Methionine oxidation under anaerobic conditions in *Escherichia coli*.

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ABSTRACT

Repairing oxidative-targeted macromolecules is a central mechanism necessary for living organisms to adapt to oxidative stress. Reactive oxygen and chlorine species preferentially oxidize sulfur-containing amino acids in proteins. Among these amino acids, methionine can be converted into methionine sulfoxide. This post-translational oxidation can be reversed by methionine sulfoxide reductases, Msr enzymes. In Gram-negative bacteria, the antioxidant MsrPQ system is involved in the repair of periplasmic oxidized-proteins. Surprisingly, in this study we observed in *Escherichia coli* that *msrPQ* was highly expressed in the absence of oxygen. We have demonstrated that the anaerobic induction of *msrPQ* was due to chlorate (ClO_3^-) contamination of the Casamino Acids. Molecular investigation led us to determine that the reduction of chlorate to the toxic oxidizing agent chlorite (ClO_2^-) by the three nitrate reductases (NarA, NarZ and Nap) led to methionine oxidation of periplasmic proteins. In response to this stress the *E. coli* HprSR two-component system was activated, leading to over-production of MsrPQ. This study therefore supports the idea that methionine oxidation in proteins is part of chlorate toxicity, and that MsrPQ can be considered as an anti-chlorate/chlorite defense system in bacteria. Finally, this study challenges the traditional view on the absence of Met-oxidation during anaerobiosis.

INTRODUCTION

Bacteria must effectively counter a myriad of stresses to survive (Fang *et al.*, 2016), oxidative stress being one of the most challenging (Imlay, 2015). Under aerobic conditions oxygen is used as the terminal electron acceptor for aerobic respiration, during which reactive oxygen species (ROS) are produced endogenously through the reaction between O_2 and univalent electron donors, such as metals, flavin and quinol (Imlay, 2013). In addition to ROS, a wide variety of reactive nitrogen species (RNS) and reactive chlorine species (RCS) act as powerful oxidants (Winterbourn and Kettle, 2013). A huge number of these types of oxidants are released in order to combat pathogens during the host immune oxidative-burst response. ROS, RNS and RCS are toxic and damage biological macromolecules (nucleic acids, proteins, lipids), ultimately becoming life threatening (Imlay, 2015).

Living organisms have developed two strategies to protect themselves from oxidants: the first one, pre-oxidation, consists in eliminating harmful oxidants and relies on ROS scavenging enzymes such as superoxide dismustases (SOD) to remove toxic O_2^- and catalases/peroxidases to prevent H_2O_2 accumulation (Imlay, 2013); the second, post-oxidation, involves the repair of oxidative-targeted macromolecules (Vaughan, 1997; Merdanovic *et al.*, 2011; Aussel and Ezraty, 2021).

Several amino acids can be targeted by oxidants leading to post-translational modifications and protein misfolding (Schramm *et al.*, 2020). Under oxidative stress, sulfur-containing methionine residues are particularly sensitive to oxidation leading to the formation of methionine sulfoxide (Met-O) (Kim *et al.*, 2014; Ezraty *et al.*, 2017). Oxidation of Met into Met-O is reversible because of specific enzymes found in most living organisms: the methionine sulfoxide reductases (Msr) (Ezraty *et al.*, 2005; Boschi-Muller and Branlant,

2014). The main representatives of this oxidized-protein repair family are MsrA and MsrB, which are complementary and ubiquitous enzymes present in all domains of life from humans to bacteria (Ezraty *et al.*, 2005). In prokaryotic cells, MsrA and MsrB are generally located in the cytoplasm with some rare exceptions: for example the extracytoplasmic MsrAB of *Neisseria* or *Streptococcus* species (Skaar *et al.*, 2002; Lowther *et al.*, 2002; Saleh *et al.*, 2013).

A recently characterized member from *Escherichia coli* is MsrP, an enzyme responsible for protein-MetO reduction in the periplasm of most Gram-negative bacteria (Gennaris *et al.*, 2015). MsrP is a molybdoenzyme containing a molybdenum cofactor of the molydopterin type (Mo-MPT), characteristic of the sulfite oxidase family (Loschi *et al.*, 2004). This reductase works with the haem-binding membrane-bound partner MsrQ, which transmits reducing power (Gennaris *et al.*, 2015). We have previously shown that the cistron *hiuH* -encoding a transthyretin-like periplasmic protein (Lee *et al.*, 2005)- and *msrPQ* genes belong to the same operon (El Hajj *et al.*, 2022). The *hiuH-msrPQ* operon is induced by RCS in a two-component system HprSR-dependent manner (El Hajj *et al.*, 2022).

An anaerobic lifestyle is not considered to produce ROS. However, many obligate anaerobes actually have ROS scavenging enzymes (superoxide dismustases or reductases; catalases/ peroxidases) and oxidative-damage repair systems (Sheng *et al.*, 2014; Imlay, 2019). Msr genes (*msrA* and *msrB*) are also present in anaerobic organisms (including *Clostridium acetobutylicum* or *perfringens* and *Chlorobium tepidum*) (Delaye *et al.*, 2007). The presence of antioxidant mechanisms in anaerobes has been suggested to be required following transient exposure to oxygen (Fu *et al.*, 2015).

In this study, we highlight the importance of the antioxidant enzyme MsrP in the absence of oxygen for the facultative anaerobic bacterium *E. coli*. We observed periplasmic protein-bound Met-O appearance during anaerobiosis following chlorate reduction to chlorite by

nitrate reductases. Mechanistically we have shown that MsrPQ production via HprSR during chlorite stress is essential for *E. coli*. Finally, our results challenge the traditional view that Met-oxidation is absent during anaerobiosis.

RESULTS

msrP expression is induced under anaerobic conditions in the presence of Casamino Acids

As reported in previous studies, we found that reactive chlorine species (HOCl and *N*-Chlorotaurine) induce the expression of the *hiuH-msrPQ* operon in *E. coli* (El Hajj *et al.*, 2022). These results corroborate the idea that the oxidized-protein repair system is necessary during oxidative stress. During this study, an unexpected observation was made: this operon is also highly up-regulated under anaerobic conditions in the absence of any apparent exogenous oxidative stress. This initial unpredicted observation suggested that an antioxidant system might be needed in the absence of oxygen and was the subject of subsequent studies. A more precise characterization of this effect led us to observe that the induction of *hiuH-msrPQ* was present in both minimal and rich medium, but only in the presence of Casamino Acids (CASA) (Fig. 1A). Indeed, by using a strain carrying the chromosomal *hiuH-lacZ* translational fusion, we showed that under anaerobic conditions the activity of the fusion increased about 20-fold in the presence of CASA in both media (Fig. 1A), whereas under aerobic conditions the expression of the fusion was not affected by the presence of CASA. This effect was confirmed on analysing MsrP protein levels by western blot (Fig. 1B). These observations led us to hypothesize that the HiuH and/or MsrPQ system might be important for cell growth under anaerobic conditions in the presence of CASA. To test this hypothesis, we plated the Δ*hiuH-msrPQ*, *hiuH*$^{-}$, Δ*msrP* and

Δ*msrQ* mutant strains onto LB-agar plates supplemented with CASA under anaerobic conditions and observed that disruption of the *hiuH-msrPQ* operon led to a defect in colony morphology (Fig. 1C). Moreover, we observed that disruption of the single *hiuH* gene had no effect on colony morphology, whereas deletions of *msrP* or *msrQ* showed a severe phenotype (Fig. 1C). Indeed, we observed that the Δ*hiuH-msrPQ,* Δ*msrP* and Δ*msrQ* mutant colonies tended to be smaller than wild type colonies. The shape was readily distinguishable, as the colonies of the mutant strains were cauliflower- or donut-shaped. This morphological defect was not dependent on the density of the colonies on the plate (Fig. S1) as previously described (Warne *et al.*, 1990). Overproduction of MsrP using the plasmid pMsrP complemented only the Δ*msrP* mutant, whereas overproduction of MsrP and MsrQ using the plasmid pMsrPQ was able to complement the mutant strains Δ*hiuH-msrPQ*, Δ*msrP* and Δ*msrQ* (Fig. 1D). These results support the conclusion that MsrPQ plays an important role in bacterial growth under anaerobic conditions in the presence of CASA.

FNR has an indirect effect on the regulation of *msrPQ* under anaerobic conditions in the presence of CASA.

We established that *msrPQ* was up-regulated under anaerobic conditions in the presence of CASA and that the absence of the MsrPQ system rendered *E. coli* highly sensitive to these conditions. However, it remained unclear why an antioxidant system appeared essential in the absence of oxygen. An understanding of the regulatory pathway could clarify the situation and therefore we initially investigated whether FNR, the master transcriptional regulator of anaerobiosis (Spiro, 1994; Kiley and Beinert, 1998), was involved. We examined *hiuH-lacZ* expression in a Δ*fnr* strain: induction of the *hiuH-lacZ* fusion under

anaerobic conditions with CASA depended on the presence of FNR since no up-regulation was observed in a Δ*fnr* mutant (Fig. 2A). Furthermore, no accumulation of MsrP was observed following immunoblotting of the Δ*fnr* mutant under anaerobic conditions with CASA (Fig. 2B). Interestingly, deletion of *fnr* was able to suppress the colony morphology phenotype of the Δ*msrP* strain. The colonies of the Δ*msrP* Δ*fnr* strain were smooth and circular compared to the rough colonies of the wild-type strain (Fig. 2C). These results suggest that FNR participates in *msrPQ* regulation. One prediction of our model is that FNR directly binds and regulates the *hiuH-msrPQ* operon. To test this, we performed an *in silico* analysis of the *hiuH-msrPQ* promoter region, using the DNA consensus binding site previously determined (Myers *et al.*, 2013), and identified a putative FNR-binding site (5'-**TTGA**AGA**T**A**ATCAA**-3') between positions -148bp and -162bp relative to the start codon of the *hiuH* gene. We then constructed a deletion mutant of this putative FNR-binding site in the *lacZ* fusion and showed that the deletion of this sequence had no impact on the induction of the *hiuH-lacZ* fusion under anaerobic conditions with CASA (Fig. 2A). These results favoured an indirect role for FNR in *msrPQ* regulation in *E. coli*.

Nitrate reductases are required for the regulation of *msrPQ* under anaerobic conditions with CASA.

