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Role of Host Factors in the Regulation of the Enterotoxin B Gene

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The levels of staphylococcal enterotoxin B (SEB) produced by various naturally occurring toxinogenic strains of *Staphylococcus aureus* are highly variable. The SEB gene (*seb*) from a high-producer strain, S6, has previously been cloned and characterized. Cloning and nucleotide sequence analysis of the upstream region of the *seb* gene from DU4916 and COL (medium- and low-level toxin-producer strains, respectively) showed that their sequence was identical to that of the *seb* gene from strain S6. Strains carrying the cloned *seb* gene from DU4916 and COL produced similar levels of SEB protein and mRNA to those produced by strains carrying the cloned *seb* gene from strain S6. An RNA encoded by the δ-lysin gene (*hld*) has been shown to regulate the genes for a number of extracellular proteins, including SEB. Northern (RNA) blot analysis showed that variable levels of *hld* RNA were present in various SEB-producer strains, with the order being S6 > DU4916 > COL. Our results suggest that differences in host factor(s), including the *hld* RNA, are responsible for the production of different amounts of SEB by many naturally occurring strains.

The genes encoding several staphylococcal enterotoxins have been cloned and characterized (1–3, 6, 12, 17). Staphylococcal enterotoxin B (SEB) consists of a single polypeptide of 239 amino acids and has a molecular weight of 28,336 (9, 12). The naturally occurring SEB-producer (Seb^+) strains S6, DU4916, and COL excrete approximately 375, 50, and 12 μg of SEB per ml, respectively, into the culture media (20, 21). The SEB gene (*seb*) has been previously cloned from the high Seb^+ strain S6 (17). The promoter sequence of the *seb* gene has been identified by nuclease S1 mapping and by comparison with the promoter consensus sequences of *Escherichia coli* (8). An upstream element located between nucleotides 58 and 93 upstream of the transcription start site has been shown to be required for the transcription of the *seb* gene (12, 13). Southern hybridization experiments have shown that all the Seb^+ strains tested carry a single copy of the *seb* gene (11). Northern (RNA) hybridization analysis of SEB mRNA from a number of naturally occurring Seb^+ strains has shown that the *seb* gene is regulated at the level of transcription or mRNA stability (8). A polycistronic locus, termed the accessory gene regulator (*agr*), coordinately regulates the synthesis of a number of extracellular proteins in *Staphylococcus aureus*, including SEB (14, 16, 18). Although the synthesis of several exoproteins is decreased in *agr* mutants, the production of some exoproteins such as protein A and coagulase is increased in these strains (10, 15, 17). The activator of the *agr* system has been shown to be the δ-lysin transcript (10). In this paper we demonstrate that host factors in the parental strains, including *hld* RNA, rather than differences in upstream sequences are responsible for the differential expression of the *seb* gene in various strains.

Cloning of the *seb* gene from *S. aureus* DU4916 and COL. The *seb* gene from strains DU4916 and COL was cloned by using procedures similar to those described earlier (17). Clones carrying plasmids similar in size to pSK161 (pC194 carrying the cloned *seb* gene from strain S6) were checked for SEB production by immunodiffusion analysis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Strains RN4220(pSK239) and RN4220(pSK323) contained the cloned *seb* gene from DU4916 and COL, respectively. The restriction maps of the pSK239 and pSK323 plasmids were identical to that of pSK161 (data not shown).

We have previously identified the promoter sequence of the *seb* gene and shown that a 93-nucleotide region upstream of the transcription initiation site is both necessary and sufficient for the transcription of this gene (13). The DNA sequence of the upstream region of the cloned *seb* gene from strains DU4916 and COL was determined by the dideoxy method (19) up to position −488 and was found to be identical to that of the *seb* gene from strain S6 (12; data not shown). These results show that the synthesis of variable levels of SEB by these strains is not due to differences in the *cis*-acting sequences of the *seb* gene.

