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 H_2O_2 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

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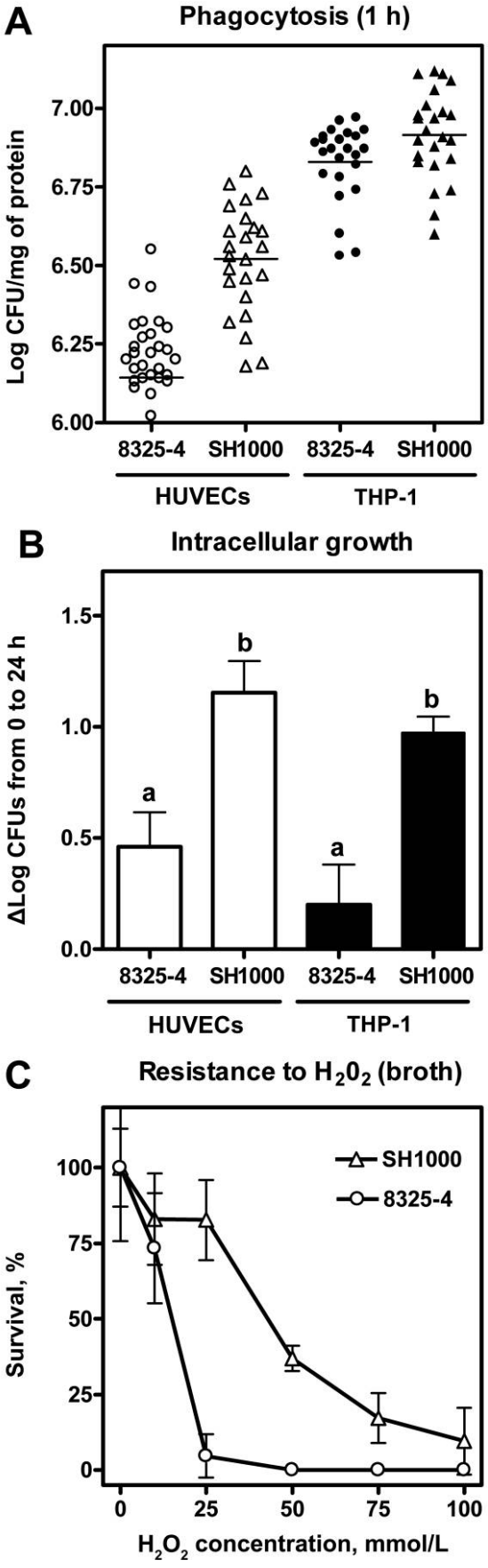

per cell, after which extracellular bacteria were removed by washing. Intracellular growth was allowed to proceed for 24 h in the presence of gentamicin at the minimum inhibitory concentration [10, 11] to minimize growth of extracellular bacteria (<10% of total bacterial counts at 24 h). Cell viability was tested at the end of the experiments by trypan blue staining (<15% stained cells in all conditions); a low initial inoculum was critical in these studies to avoid rapid cell killing by apoptosis, commonly seen with larger inocula.

To test the resistance of bacteria to hydrogen peroxide, we used the method described elsewhere [6] but with a larger concentration of catalase (100 U/mL) to fully destroy any H_2O_2 remaining at the end of the incubation period.

For in situ confocal fluorescence microscopy (MRC 1024 confocal microscope; Bio-Rad) of infected cells, bacteria were stained overnight with 0.25 mg/mL fluorescein-5-isothiocyanate (Invitrogen) prior to phagocytosis. 50 μmol/L LysoTracker Red DND 99 (Invitrogen) was added 1 h before the end of the incubation period to stain lysosomes. Images were processed with the Confocal Assistant software (version 4.02; http://www.nephrology.iupui.edu/imaging/software.htm). For electron microscopy, infected cells were harvested, washed with phosphate-buffered saline, fixed with 2% glutaraldehyde and 1% osmium tetroxide, and then stained en bloc with uranyl acetate [11].

