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Fur regulation of *Staphylococcus aureus* heme oxygenases is required for heme homeostasis

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ABSTRACT

Heme is a cofactor that is essential for cellular respiration and for the function of many enzymes. If heme levels become too low within the cell, *S. aureus* switches from producing energy via respiration to producing energy by fermentation. *S. aureus* encodes two heme oxygenases, IsdI and IsdG, which cleave the porphyrin heme ring releasing iron for use as a nutrient source. Both *isdI* and *isdG* are only expressed under low iron conditions and are regulated by the canonical Ferric Uptake Regulator (Fur). Here we demonstrate that unregulated expression of *isdI* and *isdG* within *S. aureus* leads to reduced growth under low iron conditions. Additionally, the constitutive expression of these enzymes leads to decreased heme abundance in *S. aureus*, an increase in the fermentation product lactate, and increased resistance to gentamicin. This work demonstrates that *S. aureus* has developed tuning mechanisms, such as Fur regulation, to ensure that the cell has sufficient quantities of heme for efficient ATP production through aerobic respiration.

1. Introduction

Heme is an important small molecule and an essential cofactor for a variety of enzymes (George, 1948; Morrison and Stotz, 1954; Maehly, 1952; Igo et al., 1961). During cellular respiration, heme populates cytochromes and serves as an electron acceptor in the electron transport chain (Morrison and Stotz, 1954; Hammer et al., 2013). Heme-dependent respiration is critical for many organisms (Musser and Chan, 1998). If heme is unable to populate the cytochromes, either due to genetic inactivation of the cytochromes or a lack of cellular heme, cells are unable to respire and must switch to a fermentative state (Von Eiff et al., 1997). Fermentation through glycolysis results in the production of 2 ATP molecules, compared to respiration that can generate up to 38 molecules of ATP per molecule of glucose.

Staphylococcus aureus is a Gram-positive coccoid bacterium and is the leading cause of skin and soft tissue infections (Klevens et al., 2007). In order to meet the cellular requirements for heme, *S. aureus* both biosynthesizes heme and imports heme from the extracellular milieu (Mazmanian et al., 2003; Tien and White, 1968). Heme import is mediated through the high-affinity iron-regulated surface determinant (Isd) heme acquisition system (Mazmanian et al., 2003; Torres et al., 2006). The genes in the *isd* operons are regulated by the Ferric Uptake Regulator (Fur) (Mazmanian et al., 2003; Xiong et al., 2000). Fur dimerizes when iron is present to bind Fur boxes in the promoter regions of target genes and repress transcription. This repression is alleviated under iron deplete conditions, when there is insufficient intracellular iron to allow Fur dimerization (Bagg and Neilands, 1987). Regulation by Fur is widely conserved throughout bacterial species, and Fur regulates a variety of transcripts associated with pathogenesis (Torres et al., 2010; Litwin et al., 1992; Tsolis et al., 1995; Tanui et al., 2017; Beall and Sanden, 1995).

In addition to being an important enzymatic cofactor, heme can also be used as a source of iron. Vertebrate-associated microorganisms, especially pathogenic bacteria, exploit host heme as a nutrient source. Aerobically, heme degradation is performed by heme oxygenases, while anaerobic bacteria use enzymes that rely on radical catabolism (LaMattina et al., 2016). Once heme is imported into the cell through the Isd proteins, heme is used to populate heme-binding proteins or heme is degraded by heme degrading enzymes. *S. aureus* encodes two such heme degrading enzymes, the heme oxygenases IsdI and IsdG. IsdG and IsdI facilitate the degradation of heme to produce the secondary catabolites staphylobilin and formaldehyde (Reniere et al., 2010; Matsui et al., 2013). This degraded heme also results in the release of iron, which the bacteria can use to meet their iron requirements (Skaar et al., 2004a).

Here we describe work initiated to understand the role of the heme degradation products in the context of *S. aureus*. Through an RNA-Sequencing experiment, comparing a *S. aureus* strain containing

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constitutive heme oxygenase activity to one lacking heme degradation, we found a significant increase in a number of transcripts from genes associated with oxygen-independent energy production. This led to the hypothesis that dysregulation of *isdI* causes aberrant degradation of the intracellular heme pool. Further analysis comparing constitutively and endogenously expressed *isdI* and *isdG* showed a significant decrease in intracellular heme levels and heightened fermentation. This work demonstrates the importance of Fur regulation for optimal bacterial growth in low iron conditions.

2. Results

2.1. Expression of heme oxygenases in S. aureus is essential for growth with heme as the sole source of iron

The function of the heme degradation products in *S. aureus* are unclear. To try to elucidate the roles of these catabolites in the cell, we created two strains of *S. aureus*; one lacking heme oxygenase activity (Δ*isdGI* p*lgt*) and one containing the endogenous heme oxygenase *isdI* added back *in trans* under the constitutive expression of the promoter for lipoprotein diacylglycerol transferase (*lgt)* (Selvan and Sankaran, 2008) (Δ*isdGI* p*lgt.isdI*). Removing both *isdI* and *isdG* from *S. aureus* results in a decrease in growth compared to the wildtype strain when grown in the presence of heme as the sole source of iron. Providing *isdI in trans* complemented the growth defect of Δ*isdGI* p*lgt* (Fig. 1). These data indicate that the expression of *isdI* is necessary for growth when heme is the sole source of iron, and provide an experimental condition where heme is actively degraded in the presence of the heme oxygenases but it is not degraded in their absence.