Analysis of SEB and SEB mRNA levels in agr^+ and *agr* mutant strains carrying the cloned *seb* gene from DU4916 and COL. Experiments were carried out to determine the influence of different host backgrounds on the synthesis of SEB. For this purpose, plasmids pSK239 and pSK323, carrying the cloned *seb* gene from strains DU4916 and COL, respectively, were introduced into the wild-type agr^+ strain ISP479 and the *agr* mutant strain ISP546 by electroporation (4, 7). For electroporation, overnight cultures of *S. aureus* strains were grown in CY-GP broth (14a). The cells were washed and resuspended in 7 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–272 mM sucrose to a final concentration of approximately 7×10^9 CFU/ml. The Bio-Rad Gene Pulser was used for electroporation. The voltage was set to 2.5 kV, the capacitance to 25 μF, and the pulse controller to 400 Ω. For transformation, 160-μl aliquots of frozen cells (about 1×10^9 to 1.5×10^9 cells) were thawed on ice, and 1 ng to 1 μg of the plasmid DNA was added. The DNA-cell mixtures were pulsed at the above settings in 0.2-cm electroporation cuvettes. This produced a field strength of 12.5 kV/cm and pulses with time constants of 5 to 6.5 ms. Transformed cells were plated on GL agar medium (14a) containing 5 μg of the appropriate antibiotic per ml with or without a 1-h recovery period at 37 or 32°C with shaking. The electroporation conditions described

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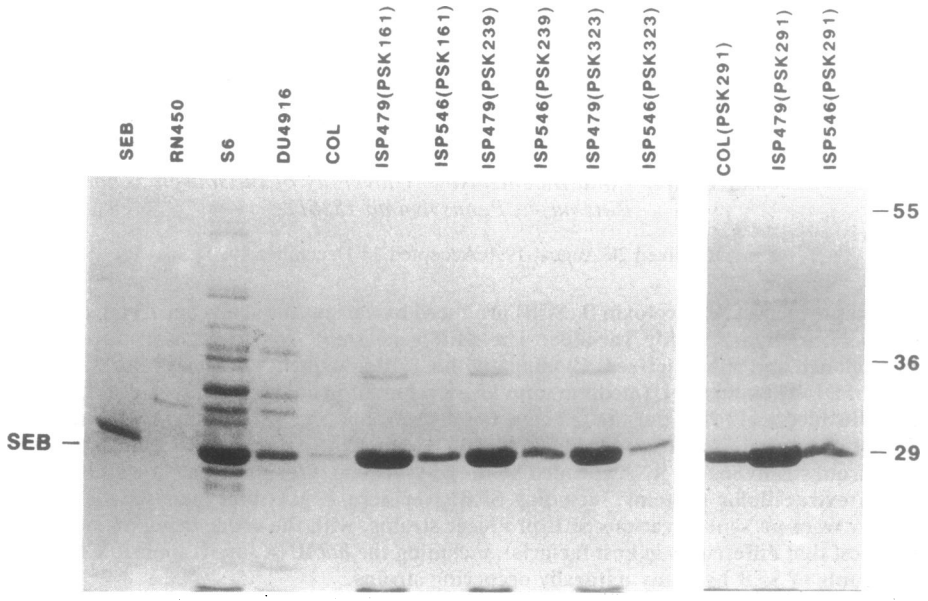

FIG. 1. SDS-polyacrylamide gel analysis of SEB levels in various strains. The culture supernatants were normalized, and 40-μl samples were run on 10% polyacrylamide gels (8). The SEB band is indicated. The positions of the protein markers are shown in kilodaltons.

above resulted in the killing of approximately 95 to 97% of the cells. The transformation efficiencies obtained with various plasmids were about 10^4 to 10^5 CFU/μg, which is about an order of magnitude better than those obtained by the protoplast transformation technique. The cells can be frozen at −70°C for several weeks with only a slight decrease in the transformation efficiencies (not shown).

