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Enterotoxin B Synthesis by Replicating and Nonreplicating Cells of *Staphylococcus aureus*

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Although 95% of the enterotoxin B produced by *Staphylococcus aureus* appears during the latter part of the exponential phase of growth, growth per se is not necessary for toxin synthesis. A procedure is described whereby a concentrated suspension (at least 6×10^{10} cells per ml) of a 16-hr culture of *S. aureus* was found to be capable of producing toxin, without replication, when air and glucose were present. This technique allows the growth requirement to be separated from toxin formation. Although higher (100 μg/ml) concentrations of toxin appeared in the medium when nitrogen was present, lower levels (30 μg/ml) were produced in the absence of N-Z-amine A. Toxin production proceeded without any net increase in deoxyribonucleic acid, ribonucleic acid, or protein. Chloramphenicol did not inhibit toxin formation in a nitrogen-free medium. The optimal *p*H for toxin production in a nitrogen-free medium was 8.0 to 8.5; for synthesis in a medium where nitrogen was available, the optimal *p*H was 7.0 to 7.5. Increasing the rate of aeration increased toxin release during growth, but decreased the amount of toxin subsequently produced when the bacteria were resuspended. These results suggest the presence of a precursor pool in the cells collected after 16 hr of growth.

Enterotoxin B, which is produced by several strains of *Staphylococcus aureus*, has been the subject of many investigations (2). Previous studies have been concerned with the preparation, identification, and assay of enterotoxin B, but the physiological factors which influence its synthesis are still poorly understood.

Since enterotoxin B formation can be inhibited in growing cultures (4, 5, 12) by concentrations of streptomycin, penicillin, and D-cycloserine which have only a slight effect on growth, there does not appear to be a direct correlation between cell growth per se and toxin formation. Friedman (5) suggested that the cell surface may contain sites involved in the synthesis of enterotoxin B. MacLean et al. (9) found that enterotoxin B production, but not cell growth, could be inhibited by growing the bacteria either at a low temperature or in a high salt medium. They showed that maximal toxin production by *S. aureus* ATCC 14458 occurs at the beginning of the stationary phase of growth; therefore, they suggested that entertoxin B, like many antibiotics, might be a secondary metabolite.

In a preliminary communication (10), we reported that nonreplicating cells of *S. aureus* can produce toxin without an exogenous nitrogen source in a medium containing only glucose and K_2HPO_4. The toxin is excreted even in the presence of chloramphenicol, thus suggesting the existence of a toxin precursor pool in the cell.

In this report, we present the results of our studies of the kinetics of enterotoxin B production by *S. aureus* S-6, as well as our observations of the influence of *p*H, temperature, aeration, and other factors on nonreplicating cells. This investigation provides further evidence for the presence of a toxin precursor pool in cells harvested at the later stages of growth.

MATERIALS AND METHODS

Media. Medium 1, as described by Rosenwald et al. (12), contained 4.0% N-Z-amine type A (Sheffield Chemical, Norwich, N.Y.), 0.4% yeast extract (Difco), and 0.1% K_2HPO_4 in distilled water.

Medium 2 contained 4.0% N-Z-amine type A and 0.1% K_2HPO_4.

Medium 3 contained 0.5% glucose and 3.5% K_2HPO_4. This medium was considered the nitrogen-free medium.

Culture propagation. Stock cultures of *S. aureus* S-6 were maintained on slants of Trypticase Soy

Agar (BBL) plus 0.5% yeast extract (Difco) and were transferred every 3 months. Inoculated slants were incubated for 24 hr at 37 C and then were stored at 4 C. For each experiment, a loopful of bacteria from the stock culture slant was transferred to 100 ml of medium 1 in a 500-ml shake flask and was incubated for 16 to 20 hr. A 1-ml amount of this culture was then used to inoculate 100 ml of fresh broth (medium 1, 500-ml flask). After further incubation for 16 to 20 hr, the bacteria were harvested by centrifugation at 9,000 × *g* for 10 min, and the supernatant fluid was stored at 4 C until analyzed.

Except where noted, bacterial incubations for shake cultures were carried out at 37 C on a rotary-action shaker, model V (New Brunswick Scientific Co., New Brunswick, N.J.), at 240 rev/min.

