by measuring NADH oxidation over time. Reactions were started by adding 100 µL

318 of NADH (200 μM final) with the indicated metal salts (200 μM final) to RclA (3 μM final)
319 at 37 ºC. NADH was quantified spectrophotometrically (ε_{340} = 6300 M^{-1} cm^{-1}).
320
321 ***Copper (I) quantification.*** Cu(I) accumulation after the course of the RclA reaction was
322 measured using bathocuproinedisulfonic acid disodium salt (BCS, Sigma Aldrich
323 B1125). NADH oxidation reactions were carried out as in the previous section. Each
324 reaction was stopped at 5 minutes with a BCS (400 μM final) and EDTA (1 mM final)
325 solution. The absorbance of the BCS/Cu(I) complex was measured at 483 nm every
326 minute for five minutes to ensure complete saturation of the BCS, after which BCS/Cu(I)
327 was quantified spectrophotometrically (ε_{483} = 13,000 M^{-1} cm^{-1}) (46).
328
329 ***Measuring sensitivity to HOCl* in vivo*.*** Copper-free MOPS was prepared by treating
330 MOPS minimal media (Teknova) containing 2 g l^{-1} glucose and 1.32 mM K_2HPO_4 with
331 Chelex 100 (Bio-Rad), then adding back the metals normally present in MOPS except
332 for copper. Overnight cultures were normalized to A_{600} = 0.02 in copper-free MOPS with
333 the indicated concentrations of HOCl and $CuCl_2$. Cultures were incubated with shaking
334 at 37 °, measuring A_{600} every 30 minutes for 27 hours. Sensitivity was determined by
335 comparing lag phase extensions (LPE; difference in hours to reach A_{600} ≥ 0.15 from the
336 no HOCl treatment control under the same $CuCl_2$ condition) for each stress condition.
337
338 ***Measuring intracellular copper after HOCl stress.*** Trace metal analysis was
339 performed using inductively coupled plasma mass spectroscopy (ICP-MS, Agilent
340 Technologies 7700x ICP-MS, Santa Clara, CA, USA). Wild-type and Δ*rclA E. coli* were

341 grown to A_{600} = 0.6 in MOPS and stressed with 400 μM HOCl for 30 mins at 37 ^{o}C with
342 shaking. Each sample was pelleted and rinsed three times with PBS before being
343 suspended in concentrated nitric acid. Samples were diluted to 2% nitric acid and
344 filtered before ICP-MS analysis. Copper concentrations were determined by comparison
345 to a standard curve using Agilent software (ICP-MS MassHunter v4.3) and normalized
346 to cell mass (wet weight).
347

348 ***NADH oxidase activity after treatment with urea and HOCl.*** RclA (3 μM) was
349 equilibrated for 24 hours at room temperature with increasing concentrations of urea (0,
350 2, 4, and 6 M) in 20 mM HEPES, 100 mM NaCl, pH 7. HOCl treatment was done by
351 mixing increasing molar ratios of HOCl (0, 5, 10, and 20-fold excess) with 35 μM RclA or
352 L-lactate dehydrogenase (LDH) (Sigma-Aldrich) in 50 mM sodium phosphate, pH 6.8,
353 150 mM NaCl and incubating on ice for 30 minutes. LDH reactions were performed
354 under the same conditions as the RclA reactions but contained no copper and were
355 started with 1.2 mM NADH and 1.2 mM pyruvate.

356

357 ***Melting temperature determination*.** CD spectra were collected on purified RclA in 20
358 mM HEPES, 100 mM NaCl, pH 7.5. Thermal CD data between 30 °C and 90 °C were
359 obtained in standard 1.0 mm quartz cells. Data were baseline corrected against the
360 appropriate buffer solution and smoothed with Jasco software.
361

362 ***Data analysis and bioinformatics.*** All statistical analyses were performed using
363 GraphPad Prism (version 7.0a). RclA conservation tree was made from amino acid