The levels of SEB produced by various strains were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (8). For the production of SEB protein and mRNA, *S. aureus* strains were grown in 4% NZ-Amine-A (enzymatic digest of casein; Sheffield Products) supplemented with 1% yeast extract at 32°C for 24 h in the presence of 5 μg of chloramphenicol or tetracycline per ml when required. The culture supernatants were used as the source of SEB (11). The proteins were stained with Coomassie brilliant blue, and the relative amounts of SEB were determined by densitometry. Analysis of strains ISP479(pSK161), ISP479(pSK239), and ISP479(pSK323) showed that they produced comparable levels of SEB (Fig. 1; Table 1). In addition, ISP546 derivatives carrying the pSK161, pSK239, and pSK323 plasmids produced similar levels of SEB. The derivatives of the *agr* mutant ISP546 produced about four- to sixfold-lower levels of SEB than their agr^+ counterparts did. Although ISP546 derivatives carrying the cloned *seb* gene from DU4916 and COL produced slightly lower levels of SEB and SEB mRNA than did the ISP546 derivative carrying the *seb* gene from S6, these differences were insignificant compared with the differences in the parental strains (Table 1). These data support the hypothesis that the production of different levels of SEB by strains S6, DU4916, and COL carrying a single copy of the *seb* gene is due to the presence of different levels of host factor(s) required for SEB synthesis. However, as discussed below, it is possible that the high copy number of the *seb* gene in strains ISP479 and ISP546 bypasses some of the normal regulatory mechanisms involved in SEB synthesis.

In another study, the pSK291 plasmid carrying the *seb* gene from S6 (12) was introduced into strain COL by electroporation. SDS-polyacrylamide gel electrophoresis of strain COL(pSK291) showed that it produced about twofold-higher levels of SEB than strain ISP546(pSK291) and about twofold lower levels of SEB than ISP479(pSK291) (Fig. 1; Table 1). The synthesis of about 10-fold-higher levels of SEB by strain COL(pSK291) (carrying multiple copies of the *seb* gene from strain S6 as well as an additional chromosomal copy) than by strain COL (Table 1) suggests that the production of low levels of SEB by COL is not due to a secretion defect in this strain.

Northern blot hybridizations were carried out to determine the levels of SEB mRNA in various strains. Whole-cell

TABLE 1. Relative SEB protein and mRNA levels in various *S. aureus* strains

Strain	Relative level of:	
	SEB[a]	SEB mRNA[b]
S6	1.0	1.0
DU4916	0.29	0.17
COL	0.07	0.09
ISP479(pSK161)	0.88	3.14
ISP546(pSK161)	0.24	1.01
ISP479(pSK239)	0.82	2.96
ISP546(pSK239)	0.17	0.69
ISP479(pSK323)	0.75	2.07
ISP546(pSK323)	0.13	0.48
COL(pSK291)	0.80[c]	ND[d]
ISP479(pSK291)	1.52[c]	ND
ISP546(pSK291)	0.49[c]	ND

[a] These values were obtained by densitometric analysis of two separate Coomassie brilliant blue-stained SDS-polyacrylamide gels. The level of SEB produced by strain S6 was arbitrarily assigned a value of 1.0, and the values for the other strains are indicated with reference to it.

[b] These values were obtained by densitometric analysis of the autoradiograms as well as by scintillation counting of the filter strips. The level of SEB mRNA produced by strain S6 was arbitrarily assigned a value of 1.0, and the values for the other strains are indicated with reference to it. The experiments were carried out in duplicate. Although the results given were obtained with 5 μg of total RNA from each strain, similar values were obtained when 1, 2, or 10 μg of RNA was used (data not shown).

[c] These values were obtained with reference to S6 in a separate experiment.

[d] ND, Not determined.

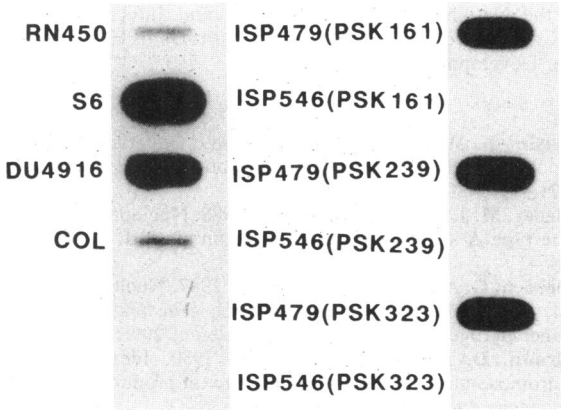

FIG. 2. Slot blot analysis of the *hld* mRNA in various strains. RNA samples (5 μg) were blotted onto nitrocellulose and hybridized to a *hld*-specific probe.