Toxin synthesis by nonreplicating cells. The harvested cells were washed twice in 0.01 M phosphate buffer (*p*H 7.0) and were diluted to 6×10^{10} to 8×10^{10} organisms/ml (5,000 to 6,000 Klett units) in either medium 2 or medium 3. Standardization of cell suspensions was accomplished with a Klett-Summerson colorimeter using a 660-nm filter. Amounts of 10 ml of suspension were shaken at 240 rev/min in 100-ml flasks at 37 C. In these experiments, it was not necessary to employ a strictly aseptic technique.

Assay methods. *S. aureus* was quantitated by the pour plate method in Trypticase Soy Agar (BBL). Serial dilutions were made with 0.1% peptone in distilled water (*p*H 7.0). Incubation was at 37 C for 24 hr.

Washed bacterial suspensions were analyzed for their protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) content according to the methods described by Hirai et al. (6), by use of a Bausch & Lomb Precision Spectrophotometer. Protein released into the media was determined according to the method of Lowry et al. (8). Since certain of the constituents of N-Z-amine type A also reacted with the Lowry reagents, only protein precipitated by 5% trichloroacetic acid was measured.

The toxin was assayed by the single-diffusion technique of Weirether et al. (13). Purified enterotoxin B and rabbit antiserum B were used. For assay of the supernatant fluids of medium 1 and medium 2, appropriate dilutions of the samples were prepared in phosphate-buffered saline plus 2% NaCl to eliminate variations in titer due to differences in ionic strengths. Medium 3, containing 3.5% K_2HPO_4, was not adjusted further. For corresponding standard calibration curves, 3.5% K_2HPO_4 or 2% NaCl in phosphate-buffered saline was employed.

Chloramphenicol-treated cells. Bacteria propagated in medium 1 were sampled at various times during growth. The cell samples were washed twice in 0.01 M phosphate buffer (*p*H 7.0) and resuspended at a concentration of 6×10^{10} to 8×10^{10} bacteria/ml in medium 3 containing 100 μg of chloramphenicol per ml (Parke, Davis & Co., Detroit, Mich.). After incubation for 4 hr (240 rev/min) at 37 C, the bacteria were removed by centrifugation and the supernatant fluid was assayed for toxin.

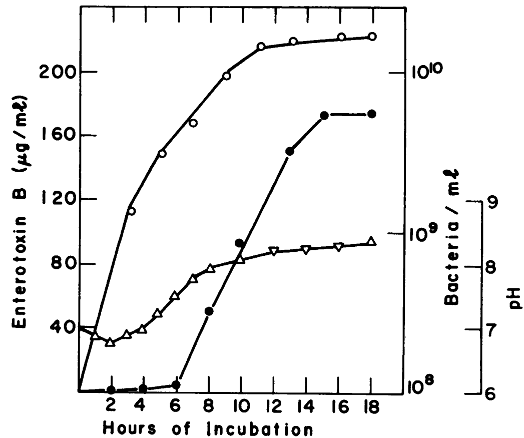

FIG. 1. *Enterotoxin B formation in shake-flask cultures of S. aureus S-6. Symbols:* ○, *bacteria;* ●, *enterotoxin B;* △, *pH.*

RESULTS

Kinetics of cell growth and toxin appearance. As can be seen in Fig. 1, 95% of the toxin produced by *S. aureus* was excreted during the late exponential and early stationary phases of growth. In agreement with the results of Friedman (4), we detected no toxin in sonic extracts prepared from bacteria obtained from either the exponential or stationary phase. This lag in toxin production could not be eliminated by propagating cells in the supernatant fluid obtained from a 4.5-hr culture, i.e., in media taken from cultures just prior to optimal toxin production. The relationship between toxin production and the phase of bacterial growth was further examined in the following experiment. Bacteria in the stationary phase of growth (propagated for 18 hr) were washed, and one-tenth of the bacterial mass was resuspended and incubated in the original and "exhausted" supernatant fluid (*p*H 8.4). The beginning of a new growth cycle was observed, but practically no additional toxin was produced. If the *p*H of the supernatant fluid was readjusted to 7.0, a normal growth cycle occurred, accompanied by the appearance of an additional 80 μg of enterotoxin B per ml. The initial lag and the pattern of toxin appearance were similar to those described in Fig. 1.