Molybdenum cofactor-dependent anaerobic respiration DMSO reductase family system genes are FNR-regulated (Myers *et al.*, 2013). Deletion of the *mobAB* operon required for the synthesis of the bis-Mo-MGD cofactor used by these systems, prevent accumulation of MsrP under anaerobic conditions with CASA and suppressed the colony morphology phenotype of the Δ*msrP* mutant strain, the colonies appeared smoother and less

147 segmented than wild-type colonies (Fig. 2B and C). This observation suggested that one or
148 more molybdenum cofactor-dependent anaerobic respiration systems are involved in this
149 phenotype. Indeed, the addition of alternative electron acceptors such as nitrate and TMAO
150 during anaerobic growth abrogated the effect of CASA on the induction of the *hiuH-lacZ*
151 fusion, while adding DMSO had no effect (Fig. 3A and B). Nitrate, and to a lesser extent
152 TMAO, chemically suppressed the Δ*msrP* colony morphology phenotype, while DMSO had
153 no impact (Fig. 3C). To perform anaerobic respiration using nitrate or TMAO as alternative
154 electron acceptors, *E. coli* relies on two cytoplasmic and one periplasmic nitrate reductase
155 (NR) (NarGHI, NarZYV and NapABC) (Fig. 4A) or TorAC respectively (Stewart, 1988;
156 Magalon and Mendel, 2015). Deletion of one NR had no effect on the induction of *msrPQ*
157 during anaerobiosis in the presence of CASA. However, the use of the Δ*narG* Δ*narZ* Δ*napA*
158 mutant strain abrogated the up-regulation of *msrPQ*, suggesting that NR systems are
159 required for the induction of *msrPQ* during anaerobiosis in the presence of CASA (Fig. 4B).
160 Furthermore, no accumulation of MsrP was observed in the Δ*narG* Δ*narZ* Δ*napA* mutant
161 strain, whereas deleting either *narG*, *narZ*, *napA* had no effect on MsrP accumulation (Fig.
162 4C). We confirmed the involvement of the three NRs by deleting the two genes encoding
163 cytoplasmic nitrate uptake proteins (NarU and NarK) (Clegg *et al.*, 2002), and by showing
164 that the Δ*narU* Δ*narK* Δ*napA* mutant strain abrogated the up regulation of *msrPQ* (Fig. 4B).
165 Given that TMAO could partially suppress the Δ*msrP* colony morphology phenotype on
166 CASA, we conducted an experiment using the Δ*torA* mutant strain. Deletion of *torA* had no
167 effect on MsrP accumulation and did not suppress the colony morphology phenotype of the
168 Δ*msrP* (Fig. 4C and D). Surprisingly, deletion of *narG* alone was able to suppress the colony
169 morphology phenotype of the Δ*msrP* strain. The Δ*msrP* Δ*narG* colonies were larger and
170 more opaque than wild-type colonies (Fig. 4D). These results suggested that the

171 requirement for MsrPQ during anaerobiosis in the presence of CASA depends on NR
172 activities.

173

174 Reduction of chlorate to chlorite by nitrate reductases induces *msrPQ*.

175 Given the multiple lines of evidence suggesting a link between MsrPQ and NRs during
176 anaerobiosis in the presence of CASA and in the absence of nitrate, we investigated
177 whether a component of the CASA could be used as a substrate for NRs. The 0.4 % CASA
178 solution was analysed by an external laboratory and a chlorate (ClO_3^-) contamination of
179 around 1.5 mg/L was detected. Chlorate, as an analogue of nitrate, is a substrate for NRs
180 which reduce chlorate to the toxic chlorite (ClO_2^-) (Glaser and DeMoss, 1971; Alefounder
181 and Ferguson, 1980; Giordano *et al.*, 1984). By analysing the expression of the *hiuH-lacZ*
182 fusion and the western blot, we confirmed that the addition of chlorate to the LB medium
183 under anaerobic conditions led to an increase in the expression of MsrP (Fig. 5A and B).
184 Moreover, the Δ*msrP* strain showed a donut-shaped colony morphology phenotype on LB
185 plates containing chlorate (Fig. 5C), as observed with CASA. These results suggested that
186 the effects observed with CASA were due to the presence of chlorate in the CASA solution.
187 To confirm that the chlorate toxicity of the Δ*msrP* strain comes from chlorate reduction to
188 chlorite by NRs, we attempted to suppress the phenotype of the Δ*msrP* mutant by
189 overproducing chlorite dismutase (Cld) from *Dechloromonas aromatica* (Goblirsch *et al.*,
190 2010). The Cld enzyme catalyses the decomposition of chlorite into molecular oxygen and
191 chloride ions. Fig. 6A shows that Cld substantially suppressesed the Δ*msrP* mutant
192 phenotype. Next, we examined the effect of chlorite on the expression of the *hiuH-lacZ*
193 fusion. We observed that ClO_2^- induced the expression of the fusion in a dose-dependent

manner (Fig. 6B). Cld co-expression abrogated *hiuH-lacZ* up-regulation during ClO_2^- stress (Fig. 6C and D). Overall, these results confirm that chlorite production via chlorate reduction by NRs led to the Δ*msrP* colony morphology phenotype and induction of *msrPQ* expression.

HprSR two-component system detects chlorite stress and induces *msrPQ*.

We have previously demonstrated that HOCl induces the expression of *msrPQ* in an HprSR-dependent manner (El Hajj *et al.*, 2022). HprSR is a two-component system in which HprS and HprR are the sensor and the regulator respectively. We thus investigated the role of HprSR in chlorite detection and performed immunodetection of MsrP from WT, Δ*hprS* and Δ*hprR* strains cultivated anaerobically in the presence of chlorate. We observed an accumulation of MsrP in the WT strain (Fig. 7), but no over-production of MsrP in the Δ*hprS* and Δ*hprR* mutants (Fig. 7). These results indicate that the induction of the *hiuH-msrPQ* operon by chlorate is dependent on the presence of a functional HprSR system and suggest that HprS is able to sense chlorite production in the cell.

Chlorite-damaged periplasmic proteins

Having established that the periplasmic oxidized-protein repair system, MsrPQ, was over-produced during chlorite stress, we sought to determine the effect of chlorite stress on the methionine-redox state of periplasmic proteins by following the primary periplasmic chaperone SurA, a specific substrate of MsrP in *E. coli* (Gennaris *et al.*, 2015). Using western blots, we observed that SurA produced in a Δ*msrP* mutant exposed to chlorite treatment migrated more slowly than SurA produced in a WT strain or in a Δ*msrP* mutant

that had not been exposed to chlorite (Fig. 8). This mobility-shift seen on SDS-PAGE was evidence for methionine oxidation in SurA. These data suggest that chlorite was able to oxidize Met residues *in vivo*. The findings presented so far suggest that periplasmic proteins are oxidized by chlorite and that MsrP is important in the repair of chlorite-induced protein damage.

Chlorate stress did not induce the expression of cytoplasmic methionine sulfoxide reductases

We also examined whether chlorate stress induced the expression of the cytoplasmic oxidized-protein repair genes, *msrA* and *msrB*. The strains carrying the chromosomal reporter fusions p*msrA-lacZ* and p*msrB-lacZ* were cultured in the absence or in the presence of chlorate under anaerobic conditions. No induction of p*msrA-lacZ* and p*msrB-lacZ* fusions was observed during chlorate stress under anaerobic conditions (Fig. 9A). Moreover, no colony morphology phenotype was observed using the Δ*msrA* Δ*msrB* mutant (Fig. 9B). Even the deletion of the *gshA* gene, that leads to decreased intracellular glutathione levels (glutathione being putative quencher of cytoplasmic chlorite), had no effect on colony morphology of the Δ*msrA* Δ*msrB* mutant (i.e absence of donut/cauliflower-shaped colonies) on LB-CASA under anaerobic conditions (Fig. S2). We conclude that cytoplasmic methionine sulfoxide reductases are not up-regulated and that their absence does not lead to morphological colony defects under chlorate stress.

DISCUSSION

Chlorate toxicity in bacteria has been studied for many decades, especially in relation to anaerobic respiratory systems (Stewart, 1988). This study provides new insight into the molecular mechanism of chlorate toxicity and has characterized, for the first time, protection mechanisms against this stress in *E. coli*. We demonstrated that reduction of chlorate to toxic chlorite leads to periplasmic protein oxidation. In response to this stress, the *E. coli* HprSR two-component system is activated leading to the over-production of the periplasmic oxidized-protein repair system, MsrPQ.

A word of caution on chlorate contaminated Casamino Acids

A fortuitous observation led us to discover that the Casamino Acids were contaminated with chlorate, which represented the starting point for this study. This contamination does not seem to be anecdotal since another global transcriptomic analysis study reported an induction of *hiuH*, *yedY* and *yedZ* gene expression (as *msrP* and *msrQ* were previously named) under anaerobic conditions (Covert *et al.*, 2004). CASA are a product of hydrochloric acid hydrolysis of casein of animal origin and a dedicated study will be necessary to investigate the presence of chlorate depending on the commercial origin of the CASA. We would like to alert the wider population of CASA users to the possibility of chlorite production during anaerobic experiments.

New insight into chlorate reduction in *E. coli*

The ability of nitrate reductases to reduce chlorate was discovered in the sixties through the isolation of chlorate-resistant (Chl^r) mutants and subsequent genetic studies (Glaser and DeMoss, 1971; Alefounder and Ferguson, 1980; Giordano *et al.*, 1984). Retrospectively, the analysis of this plethora of mutants has made it possible to identify all the steps from

gene expression to enzyme maturation involved in the activity of nitrate reductases. The current model for chlorate reduction in *E. coli* is that chlorate is a substrate for the cytoplasmic nitrate reductase A (NarA), but not for the second cytoplasmic reductase (NarZ) or the periplasmic one (Nap). Moreover, it has been proposed that chlorate transport into *E. coli* occurs independently of the nitrate transporters NarK and NarU (Clegg *et al.*, 2002), however, these conclusions were made based on studies using high chlorate concentrations (1 to 20 mM). Here, for the first time, we highlight an *E. coli* chlorate phenotype at low chlorate concentrations (10 to 200 µM), which allowed reinvestigation of the chlorate reduction model. Our results showed that the three nitrate reductases were able to reduce chlorate, as the concomitant deletion of the three nitrate reductases was necessary to avoid chlorate induction of *msrP*. Moreover, given that the Δ*narU* Δ*narK* Δ*napA* mutant phenocopy the Δ*narG* Δ*narZ* Δ*napA* mutant strain, we propose that the nitrate transporters NarK and NarU are involved in chlorate import.

New insight into chlorate/chlorite toxicity

While chlorite production is known to be toxic to bacteria, the molecular mechanism behind the toxicity of this oxidizing agent was unknown. Previous *in vitro* studies showed that chlorite was able to oxidize protein-bound methionine (Melnyk *et al.*, 2015). In this study, we show that chlorite production by the anaerobic respiratory complex NRs damages periplasmic proteins, such as the primary chaperone SurA. Moreover, we show that chlorite activates the HprRS two-component system, whose activation has been proposed to be mediated through a methionine redox switch located in the periplasmic loop of the sensor (El Hajj *et al.*, 2022). Chlorite-HprRS activation leads to expression of the periplasmic methionine sulfoxide reductase *msrPQ*. Together, these elements suggest that protein methionine oxidation contributes to the molecular mechanisms of chlorate

toxicity. While the expression of cytoplasmic methionine sulfoxide reductases (*msrA* and *msrB*) is not induced by chlorate treatment and that there is no pronounced morphological defect in the Δ*msrA* Δ*msrB* double mutant, the possibility that cytoplasmic proteins are damaged by chlorite cannot be rule out, especially since NarA and NarZ have a cytoplasmic-facing orientation, implying that chlorite is produced in the cytoplasm. However, the fact that a strain lacking the periplasmic nitrate reductase, NapA, is still able to activate *msrPQ* expression implies that cytoplasmic chlorite is, at least partially, transferred to the periplasm, most likely driven by the membrane potential and the nitrate/nitrite antiporter. Under high chlorate content, the rate of chlorite production could be higher than the rate of periplasmic transfer, leading to oxidation of cytoplasmic proteins and therefore MsrA/B could be important for chlorate resistance.