364 sequence alignments using MUSCLE of RclA (BLAST e value < $1x10^{-90}$ in 284 species),
365 RclB (BLAST e value < $1x10^{-1}$ in 61 species), RclC, (BLAST e value < $1x10^{-80}$ in 49
366 species), and RclR (BLAST e value < $1x10^{-40}$ in 43 species). BLAST searches were
367 done by comparing to each respective protein in *E. coli* MG1655. Tree graphic was
368 made using the interactive tree of life (73). Active site alignment of *E. coli* RclA
369 (ADC80840.1) and MerA amino acid sequences from seven bacterial species was
370 made using CLUSTAL O (1.2.4) and graphic was made with WEBLOGO
371 (weblogo.berkeley.edu/logo.cgi). Full length alignment of *E. coli* RclA (ADC80840.1)
372 and MerA (ADC80840.1), conservation scoring, and graphic were made using
373 PRALINE.
374

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383

384 CONFLICTS OF INTEREST

385 The authors have no conflicts of interest to declare.
386

387 AUTHOR CONTRIBUTIONS

388 R.M.D., A.J.B., and I.F.C. performed research, and R.M.D., A.J.B, S.E.L., N.A.B, and
389 M.J.G. contributed to designing research, analyzing data, and writing the paper.

390

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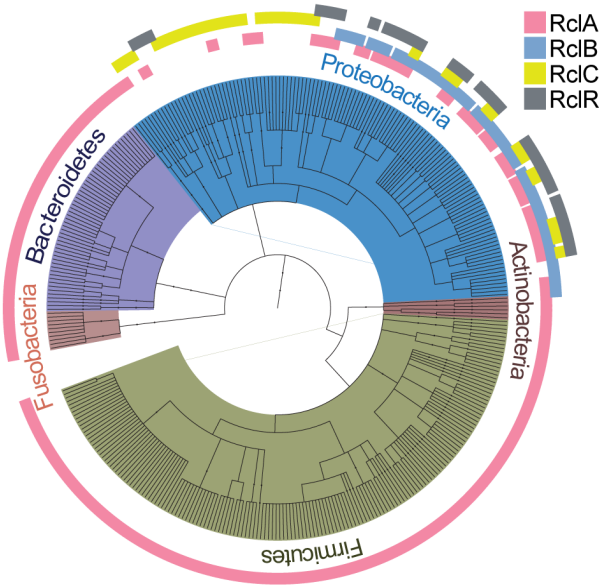
572 FIGURES



573

574 **Figure 1: RclA is widely conserved among bacteria that colonize epithelial**
575 **surfaces.** Phylogenetic tree made from amino acid sequence alignments of RclA (284
576 species), RclB (61 species), RclC, (49 species), and RclR (43 species). See SI Dataset
577 S1 for lists of each hit used in the phylogenetic tree. Tree graphic was made using the
578 interactive tree of life (73).
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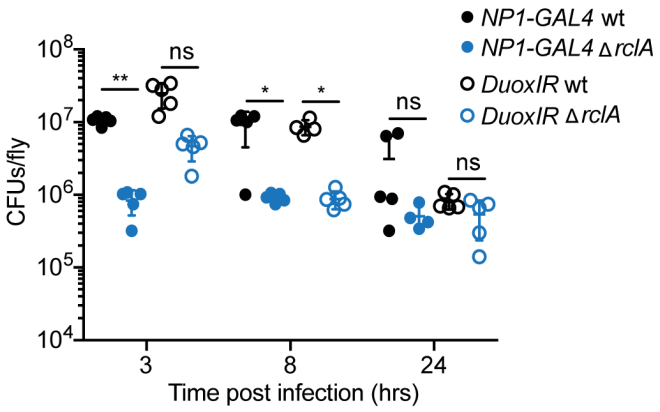


Figure 2: RclA contributes to host colonization in the presence of Duox-mediated oxidative stress. *E. coli* Nissle 1917 (EcN) Δ*rclA* mutants had a significant defect in their ability to colonize *D. melanogaster* compared to wild-type (wt) EcN. *NP1-GAL4* control or *DuoxIR* (Duox-inhibited) *D. melanogaster* were fed either wt or Δ*rclA* EcN and bacterial loads were measured (n = 4 - 5, ± SD). Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparisons test (**** = P < 0.0001, ** = P < 0.01, ns = not significant).