Toxin production by nongrowing cultures. A comparison was made between the ability of replicating (Fig. 2) and nonreplicating (Fig. 3) cells to produce toxin. The nonreplicating cells initiated toxin production without any delay and without any change in bacterial concentration or in the DNA, RNA, and protein content. The final concentration of toxin after 4 hr was 100

μg/ml. In contrast, in the first 4 hr the replicating cells produced a small proportion of the total amount of toxin they excreted during the entire growth cycle, in spite of significant increases in DNA, RNA, protein, and cell turbidity. The potential of nongrowing cells to produce toxin was further investigated with cells collected from other stages of growth (Table 1). There was a correlation between the stage of growth of the cells and the amount of toxin produced. Significant amounts of toxin were produced only by cells that had been grown for 4 hr or more. Without a nitrogen source (medium 3), significant amounts of toxin could still be produced, but only from a 16-hr culture. This toxin appeared only in the first 4 hr of incubation (Fig. 4), indicating that the toxin precursor pool was limited even though overall protein excretion continued to increase. The relationship between the concentration of resuspended cells and the amount of enterotoxin B produced was also

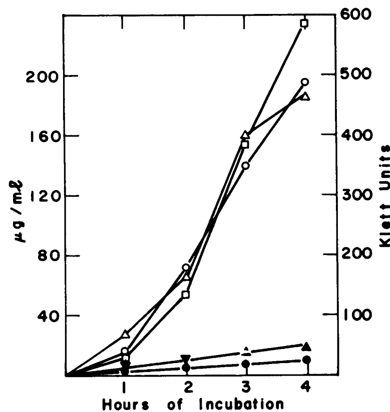

FIG. 2. *Enterotoxin B, DNA, RNA, and protein formation by replicating cells of S. aureus S-6. Symbols:* ○, *protein;* ●, *enterotoxin B;* ▲, *DNA;* △, *RNA;* □, *Klett units.*

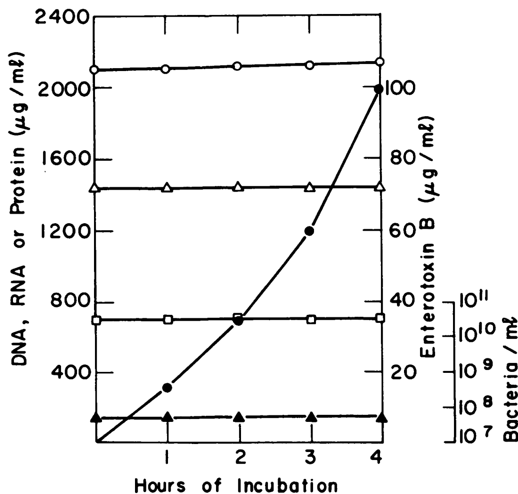

FIG. 3. *Enterotoxin B, DNA, RNA, and protein formation by nonreplicating cells of S. aureus S-6. Symbols:* ○, *protein;* ●, *enterotoxin B;* ▲, *DNA;* △, *RNA;* □, *bacteria.*

TABLE 1. *Toxin production by nongrowing resuspended cells*[a]

Age of resuspended culture	Toxin produced	
	Medium 2	Medium 3[b]
hr	*μg/ml*	*μg/ml*
2	15	0
4	50	2
6	70	4
16	120	30
16[c]	35	—

[a] At the time intervals specified, cells were collected, washed, resuspended in medium 2 or 3 at a concentration of 5,000 to 6,000 Klett units as designated for nonreplicating cells, and incubated for 4 hr.

[b] No difference in toxin synthesis was found when the cells were resuspended in the presence of 100 μg of chloramphenicol per ml.

[c] With 100 μg of chloramphenicol per ml.

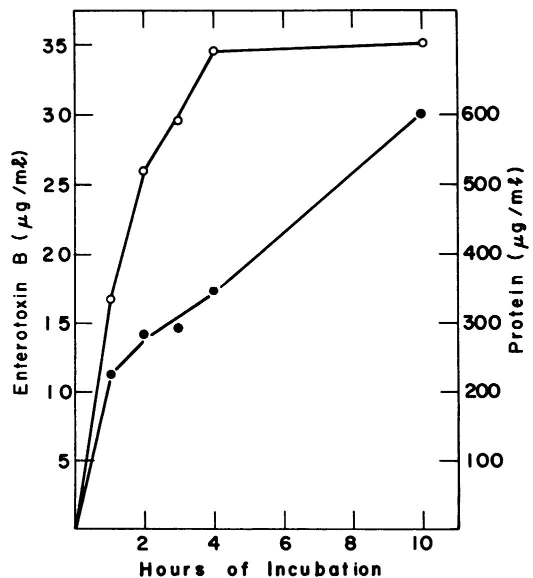

FIG. 4. *Enterotoxin B formation in a nitrogen-free medium by nonreplicating cells of S. aureus S-6. Bacteria incubated in medium 3. Symbols:* ○, *toxin;* ●, *protein.*

investigated (Table 2). This experiment emphasizes the necessity of using a bacterial concentration in the range of 3×10^{10} to 6×10^{10} cells/ml to obtain significant toxin release.