The dehydrosqualene synthase inhibitor BPH-652, which blocks STX biosynthesis, was prepared as described elsewhere [9, 12]. Gentamicin was obtained from GlaxoSmithKline as the commercial product registered for human administration. Gelatin and H_2O_2 were obtained from Sigma-Aldrich. Statistical

Figure 1. Phagocytosis, intracellular growth, and susceptibility to H_2O_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 ($n = 30$ for 8325-4 in HUVECs and 24 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 ($P < .001$ 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 ($P < .001$). *C,* Susceptibilities of 8325-4 (*circles*) or SH1000 (*triangles*) strains to H_2O_2 in broth. Bacteria were incubated for 45 min with increasing concentrations of H_2O_2 (0–100 mmol/L). Values are expressed as the percentage of CFUs compared with controls (no H_2O_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 1*A*, 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 1*B*). 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 ~10^9 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 H_2O_2 than 8325-4. Figure 1*C* shows that this is indeed the case, with concentrations of H_2O_2 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 2*A*) 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 $\log_{10}$ CFU in both cell types) (Figure 2*B*). BPH-652 also reduced the growth of 8325-4 but

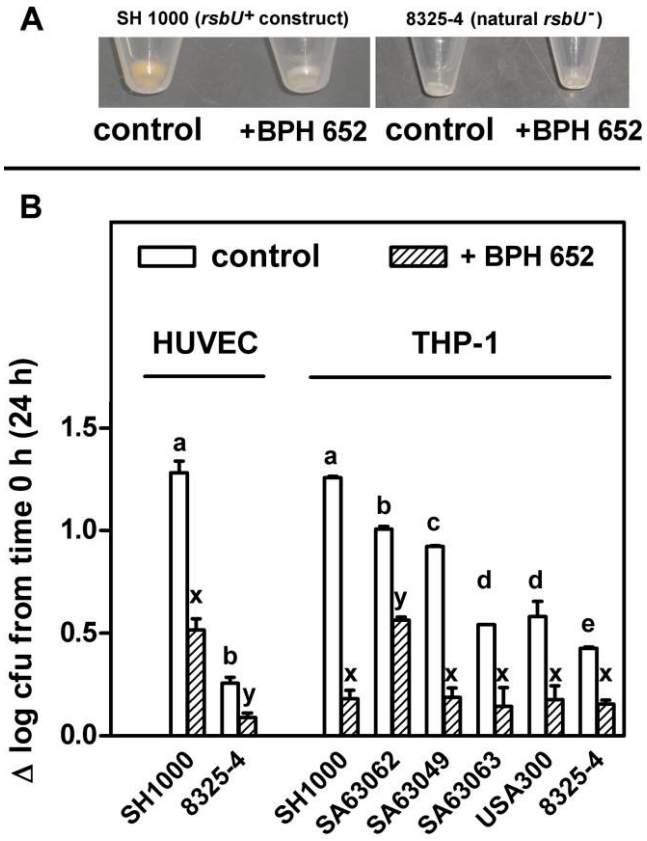

Figure 2. *A*, Influence of the dehydrosqualene synthase inhibitor BPH-652 on the pigmentation of the SH1000 strain grown in broth. Bacteria were grown for 2 days in the absence (control) or in the presence of the inhibitor (100 μmol/L) before being pelleted for photography; the 8325-4 strain subjected to the same treatments is also shown, to demonstrate the near absence of pigmentation under all conditions. *B*, Intracellular growth of SH1000 and 8325-4 in human umbilical vein endothelial cells (HUVECs), and of SH1000, SA63062, SA63049, SA63063, USA300 (4 clinical isolates) and 8325-4 in THP-1 macrophages in the absence (control; strains are ranked according to the intensity of their pigmentation as assessed by visual inspection of colonies) or presence of BPH-652. Bacteria, treated as in panel *A*, were opsonized and phagocytized by the cells by means of the same protocol as in the experiments shown in Figure 1, with inhibitors maintained at 100 μmol/L in the culture medium for the whole incubation period. Data are presented as in Figure 1*B*. Statistical analysis was as follows: (1) paired comparisons for each strain between control and BPH-652 (2-tailed unpaired *t* test) indicate that all differences are significant at $P < .002$; (2) multiple comparisons between strains in control conditions or in the presence of BPH-652 (1-way analysis of variance with Tukey-Kramer multiple comparison test applied independently for HUVECs and THP-1 cells) indicate that the bars with different letters (*a* to *e* for controls; *x* and *y* for BPH-652) were significantly different from each other at $P < .01$.

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 level 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 *S. 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 σ^B. 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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