Repair of Iron Centers RIC protein contributes to the virulence of Staphylococcus aureus

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Staphylococcus aureus colonizes the upper respiratory tract and skin of humans, which constitutes a risk factor for the development of invasive diseases such as pneumonia, meningitis and septicemia, especially in immune-compromised people. The success of S. aureus infections depends on the ability of the pathogen to escape the host’s protective mechanisms, allowing invasion and pathogen proliferation. The multitude of evasive mechanisms and the increase in antibiotic resistance have made S. aureus a serious human threat. Moreover, methicillin-resistant S. aureus strains (MRSA) are no longer restricted to the hospital setting and are widespread in the community.

The innate immune system is the first barrier encountered by the pathogen during host infection. Several studies have reported that internalisation by epithelial cells and phagocytosis by macrophages exposes the microbes to reactive oxygen species (ROS). Therefore, the defences against oxidative stress play an important role in pathogen survival. The wax moth larva Galleria mellonella, that only has innate immunity, is a recognized in vivo model for the study of bacterial virulence of several pathogens including S. aureus. G. mellonella presents advantages over conventional mammalian models, due to the higher temperature required for infection (37°C) and the possibility of the direct injection of a precise inoculum.

Repair of Iron Centers (RIC) proteins are a widespread family of bacterial proteins, which are also present in the genomes of Trichomonas vaginalis and Cryptococcus neoformans eukaryotes. The first RIC protein was found in E. coli due to the marked induction of the encoding gene (formerly named ytfE) in cells grown under nitrosative stress conditions. Consistent with these results, transcriptomic studies done in several organisms have consistently shown that the expression of ric is induced in stressed cells, and that the ric gene deletion generates strains with lower resistance to nitrosative stress. However, in vitro studies done in S. aureus indicated that the protein protects from oxidative stress as inactivation of ric decreased the viability of S. aureus when exposed to hydrogen peroxide. Furthermore, the S. aureus Δric mutant strain exhibited reduced activity of important iron-sulfur (FeS)-containing enzymes such as aconitase and fumarase, activities that could be recovered to the levels observed in the wild-type strain by addition of the recombinant RIC protein to the cell extracts. Following these studies, in this work we have analysed the contribution of RIC to the survival of S. aureus during infection of macrophages, lung epithelial cells, and G. mellonella larvae.

Therefore to investigate the in vivo role of S. aureus RIC, we first tested the behaviour of a strain lacking ric during infection of innate immune cells. For this purpose, macrophages J774A.1 were incubated with S. aureus wild-type and an isogenic Δric mutant, and the survival rate of each strain was determined (Fig. 1). When compared with the parental strain, S. aureus Δric exhibited lower resistance to macrophage killing. Moreover, the susceptibility of the mutant was shown to be dependent on the infection time, i.e., during the first half-hour post-infection no major differences were observed in the number of viable colonies between the two strains, whereas after 6 h a decrease of ~40% in the survival rate was noted for the Δric mutant strain. Additionally, expression in trans of RIC abolished the increased susceptibility of the mutant strain, that under...
In the contrary, the survival of the larvae was lower than that of the wild-type strain (Fig. 2B). These results show that RIC contributes to the successful infection of non-phagocytic cells, such as the lung epithelial cells, by S. aureus.

Galleria mellonella, that is a model organism for the study of innate immunity, was here used to determine the virulence of S. aureus. For this purpose, groups of larvae were infected with S. aureus JE2, incubated at 37°C, and the survival rate was recorded daily for up to 4 days. While inoculation of PBS exhibited no effect (data not shown), the administration of live MRSA (JE2) strain reduced the larval survival in a dose-dependent manner. Inoculation of 10³ CFU/larvae reduced larval viability by 30% after 3 days and 10⁶ CFU/larva caused the death of all larvae after 2 days (Fig. 2A). The lethal dose of S. aureus showed to cause the death of 50% (LD₅₀) of the G. mellonella population at 48 h was found to be ~10⁷ CFU/larva (Figure S2).