The influence of glucose concentration on toxin production is summarized in Table 3. It is apparent that higher glucose concentrations inhibited toxin synthesis but had no quantitative effect on the level of protein excretion into medium 3. The *p*H for maximal toxin formation depended on the composition of the menstruum (Table 4). Maximal toxin production occurred in the *p*H range of 7.0 to 7.5 for medium 2 and 8.0 to 8.5 for medium 3. Although the *p*H in these experiments was not strictly controlled, the changes after incubation were found to be small, owing to the strong phosphate buffer present in the media.

We previously reported that several metabolic inhibitors, such as chloramphenicol, penicillin, and streptomycin, which are known to interfere with toxin production in growing cells, do not prevent enterotoxin B formation by nongrowing cells suspended in medium 3 (10). Chloramphenicol (100 μg/ml), an inhibitor of protein synthesis, was added to cells collected during the logarithmic stages of growth. No significant effect on either the rate or the total quantity of toxin produced by resuspended, nonreplicating cells could be detected in medium 3, which was devoid of nitrogen (Table 1). With nitrogen present, the amount of toxin produced in the presence of chloramphenicol was appreciably decreased.

When cells grown at 20 C for 72 hr were resuspended at 37 C in medium 2, a small amount of toxin was produced by this nonreplicating culture (Fig. 5). Both the rate of toxin release and the amount accumulated after 4 hr were much lower for bacteria initially propagated at 20 C.

TABLE 2. *Influence of cell concentration on enterotoxin B production*[a]

Bacterial concentration	Enterotoxin B
cells/ml	*µg/ml*
7.9×10^{9}	0
1.5×10^{10}	3
3×10^{10}	15
6×10^{10}	28

[a] Cells from the stationary phase of growth were resuspended in medium 3 by the procedure described in Fig. 1.

TABLE 3. *Influence of glucose concentration on enterotoxin B production*[a]

Glucose concn	Enterotoxin B	Protein
%	*µg/ml*	*µg/ml*
0.1	15	510
0.5	20	510
1.0	10	750
2.0	4	860

[a] Cells from the stationary phase of growth were resuspended in medium 3 by the procedure described in Fig. 1.

TABLE 4. *Effect of initial pH on enterotoxin B formation*[a]

*p*H	Toxin produced	
	Medium 2[b]	Medium 3
	µg/ml	*µg/ml*
5.5	4	4
6.5	17	8
7.0	100	10
8.0	35	13
8.5	17	20
9.0	2	10

[a] Both media contained 0.2 M K_2HPO_4 and were adjusted initially to the appropriate *p*H with 0.5 M HCl or NaOH. Bacteria from the stationary phase of growth were then resuspended in the designated media by the procedure described in Fig. 1.

[b] The K_2HPO_4 concentration of medium 2 was adjusted to 0.2 M.

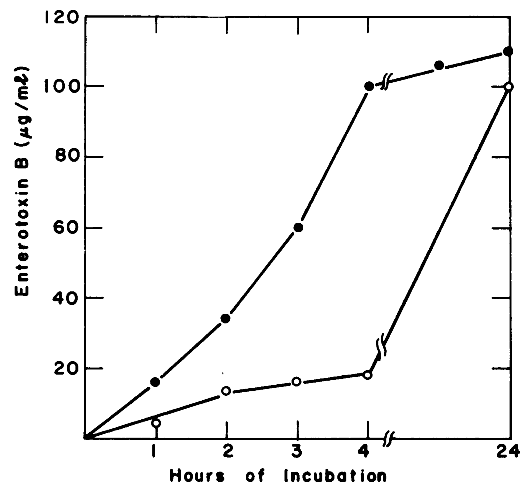

FIG. 5. *Effect of propagation temperature on enterotoxin B formation by nonreplicating cells of S. aureus S-6. Bacteria propagated at either 20 C or 37 C were resuspended in medium 2 and incubated at 37 C. Cells propagated at 20 C and resuspended at 37 C in the presence of chloramphenicol (100 µg/ml) excreted no toxin in 4 hr and only 3 µg/ml in 24 hr. Symbols:* O, *cells propagated at 20 C;* ●, *cells propagated at 37 C.*

However, if these bacteria were incubated overnight, they then produced significantly greater amounts of toxin. In the presence of chloramphenicol (100 μg/ml), these cells excreted no toxin in 4 hr and only 3 μg/ml in 24 hr.

