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Short communication

Differential expression from two iron-responsive promoters in *Salmonella enterica* serovar Typhimurium reveals the presence of iron in macrophage-phagosomes

Clare M. Taylor [1], Deenah Osman, Jennifer S. Cavet*

Life Sciences, Michael Smith Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK

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ABSTRACT

The metal status of macrophage-phagosomes during *Salmonella* infection is largely unknown. In this study, we have precisely calibrated the metal-specificities of two metal-responsive promoters, $P_{iroBCDE}$ and P_{sodB}, from *Salmonella enterica* serovar Typhimurium and used these to directly monitor iron-levels in *Salmonella*-containing macrophage-phagosomes. Expression from the $P_{iroBCDE}$ promoter is highly elevated in metal-depleted media but low in media supplemented with iron or cobalt, and to a lesser extent manganese. In contrast, P_{sodB} shows low levels of expression in metal-depleted media but is induced in media supplemented with iron but no other metals at maximum permissive concentrations. In both cases, iron-responsive expression corresponds to changes in the number of iron atoms per bacterial cell and is unaffected by pH or the presence of reactive oxygen species (hydrogen peroxide and superoxide). Importantly, expression from $P_{iroBCDE}$ remained low while expression from P_{sodB} was elevated during infection of both Nramp1$^{+/+}$ and Nramp1$^{-/-}$ macrophages. Expression from a control promoter, P_{polA}, unaffected by metal ions, remained unchanged. These findings are therefore consistent with the presence of iron within *Salmonella*-containing macrophage-phagosomes and support a model in which the toxic potential of iron may be exploited as a component of the respiratory burst killing mechanism.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) infections cause serious medical and veterinary problems worldwide resulting in significant morbidity and mortality. Iron is required by *S.* Typhimurium as a cofactor for several fundamental enzymes and competition with the host for these ions is considered a key factor for infection [1]. However, when unbound within a cell, redox cycling means iron is extremely toxic, largely due to its ability to catalyze Fenton chemistry generating highly reactive hydroxyl radicals that damage biomolecules such as DNA, proteins and lipids. The toxic nature of these ions is suggested to be exploited by macrophages constituting a crucial component of the respiratory burst killing mechanism [2,3]. Furthermore, the divalent cation transporter natural resistance associated macrophage protein 1 (Nramp1, alias SLC11A1) is thought to confer resistance to *S.* Typhimurium by altering metal-levels within bacteria-containing macrophage-phagosomes [4–10], although the direction of flux, metal substrates *in vivo*, and precise mechanism of pathogen killing are not known. Survival in macrophage-phagosomes is critical for *S.* Typhimurium virulence [11], but what is the nature of the iron stress encountered within these vesicles?

In this study, we have precisely calibrated the metal-specificities of two metal-responsive promoter regions, $P_{iroBCDE}$ and P_{sodB}, from *S.* Typhimurium and used these to directly monitor iron-levels in both Nramp1$^{+/+}$ and Nramp1$^{-/-}$ macrophages during *Salmonella* infection.

2. Results and discussion

2.1. Calibration of the metal-specificities of $P_{iroBCDE}$ and P_{sodB} from S. Typhimurium

The products of the *iroBCDE* operon (Fig. 1A) are required for converting the enterobactin siderophore into salmochelin, involved in iron-uptake [12–15]. Expression from $P_{iroBCDE}$ is under the control of the iron-responsive transcription factor Fur and induced by iron-deficiency [12]. In contrast, the abundance of *sodB* transcripts (Fig. 1B), encoding an iron-superoxide dismutase, are substantially reduced in cells depleted of iron due to the action of two anti-sense regulatory small RNAs, *rfrA* and *rfrB*, which are

* Corresponding author. Tel.: +44 161 275 51543; fax: +44 161 275 5082.
E-mail address: jennifer.s.cavet@manchester.ac.uk (J.S. Cavet).
[1] Present address: School of Life Sciences, Napier University, 10 Colinton Road, Edinburgh EH10 5DT, UK.