MsrPQ appears as an anti-chlorite defense system

The up-regulation of *msrPQ* during chlorate stress in *E. coli* is reminiscent of the response found in the dissimilatory (per)chlorate-reducing bacteria (DPRB) which use (per)chlorate as an electron acceptor (Wang and Coates, 2017). In the canonical perchlorate (ClO_4^-) reduction pathway, perchlorate reductase (PcrAB) reduces ClO_4^- to chlorate (ClO_3^-) and then to chlorite (ClO_2^-) in the periplasm. Chlorite dismutase (Cld) detoxifies chlorite by generating chloride (Cl^-) and oxygen (O_2), and then a terminal oxygen reductase reduces oxygen to water. In addition to the periplasmic anti-chlorite enzyme Cld, it has been shown in the DPRB *Azospira suillum* that the *msrQP-mrpX* operon is induced during chlorite stress via a sigma/anti-sigma factor system (SigF/NrsF). MrpX is a methionine-rich periplasmic protein suggested to be an MsrP-dependent recyclable 'sponge' for oxidants (Melnyk *et al.*, 2015). Moreover, chlorate reduction in *Shewanella algae* ACDC has been shown to induce the expression of *msrPQ* (Clark *et al.*, 2014). In this study, the *huiH-msrPQ* operon in *E. coli*

315 is not shown to be regulated by FNR, in accordance with previous studies which have not
316 reported *msrPQ* as direct FNR target genes (Myers *et al.*, 2013). Interestingly, in *Shewanella*
317 *oneidensis* the *msrPQ* operon has been suggested to be under the direct control of the FNR
318 homolog, EtrA (Zupok *et al.*, 2019), but to our knowledge this has not been experimentally
319 demonstrated. Therefore, it appears that different regulatory pathways (HprRS, SigF/NrsF,
320 FNR) are involved in adaptive regulation to chlorite, leading to the production of MsrPQ,
321 which can be considered as a necessary and efficient anti-chlorite defense system for
322 bacteria. The conservation of *msrPQ* (*yedYZ*) genes throughout Gram-negative bacteria
323 (Gennaris *et al.*, 2015) and more precisely in chlorate-respiring bacteria (Barnum *et al.*,
324 2018) illustrates the importance of having a periplasmic methionine sulfoxide reductase
325 system to overcome chlorite production.
326
327 **Methionine oxidation under anaerobic conditions**
328 This study reports for the first time a molecular explanation for the importance of
329 methionine sulfoxide reductases under physiological anaerobic conditions. This finding is
330 reminiscent of the study placing MsrA/B at the centre of the *Bacillus cereus* antioxidant
331 system under anaerobic fermentative conditions, however the molecular source of
332 methionine oxidation was not demonstrated (Duport *et al.*, 2021). Therefore, our study
333 shows that methionine redox control is not a phenomenon that is solely linked to a
334 reaction with reactive oxygen species.
335
336 Finally, these results become relevant if we assume that the natural life cycle of *E. coli* takes
337 place in the gastrointestinal tract and, most of its existence is spent under anaerobic
338 conditions. Chlorate contamination of drinking water could lead to the presence of chlorate

339 in the human gut and therefore put MsrPQ at the forefront of adaptive responses in
340 bacteria.
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342
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349
350 The authors declare no conflict of interest.
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EXPERIMENTAL PROCEDURES

Chemical reagents and antibiotics

All chemicals used were purchased from Sigma, Euromedex, or Fisher Scientific. CASA used was purchased from Gibco™ Ref: #223050; batch n° 6266538. Sodium chlorate used was purchased from Acros Organics Ref: #223222500. Antibiotics were used at the following concentrations: Ampicillin (50 µg/ml) and kanamycin (25 µg/ml). Arabinose was used at 0.02%. Nitrate (40 mM), and trimethyl amine oxide (TMAO) (40 mM) were used as electron acceptors.

Strains and microbial techniques

The strains used in this study are listed in Table 1. The *fnr*::Kan^R, *narG*::Kan^R, *narZ*::Kan^R, *napA*::Kan^R, *narU*::Kan^R, *narK*::Kan^R and *torA*::Kan^R mutations from the BW25113 strain (Keio library) (Baba *et al.*, 2006) were transduced into the MG1655 wild-type strain by P1 transduction standard procedure and checked by PCR. Strains were cured using pCP20 plasmid for successive transductions in order to obtain double and triple mutants. Primer sequences used in this study are listed in Supplementary Table 2.

The *ΔhiuH-msrPQ* scarless mutant (strain LL996) was generated by lambda red recombination adapted from Blank et al. (Blank *et al.*, 2011). Briefly, the DNA fragment was amplified by PCR using primers 5-yedX-scarless and 3-wanner-msrQ (see Table 2) with plasmid pWRG100 as a template. The resulting PCR product was electroporated into BW25113 cells carrying pKD46 plasmid. After 1 h of expression, the cells were plated on LB containing 25 µg/mL chloramphenicol. Selected colonies contained a Δ*hiuH-msrPQ sce-I*::cm cassette which has been transduced in MG1655 cells harboring the pWRG99 plasmid. The chloramphenicol cassette was then removed by counter selection using lambda red recombination to insert a PCR product complementary to the flanking regions of the *sce-I*::cm cassette on the chromosome. DsDNA is prepared from two complementary 96 mer oligonucleotides generated with primers 5-scarlessyedXPQ and 3-scarlessyedXPQ. DsDNA was then electroporated into MG1655 Δ*hiuH-msrPQ sce-I*::cm expressing lambda recombinase from pWRG99, and after 1 hour expression serially diluted and plated on LB containing 100 µg/mL ampicillin and 1 µg/mL anhydrotetracycline. pWRG99 also encoded for the meganuclease I-Sce-I under the control of an anhydrotetracycline (aTc) inducible

promoter. The proper integration of the Δ*hiuH-msrPQ* was confirmed by diagnostic PCR and sequenced.

The *hiuH*- 3stop mutant (strain LL1913) was performed using the same method *as* described above. Briefly, a Δ*yedX* sce-I::cm mutant was obtained by PCR using primers 5-yedX-scarless and 3-yedX-scarless with plasmid pWRG100 as a template. The chloramphenicol cassette was then removed by counter selection using lambda red recombination to insert a *hiuH*- 3stop PCR product amplified by PCR using primers *yedX*_ATG_TAAx3_F and *yedX*_ATG_TAAx3_R.

The ΔFNR-putative-box*::cat-hiuH-lacZ* mutation was constructed in a one-step inactivation by using lambda red recombination. A *cat* gene *cassette* was inserted into the *putative FNR box* upstream *hiuH* gene. A DNA fragment containing the *cat* gene flanked with 5′ and 3′ regions bordering the *E. coli* putative FNR box contained in the *hiuH-lacZ* construction was amplified by PCR using pKD3 as a template and oligonucleotides 5del-wanner-*yedW* and 3del-wanner-*yedW*. PM1205 *hiuH-lacZ* strain, carrying the pKD46 plasmid, was transformed by electroporation with the amplified fragment and Cm^r colonies were selected.

Growth conditions

Cells were grown in Lysogeny Broth (LB) medium or in minimal medium (M9) supplied with 0.2% glucose; for solid medium, 15 g/L of agar was supplemented. In aerobic conditions, cells were grown in flask with a 1/10 volume of the flask capacity at 37 °C with shaking at 150 rpm. In anaerobic conditions, cells were grown in 2 ml tubes full to the brim, and for solid medium by using the GENbox Anaer generator (bioMérieux) in a dedicated chamber.

CASA induction assays

The *hiuH-lacZ* -containing strain (CH184) was grown over night at 37 °C under aerobic or anaerobic conditions in M9-glucose minimal medium or LB supplemented or not with CASA (0.4% w/v). Levels of β–galactosidase were measured as previously described (Miller, 1992). Growth under anaerobiosis was achieved by using 2 ml tubes full to the brim.

Colony morphology assays

Strains were spread onto LB-agar plates containing CASA (0.4 % w/v) or $NaClO_3$ (16 µM). The plates were incubated at 37 °C in anaerobic conditions for 48 hours. Growth under anaerobiosis was achieved by using the GENbox Anaer generator (bioMérieux) in a dedicated chamber. Ampicillin (50 µg/ml) and arabinose (0.02 %) were added to solid media when required. The plates are then scanned using CanonScan 4200F and colony morphology are observed using binocular magnifier Nikon SMZ800N. Colony shape homogeneity was verified, and a representative colony was shown in the figure.

Chlorate detection in CASA solution

CASA solution (0.4 %) was analysed by Flandres-analyses laboratory (59180 Cappelle la Grande) by liquid phase ion chromatography approach according to AFNOR NF EN ISO 10304-1 protocol.

Heterologous expression of the chlorite dismutase enzyme.

Genewiz from Azenta Life Sciences synthesized the pBAD24-clD-6His. The synthetic gene was inserted into the *NcoI*/*SalI* sites of pBAD24 vector. This chimeric gene encode for a protein composed by the 1-44 amino acids of the TAT signal sequence of MsrP of *E. coli* and the 40-282 amino acids of chlorite dismutase of *Dechloromonas aromatica.* A His6-tag at the *C terminus* of the polypeptide chain was introduced during the synthesis. 0.02 % arabinose was used to induce the expression of *cld* gene. Immunoblot analysis using an anti-His-tag antibody show the production of the non-mature form of Cld, suggesting that the enzyme is not efficiently translocated the periplasm. However, this Cld form is able to attenuate chlorite toxicity. MG1655 or CH184 strains harbouring pCld plasmid was grown aerobically at 37 °C in LB supplemented with ampicillin (50 µg/ml) and arabinose (0.02%). When cells reached an OD_{600nm} of 0.1, $NaClO_2$ (200 µM) was then added for 3.5 hours, followed by ß-galactosidase assays or immunoblot analysis using an anti-His-tag antibody.

Immunoblot analysis of MsrP production.

To assess MsrP production by CASA (0.04%) or chlorate (1, 10, 100 and 200 µM), cells from overnight anaerobically cultures of the CH184, Δ*hprR* and Δ*hprS* strains were harvested. The pellets were suspended in Laemmli buffer (2 % SDS, 10 % glycerol, 60 mM Tris-HCl [pH 7.4], 0.01% bromophenol blue). The amount of protein loaded onto the gel was standardized for each culture based on its A600 value. Samples were then heated for

10 min at 95 °C and loaded on SDS-PAGE. Immunoblot analysis was performed according to standard procedures: the primary antibody was a guinea pig anti-MsrP antibody (provided by Jean-François Collet Lab, De Duve Institute, Belgium). The secondary antibody was an anti-guinea pig IgG conjugated to horseradish peroxidase (HRP) (Promega). An ImageQuant LAS4000 camera (GE Healthcare Life Sciences) was used for image chemiluminescence.

In vivo repair of oxidized SurA by MsrP.

