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The σ^B Regulon Influences Internalization of *Staphylococcus aureus* by Osteoblasts

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Individual strains of *Staphylococcus aureus* have different capacities to become internalized by osteoblasts. Here we report that the levels of σ^B expressed by *S. aureus* correlate with the capacity of this bacterium to be internalized by osteoblasts. However, σ^B is not essential for internalization and does not necessarily account for the differences in the capacities of strains to be internalized.

Staphylococcus aureus is an important human pathogen that causes a range of infections, including those of bone. *S. aureus* bone infections can be extremely difficult to treat, requiring prolonged antibiotic treatment and surgical intervention (18). We, and others, have shown previously that *S. aureus* is internalized by the bone-forming cells, osteoblasts (10, 14, 15, 19). The capacity of *S. aureus* to be internalized by osteoblasts has been proposed as an explanation for the recurrent nature of, and the difficulty in treating, bone infections with this organism.

We have previously shown that osteoblasts employ a receptor-mediated pathway in the uptake of *S. aureus* (15) and that the expression of fibronectin binding proteins (FnBPs) by the bacterium is essential to the process of internalization (1). However, the capacities of different isolates of *S. aureus* to be internalized by osteoblasts varied and could not be accounted for by differences in the levels of expression of FnBPs by these strains. In fact, *S. aureus* strain LS-1, which expressed lower levels of FnBPs, was internalized to a greater extent than strain 8325-4 (1). Although the mechanism underlying the differences in the capacities of these two strains to be internalized is unknown, it is interesting that strain 8235-4 is a derivative of NCTC8325, which is a natural *rsbU* mutant (13, 17).

In *Bacillus subtilis*, a network of protein-protein interactions regulates the activity of the alternative sigma factor, σ^B, post-translationally. One of the proteins in this network, RsbU, is essential for the activation of σ^B. It has recently been shown that σ^B activity in *S. aureus* also depends on RsbU (13). Derivatives of *S. aureus* NCTC8325 have an 11-bp deletion in *rsbU*, resulting in the introduction of a stop codon into this gene, and hence these strains are essentially deficient in σ^B activity.

In many bacteria the alternative sigma factors of RNA polymerase are important in cell adaptation to environmental stress (20). Association of σ^B with the core RNA polymerase results in the recognition of a specific subset of promoters and to the initiation of transcription of their genes. To date only one alternative sigma factor has been identified in *S. aureus*, σ^B. Although σ^B has primarily been associated with responses to environmental stress, it has been shown that expression of the global virulence regulator SarA is influenced by σ^B in *S. aureus* (8, 9, 12, 17), and it has been suggested that σ^B functions as a global regulator of virulence genes in this bacterium (16). It has recently been demonstrated that σ^B expression influences transcription of both the *sar* and *agr* loci, and it has been suggested that σ^B might prolong the production of cell surface proteins such as the FnBPs, while preventing upregulation of secreted exoproteins (4).

Given the apparent importance of σ^B in the regulation of virulence genes in *S. aureus*, we have examined the hypothesis that σ^B activity may influence the capacity of *S. aureus* to be internalized by osteoblasts and thus account for our previous observation of strain-dependent differences in this capacity (1).

The bacterial strains used in this study are listed in Table 1. Strain BB1591 was obtained by phage 80α-mediated transduction of (Δ*rsbUVWsigB*)::*ermB* from IK181 (16) into strain LS-1, selecting for erythromycin resistance. Strain MB258 was obtained by transduction of the reporter construct *asp23p*::pBT*asp23p-luc*$^+$ from MB61 (13) into strain LS-1, selecting for tetracycline resistance. The levels of σ^B activity in *S. aureus* strains were analyzed during growth by using an *asp23* reporter gene system as previously described (13). *S. aureus* strain BB255 is our laboratory stock of NCTC8325 and carries a mutation in *rsbU*5. Table 2 shows that the MB33 reporter strain of BB255 expressed very low levels of σ^B activity. Complementation of this strain with an intact *rsbU* allele from strain COL, as in strain GP268, resulted in expression of significant levels of σ^B activity, as indicated by the reporter strain MB49 (Table 2). The level of σ^B activity in MB49 is similar to that found in other RsbU$^+$ strains of *S. aureus* (13). Replacement of the σ^B operon in *S. aureus* strain BB255 with an erythromycin cassette, as in strain IK181, resulted in no expression of σ^B activity as shown by the reporter strain MB90 (Table 2). *S. aureus* strain MB138, BB255 with a point mutation in the anti-sigma factor *rsbW*, produced high levels of σ^B activity (Table 2). *S. aureus* strain LS-1, represented by strain MB258, produced levels of σ^B activity which were similar to those of strain GP268, as indicated by the reporter strain