RNA was isolated by treatment of *S. aureus* cells with lysostaphin, followed by guanidinium thiocyanate-phenol-chloroform extraction (5). Hybridizations were carried out by blotting 5 μg of RNA samples onto nitrocellulose filters by using the Schleicher & Schuell Slot-Blot apparatus. The filters were hybridized to an RNA probe (1×10^7 cpm) that was complementary to the SEB transcript as described earlier (8). The relative levels of SEB mRNA were determined by performing densitometry of the autoradiograms as well as by counting the radioactivity in filter strips (8). The levels of SEB mRNA present in the agr^+ strains ISP479 (pSK161), ISP479(pSK239), and ISP479(pSK323) were comparable (Table 1). The levels of SEB mRNA present in the *agr* mutants ISP546(pSK161), ISP546(pSK239), and ISP546 (pSK323) were also similar (Table 1). Northern blot analysis of SEB mRNA on agarose gels showed that the size of the SEB transcript in the above strains was the same (data not shown). This indicated that the cloned *seb* gene was being transcribed from its own promoter in various host strains. The levels of SEB mRNA present in the agr^+ strains were about fourfold higher than those present in the *agr* mutants (Table 1). These results show that the cloned *seb* gene from strains S6, DU4916, and COL directs the synthesis of similar levels of SEB mRNA when introduced into identical host backgrounds.

Determination of the levels of *hld* mRNA in Seb$^+$ strains. To examine the relationship between *hld* transcription and SEB production, we determined the levels of *hld* mRNA in various Seb$^+$ strains. A *hld*-specific oligonucleotide probe having the sequence 5′-dGGTTATTAAGTTGGGATGGC-3′, which is complementary to a region internal to the *hld* mRNA, was used (10). This probe was 5′ end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase to a specific activity of 2×10^9 cpm/μg. Hybridizations with 5 μg of total RNA and 1×10^7 cpm of the oligonucleotide probe were carried out overnight at 50°C in 6× SET buffer (17). Filters were washed with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS four times at 37°C for 15 min each and once at 50°C for 1 min. The relative levels of *hld* mRNA were determined as described above for SEB mRNA. Strain RN450, which is exoprotein deficient, produced very low levels of *hld* mRNA (Fig. 2). The levels of *hld* transcript in strains S6, DU4916, and COL (Fig. 2) paralleled the expression of the *seb* gene, although this correlation was not absolute. For example, although strain S6 produced about 50 times more *hld* mRNA than strain COL, it synthesized only about 12-fold more SEB mRNA than COL. Similarly, although strain DU4916 produced about 20-fold-higher levels of *hld* mRNA than strain COL, it synthesized only about 2-fold-higher levels of SEB mRNA than COL. Since the level of *hld* mRNA in strain COL is very low, the increased production of SEB by strain COL(pSK291) carrying multiple copies of the *seb* gene (Table 1) is likely to be due to an *agr*-independent mechanism. Similarly, the *agr* mutants carrying the cloned *seb* gene synthesized significant quantities of SEB and SEB mRNA, even though the *hld* gene was not detectably expressed in these strains (Table 1; Fig. 2). These results show that SEB synthesis in strains carrying multiple copies of the *seb* gene can bypass regulation by *agr* to a significant extent. These results are in contrast to those obtained for toxic shock syndrome toxin-1, which show that little or no toxin is produced by *agr* mutants carrying multiple copies of the *tsst-1* gene (18). The strains carrying multiple copies of the *seb* gene produce relatively higher levels of SEB mRNA than SEB (Table 1). Recent results in our laboratory with strains ISP479(pSK161) and ISP546(pSK161) carrying multiple copies of the *seb* gene have shown that the *agr* system may regulate the *seb* gene at the level of mRNA stability (unpublished data).