MG1655 and Δ*msrP* strains were grown in LB at 37 °C in aerobic conditions. At OD_{600} of 0.1 cells were treated or not with $NaClO_2$ (200 µM). Cells were harvested after 2 hours of $NaClO_2$ addition. The pellets were suspended in Laemmli buffer. The amount of protein loaded onto the gel was standardized for each culture based on its A600 value. Samples were then heated for 10 min at 95 °C and loaded on SDS-PAGE for immunoblot analysis using anti-SurA antibody (provided by Jean-François Collet Lab, De Duve Institute, Belgium).

464 **Table 1. Strains used in this study**

465 This table contains the information regarding the strains used in this study, including
466 strain names, genotypes, description and source.

467

Strain	Genotype and description	Source
MG1655	WT	Laboratory collection
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	(Datsenko and Wanner, 2000)
PM1205	MG1655 *mal::lacI*^{q} *ΔaraBAD araC*$^{+}$ *lacI′::P*$_{BAD}$*-cat-sacB-lacZ, mini λ tet*^{R}	(Mandin and Gottesman, 2009)
LL996	MG1655 *ΔhiuH-msrPQ*	This study
LL1913	MG1655 *hiuH*$^{-}$ *3stop mutant*	This study
BE107	MG1655 Δ*msrP*::Kan^{r}	(Gennaris *et al.*, 2015)
CH380	MG1655 Δ*msrP*	This study
BE206	MG1655 Δ*msrQ*::Kan^{r}	This study
CH184	PM1205 *hiuH-lacZ*	(El Hajj *et al.*, 2022)
LL1103	PM1205 *hiuH-lacZ* Δ*fnr*::Kan^{r}	This study
LL1699	PM1205 *(lacI-yedW::cat)-hiuH-lacZ* (deletion of the putative FNR box)	This study
LL216	MG1655 Δ*fnr*::Kan^{r}	This study
TP1004	RK4353 Δ*mobAB*::Kan^{r}	Received from Tracy Palmer
LL1119	MG1655 Δ*msrP* Δ*fnr*::Kan^{r}	This study
LL1120	MG1655 Δ*msrP* Δ*mobAB*::Kan^{r}	This study
LL1138	MG1655 Δ*narG*::Kan^{r}	This study
LL1170	LL1138 *(lacI-yedW::cat)-hiuH-lacZ*	This study
LL1130	MG1655 Δ*narZ*::Kan^{r}	This study
LL1168	CH184 Δ*narZ*::Kan^{r}	This study
LL1129	MG1655 Δ*napA*::Kan^{r}	This study
LL1166	CH184 Δ*napA*::Kan^{r}	This study
LL1144	MG1655 Δ*narG*::Kan^{r} Δ*narZ* Δ*napA*	This study
LL1171	LL1144 *(lacI-yedW::cat)-hiuH-lacZ*	This study

LL1169	MG1655 Δ*narU* Δ*narK*::Kan^r	This study
LL1231	LL1169 *(lacI-yedW::cat)-hiuH-lacZ*	This study
LL1187	MG1655 Δ*napA* Δ*narU* Δ*narK*::Kan^r	This study
LL1232	LL1187 *(lacI-yedW::cat)-hiuH-lacZ*	This study
LL1146	MG1655 Δ*msrP* Δ*narZ*::Kan^r	This study
LL1147	MG1655 Δ*msrP* Δ*napA*::Kan^r	This study
LL1148	MG1655 Δ*msrP* Δ*narG*::Kan^r	This study
LL1145	MG1655 Δ*torA*::Kan^r	This study
LL1153	MG1655 Δ*msrP* Δ*torA*::Kan^r	This study
BE152	MG1655 Δ*msrA* Δ*msrB*	(Henry *et al.*, 2021)
LL1296	MG1655 Δ*msrA* Δ*msrB* Δ*gshA*::Kan^r	This study
JB56	IBPC5321 *msrA'-'lacZ*	(Bos *et al.*, 2013)
JB44	IBPC5321 *msrB'-'lacZ*	(Bos *et al.*, 2013)
BE255	MG1655 Δ*hprR*::Kan^r	This study
BE254	MG1655 Δ*hprS*::Kan^r	This study

471 **Table 2. Primers used in this study**

472 This table contains the information regarding the primers used in this study, including
473 primer names and sequences.

474

Name	Sequence (5' to 3')
5-yedX-scarless	cgctatcctcaaaactcattcacatgacaaggatataaaccgccttacgccccgccctgc
3-wanner-msrQ	acatccggcagggataacgcgtactttgtcggcaatatgaagcatatgaatatcctcctta
5-scarlessyedXPQ	gctttttcgctatcctcaaaactcattcacatgacaaggatataaacttcatattgccgacaaagtacgc gttatccctgccggatgtggcgcgag
3-scarlessyedXPQ	ctcgcgccacatccggcagggataacgcgtactttgtcggcaatatgaagtttatatccttgtcatgtga atgagttttgaggatagcgaaaaagc
3- yedX-scarless	ccgtaacatctgattcttttaaaaattgattctttttcatctagactatattaccctgtt
*yedX*_ATG_TAAx3_F	cgctatcctcaaaactcattcacatgacaaggatataaacatgtaataataatatttagtactctcggta gcaacg
*yedX*_ATG_TAAx3_R	ccctaacagtagcagagatctgcttttacatatttatgcattaactgccacgataggttgaataccc
5del-wanner-*yedW*	cgaagcggcatgcatttacgttgacaccatcgaatggcgcgtgtaggctggagctgcttc
3del-wanner-*yedW*	ggctgtttctataacatatgatttatggcatattattttcacatatgaatatcctcctta

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478 **Table 3. Plasmids used in this study**

479 This table contains the information regarding the plasmids used in this study, including
480 plasmid names, genotypes, description and source.

481

Plasmid	Genotype and description	Source
pAG177 (p-empty)	pTAC-MAT-Tag-2-NdeI, Amp^R	(Gennaris *et al.*, 2015)
pAG192 (pMsrP)	pTAC-MAT-Tag-2-NdeI MsrP, Amp^R	(Gennaris *et al.*, 2015)
pAG195 (pMsrPQ)	pTAC-MAT-Tag-2-NdeI MsrP-MsrQ, Amp^R	(Gennaris *et al.*, 2015)
pKD46	λ Red-expression under control of arabinose-inducible promoter, temperature-sensitive, Amp^R	(Datsenko and Wanner, 2000)
pCP20	*FLP*$^+$, □□cI857$^+$, □□P_R Rep^{ts}, Amp^R, Cm^R	(Cherepanov and Wackernagel, 1995)
pWRG99	pKD46 with I-*Sce*I endonuclease under control of tetracycline-inducible promoter (P_{tetA}), temperature-sensitive, orientation 2 (5'->3': I-*Sce*I-*tetR*), Amp^R	(Blank *et al.*, 2011)
pWRG100	pKD3 with I-*Sce*I recognition site, Cm^R	(Blank *et al.*, 2011)
pBAD24	PBAD promoter, pACYC184-based, arabinose inducible, Amp^R	Laboratory collection
pBAD24-cld-6His	pBAD24-NcoI/SalI containing *cld* gene from *Dechloromonas aromatic*, Amp^R	Genewiz/Azenta Life Sciences

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485 **LEGENDS**

486 **Figure 1. A central role for MsrPQ under anaerobic conditions in the presence of**
487 **CASA.**
488 **A)** Evaluation of *hiuH-msrPQ* expression under aerobic and anaerobic conditions. The
489 expression of the *hiuH-lacZ* fusion, in strain CH184, was used as a proxy for *msrPQ*
490 expression. Strain CH184 was cultured overnight at 37°C in M9-Glucose or LB
491 supplemented or not with 0.4 % CASA, under aerobic or anaerobic conditions, followed by
492 ß-galactosidase assays. Results are expressed as means +/- SD (n = 3). Asterisks indicate a
493 statistically significant difference between the absence and the presence of CASA,
494 ***P<0.001 (Ordinary one-way ANOVA with Tukey's multiple comparison test) **B)**
495 Immunoblot analysis using an anti-MsrP antibody showing the production of MsrP under
496 anaerobic conditions in the presence of 0.4 % CASA. The image is representative of
497 experiments carried out in triplicate. **C)** Evaluation of colony morphology on LB-CASA agar
498 plates. Wild-type (MG1655), Δ*hiuH-msrPQ* (LL996), *hiuH*$^{-}$ (LL1913), Δ*msrP* (CH380) and
499 Δ*msrQ* (BE206) strains were plated onto LB-agar containing 0.4 % CASA. The plates were
500 incubated at 37 °C under anaerobic conditions for 48 hours. The plates were then scanned
501 (left panel) and observed using a binocular magnifier (right panel). The images are
502 representative of experiments carried out (at least) in triplicate. **D)** Colony morphology of
503 Δ*hiuH-msrPQ* (LL996), Δ*msrP* (CH380) and Δ*msrQ* (BE206) strains carrying either an
504 empty vector, pMsrP or pMsrQ plasmids. The strains were plated onto LB-ampicillin-agar
505 containing 0.4 % CASA. The plates were incubated at 37 °C under anaerobic conditions for
506 48 hours before being observed using a binocular magnifier. The images are
507 representative of experiments carried out at least three times.
508
509 **Figure 2. An indirect role for FNR in *msrPQ* expression.**
510 **A)** Effect of *fnr* deletion on *hiuH-msrPQ* expression. Wild-type and Δ*fnr* strains carrying the
511 *hiuH-lacZ* fusion and the wild-type strain carrying the p**hiuH-lacZ* fusion (deletion of the
512 putative FNR box) were cultured overnight in LB supplemented with 0.4 % CASA under
513 anaerobic conditions, followed by ß-galactosidase assays. Results are expressed as means
514 +/- SD (n = 3). Asterisks indicate a statistically significant difference between mutant and
515 the WT, ***P<0.001 (Ordinary one-way ANOVA with Tukey's multiple comparison test) **B)**
516 Immunoblot analysis using an anti-MsrP antibody, showing the production of MsrP from
517 wild-type, Δ*msrP* (CH380), Δ*fnr* (LL216) and Δ*mobAB* (TP1004) strains cultivated under

anaerobic conditions in the presence of 0.4 % CASA. The image is representative of experiments carried out in triplicate. **C)** Evaluation of colony morphology on LB-CASA agar plates. Wild-type (MG1655), Δ*msrP* (CH380), Δ*msrP* Δ*fnr* (LL1119) and Δ*msrP* Δ*mobAB* (LL1120) strains were plated onto LB-CASA (0.4 %) agar and incubated at 37 °C under anaerobic conditions for 48 hours before being scanned (upper part) and observed using a binocular magnifier (lower panel). The images are representative of experiments carried out at least three times.

Figure 3. Nitrate chemically suppresses the effect of CASA on MsrP.

A) Effect of the addition of alternative electron acceptors (nitrate, DMSO and TMAO) on *hiuH-msrPQ* expression. Wild-type strain carrying the *hiuH-lacZ* fusion was cultured overnight in LB supplemented with 0.4 % CASA in the presence of nitrate, DMSO or TMAO (40 mM) under anaerobic conditions, followed by ß-galactosidase assays. Results are expressed as means +/- SD (n = 3). Asterisks indicate a statistically significant difference between the absence and the presence of electron acceptors, **P<0.01 and ***P<0.001 (Ordinary one-way ANOVA with Tukey's multiple comparison test) **B)** Immunoblot analysis using an anti-MsrP antibody showing the production of MsrP from wild-type cultivated following the procedure described for panel (A). The image is representative of experiments carried out in triplicate. **C)** Evaluation of colony morphology on LB-CASA agar plates supplemented with alternative electron acceptors. The Δ*msrP* (CH380), strain was plated onto LB-CASA (0.4 %) agar containing nitrate, DMSO or TMAO (40 mM). Plates were incubated at 37 °C under anaerobic conditions for 48 hours before scanning. The images are representative of experiments carried out at least three times.

