Role of rsbU and Staphyloxanthin in Phagocytosis and Intracellular Growth of Staphylococcus aureus in Human Macrophages and Endothelial Cells

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In Staphylococcus aureus, rsbU down-regulates agr and stimulates production of staphyloxanthin (STX), an antioxidant that may contribute to intracellular survival after phagocytosis. Using isogenic rsbU− and rsbU+ strains, we show that rsbU causes increased internalization and intracellular growth in both THP-1 macrophages and human umbilical vein endothelial cells (more so for the latter) without change in subcellular localization and that inhibition of STX biosynthesis markedly reduces intracellular growth of the rsbU+ strain (and of clinical isolates, including USA300; tested with macrophages only) without affecting internalization. Thus, rsbU is important for uptake and for STX biosynthesis and is critical for intracellular multiplication of S. aureus.

Staphylococcus aureus, a frequent cause of severe nosocomial and community-acquired bacterial infections in humans, produces a large collection of virulence factors that induce immediate local and general damage during infection. In this context, we have examined the role of RsbU, a phosphatase that positively controls the transcriptional factor sigma B, which itself down-regulates the expression of agr [1]. Indeed, agr is a regulator of S. aureus virulence and sigma B is a transcriptional factor that plays a central role in stress response [2] and persistence of infection in vivo [3]. In several studies, the persistent and recurrent nature of staphylococcal infections has been related to the existence of an intracellular pool of bacteria in both professional and nonprofessional phagocytes. The expression of virulence factors that damage the host is reduced in intracellular S. aureus (in order to maintain itself in the intracellular milieu [4]), but expression of other genes appears to be essential for resistance to host defenses. The lack of expression of the corresponding gene (rsbU) is also associated with reduced H2O2 tolerance related to impairment of the biosynthesis of staphyloxanthin (STX) [5], a carotenoid pigment that acts as an antioxidant, blocking attack by host reactive oxygen species [6].

Here, we take advantage of the fact that rsbU is absent in the common S. aureus laboratory strain 8325-4 but is present in the isogenic variant SH1000. This enables an investigation of the internalization and intracellular growth of both strains in professional phagocytes (THP-1 macrophages), as well as in human umbilical vein endothelial cells (HUVECs). In addition to enhanced pigmentation by STX, SH1000 also shows reduced secretion of exoproteins and down-regulation of agr [7, 8]. Therefore, we complemented our investigation by using a newly described inhibitor of STX biosynthesis, BPH-652 [9], to determine the role of STX in intracellular growth in 8325-4, SH1000, and several clinical strains, including USA300, one of the more virulent community-associated methicillin-resistant Staphylococcus aureus (MRSA) strains.

Methods. Experiments were performed with S. aureus 8325-4, a weak producer of STX with a natural deletion in rsbU, and with SH1000, a highly pigmented rsbU+ construct obtained from S. J. Foster [7]. We also tested 3 MRSA clinical isolates showing marked pigmentation (obtained from the Statens Serum Institute, Copenhagen, Denmark), and the community-associated MRSA strain USA300 (NRS384, obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus; Focus Technologies). Bacteria were grown in Mueller-Hinton broth, and colony-forming unit (CFU) counting was done by plating on tryptic soy agar.

Human THP-1 cells (ATCC TIB-202; LGC Promochem) were cultivated using Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum (Invitrogen) and infected as described elsewhere [10]. HUVECs (Lonza) with <8 passages were seeded in gelatin-coated plates in Dulbecco modified Eagle medium-glutamax medium (Invitrogen) supplemented with 10% fetal calf serum, and infected following our protocol for adherent cells [11]. Phagocytosis was continued for 1 h at an initial inoculum of 4 preopsonized bacteria.
Figure 1. Phagocytosis, intracellular growth, and susceptibility to H$_2$O$_2$ of the isogenic S. aureus 8325-4 rsbU$^-$ and SH1000 rsbU$^+$ strains. A, Enumeration of cell-associated colony-forming units (CFUs) in human umbilical vein endothelial cells (HUVECs) (white symbols) and THP-1 macrophages (black symbols) after 1 h of phagocytosis of 8325-4 (circles) or SH1000 strains (triangles). Each data point corresponds to the actual counts from independent samples (for 8325-4 in HUVECs and 24 n$^p$30 for all other conditions). The horizontal bar represents the corresponding mean values. Statistical analysis (by analysis of variance [ANOVA], Tukey post hoc test) indicated that all conditions were significantly different from one another (for all comparisons except THP-1 8325-4 vs THP-1 SH1000, for which $P < .01$). B, Intracellular growth of 8325-4 or SH1000 in HUVECs (white bars) or in THP-1 (black bars) macrophages over the 24 h after phagocytosis. Values are expressed as the change in CFUs per milligram of protein over the incubation period of 3 independent samples; error bars represent standard deviations (SDs). Statistical analysis (ANOVA, Tukey post hoc test) indicated that bars with different letters are significantly different from one another ( ). C, Susceptibilities of 8325-4 (circles) or SH1000 (triangles) strains to H$_2$O$_2$ in broth. Bacteria were incubated for 45 min with increasing concentrations of H$_2$O$_2$ (0–100 mmol/L). Values are expressed as the percentage of CFUs compared with controls (no H$_2$O$_2$) and are the means ± SD for 3 independent samples.
analyses (analysis of variance [ANOVA]) were performed with GraphPad Instat software (version 3.06; GraphPad).