Preliminary experiments were carried out to determine whether the levels of *hld* mRNA in strains S6, DU4916, and COL were correlated with the levels of other extracellular proteins. Two proteins, alpha-toxin, which is positively regulated by the *agr* system, and coagulase, which is negatively regulated by the *agr* system (10, 16, 18), were studied. The coagulase activity was determined by mixing 1-ml aliquots of fivefold-concentrated, undiluted, and diluted culture supernatants with 0.25 ml of rabbit plasma as described previously (15). Whereas 1:50 dilutions of COL and RN450 culture supernatants showed coagulase activity, fivefold-concentrated cultures of S6 and DU4916 gave negative results (data not shown). These results showed that strains COL and RN450, which contain relatively low levels of *hld* mRNA, have at least 250-fold-higher levels of coagulase activity than strains S6 and DU4916, which produce high levels of *hld* mRNA. The relative levels of alpha-toxin produced by various strains were determined by incubating 0.5 ml of undiluted or diluted culture supernatants with 0.5 ml of 4% whole rabbit blood as described previously (16). Strains S6 was found to have high levels of α-hemolysin (50 U/ml), whereas strains DU4916, RN450, and COL contained 4-, 20-, and 33-fold-lower levels, respectively, than S6 (data not shown). These results suggest that the synthesis of alpha-toxin and coagulase may also be dependent upon the levels of *hld* expression in various strains. However, the possibility of additional regulatory mechanisms involved in the production of these exoproteins cannot be ruled out.

The mechanism of action of *hld* mRNA in the activation of the genes for extracellular proteins is currently not understood. Since the nucleotide sequence of the upstream region is different for several of these genes (10, 16), this RNA is unlikely to act simply by an antisense mechanism involving transcription termination or translation inhibition. Such a mechanism is also unlikely since the *hld* transcript has both a positive and a negative effect on the expression of various genes. It is possible that the *hld* transcript indirectly regulates the synthesis of extracellular proteins by its effect on an as yet unidentified regulatory gene. We have recently shown that deletion of a region between nucleotides 58 and 93 upstream of the transcription start site of the *seb* gene totally

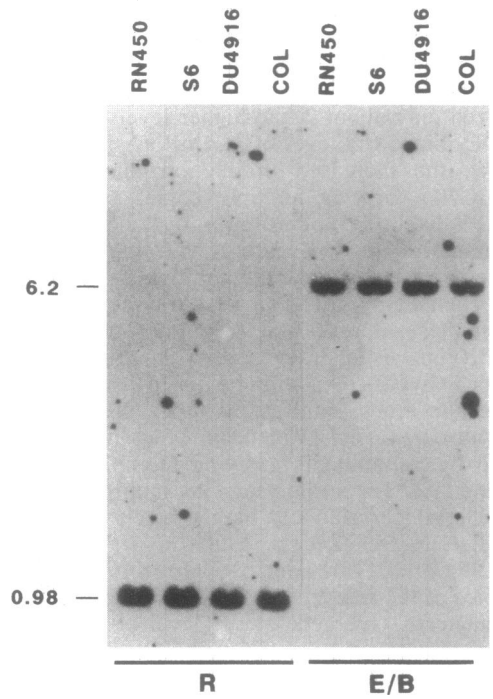

FIG. 3. Southern blot analysis of the *hld* gene in various Seb$^+$ strains. Chromosomal DNA from the indicated strain was digested with *Eco*RI plus *Bgl*II (E/B) or with *Eco*RV (R). The sizes of the bands are indicated in kilobases.

abolishes transcription of this gene (16). A cellular protein that was shown to bind to this region may activate transcription of the *seb* gene (13).

Southern hybridization analysis of the *hld* gene. To determine whether the differences in the levels of *hld* transcript in various strains were due to differences in the copy number of the *hld* gene present in these strains, we carried out Southern hybridization experiments. The restriction map of the *agr* region, including the *hld* gene, has been reported (10). Chromosomal DNA (10 μg) from various strains was double digested with *Eco*RI and *Bgl*II. Since these enzymes do not cleave within the *agr* locus, the presence of multiple chromosomal copies of the *agr* region will yield multiple bands. However, if a single copy of the *agr* gene is present at its normal location, the oligonucleotide probe is expected to hybridize to an approximately 6.2-kb band (10). The DNA was also digested with *Eco*RV, which cleaves at multiple sites within the *agr* region, and the probe is expected to hybridize to a 0.98-kb band (10). The DNA digests were electrophoresed on a 1% agarose gel, transferred to GeneScreen, and hybridized to the *hld*-specific probe as described above. The probe hybridized to a single 6.2-kb *Eco*RI-*Bgl*II fragment and a 0.98-kb *Eco*RV fragment in all the strains tested (Fig. 3). These results showed that a single copy of the *agr* locus is present in all the strains tested at the same chromosomal locus and that the variation in the levels of *hld* mRNA is not due to differences in the copy number or location of this gene.

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