2.2. Constitutive expression of isdI leads to an increase in expression of transcripts associated with oxygen-independent energy production

In order to elucidate the role of heme catabolites in *S. aureus*, Δ*isdGI* p*lgt* and Δ*isdGI* p*lgt.isdI* were grown with heme as the sole iron source. Samples were collected for RNA isolation at mid-log phase growth and RNA from these samples was submitted for RNA-Sequencing analysis. In total, the abundance of 74 transcripts were significantly different between Δ*isdGI* p*lgt.isdI* and Δ*isdGI* p*lgt* ($q \leq 0.05$) (Fig. 2A). Of these, 23 were increased in abundance and 51 were decreased in abundance when Δ*isdGI* p*lgt.isdI* transcripts were compared to Δ*isdGI* p*lgt* (Fig. 2B). As expected, there were significant changes in abundance of transcripts for genes associated with iron acquisition (*sbnA-C (NWMN_0060-_0062), sbnE-I (NWMN_0064-_0068))* and genes known to be regulated by heme, such as the heme regulated transporter (*hrtAB (NWMN_2261, _2262)*) genes, which are regulated by the heme sensing two component system (HssRS) (Torres et al., 2010; Torres et al., 2007) (Fig. 2C, D). However, there was also a significant increase in the expression of genes associated with oxygen-independent energy production (Fig. 2C, D, Table S3). This result was surprising because heme oxygenases require oxygen to function (Streit et al., 2016) and the cultures were grown aerobically.

To confirm the results of the RNA-Seq, quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed on transcripts from a subset of the genes linked with oxygen-independent energy production. The nitrite reductase operon contains three genes (*nirD* (NWMN_2299)*, nirB* (NWMN_2300), and *nirR* (NWMN_2301) known to be upregulated under anaerobic conditions in various bacteria, including the closely related *Staphylococcus carnosus* (Neubauer et al., 1998; Jayaraman et al., 1987; Goldman and Roth, 1992). NirBD form the nitrite reductase, which acts to reduce nitrate to nitrite. Nitrite can be used as an alternative electron acceptor during growth under anaerobic conditions. While nitrite is likely not being used as the terminal electron acceptor since the cultures are grown aerobically, the reduction of nitrate to nitrite performed by NirBD consumes NADH, and therefor may be involved in recycling reduction equivalents. Analysis by qRT-PCR confirmed that these transcripts had a 17–20 fold increase in transcript abundance in the Δ*isdGI* p*lgt.isdI* strain compared to Δ*isdGI* p*lgt* strain.

Due to this increased expression of genes associated with oxygen-independent energy production, we hypothesized that the over expression of *isdI* under the control of the *lgt* promoter may lead to unregulated production of the heme oxygenase, unregulated heme degradation, and disruption of heme homeostasis.

2.3. Constitutive expression of S. aureus heme oxygenases leads to heme-dependent growth inhibition

Since the heme oxygenases of *S. aureus* are regulated by Fur, additional strains were created expressing both of the heme oxygenases, *isdI* and *isdG*, on the pOS1 plasmid controlled by either the *lgt* promoter (p*lgt*) or their endogenous *isdI/isdG* promoters (p*isd*). The strains were grown in heme as the sole source of iron. Under these conditions, the strains expressing the heme oxygenases from their native promoters (p*isd.isdI* and p*isd.isdG*) grew to slightly higher optical densities than the strains containing the constitutively expressed heme oxygenases (p*lgt.isdI* and p*lgt.isdG*) (Fig. 3B). Additionally, when the strains were grown under iron limiting media lacking heme, the strain expressing the constitutively expressed *isdI* grew significantly worse than all of the other strains (Fig. 3A). Unlike the strain constitutively expressing *isdI*, the strain constitutively expressing *isdG* did not exhibit reduced growth under these conditions. This is consistent with the fact that IsdG requires heme for protein stability, and in the absence of heme, IsdG is rapidly degraded post-translationally (Reniere and Skaar, 2008). These data indicate that while heme oxygenases are active under iron limited conditions, constitutive *isdI* expression results in reduced growth.

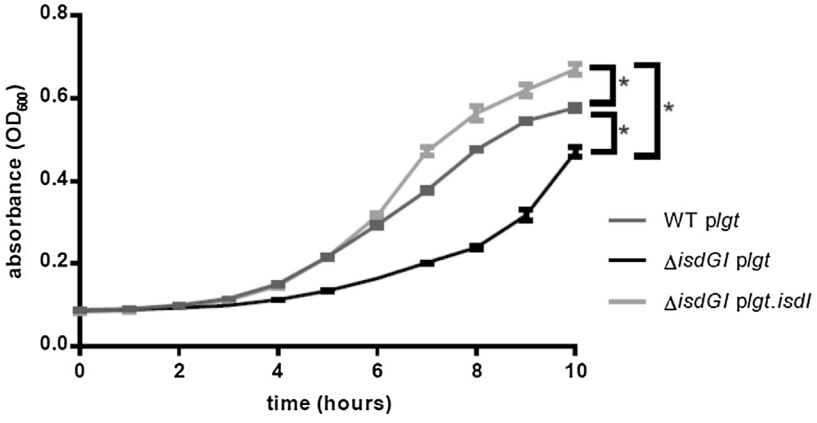

Fig. 1. Expression of *isdI* is required for growth of *S. aureus* with heme as the sole source of iron. Growth of *S. aureus* strains in Chelex treated RPMI with 0.75 mM EDDHA and 1 μM heme. 2-way ANOVA analysis was applied to the 10 h time point ($p < .05$), error bars represent standard error of the mean (S.E.M.).