Results. In a first series of experiments, we compared the susceptibility to phagocytosis and intracellular growth of 8325-4 (rsbU- ) and SH1000 (rsbU+ ) strains. To measure phagocytosis, cells were exposed to opsonized bacteria for 1 h and then collected after extensive washing (to remove noninternalized S. aureus). As shown in Figure 1A, phagocytosis by HUVECs was significantly more important with SH1000 (~1.8-fold increase) than with 8325-4. With THP-1 cells, phagocytosis was globally more efficient but, again, with a significant difference between the 2 strains (~1.5-fold). To measure intracellular growth, cells exposed to bacteria as indicated above were returned to bacteria-free medium and incubated for 24 h before being collected (also after extensive washing). Intracellular infection could be obtained for both strains in both cell types, but there was significantly more growth of SH1000 than 8325-4 (Figure 1B). In light of this result, 3 additional experiments were performed. First, we examined whether SH1000 and 8325-4 would not grow at different rates in broth under conditions mimicking what could take place in cells (ie, logarithmic growth and up to densities of ~10⁶ CFUs/mL; for details, see Barcia-Macay et al [10]), but no difference was seen. Second, confocal and electron microscopy were used to detect potential differences in the subcellular localization of both strains. These were found confined to phagolysosomes in THP-1 cells at 3 and 24 h, as reported previously for other strains of S. aureus [11]. In HUVECs, most bacteria were also found in phagolysosomes, although a small number were observed in the cytosol (but with no difference between strains). Third—and because STX had been reported to protect phagocytized S. aureus against reactive oxygen species–dependent cell defense mechanisms [6]—we tested whether SH1000 in broth was more resistant to H₂O₂ than 8325-4. Figure 1C shows that this is indeed the case, with concentrations of H₂O₂ required to kill 50% and 90% of bacteria being ~42 and 100 mmol/L for SH1000, versus 16 and 22 mmol/L for 8325-4.

In a second series of experiments, we examined the influence of the STX biosynthesis inhibitor BPH-652 on bacterial phagocytosis and growth of SH1000 in HUVEC and THP-1 macrophages, as well as on the growth of other pigmented strains inside THP-1 macrophages. We first verified that BPH-652 (100 μmol/L) added to broth (1) impaired the biosynthesis of staphyloxanthin (illustrated for SH1000 in Figure 2A) and (2) did not affect bacterial growth (checked by change in optical density read at 620 nm [turbidimetry] and bacterial counts over 24 h). Bacteria preexposed to BPH-652 were then used to infect HUVECs or THP-1 macrophages, and intracellular growth was measured as described above. BPH-652 was maintained at 100 μmol/L throughout the experiment, which neither significantly modified the extent of phagocytosis (~1% difference in 2 independent experiments) nor affected cell viability (≥85%, on the basis of the trypan blue exclusion test; no significant difference in total protein content of samples prepared from treated vs control cells). However, it significantly reduced the intracellular growth of SH1000 (~1 log₁₀ CFU in both cell types) (Figure 2B). BPH-652 also reduced the growth of 8325-4 but...
to a considerably lesser extent in relation to the lower production of STX (as evidenced by pigment loss). We also compared in THP-1 cells the growth of a series of clinical isolates of various levels of pigmentation (assessed by visual inspection). As shown in Figure 2, their growth was proportional to pigmentation and was markedly reduced in the presence of BPH-652 (except for SA63062), to a level that was similar for all strains, including 8325-4.

Discussion. The main conclusions that can be drawn from the present study are (1) that restoration of rsbU in the naturally deficient Staphylococcus aureus laboratory strain 8325-4 enhances its internalization and intracellular growth in both HUVECs and THP-1 cells without change in subcellular localization and (2) that inhibition of STX biosynthesis impairs the intracellular growth of SH1000 in both HUVECs and macrophages. A similar effect was seen in macrophages for several other pigmented strains of clinical interest. Thus, rsbU appears to control 2 key steps in the establishment of an intracellular infection—phagocytosis and intracellular growth—but by 2 distinct downstream mechanisms.

Phagocytosis of S. aureus involves binding of the bacterium to the cell surface, which in endothelial cells takes place via fibronectin-binding proteins that in agr mutants are up-regulated and show improved binding [13]. This may explain the larger internalization seen with the SH1000 strain, since RsBU negatively controls agr expression through σδ. A similar influence of agr on S. aureus internalization is probably also important in THP-1 macrophages, although the basal phagocytic activity of these cells is globally higher, since internalization in professional phagocytes operates through a zipper mechanism in which cells produce pseudopodes, making the overall process more efficient [14].

We showed previously with Listeria-infected macrophages and epithelial cells that intracellular growth (of an intracellular bacterium) is the result of a dynamic balance between bacterial multiplication capabilities and host defense destructive mechanisms that is mainly related to the production of reactive oxygen and reactive nitrogen species [15]. The present experiments suggest that the same phenomenon is taking place in S. aureus, with STX providing a primary defense against oxidative damage. This STX-related mechanism is not expected to affect phagocytosis, as was indeed observed. These results are therefore consistent with in vivo observations that show that STX accumulation plays a critical role in triggering sustained tissue infections with abscess formation but does not affect mucosal colonization [9]. Additionally, while intracellular survival of S. aureus is determined by multiple virulence factors [1], our results point to a critical role for rsbU in the control of virulence in clinical isolates from persistent infections collected from intracellular foci. While generalization to more clinical isolates and more cell types is desirable, the present data already suggest new therapeutic approaches based on the control of STX biosynthesis in S. aureus, as a complement or alternative to direct intervention at the level of this gene product and the corresponding downstream products.

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References