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TABLE 1. Strains used in this study

Strain	Relevant genotype and phenotype[a]	Reference(s) or source
BB255	Laboratory stock of NCTC8325, *rsbU*	2
MB33	BB255 *asp23*$^+$ *asp23p*::pE*Casp23p-luc*$^+$ Em^r	13
IK181	BB255 (Δ*rsbUVWsigB*)::*erm*(B) Em^r	16
MB61	RN4220 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r	13
MB90	IK181 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r Em^r	13
GP268	BB255 (*rsbU*$^+$*V*$^+$*W*$^+$*sigB*$^+$)-*tetL* Tc^r	13
MB49	GP268 *asp23*$^+$ *asp23*$^+$ *asp23p*::pEC*asp23p-luc*$^+$ Em^r Tc^r	13
MB138	MB33 *rsbW7* Em^r	3
LS-1		6, 7
MB258	LS-1-*asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r	This study
BB1591	LS-1 (Δ*rsbUVWsigB*)::*erm*(B) Em^r	This study
MB259	BB1591 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r Em^r	This study

[a] *asp23* is the gene for the 23-kDa alkaline shock protein of *S. aureus.*

MB49 (Table 2). Replacement of the σ^B operon in *S. aureus* LS-1 with an erythromycin cassette, as in strain BB1591, led to a complete loss of σ^B activity as indicated by the reporter strain MB259 (Table 2).

The capacity of *S. aureus* strains to be internalized by the osteoblastic cell line MG63 was determined as previously described (1) with the modification that overnight cultures of bacteria were diluted 1:20 in 10 ml of fresh brain heart infusion and grown to an optical density at 600 nm of approximately 1 before use. *S. aureus* strain BB255 was internalized by osteoblasts as shown in Fig. 1. Strain GP268 (BB255 *rsbU*$^+$) had a twofold-greater capacity to be internalized by osteoblasts than did BB255 (Fig. 1). The capacity of strain IK181 (BB255 Δ*rsbUVWsigB*) to be internalized by osteoblasts was slightly lower than that of strain BB255, although the difference was not statistically significant (Fig. 1). *S. aureus* strain MB138 (BB255 *rsbW*) had a 10-fold-greater capacity to become internalized by osteoblasts than did strain BB255 (Fig. 1). To determine if σ^B activity had an effect on the ability of *S. aureus* to grow and/or survive within osteoblasts, cocultures were extended for a period of up to 6 h as previously described (1). There was no significant difference in the percentages of the natural *rsbU* mutant BB255 and its isogenic mutants GP268 and MB138 recovered, at hourly intervals up to 6 h, from the number recovered after 2 h (data not shown).

It has previously been shown that *S. aureus* strain LS-1 has a

TABLE 2. Strain-dependent differences in σ^B activity

Strain	Relevant genotype[a]	σ^B activity[b]
MB33	BB255	34.0 ± 3.00
MB49	BB255 *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	301.3 ± 23.8
MB90	BB255 Δ*rsbUVWsigB*	0.62 ± 0.10
MB138	BB255 *rsbW7*	1,084.0 ± 46.5
MB258	LS1 *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	256.6 ± 17.2
MB259	LS1 Δ*rsbUVWsigB*	0.31 ± 0.14

[a] Detailed relevant genotypes and phenotypes are listed in Table 1.

[b] σ^B transcriptional activity (relative light units) was determined from cells grown to an optical density at 600 nm of 1.5 by measuring the luciferase activity of Luc$^+$, the product of the *luc*$^+$ reporter gene fused to the σ^B-dependent promoters of *asp23.* The values shown are the results of four independent assays.