Figure 4. The three nitrate reductases are required for the regulation of *msrPQ* under anaerobic conditions with CASA.

A) Schematic representation of the respiratory nitrate reductase systems in *E. coli*. The cytoplasmic nitrate reductases NarA (in blue) and NarZ (in orange) composed of NarGHI and NarZYV subunits respectively, are able to reduce nitrate (NO_3^-) to nitrite (NO_2^-) in the cytoplasm. The periplasmic nitrate reductase NapA (in green) composed of NapABC subunits, reduce NO_3^- in the periplasmic compartment. The nitrate importers NarU (in yellow) and NarK (in purple) move nitrate into the cytoplasm. **B)** Effect of *nar* and *nap* deletions on *hiuH-msrPQ* expression. Wild-type, Δ*narG* (LL1170), Δ*narZ* (1168), Δ*napA*

(LL1166), Δ*narG* Δ*narZ* Δ*napA* (LL1171), Δ*narU* Δ*narK* (LL1231) and Δ*narU* Δ*narK* Δ*napA* (LL1232) strains carrying the *hiuH-lacZ* fusion were cultured overnight in LB supplemented with 0.4 % CASA under anaerobic conditions, followed by ß-galactosidase assays. Results are expressed as means +/- SD (n = 3). Asterisks indicate a statistically significant difference between mutants and the WT, ***P<0.001 and ns, not significant (Ordinary one-way ANOVA with Tukey's multiple comparison test). **C)** Immunoblot analysis using an anti-MsrP antibody showing the production of MsrP from strains indicated below the panel, cultivated following the same procedure as in panel (B). The image is representative of experiments carried out in triplicate. **D)** Evaluation of colony morphology on LB-CASA agar. Wild type (MG16555), Δ*msrP* (CH380), Δ*msrP* Δ*narG* (LL1148), Δ*msrP* Δ*narZ* (LL1146), Δ*msrP* Δ*napA* (LL1147) and Δ*msrP* Δ*torA* (LL1153) strains were plated onto LB-CASA (0.4 %) agar. The plates were incubated at 37 °C under anaerobic conditions for 48 hours before being scanned (upper panel) and observed using a binocular magnifier (lower panel). The images are representative of experiments carried out at least three times.

Figure 5. Chlorate (ClO_3^-) contaminated CASA.

A) Effect of chlorate on *hiuH-msrPQ* expression under anaerobic conditions. The expression of the *hiuH-lacZ* fusion in strain CH184 was used as a proxy for *msrPQ* expression. Strain CH184 was cultured overnight at 37 °C in LB supplemented or not with $NaClO_3$ (10 µM), under anaerobic conditions, followed by ß-galactosidase assays. Results are expressed as means +/- SD (n = 3). Asterisks indicate a statistically significant difference between the absence and the presence of $NaClO_3$, ***P<0.001 (Ordinary one-way ANOVA with Tukey's multiple comparison test). **B)** Immunoblot analysis using an anti-MsrP antibody showing the production of MsrP from the wild-type strain cultivated overnight at 37 °C in LB supplemented with $NaClO_3$ (1 to 100 µM) under anaerobic conditions. The image is representative of experiments carried out in triplicate. **C)** The wild type (MG16555) strain was plated onto LB-agarose supplemented or not with $NaClO_3$ (16 µM). The plates were incubated at 37 °C under anaerobic conditions for 48 hours before being observed using a binocular magnifier. The images are representative of experiments carried out at least three times.

Figure 6. Effect of the heterologous expression of chlorite dismutase.

A) Chlorite dismutase (Cld) from *Dechloromonas aromatica* was expressed using the pCld plasmid. The Δ*msrP* strain carrying the empty vector (pBad24) or pCld was spread onto LB-agar plates supplemented or not with $NaClO_3$ (16 µM), containing ampicillin and arabinose (0.02 %). The plates were incubated at 37 °C under anaerobic conditions for 48 hours before being observed using a binocular magnifier. The images are representative of experiments carried out at least three times. **B)** Effect of chlorite (ClO_2^-) on *hiuH-msrPQ* expression under aerobic conditions. The expression of the *hiuH-lacZ* fusion in strain CH184 was used as a proxy for *msrPQ* expression. Strain CH184 was cultured at 37°C in LB to OD_{600} ≈ 1.5 under aerobic conditions, $NaClO_2$ (20 or 200 µM) was then added and ß-galactosidase assays were conducted at different time points for each condition. **C)** Effect of the production of Cld on *hiuH-lacZ* fusion during chlorite stress. The CH184 strain carrying empty vector (pBad24) or pCld was cultured at 37°C in LB (supplemented with ampicillin and arabinose 0.02 %) to OD_{600} ≈0.1 under aerobic conditions. $NaClO_2$ (200 µM) was then added for 3.5 hours, followed by ß-galactosidase assays. Results are expressed as means +/- SD (n = 3). Asterisks indicate a statistically significant difference between the absence and the presence of Cld, ***P<0.001 (Ordinary one-way ANOVA with Tukey's multiple comparison test). **D)** Immunoblot analysis using an anti-MsrP antibody, showing the production of MsrP from the strains indicated above the panel, cultivated following the same procedure as described for panel (C).

Figure 7. Chlorate regulates *msrPQ* expression in an HprSR-dependent manner.
Immunoblot analysis using an anti-MsrP antibody showing the production of MsrP from wild-type (MG1655), Δ*hprR* (BE255) and Δ*hprS* (BE254) strains cultivated overnight at 37 °C in LB supplemented with $NaClO_3$ (20 µM) under anaerobic conditions. The image is representative of experiments carried out in triplicate.

Figure 8. MsrP repairs the chlorite-oxidized SurA.
Wild type and Δ*msrP* cells were grown in LB at 37°C under aerobic conditions. At an OD_{600} of 0.1 cells were treated or not with $NaClO_2$ (200 µM) for 2 hours and the SurA protein was analysed by immunoblotting. In the Δ*msrP* strain, chlorite treatment led to the appearance of the oxidized form of SurA (SurAox) which exhibits a mobility shift on SDS-PAGE resulting from Met-O formation. This blot is representative of three independent experiments.

Figure 9. Cytoplasmic methionine sulfoxide reductases are not induced by chlorate. **A)** Effect of chlorate on *msrA* and *msrB* expression under anaerobic conditions. The strains JB56 and JB44, harbouring the p*msrA-lacZ* and p*msrB-lacZ* fusions respectively, were cultured overnight at 37°C in LB supplemented or not with $NaClO_3$ (10 µM), under anaerobic conditions, followed by ß-galactosidase assays. The *hiuH-lacZ* fusion (CH184) was used as a control. Results are expressed as means +/- SD (n = 3). Asterisks indicate a statistically significant difference between the absence and the presence of $NaClO_3$, ***P<0.001 and ns, not significant (Ordinary one-way ANOVA with Tukey's multiple comparison test). B) Colony morphology of Δ*msrP* (CH380) and Δ*msrA* Δ*msrB* (BE152) strains. The strains were plated onto LB-CASA (0.4 %) agar and incubated at 37 °C under anaerobic conditions for 48 hours. The plates were then scanned (upper panel) and observed using a binocular magnifier (lower panel). The images are representative of experiments carried out at least three times.

REFERENCES

Alefounder, P.R., and Ferguson, S.J. (1980) The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*. *Biochem J* **192**: 231–240.

Aussel, L., and Ezraty, B. (2021) Methionine Redox Homeostasis in Protein Quality Control. *Front Mol Biosci* **8**: 1–8.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* **2**.

Barnum, T.P., Figueroa, I.A., Carlström, C.I., Lucas, L.N., Engelbrektson, A.L., and Coates, J.D. (2018) Genome-resolved metagenomics identifies genetic mobility, metabolic interactions, and unexpected diversity in perchlorate-reducing communities. *ISME J* **12**: 1568–1581 http://www.nature.com/articles/s41396-018-0081-5.

Blank, K., Hensel, M., and Gerlach, R.G. (2011) Rapid and Highly Efficient Method for Scarless Mutagenesis within the Salmonella enterica Chromosome. *PLoS One* **6**: e15763.

Bos, J., Duverger, Y., Thouvenot, B., Chiaruttini, C., Branlant, C., Springer, M., *et al.* (2013) The sRNA RyhB Regulates the Synthesis of the Escherichia coli Methionine Sulfoxide Reductase MsrB but Not MsrA. *PLoS One* **8**.

Boschi-Muller, S., and Branlant, G. (2014) Methionine sulfoxide reductase: Chemistry, substrate binding, recycling process and oxidase activity. *Bioorg Chem* **57**: 222–230.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9–14.

Clark, I.C., Melnyk, R.A., Iavarone, A.T., Novichkov, P.S., and Coates, J.D. (2014) Chlorate

reduction in *S hewanella algae* ACDC is a recently acquired metabolism characterized by gene loss, suboptimal regulation and oxidative stress. *Mol Microbiol* **94**: 107–125.

Clegg, S., Yu, F., Griffiths, L., and Cole, J.A. (2002) The roles of the polytopic membrane proteins NarK, NarU and NirC in Escherichia coli K-12: two nitrate and three nitrite transporters. *Mol Microbiol* **44**: 143–155.

Covert, M.W., Knight, E.M., Reed, J.L., Herrgard, M.J., and Palsson, B.O. (2004) Integrating high-throughput and computational data elucidates bacterial networks. *Nature* **429**: 92–96.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–5 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=18686&tool=pmcentrez&rendertype=abstract.

Delaye, L., Becerra, A., Orgel, L., and Lazcano, A. (2007) Molecular Evolution of Peptide Methionine Sulfoxide Reductases (MsrA and MsrB): On the Early Development of a Mechanism That Protects Against Oxidative Damage. *J Mol Evol* **64**: 15–32.

Duport, C., Madeira, J.-P., Farjad, M., Alpha-Bazin, B., and Armengaud, J. (2021) Methionine Sulfoxide Reductases Contribute to Anaerobic Fermentative Metabolism in Bacillus cereus. *Antioxidants* **10**: 819.

Ezraty, B., Aussel, L., and Barras, F. (2005) Methionine sulfoxide reductases in prokaryotes. *Biochim Biophys Acta* **1703**: 221–9 http://www.ncbi.nlm.nih.gov/pubmed/15680230.

Ezraty, B., Gennaris, A., Barras, F., and Collet, J.-F. (2017) Oxidative stress, protein damage and repair in bacteria. *Nat Rev Microbiol* **15**: 385–396 http://www.nature.com/doifinder/10.1038/nrmicro.2017.26.

Fang, F.C., Frawley, E.R., Tapscott, T., and Vázquez-Torres, A. (2016) Bacterial Stress Responses during Host Infection. *Cell Host Microbe* **20**: 133–143.