Bacteria propagated at 37 C for 16 hr produced 2 μg of toxin per ml when resuspended for 4 hr at 20 C in medium 3. This represents only 10% of the toxin normally obtained when *S. aureus* was grown and resuspended at 37 C.

The effect of aeration on the pattern of toxin production was also investigated. Cells propagated at various aeration rates were tested for their ability to form toxin after resuspension. As can be seen from Table 5, increasing the rate of aeration increased the amount of toxin produced by the propagating bacteria. However, after resuspension, cells which had been subjected to high aeration rates during propagation subsequently produced the smallest amount of toxin.

DISCUSSION

The kinetics of enterotoxin B production by *S. aureus* S-6 are similar to those described for strain ATCC 14458 (9). In both strains, the capacity for toxin formation appears only at the end of the exponential phase of growth. Toxin production thus occurs in what is presumably a partially exhausted medium. This would suggest that the growing bacteria trigger toxin production by altering their medium environment, either through removal of an inhibitor present in the medium or by an increase of the *p*H. However, the results obtained with strain S-6 inoculated into used medium tend to discount these possibilities. At 4.5 hr, the medium has presumably attained a composition and *p*H suitable for toxin formation, since the rate of toxin release increases greatly 5 to 6 hr after inoculation. However, the lag period for toxin appearance reoccurred when cells were propagated in "used" medium obtained either 4 to 5 hr after inoculation or after the stationary state had been reached and the *p*H had been readjusted to 7.0. This lag apparent in used media would also tend to argue against an extracellular accumulation of toxin precursors capable of being transformed to an active form.

The S-6 strain would not multiply when suspended at a concentration of 6×10^{10} to 8×10^{10} cells/ml in the nitrogen-containing medium. This can presumably be attributed to the absence of yeast extract or to the fact that bacteria will grow only to a specific concentration characteristic of a given species (11), or to a combination of these factors.

Bacteria harvested from the stationary phase of growth formed toxin when resuspended in a nitrogen-free medium, and chloramphenicol had no effect on the amount of enterotoxin B produced. If instead the bacteria were supplied with a nitrogen source, additional toxin was formed; however, chloramphenicol inhibited this net increase. This is a strong indication that cells which are harvested from the stationary phase can contain a toxin precursor pool. This metabolic pool can be converted into toxin without the necessity of de novo protein synthesis.

The existence of a toxin precursor pool in cells entering the stationary phase of growth is further supported by the following experiments. Bacteria harvested during the exponential phase, if forced into the stationary phase by resuspension at a concentration of 6×10^{10} to 8×10^{10} cells/ml, formed toxin. However, the quantity of toxin

TABLE 5. *Effect of aeration on enterotoxin B formation*

Aeration rate during propagation[a]	Toxin concn in the medium	Toxin release/1,000 Klett units[b]			Total toxin synthesis by replicating cells[c]	Net toxin synthesis by nonreplicating cells[d]
		After propagation, medium 1	After resuspension			
			Medium 2	Medium 3		
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
Low	30	35.0	22	3	38	19.0
Medium	135	65.4	24	6.4	71.8	17.6
High	200	100.0	12	0	100.0	12.0

[a] Medium aeration rate was obtained by following the standard propagation procedure as described in Materials and Methods. Low aeration was obtained by decreasing the agitation to 125 rev/min and high aeration by agitating 50 ml instead of 100 ml of medium and covering the flasks with cheese cloth in place of cotton plugs. The propagation time was 24 hr, and in all aeration rates the cells were in the stationary phase of growth.

[b] The resuspension procedure was the same as that described for Fig. 1.

[c] Sum of toxin produced in medium 1 plus medium 3/1,000 Klett units.