The role of RIC in S. aureus virulence was examined in G. mellonella by inoculating larvae with the S. aureus wild-type and the correspondent isogenic ric mutant. Inoculation of G. mellonella with equivalent doses of S. aureus JE2 and Δric mutant showed that the latter had a higher survival rate (Fig. 2B). Four days following infection, only ~20% of the S. aureus JE2 wild-type-infected larvae were still alive, whereas survival of larvae infected with the S. aureus ric mutant was approximately two 2.5-times higher. Complementation in trans with a ric plasmid-borne restored the lethality of the mutant strain to levels similar to those induced by the wild-type strain (Fig. 2B).

We also assessed the proliferation of S. aureus within G. mellonella by determination of the bacterial loads in the larvae hemolymph. S. aureus wild-type and Δric mutant were used at concentrations of ~1 x 10⁷ CFU/larva, and the hemolymph bacterial load was determined at 1 h, 4 h and 8 h post-infection. The viability of the two strains did not decrease significantly after 4 h of infection, but 8 h post-infection the ric mutant exhibited an intracellular viability lower than the wild-type strain.
Moreover, expression of a RIC plasmid-borne in the ric mutant cells led to an increase of the mutant strain viability to levels comparable to the wild-type (Fig. 3A).

To further investigate the role of ric to the S. aureus survival during infection of G. mellonella, we have determined the expression of the S. aureus ric gene in the larvae by quantitative RT-PCR analysis. For this purpose, total RNA was extracted from G. mellonella after infection with S. aureus wild-type up to 8 h. The expression of the S. aureus ric gene within the larvae increased along the course of infection, been the highest after 8 h of infection (Fig. 3B). These results are consistent with the observed contribution of RIC to the survival of S. aureus within the larvae (Fig. 3A).

Previous studies done in E. coli and H. influenzae have implicated RIC in nitrosative stress resistance on the basis of marked increase of the ric gene expression and enhanced sensitivity of the Δric mutants to nitrosative stress. However, in NO-treated S. aureus cells, the ric mRNA levels were only slightly increased and the growth of the S. aureus Δric mutant was not significantly compromised. Although, it is not possible to exclude that the presence in S. aureus of other RNS defences, particularly flavohaemoglobin, may mask the role of RIC in NO stress resistance of this microorganism, several results indicate the involvement of RIC in oxidative stress resistance. RIC is herein shown to defend S. aureus from the effects caused by oxidative stress imposed by macrophages and consistent with these results, we previously reported that S. aureus ric mutant is more sensitive to oxidative stress. Also, the mRNA ric levels increase upon exposure of S. aureus to hydrogen peroxide, and a marked up-regulation of the ric gene was detected in Salmonella enterica and Yersinia pestis upon infection of macrophages and rats, respectively. In addition, inactivation of ric decreased the survival of H. influenzae within macrophages.

S. aureus uptake by epithelial cells is a rapid process that is dose-dependent. We observed that the deletion of ric reduced the rate of S. aureus internalization and survival into human epithelial cells. Interestingly, the S. aureus ric gene is upstream of the lytSR gene cluster that encodes a two-component system regulating murein hydrolase activity and cell death. Moreover, Brunskill and co-workers previously reported that the absence of
ric in *S. aureus* causes morphological defects, while other authors reported that the ric transcription increases upon internalization of *S. aureus* by epithelial A549 cells, along with other genes involved in iron metabolism and oxidative stress resistance.

*Galleria mellonella* wax moth larval infection model has been used to study bacterial pathogenesis, including infections by *S. aureus*. In particular, *G. mellonella* allowed revealing several *S. aureus* virulence factors, such as the accessory gene regulator (Agr), a global regulator of the staphylococcal virulon that includes secreted virulence factors and surface proteins, RelA, a guanosine pentaphosphate synthase that regulates amino acid biosynthesis under nutrient-limited conditions, and two glyceraldehyde-3-phosphate dehydrogenase homologs called GapAB, involved in glycolysis and gluconeogenesis, respectively. Our data show that RIC also contributes to the virulence of *S. aureus*.

The inactivation of RIC in *S. aureus* was previously reported to decrease the activity of oxidatively damaged Fe-S containing enzymes such as aconitate and fumarase, enzymes that are required for a functional TCA and fully respiratory activity of *S. aureus*. The contribution of RIC to the survival in phagocytes and epithelial cells and to the virulence towards *G. mellonella* shows the in vivo importance of this protein, which is most probably related with protection that it confers against the oxidative stress imposed by the host cells.