Fu, H., Yuan, J., and Gao, H. (2015) Microbial oxidative stress response: Novel insights from environmental facultative anaerobic bacteria. *Arch Biochem Biophys* **584**: 28–35.

Gennaris, A., Ezraty, B., Henry, C., Agrebi, R., Vergnes, A., Oheix, E., *et al.* (2015) Repairing oxidized proteins in the bacterial envelope using respiratory chain electrons. *Nature* **528**: 409–412 http://www.nature.com/doifinder/10.1038/nature15764%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/26641313.

Giordano, G., Violet, M., Medani, C., and Pommier, J. (1984) A common pathway for the activation of several molybdoenzymes in Escherichia coli K12. *Biochim Biophys Acta - Gen Subj* **798**: 216–225.

Glaser, J.H., and DeMoss, J.A. (1971) Phenotypic Restoration by Molybdate of Nitrate Reductase Activity in *chlD* Mutants of *Escherichia coli*. *J Bacteriol* **108**: 854–860.

Goblirsch, B.R., Streit, B.R., DuBois, J.L., and Wilmot, C.M. (2010) Structural features promoting dioxygen production by Dechloromonas aromatica chlorite dismutase. *JBIC J Biol Inorg Chem* **15**: 879–888.

Hajj, S. El, Henry, C., Andrieu, C., Vergnes, A., Loiseau, L., Brasseur, G., *et al.* (2022) HprSR Is a Reactive Chlorine Species-Sensing, Two-Component System in Escherichia coli. *J Bacteriol* **204**.

Henry, C., Loiseau, L., Vergnes, A., Vertommen, D., Mérida-Floriano, A., Chitteni-Pattu, S., *et al.* (2021) Redox controls reca protein activity via reversible oxidation of its methionine residues. *Elife* **10**: 1–59.

Imlay, J. a (2015) Diagnosing oxidative stress in bacteria: not as easy as you might think. *Curr Opin Microbiol* **24**: 124–131 http://linkinghub.elsevier.com/retrieve/pii/S1369527415000053.

Imlay, J.A. (2013) The molecular mechanisms and physiological consequences of oxidative

stress: lessons from a model bacterium. *Nat Rev Microbiol* **11**: 443–454.

Imlay, J.A. (2019) Where in the world do bacteria experience oxidative stress? *Environ Microbiol* **21**: 521–530.

Kiley, P.J., and Beinert, H. (1998) Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol Rev* **22**: 341–352.

Kim, G., Weiss, S.J., and Levine, R.L. (2014) Methionine oxidation and reduction in proteins. *Biochim Biophys Acta - Gen Subj* **1840**: 901–905 http://dx.doi.org/10.1016/j.bbagen.2013.04.038.

Lee, Y., Do, H.L., Chang, W.K., Ah, Y.L., Jang, M., Cho, S., *et al.* (2005) Transthyretin-related proteins function to facilitate the hydrolysis of 5-hydroxyisourate, the end product of the uricase reaction. *FEBS Lett* **579**: 4769–4774.

Loschi, L., Brokx, S.J., Hills, T.L., Zhang, G., Bertero, M.G., Lovering, A.L., *et al.* (2004) Structural and biochemical identification of a novel bacterial oxidoreductase. *J Biol Chem* **279**: 50391–400 http://www.ncbi.nlm.nih.gov/pubmed/15355966.

Lowther, W.T., Weissbach, H., Etienne, F., Brot, N., and Matthews, B.W. (2002) The mirrored methionine sulfoxide reductases of Neisseria gonorrhoeae pilB. *Nat Struct Biol* .

Magalon, A., and Mendel, R.R. (2015) Biosynthesis and Insertion of the Molybdenum Cofactor. *EcoSal Plus* **6**.

Mandin, P., and Gottesman, S. (2009) A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. *Mol Microbiol* **72**: 551–565.

Melnyk, R.A., Youngblut, M.D., Clark, I.C., Carlson, H.K., Wetmore, K.M., Price, M.N., *et al.* (2015) Novel Mechanism for Scavenging of Hypochlorite Involving a Periplasmic Methionine-Rich Peptide and Methionine Sulfoxide Reductase. *MBio* **6**: 1–8.

764 Merdanovic, M., Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) Protein quality
765 control in the bacterial periplasm. *Annu Rev Microbiol* **65**: 149–68
766 http://www.ncbi.nlm.nih.gov/pubmed/21639788.
767
768 Miller, J.H. (1992) *A short course in bacterial genetics: a laboratory manual and handbook*
769 *for Escherichia coli and related bacteria.* Cold Spring Harbor Laboratory Press, Cold Spring
770 Harbor, NY, .
771
772 Myers, K.S., Yan, H., Ong, I.M., Chung, D., Liang, K., Tran, F., *et al.* (2013) Genome-scale
773 Analysis of Escherichia coli FNR Reveals Complex Features of Transcription Factor
774 Binding. *PLoS Genet* **9**: e1003565.
775
776 Saleh, M., Bartual, S.G., Abdullah, M.R., Jensch, I., Asmat, T.M., Petruschka, L., *et al.* (2013)
777 Molecular architecture of streptococcus pneumoniae surface thioredoxin-fold lipoproteins
778 crucial for extracellular oxidative stress resistance and maintenance of virulence. *EMBO*
779 *Mol Med* **5**: 1852–1870.
780
781 Schramm, F.D., Schroeder, K., and Jonas, K. (2020) Protein aggregation in bacteria. *FEMS*
782 *Microbiol Rev* **026**: 54–72 https://orcid.org/0000-0002-6271-4530.
783
784 Sheng, Y., Abreu, I.A., Cabelli, D.E., Maroney, M.J., Miller, A.-F., Teixeira, M., and Valentine, J.S.
785 (2014) Superoxide Dismutases and Superoxide Reductases. *Chem Rev* **114**: 3854–3918.
786
787 Skaar, E.P., Tobiason, D.M., Quick, J., Judd, R.C., Weissbach, H., Etienne, F., *et al.* (2002) The
788 outer membrane localization of the Neisseria gonorrhoeae MsrA/B is involved in survival
789 against reactive oxygen species. *Proc Natl Acad Sci U S A* **99**: 10108–13
790 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=126632&tool=pmcentrez&re
791 ndertype=abstract.
792
793 Spiro, S. (1994) The FNR family of transcriptional regulators. *Antonie Van Leeuwenhoek* **66**:
794 23–36.
795
796 Stewart, V. (1988) Nitrate respiration in relation to facultative metabolism in

enterobacteria. *Microbiol Rev* **52**: 190–232.

Vaughan, M. (1997) Oxidative Modification of Macromolecules Minireview Series. *J Biol Chem* **272**: 18513.

Wang, O., and Coates, J. (2017) Biotechnological Applications of Microbial (Per)chlorate Reduction. *Microorganisms* **5**: 76.

Warne, S.R., Varley, J.M., Boulnois, G.J., and Norton, M.G. (1990) Identification and Characterization of a Gene that Controls Colony Morphology and Auto-Aggregation in Escherichia Coli K12. *J Gen Microbiol* **136**: 455–462 https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-136-3-455.

Winterbourn, C.C., and Kettle, A.J. (2013) Redox Reactions and Microbial Killing in the Neutrophil Phagosome. *Antioxid Redox Signal* **18**: 642–660.

Zupok, A., Iobbi-Nivol, C., Méjean, V., and Leimkühler, S. (2019) The regulation of Moco biosynthesis and molybdoenzyme gene expression by molybdenum and iron in bacteria. *Metallomics* **11**: 1602–1624.