Figure 3: RclA and Mercuric reductase (MerA) share a conserved active site. Active site consensus sequence of *E. coli* RclA and MerA amino acid sequences from seven bacterial species (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Serratia marcescens)*. Alignment was made using CLUSTAL O (1.2.4) and graphic was made with WEBLOGO.

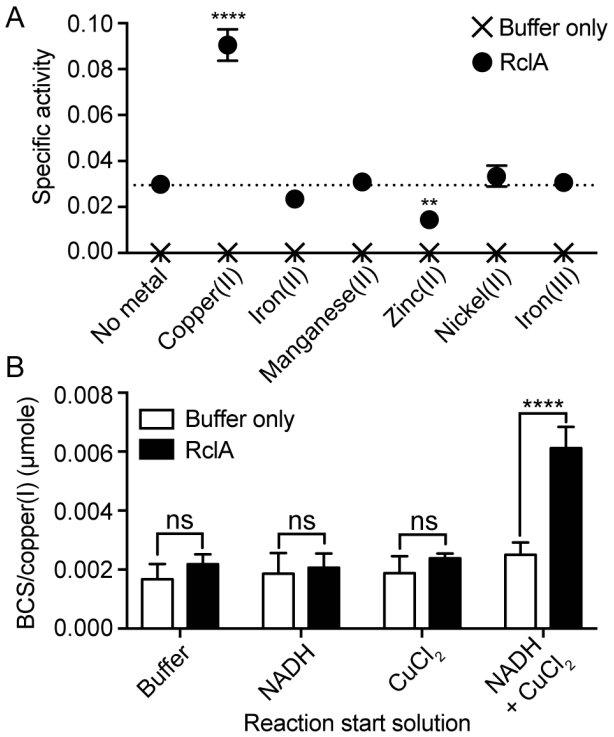


Figure 4: RclA is Cu(II) reductase. (A) RclA specific activity (SA) increases in the presence of Cu(II). Specific activity (µmole NAD^+ min^{-1} mg^{-1} RclA) of RclA was assayed by measuring NADH oxidation over time spectrophotometrically (n = 6, ± SD). Differences in SA in the presence of each metal were analyzed using a two-way ANOVA with Dunnet's multiple comparison test using the no metal reaction as the control (****= P < 0.0001, ** = P < 0.01). (B) Cu(I) accumulates after the RclA and NADH/copper (II) reaction, as measured by BCS/Cu(I)-complex absorption. RclA reactions were started with the indicated solutions and carried out as described in A (n = 6 for each reaction type, ± SD). Differences in the amount of BCS/Cu(I) complex between the buffer only and RclA reactions were analyzed using a two-way ANOVA with Sidak's multiple comparison test (****= P < 0.0001, ns = not significant).