Experimentally the study included the *S. aureus* strains listed in Table S1, namely the methicillin-resistant *S. aureus* (MRSA) strain JE2 (wild-type), derived from community-associated methicillin-resistant *S. aureus* strain USA300 LAC, and the JE2 Δric strain, which were provided by the Nebraska Transposon Mutant Library. The media used for growth of bacteria was purchased from BD Difco and antibiotics from Sigma. *S. aureus* was cultured overnight in Tryptic Soy Broth (TSB), at 37°C, and used to inoculate fresh Luria-Bertani (LB) medium and grown to the indicated optical density at 600 nm (OD600). Bacterial viability was determined by CFUs counting by performing serial dilutions of *S. aureus* in phosphate-buffered saline (PBS), plating on Tryptic Soy Agar (TSA) and incubation overnight at 37°C.

The infecting dose was optimized by injecting *G. mellonella* with increasing inocula of *S. aureus* JE2 cell suspensions (~10^7–10^8 cells/larva). For LD_{50} determination, eight bacterial concentrations were tested and the number of dead larvae was evaluated after 48 h, 72 h and 96 h. GraphPad Prism program was used to plot a non-linear fitting curve and obtain the LD_{50} value.

*S. aureus* JE2 and the isogenic Δric mutant derivative were grown overnight and diluted in PBS (to final 10^7–10^8 CFU/mL).

A DNA fragment of ~1500 bp containing the complete coding region of the *S. aureus* ric gene plus a ~1000 bp upstream region, that contains the ric own promotor and lacks any other complete open reading frame, was PCR-amplified from *S. aureus* NCTC8325 genomic DNA using the oligonucleotides SA_RICcomp_fw and SA_RICcomp_rev (Table S2) and ligated into pMK4 digested with EcoRI and SalI. After confirming the integrity of the cloned gene, the resulting vector (pMK4-RIC) was electroporated (~100 ng) into *S. aureus* RN4220 and transformants were selected on TSA medium containing 5 μg/mL chloramphenicol. The recombinant plasmid extracted from *S. aureus* RN4220 cells was electroporated (~20 μg) into *S. aureus* JE2 Δric, and its integration confirmed by colony PCR and digestion with appropriated enzymes. Restriction enzymes were obtained from New England Biolabs, DNA polymerase from Roche.

For the eukaryotic cell culture assays, murine macrophages J774.1 and human lung epithelial A549 cells (ATCC CCL 185) were routinely cultivated for 2 days in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (DMEMi), 50 U/mL penicillin and 50 μg/mL streptomycin (DMEMc), at 37°C in a 5% CO₂-air atmosphere. *S. aureus* JE2 and JE2 Δric mutant cells grown to OD_{600} of ~0.4 were collected, washed with PBS and diluted in DMEMi to obtain a culture at OD_{600} ~0.03. Prior to infection, macrophages (~5 × 10^5 cells/mL) were seeded in 24-well plates and cultivated under 5% CO₂-air atmosphere at 37°C, for 48 h, and activated by incubation with 1 μg/mL gamma interferon (IFN-γ) and 5 μg/mL E. coli lipopolysaccharides (LPS), for 5 h; when indicated, 800 μM NG-monomethyl-l-arginine acetate salt (L-NAME) or 400 μM apocynin were added to inhibit inducible nitric oxide synthase or NADPH phagocyte oxidase, respectively. Bacterial suspensions at ~10^7 cells/mL were used to infect macrophages at a multiplicity of infection (MOI) of 5. After a 30 min incubation period, non-phagocytosed bacterial cells were removed by incubation with 50 μg/mL gentamicin for 10 min. Macrophages were washed with PBS, lysed (2% saponin, Sigma), and the intracellular bacterial CFUs determined after 30 min and 6 h of infection. Nitric oxide levels were inferred by the oxidative product nitrite using the Griess assay.