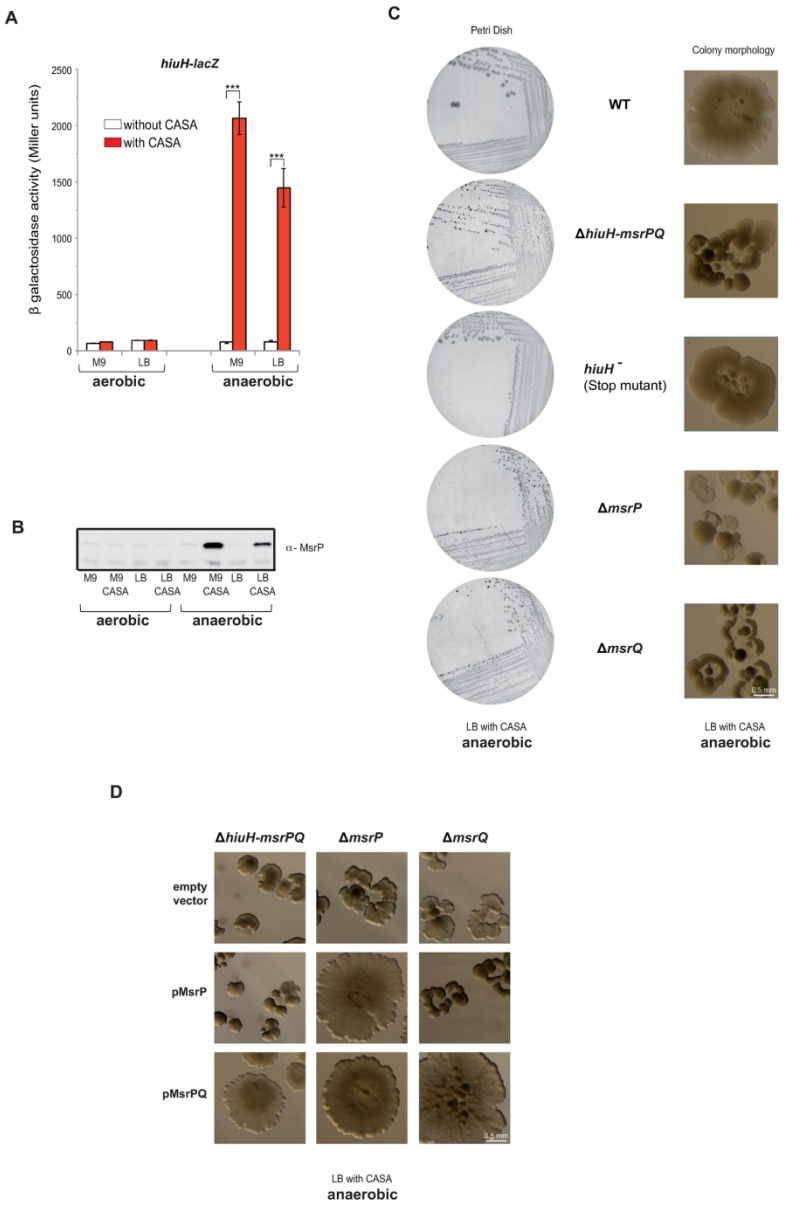

figure 1

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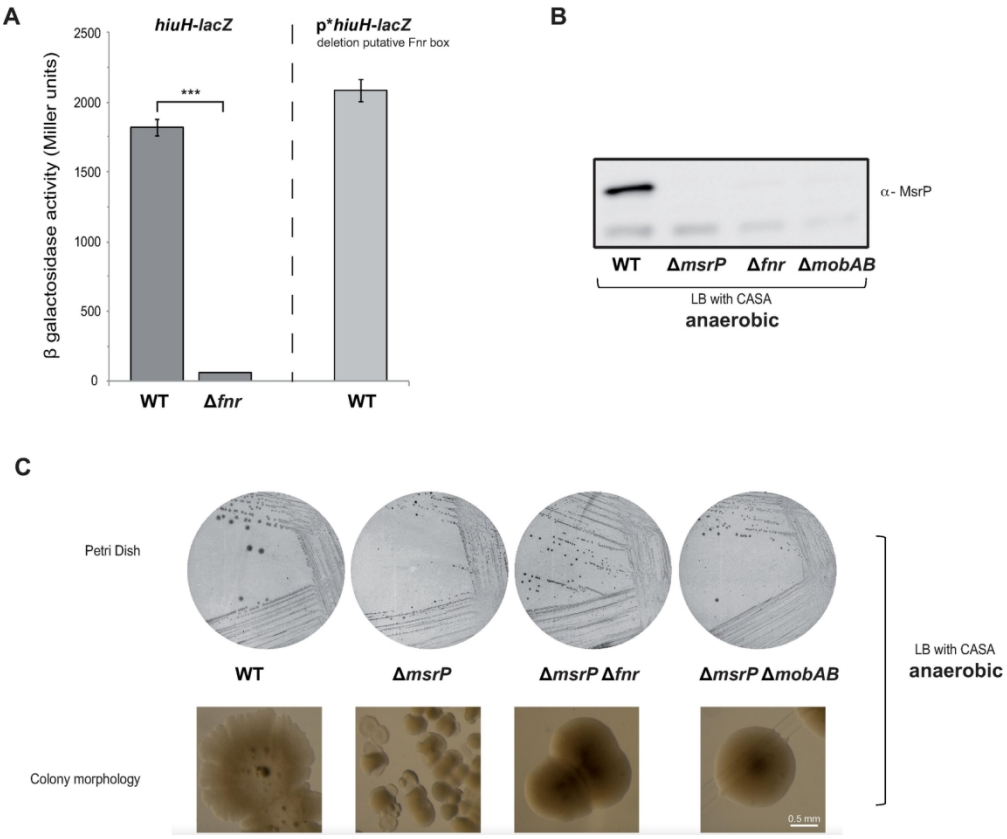

figure 2

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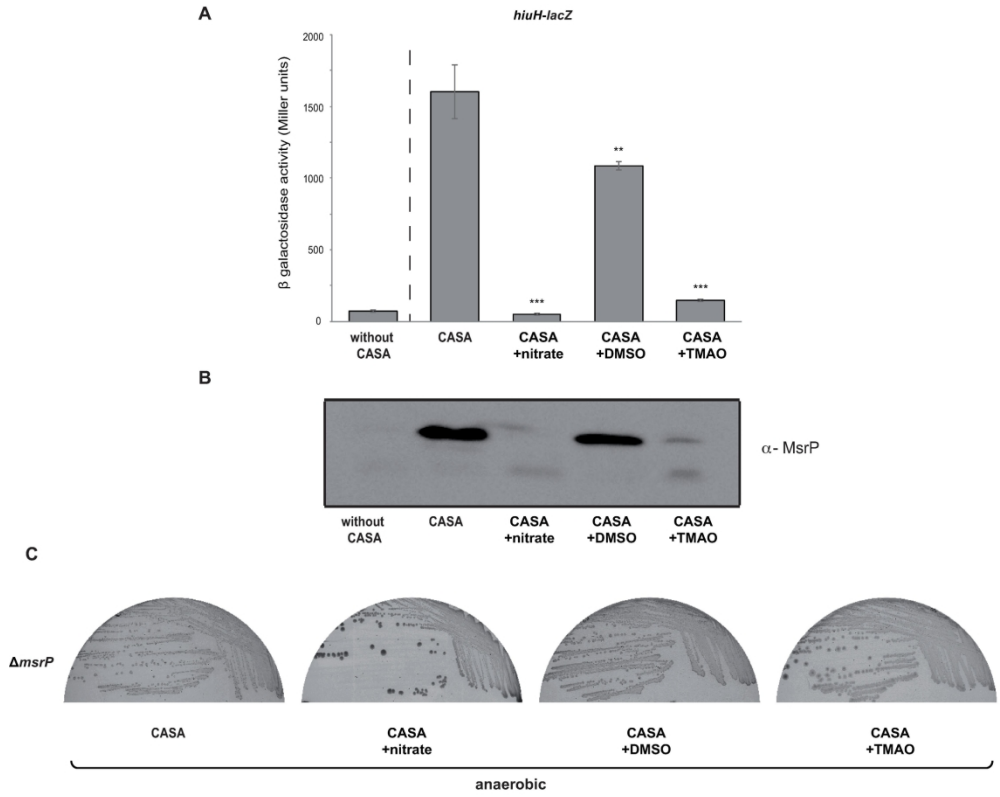

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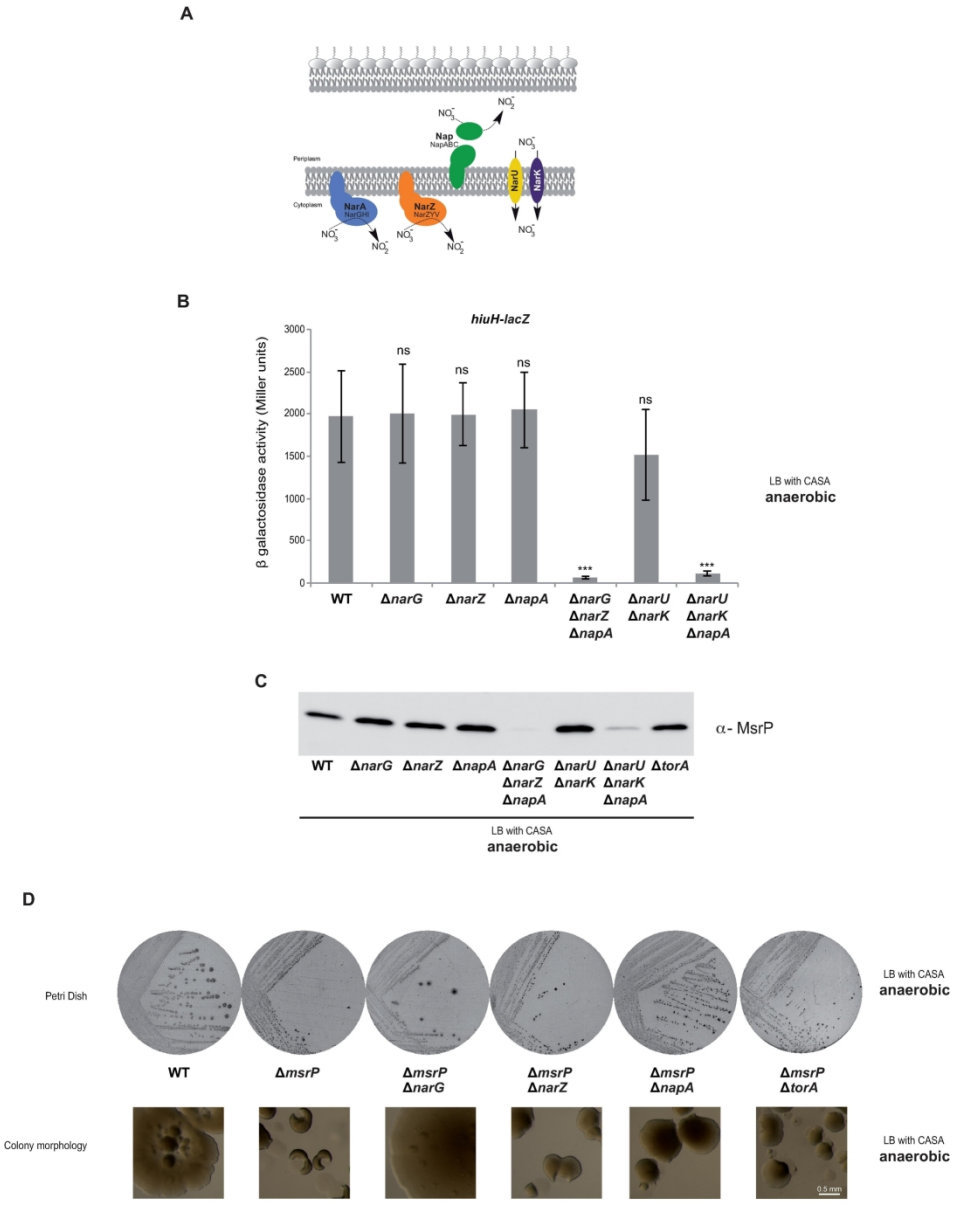

figure 4

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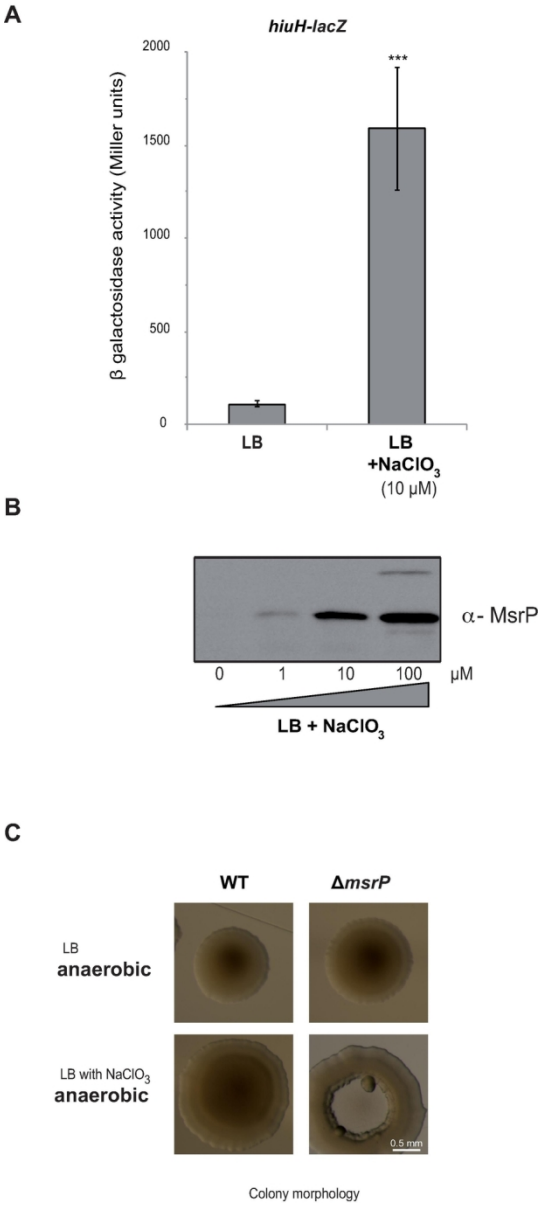