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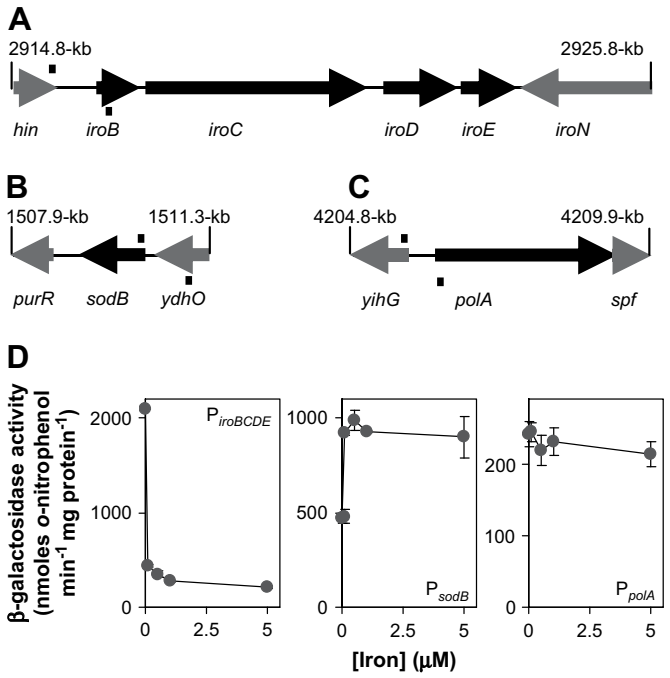

Fig. 1. Expression from $P_{iroBCDE}$ and P_{sodB} is regulated by iron. Physical map of the *iroBCDE* (A), *sodB* (B) and *polA* (C) gene regions with nucleotide positions in the *S.* Typhimurium LT2 genome (vertical lines) and annealing positions of primers used to amplify promoter regions (black boxes). (D), β-galactosidase activity in *S.* Typhimurium SL1344 containing $P_{iroBCDE}$, P_{sodB} or P_{polA} fused to *lacZ* grown in M9 minimal medium supplemented with increasing concentrations of iron.

regulated by Fur [16]. To examine iron-responsiveness further, $P_{iroBCDE}$ and P_{sodB} were fused to the reporter gene *lacZ* in vector pRS415 [17], introduced into *S.* Typhimurium strain SL1344 and β-galactosidase activity monitored following growth in metal-depleted M9 minimal medium supplemented with increasing concentrations of iron. Expression from $P_{iroBCDE}$ was highly elevated in cells grown in minimal medium and substantially reduced following iron-supplementation (Fig. 1D). In contrast, expression from P_{sodB} was substantially increased in response to iron, with maximal expression observed at ≥0.1 μM iron. Consistent with previous findings [18] expression from a control promoter, P_{polA} (Fig. 1C) encoding DNA polymerase I, was unaffected by iron-levels (Fig. 1D). Determination of the iron quotas of chelate-washed *S.* Typhimurium revealed a substantial increase in iron content upon supplementation of the medium with iron (Table 1), confirming that altered expression from $P_{iroBCDE}$ and P_{sodB} relates to changes in cytosolic iron-levels.

In addition to ferrous iron, Fur is known to bind other divalent metal ions *in vitro* which may also act as effectors *in vivo* [19–21]. Hence, we examined which, if any, other metals alter expression from $P_{iroBCDE}$ and P_{sodB}. In addition to iron, expression from $P_{iroBCDE}$ was reduced in response to maximum permissive concentrations of cobalt and, to a lesser extent, manganese while expression from P_{sodB} was solely responsive to elevated iron-levels (Fig. 2A). Supplementation of the M9 minimal medium with the iron chelator diethylenetriamine pentaacetic acid (DTPA) did not affect expression from either promoter consistent with metal-depletion of the medium. Expression from the control promoter P_{polA} was unaffected by any metal ions tested (Fig. 2A).

Table 1
Iron contents of *S.* Typhimurium grown in M9 minimal medium in the presence or absence of the iron chelator DTPA or iron-supplementation.