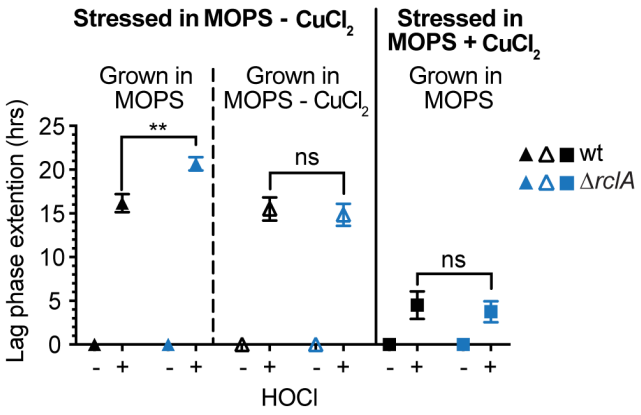


Figure 5: Extracellular $CuCl_2$ protects both wild-type and Δ*rclA E. coli* against HOCl and *rclA* is required to resist the combination of HOCl and intracellular Cu. Comparing LPE of wild-type (wt) and Δ*rclA E. coli* strains in various combinations of HOCl (132 μM) and $CuCl_2$ (n = 3, ± SD). Media conditions are as follows: "stressed/grown in MOPS" = background copper in the media (9 nM $CuCl_2$), "stressed in MOPS + $CuCl_2$" = background copper in the media (9 nM $CuCl_2$) + 10 μM $CuCl_2$ added (copper was added to media and mixed with HOCl before resuspending cells in the mixture), "stressed/grown in MOPS – $CuCl_2$" = media treated with a metal chelator, then supplemented with all metals usually present in MOPS excluding copper. Differences between strain LPE were determined by comparing LPE for each $CuCl_2$ concentration at each growth condition between strains (two-way ANOVA with Tukey's multiple comparison test; *** = $P < 0.001$, ** = $P < 0.01$, ns = not significant).

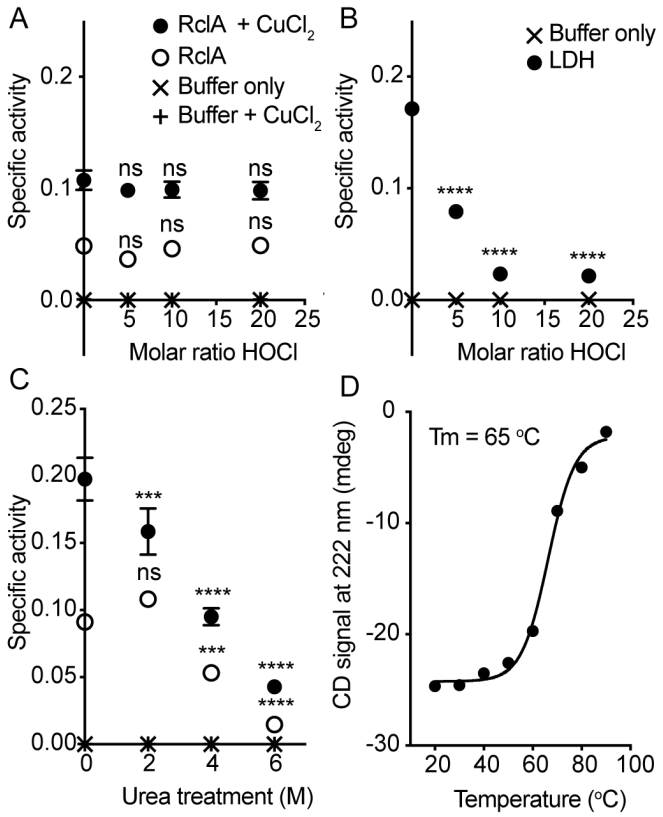
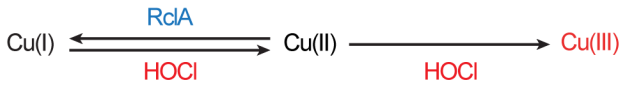


Figure 6. RclA is resistant to denaturation. (A) SA of RclA (μmoles NAD^+/min^{-1} mg $RclA^{-1}$), with and without $CuCl_2$, after being treated with the indicated molar ratios of HOCl to RclA. (B) SA of lactate dehydrogenase (LDH) (μmoles NAD^+/min^{-1} U LDH^{-1}) used as control reactions for HOCl degradation of enzymatic activity. (C) RclA with and without $CuCl_2$ (200 μM final) after being treated with the indicated concentrations of urea. (D) CD signals at 222 nm (mdeg) (raw data shown in Supp Figure 6) at each temperature used to determine the T_m of RclA (65 ˚C). Differences in SA (n = 6, ± SD) after treatment were analyzed using a two-way ANOVA with Sidak's multiple comparison test for HOCl treatment (A and B) and Dunnet's test using the buffer only reaction as the control for the urea treated samples (C) (**** = P < 0.0001, * = P < 0.05 ns = not significant).

636 

637 **Figure 7. Proposed model for how RclA protects *E. coli* against combined HOCl**

638 **and copper stress.** The Cu(II) reductase activity of RclA may be important in reversing

639 the oxidation of copper by HOCl, preventing the production of toxic Cu(III).