For the internalization assays in lung epithelial cells, 24 h prior to infection, human lung epithelial cells A549 (ATCC CCL 185) (~2 × 10^5 cells/mL) were seeded in 24-well plates and cultured in 5% CO₂-air atmosphere at 37°C. *S. aureus* wild-type and ric mutant strains grown in LB to OD_{600} of ~0.5–0.6 were diluted in DMEMi to OD_{600} ~0.05 (~10^7 cells/mL) and incubated for 30 min and 2 h in A549 cells (MOI of 20). Extracellular bacteria
were removed by incubation with 4 μg/mL lysostaphin (Sigma) at 37 °C for 20 min. Lung epithelial cells were PBS-washed, trypsinised and lysed with cold Triton X-100 (0.1%), and viable intracellular bacteria were counted.

Larval infection assays were performed with G. mellonella larvae reared in an iBB insectarium and maintained on a beeswax and pollen grains diet at 25 °C in darkness, and used in killing assays at the final instar larval stage. The infecting dose was optimized by injecting G. mellonella with increasing inocula of S. aureus JE2 cell suspensions (~10^3–10^8 cells/larva). S. aureus JE2 and the isogenic Δric mutant were grown overnight and diluted in PBS (to final 10^7–10^8 CFU/mL). For each bacterial dilution, 3.5 μL aliquots were injected into the hindmost left proleg of each larva using a microsyringe adapted to a micrometer that controls the volume of injection. Control larvae injected with equal volumes of PBS were also monitored. Larvae were placed in Petri dishes and stored in the dark at 37 °C up to 4 days. Larval survival was monitored daily by inspection for dead organisms which were identified by development of a black colour resultant from larval melanisation, and immobility.

Bacterial load in the G. mellonella hemolymph was evaluated in three living larvae that were punctured in the abdomen with a sterile needle and after 1 h (time zero of infection), 4 h and 8 h of infection the plasma was collected to a sterile microtube containing a few crystals of phenylthiourea. The hemolymph was serially diluted in PBS, plated on TSA plates and CFU were determined after incubation at 37 °C, and used in killing assays at the final instar larval stage. The infecting dose was optimized by injecting G. mellonella with increasing inocula of S. aureus JE2 cell suspensions (~10^3–10^8 cells/larva). S. aureus JE2 and the isogenic Δric mutant were grown overnight and diluted in PBS (to final 10^7–10^8 CFU/mL). For each bacterial dilution, 3.5 μL aliquots were injected into the hindmost left proleg of each larva using a microsyringe adapted to a micrometer that controls the volume of injection. Control larvae injected with equal volumes of PBS were also monitored. Larvae were placed in Petri dishes and stored in the dark at 37 °C up to 4 days. Larval survival was monitored daily by inspection for dead organisms which were identified by development of a black colour resultant from larval melanisation, and immobility.

Statistical analysis was carried out using GraphPad Prism version 5.01 for Windows. Survival curves were plotted using the Kaplan-Meier method, and differences in survival were calculated using the Mantel-Cox test for curve comparisons. Ten larvae were examined for each condition, and each experiment was repeated at least three times, over two different weeks and for up to four weeks. Differences between mean values were tested for significance by performing unpaired two-tailed Student’s t-test with P<0.05.

The S. aureus ric expression during G. mellonella infection was analysed by incubating the larvae with S. aureus (~10^7 CFU/larva). For each time point (0 h, 4 h and 8 h after infection), three living larvae were cryopreserved, sliced and homogenized in 1 mL TRIZol (ThermoFisher Scientific). Total animal/bacteria RNA was extracted according the manufacturer’s protocol, treated with RNase-Free DNase (Quiaegen), and its concentration and purity was evaluated in a Nanodrop ND-1000 UV–visible spectrophotometer (Thermo Fisher Scientific) and agarose gel. Total RNA (900 ng) was reverse transcribed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) using the Anchored-oligo (dT)18 and Random Hexamer primers. Quantitative real-time RT-PCR assays were done in a LightCycler® 480 (Roche) using the oligonucleotides listed in Table S2 and the LightCycler® 480 SYBR Green I Master kit (Roche). Relative quantification of ric gene is shown in relation to the 16S rRNA reference gene, whose expression does not vary under the tested conditions, and using the comparative CT method.

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