Metal supplementation	Iron atoms × 10^5 cell^{-1}	Iron atoms × 10^{14} mg^{-1} protein
0.1 μM DTPA	2.3 (±0.4)	2.4 (±0.2)
0	2.8 (±0.1)	2.7 (±0.1)
1 μM iron	9.1 (±1.7)	5.9 (±0.4)
10 μM iron	59 (±12.1)	29.1 (±0.4)

To further investigate the response of $P_{iroBCDE}$ to manganese and cobalt, β-galactosidase activity was measured in response to a range of metal concentrations up to inhibitory doses. Elevated cobalt-levels caused a similar fold reduction in expression as iron, with essentially full repression observed at ≥0.5 μM cobalt (Figs. 1B and 2B). However, although manganese caused a reduction in $P_{iroBCDE}$ expression, the magnitude of repression remained less at all viable levels of manganese (Fig. 2B). These data are consistent with cobalt and manganese, in addition to iron, being able to act as co-repressors of Fur, although the response to manganese is less. Similar effects of iron, cobalt and manganese on Fur-mediated regulation of the *S. Typhimurium sitABCD* operon have been observed [21]. In contrast to $P_{iroBCDE}$, no viable concentration of cobalt or manganese altered expression from P_{sodB} (data not shown), despite P_{sodB} being regulated by Fur, albeit indirectly. Regulation of $P_{iroBCDE}$ by other metal-responsive regulators such as MntR, direct or indirect, cannot be excluded.

2.2. Expression from P_{iroBCDE} and P_{sodB} is unaffected by other environmental conditions encountered within macrophage-phagosomes

In order to use $P_{iroBCDE}$ and P_{sodB} to monitor phagosomal iron-levels it is important that their expression is unaffected by other environmental stresses that *S.* Typhimurium might face inside macrophages. Expression from $P_{iroBCDE}$ and P_{sodB} was therefore examined in response to acidic pH and oxidative stress to mimic conditions in the micro-environment of the phagosome. Importantly, iron-responsiveness was retained in all cases (Fig. 3) and no substantial change in expression was detected in the presence of hydrogen peroxide and superoxide. Some reduction in expression was detected as the pH decreased from 7.5 to 4.5 (Fig. 3C, F and I). In the case of $P_{iroBCDE}$, it is possible that this relates to an increase in iron-availability in acid conditions. However, a similar decrease in expression was observed for both P_{sodB} and P_{polA} and was coincident with reduced growth. Hence, the inhibitory effects of low pH on expression from these promoters most likely relates to toxicity. The findings above confirm the feasibility of using $P_{iroBCDE}$ with P_{sodB} to report phagosomal iron-levels.

2.3. Monitoring iron-levels within Salmonella-containing phagsosomes using P_{iroBCDE} and P_{sodB}

S. Typhimurium possessing the *lacZ* fusion constructs were used to infect RAW264.7 derived macrophage cell lines 7.5R and 10S, which are stably transfected with wild-type $Nramp1^{Gly169}$ or mutant (functional null) $Nramp1^{Asp169}$ alleles, respectively [22]. Intracellular *S.* Typhimurium were isolated at various time points post-infection, the number of bacteria assessed by viable counts and β-galactosidase activity determined. Maintenance of the reporter constructs during this time course and iron-responsive β-galactosidase activity in recovered bacteria was confirmed (data not shown). Furthermore, Nramp1 localisation with *Salmonella*-containing vesicles in $Nramp1^{+/+}$ but not $Nramp1^{-/-}$ cells was demonstrated by fluorescence microscopy in parallel infection experiments using RAW264.7 cells containing EGFP-tagged $Nramp1^{Gly169}$ or $Nramp1^{Asp169}$ stably integrated into the chromosome [23] (data not shown). Importantly, $P_{iroBCDE}$ expression remained low in *S.* Typhimurium isolated from macrophages with and without functional Nramp1, while expression from P_{sodB} was increased by up to 3.9- and 3.5-fold in 7.5R and 10S macrophages, respectively, over the 12-h infection period (Fig. 4). No change in expression from either promoter was observed in bacteria