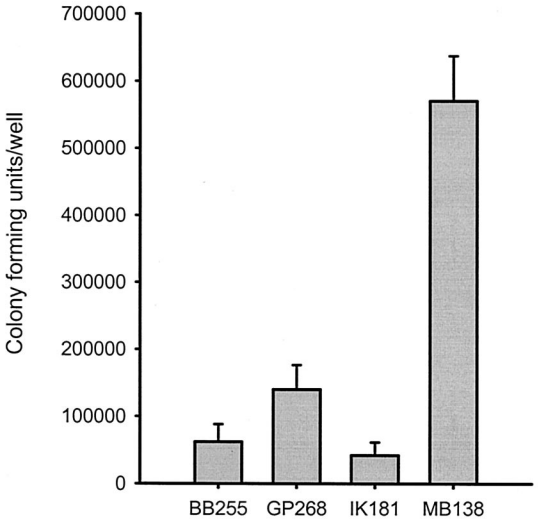

FIG. 1. Capacity of *S. aureus* strain BB255 and its isogenic mutants to be internalized by osteoblasts. The isogenic strains BB255 (*rsbU*), GP268 (BB255 *rsbU*$^+$), IK181 (BB255 Δ*rsbUVWsigB*), and MB138 (BB255 *rsbW*) were cocultured with osteoblasts at a multiplicity of infection of 100:1. The figure shows the results from one representative experiment of at least three, and the data are the means and standard deviations of three replicate cultures.

10-fold-greater capacity to be internalized by osteoblasts than does the NCTC8325 derivative 8325-4 (1). In this study we compared the capacity of the NCTC8325 derivative BB255 to be internalized by osteoblasts with that of LS-1. Figure 2 shows that *S. aureus* strain LS-1 had a greater capacity to be internalized than did strain BB255 (sevenfold) or its *rsbU*$^+$ derivative GP268. Strain BB1591, LS-1 in which the σ^B operon had

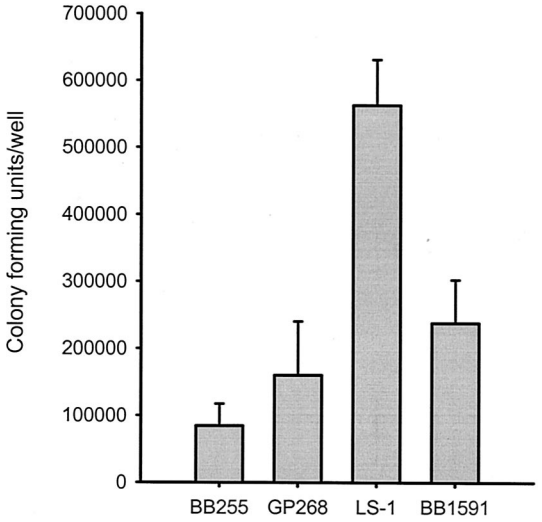

FIG. 2. Comparison of the capacities of *S. aureus* strains BB255 and LS-1 and their respective isogenic mutants to be internalized by osteoblasts. *S. aureus* strains BB255 (*rsbU*), GP268 (BB255 *rsbUVWsigB*$^+$), LS-1, and BB1591 (LS-1 Δ*rsbUVWsigB*) were cocultured with osteoblasts at a multiplicity of infection of 100:1. The figure shows the results from one representative experiment of at least three, and the data are the means and standard deviations of three replicate cultures.

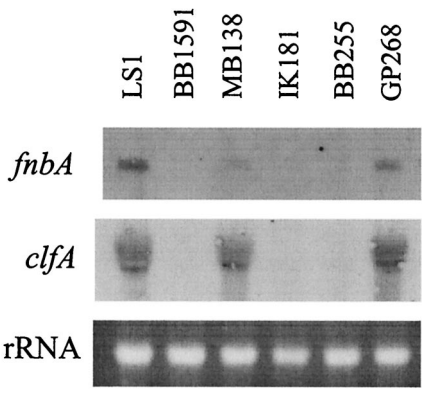

FIG. 3. Northern blot analyses of *fnbA* and *clfA* expression by *S. aureus* strains LS1, BB1591 (LS1 Δ*rsbUVWsigB*), MB138 (BB255 *rsbW*), IK181 (BB255 Δ*rsbUVWsigB*), BB255 (*rsbU*), and GP268 (BB255 *rsbU*$^+$).

been deleted, had a twofold-lower capacity to be internalized by osteoblasts than did its parent LS-1 (Fig. 2).

One mechanism by which the σ^B operon may influence internalization of *S. aureus* by osteoblasts is alteration of the expression of bacterial cell surface proteins such as the FnBPs. To investigate this possibility, Northern blot analysis for *fnbA* and *clfA* expression was performed. Bacterial overnight cultures were diluted to an optical density at 600 nm of 0.1 and grown at 37°C. RNA samples were prepared from cells collected after 1 h and after 8 h. Specific, digoxigenin-labeled *fnbA* and *clfA* probes were used for hybridizing 1-h and 8-h RNA extracts (7 μg), respectively. As can be seen in Fig. 3, the levels of expression of *fnbA* and *clfA* were highest in strains LS-1, MB138, GP268, and MB79, all of which possessed high σ^B activity. The capacities of *S. aureus* strains to bind to fibronectin were determined as previously described (1). The capacity of the *rsbU* mutant BB1591 to bind fibronectin was reduced to 24% ± 6% (mean ± standard deviation) of that of the wild-type *rsbU*$^+$ strain LS-1. Similarly the capacity of the natural *rsbU* mutant BB255 to bind to fibronectin was only 35% ± 9% (mean ± standard deviation) of that of the *rsbU*-complemented strain GP268.