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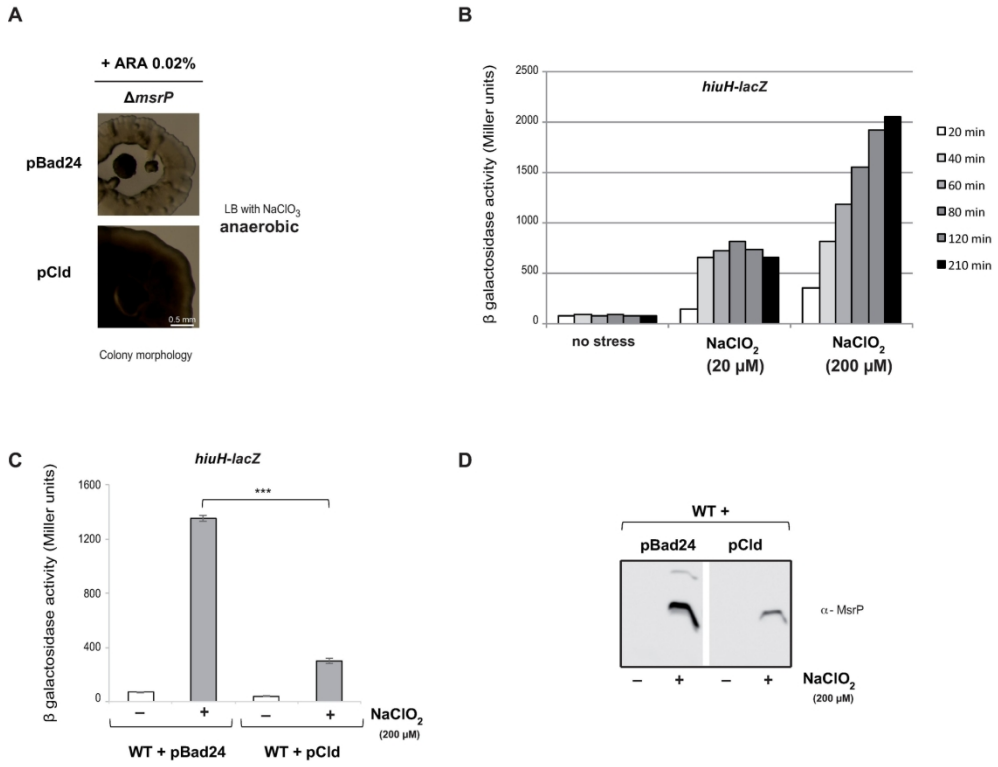

figure 6

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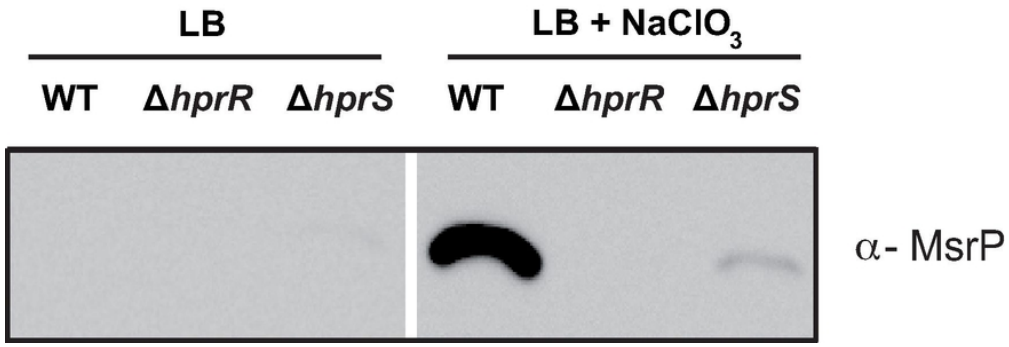

figure 7

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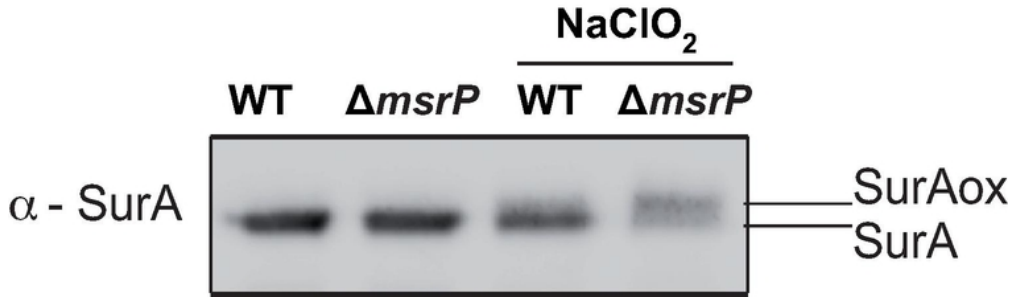

figure 8

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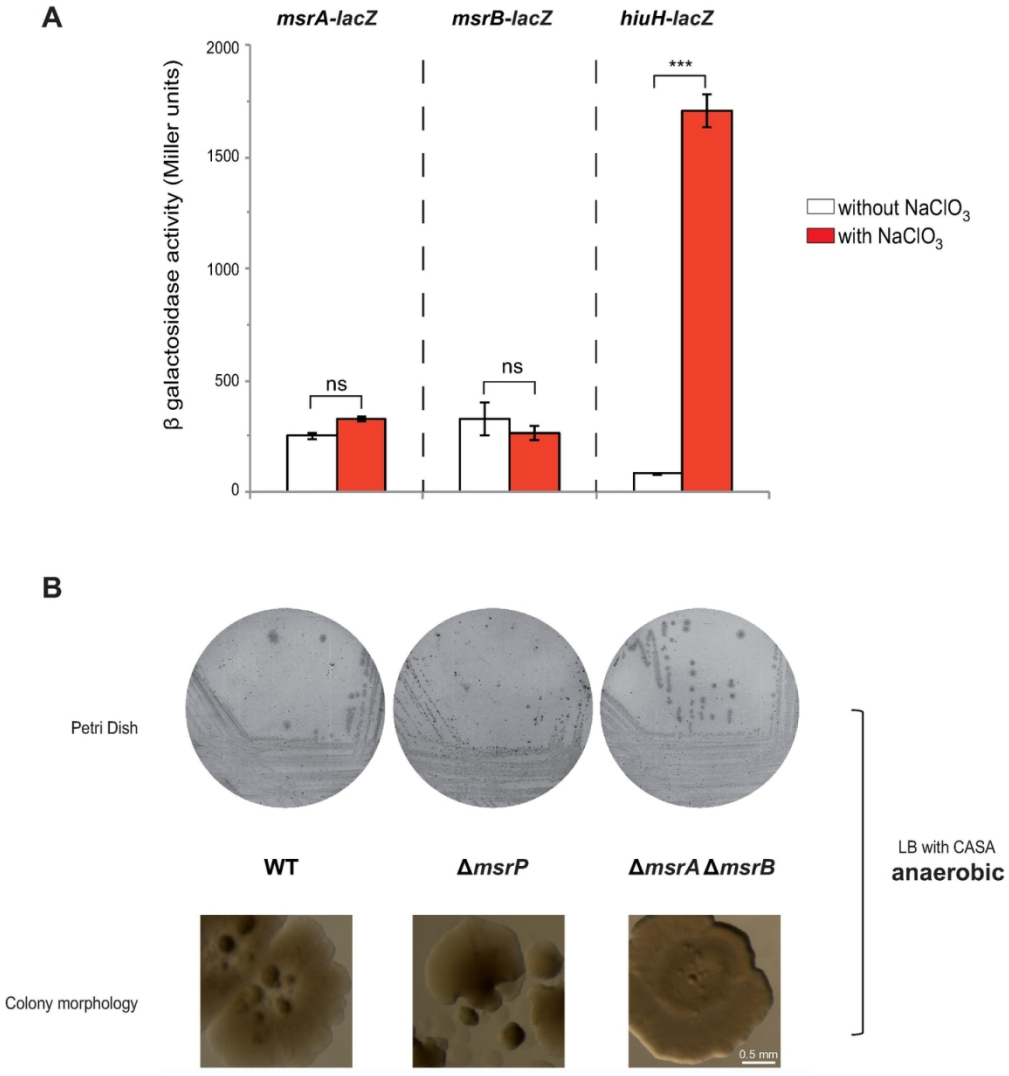

figure 9

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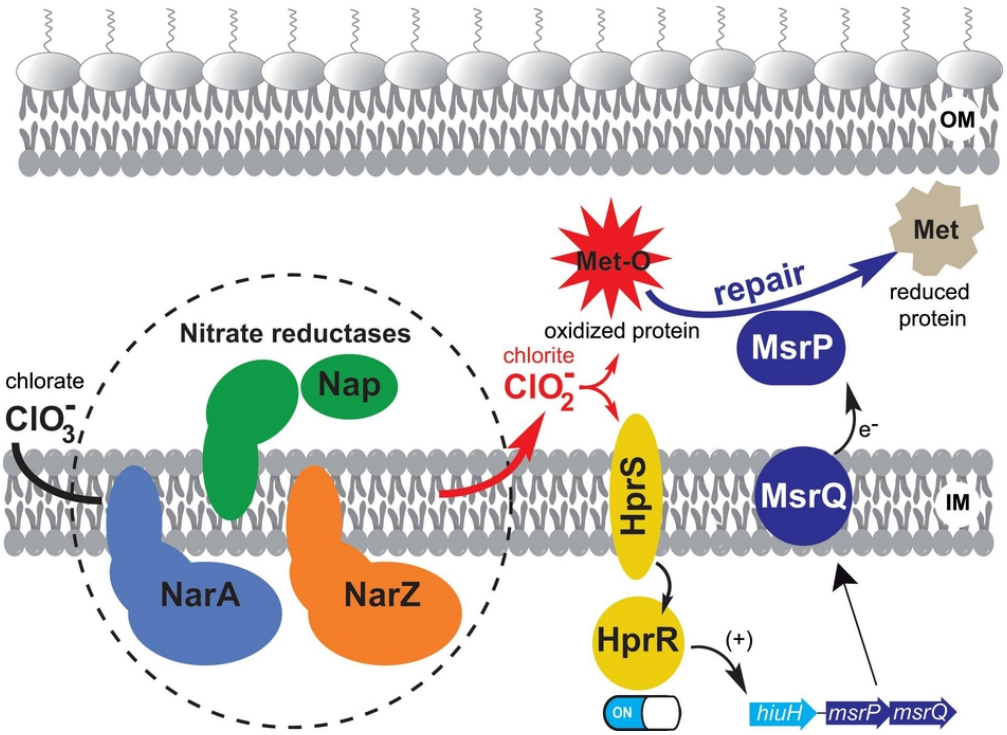

In anaerobic condition, reduction of chlorate (ClO3-) to the toxic oxidizing agent chlorite (ClO2-) by the three nitrate reductases (NarA, NarZ and Nap) led to methionine oxidation of periplasmic proteins. In response to this stress the E. coli HprSR two-component system was activated, leading to over-production of the periplasmic methionine sulfoxide reductase (MsrPQ) and the repair of the oxidized proteins.

80x57mm (300 x 300 DPI)

Summary graphical abstract :

In anaerobic condition, reduction of chlorate (ClO_3^-) to the toxic oxidizing agent chlorite (ClO_2^-) by the three nitrate reductases (NarA, NarZ and Nap) led to methionine oxidation of periplasmic proteins. In response to this stress the *E. coli* HprSR two-component system was activated, leading to over-production of the periplasmic methionine sulfoxide reductase (MsrPQ) and the repair of the oxidized proteins.