[d] Difference of toxin produced in medium 2 minus medium 3/1,000 Klett units.

produced by these resuspended cells was directly related to their stage of growth (Table 1). The increased ability of "older" cells to produce toxin can presumably be due to the accumulation of an appropriate precursor pool.

According to our data, the cells grown under higher aeration rates synthesized the highest toxin concentration in the propagation medium, and the lowest one when maintained in a nonreplicating condition (Table 5). This observation serves as a further support for the theory of a toxin precursor pool. We assume that toxin formed in the nitrogen-free medium represents the precursor pool produced during bacterial growth. This is why total toxin production was calculated by summing toxin produced both during growth and after resuspension in the nitrogen-free medium. Values for net toxin synthesis by nonpropagating cells were also obtained if the existence of a toxin precursor pool was assumed. The relationship between the cell metabolic pool, the toxin precursor pool, and the synthesis of toxin is unknown and is receiving further attention.

It is highly unlikely that the appearance of enterotoxin B in the absence of a nitrogen source (medium 3) is due to the release by the more alkaline *p*H and agitation of toxin attached to the cell surface rather than to a precursor pool. The necessary presence of a fermentable carbohydrate for toxin excretion (little or none is excreted in 0.2 M phosphate buffer, *p*H 8.5) and the inhibition of toxin production by different enzyme poisons (10) demonstrate that enterotoxin B is an integral part of the metabolism of *S. aureus* S-6. The experiment described in Table 5 for a variety of propagating conditions and resuspension menstruums yielded results that cannot be explained as simple desorption phenomena but must be regarded as regulated microbial activities. It seems that in the case of *S. aureus*, as previously described for streptolysin S (3), enterotoxin B appearance "is limited by the rate at which toxin precursor is synthesized and . . . the latter in turn is limited by energy-yielding or other metabolic processes."

In agreement with the results published by MacLean (9) for strain ATCC 14458, little toxin was produced in cultures propagated at 20 C (10 μg/ml). These cells, though, had a delayed potential for greater toxin formation. The bacterial metabolism seemingly incapable of producing toxin in the first 4 hr at 37 C could be redirected so that at the end of 24 hr normal amounts of toxin were formed. Inhibition by chloramphenicol indicates that this is due to newly formed metabolic capabilities.

The *p*H of the medium influences not only the growth rate of *S. aureus* but also toxin synthesis and release. Distinctions in the *p*H required for these mechanisms were not determined in previous studies, but instead the initial *p*H of the medium used for growth and toxin excretion was studied. In this manner, a *p*H of 6.0 to 6.5 was determined to be optimal for enterotoxin B production (7). With the nonreplicating culture technique, a distinction in optimal *p*H can be made between toxin synthesis and release in a nitrogen-rich medium (*p*H 7.0 to 7.5) and in a simple glucose-phosphate medium (*p*H 8.0 to 8.5) in which it is postulated that conversion from a precursor pool and subsequent release occur.

Catabolic repression is perhaps involved in the inhibition of toxin formation by higher glucose concentrations. Such inhibition has also been described for enterotoxin B produced by growing cultures (S. A. Morse, R. A. Mah, and W. J. Dobrogosz, Bacteriol. Proc., p. 3, 1968).

The nonreplicating toxin-producing system described earlier provides a useful tool for examining medium composition, temperature, aeration, and other factors concerned with toxin production. This system thus minimizes factors which inhibit toxin formation primarily by interfering with cell growth.

It was found that ultraviolet mutants of S-6 selected for decreased coagulase production also produced less enterotoxin B (*unpublished data*). This seems to indicate that there is some relation between the synthesis of these seemingly disparate extracellular proteins. It should be noted that, for the majority of *S. aureus* strains described in the literature, enterotoxin producers are strongly coagulase-positive, and, when tested according to the recommended procedure (1), they coagulate plasma in less than 4 hr. Many strains considered coagulase-negative are in reality low coagulase producers. Our laboratory mutants, which are coagulase-negative according to the accepted definition, still produced significant amounts of enterotoxin B. Although they produced less coagulase and toxin, there was no detectable change in the total quantity of proteins excreted. In the wild state, approximately 25% of the protein released by S-6 is accounted for by coagulase and enterotoxin B. Since overall protein release has not changed, it appears possible that the isolates release the toxin in an inactive form. However, the high specificity of the antigen-antibody reaction prevents the detection of the toxin precursor or any otherwise modified toxin protein.

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