It has been postulated that the capacity of *S. aureus* to be internalized by mammalian cells may contribute to the pathogenicity of this organism. In particular this capacity could account for the persistent nature of some *S. aureus* infections. The molecular details of the bacterial-host cell interactions that result in uptake of *S. aureus* by host cells are beginning to be elucidated. It has become evident that the FnBPs of *S. aureus* play an important, if not essential, role in the uptake process. However, we have previously reported that strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts do not necessarily correlate with the levels of expression of the FnBPs, suggesting that other factors are also important in this process (1). Since we were aware that one of the strains used in the previous study was an *rsbU* mutant that was deficient in σ^B expression, we have examined the possibility that this may account for strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts.

Complementation of the natural *rsbU* mutant BB255 with a functional *rsbU* gene resulted in the expression of significant levels of σ^B activity and increased the capacity of this strain to be internalized by osteoblasts by twofold. Deletion of the entire σ^B operon in BB255 resulted in no expression of σ^B activity and had little effect on the capacity of this strain to be internalized by osteoblasts. On the other hand, an isogenic strain with a point mutation in *rsbW*, which resulted in the expression of very high levels of σ^B activity, had a significantly increased capacity (5- to 10-fold) to be internalized by osteoblasts compared to that of the parental strain. Upon entry into the osteoblast *S. aureus* is found within intracellular vacuoles, as well as free within the cytosol. If the intracellular vacuoles containing *S. aureus* fuse with the lysosomal system, then the bacteria may be subject to environmental stresses such as low pH. Since the alternative sigma factor, σ^B, regulates the expression of general stress response genes (11, 12), it might affect the ability of *S. aureus* to survive and/or grow within the intracellular environment of the osteoblast. If this were the case, the numbers of staphylococci recovered from within osteoblasts incubated with isogenic strains producing high levels of σ^B activity would not be a reflection of the capacity of these bacteria to be internalized by osteoblasts. However, our studies show that the levels of σ^B expressed by isogenic mutants do not affect the ability of *S. aureus* to survive and/or grow within the intracellular environment of the osteoblasts. These data taken together clearly demonstrate that the level of σ^B activity expressed by *S. aureus* affects the capacity of this bacterium to be internalized by osteoblasts.

Given that the level of expression of σ^B activity in *S. aureus* affects the capacity of this bacterium to be internalized by osteoblasts, it could account for strain-dependent differences in this capacity. Our previous studies and data presented herein show that *S. aureus* strain LS-1 has a much higher capacity (7- to 10-fold) to be internalized by osteoblasts than do the NCTC8325 derivatives 8325-4 (1) and BB255. *S. aureus* strain LS-1 also produces significant amounts of σ^B activity in comparison to the NCTC8325 derivatives. In fact the levels of σ^B produced by LS-1 are equivalent to those produced by BB255 complemented with an intact *rsbU* gene as in GP268. However LS-1 has a three- to fourfold-higher capacity to be internalized by osteoblasts than does strain GP268. Deletion of the σ^B operon in LS-1 resulted in a complete loss of σ^B activity but only halved its capacity to be internalized by osteoblasts. These data demonstrate that, while σ^B activity affects the capacity of *S. aureus* to be internalized by osteoblasts, it cannot fully account for the strain-dependent differences that have previously been reported (1). Thus, other as-yet-undefined factors must also play a role in the strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts. In the present study we have also shown that the σ^B operon affects the level of expression of two microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), *fnbA* and *clfA*. Furthermore we have found that the capacities of *S. aureus* strains to bind to fibronectin correlate with their σ^B activities. We have previously reported that at least two MSCRAMMs, the FnBPs A and B, play an important role in the process of internalization of *S. aureus* by osteoblasts. This suggests that one possible mechanism by which the σ^B regulon may influence the internalization of *S. aureus* by osteoblasts is through alteration in the level of expression of MSCRAMMs.

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