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Effect of Shaking Speed on the Secretion of Enterotoxin B by *Staphylococcus aureus*

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The concentration of enterotoxin B secreted by four strains of *Staphylococcus aureus* was dependent upon the shaking speed. For the conditions established, each strain demonstrated an optimal shaking speed, and speeds in excess of the optimum resulted in decreased secretion of toxin. At the optimal shaking speed, maximum secretion occurred at 37 C. At 45 C, both growth and toxin secretion were absent. By using agar containing antienterotoxin B sera, studies with strain S-6 at optimal and suboptimal shaking speeds demonstrated that individual cells vary in their toxin-synthesizing ability and that the relative numbers of high and low producers change during the growth cycle. Although most of the toxin was secreted during the first 12 hr of growth, a portion was secreted during the subsequent 6 hr, even though growth as measured by colony-forming units per milliliter decreased and Klett units increased.

To obtain appreciable enterotoxin B yields, it is necessary to aerate cultures of *Staphylococcus aureus* (2, 5, 10, 12, 15). In practice, a variety of cultural techniques have been used, resulting in different levels of enterotoxin B secretion.

Reiser and Weiss (12) studied the production of enterotoxins A, B, and C, and Kato et al. (3) studied A and B production by shaking 50 ml of medium in a 250-ml Erlenmeyer flask on a gyratory incubator shaker. Markus and Silverman (6, 7; Z. Markus, Bacteriol. Proc., p. 114, 1970) normally used a 500-ml Erlenmeyer flask containing 100 ml of medium shaken at 250 rev/min on a gyratory shaker for studying the secretion of enterotoxins A and B. They also studied, in a limited manner, the influence of three different shaking rates on the secretion of enterotoxin B (6) and found that the highest shaking rate during growth resulted in the greatest secretion. McLean et al. (8), in their investigation of the effects of salt and temperature on enterotoxin B production, used a 1-liter Erlenmeyer flask containing 100 ml of medium on a reciprocating shaker bath at 90 rev/min.

A comparison of the data from the above studies suggests that, although enterotoxin B secretion and cell density are dependent upon agitation, there is no direct correlation among these three factors. The fact that the secretion of appreciable concentrations of enterotoxin B does not appear to be essential for growth but is dependent upon the presence of aeration supports the suggestion of Lockhart and Squires (4) that metabolic events and the genetic nature of the cells in a given population can be governed by this factor. To our knowledge, no publication exists of studies designed to evaluate the relationship of shaking to enterotoxin B secretion. Kato et al (3) studied this effect with strain 100, an enterotoxin A producer. They found that both shaking speed and the ratio of fluid-to-flask volume influenced toxin secretion, and that once maximum toxin secretion was achieved, faster shaking rates were without effect. Preliminary experiments verified their results for enterotoxin A but indicated that secretion by B producers was adversely affected by extremely high shaking rates.

This study was undertaken to investigate the importance of shaking rate in the production of enterotoxin B by several strains of *S. aureus*. The rate of enterotoxin secretion of strains S-6 and 135 at optimal shaking rates was obtained, and the effect of growth temperature at the optimal shaking rate on enterotoxin B secretion by strain S-6 was ascertained. Plates containing antienterotoxin B sera were used to examine cells grown at optimal and suboptimal shaking rates for their potential to secrete enterotoxin, in an attempt to relate this ability to that of the culture under actual growth conditions.

MATERIALS AND METHODS

Organism. *S. aureus* S-6 and 243, prototype strains for enterotoxin B production, were obtained

from M. S. Bergdoll, Food Research Institute, Madison, Wis., and H. D. Lilly, Eastern Utilization R & D Division, USDA, Beltsville, Md., respectively. *S. aureus* S-6R, a nontoxigenic mutant of S-6, was supplied by M.S. Bergdoll. *S. aureus* 295, a low enterotoxin B producer, and 135, a high enterotoxin-producing strain, were obtained from stock-cultures in our laboratory.

Medium. The basal medium used for growth and toxin production was that described by Rosenwald and Lincoln (13) and contained 4% N-Z Amine type A (Sheffield Chemical, Norwich, N.Y.), 0.4% yeast extract (Baltimore Biological Laboratory), and 0.1% K_2HPO_4 in distilled water, *p*H 7.0.

Cultural method. The methods for the preparation of inocula and shake-flask cultures were those described previously (6). For varying aeration rates in shake-flask cultures at 37 C for 18 to 24 hr, a rotary shaker model G2 (New Brunswick Scientific Co., New Brunswick, N.J.) was employed.

Assay procedures. A 5-ml sample of the shake-flask culture was removed at 2-hr intervals. For plate counts, serial dilutions were made, and the culture was measured on prepoured plates of Trypticase soy agar (Difco) containing 0.5% yeast extract (BBL, TSY agar). All plates were incubated at 37 C for 24 hr. Results are expressed as colony-forming units (CFU) per milliliter.

Bacterial density was measured with a Klett-Summerson colorimeter using a 660-nm filter. When necessary, samples were diluted 1:10 in distilled water to obtain readings ranging from 20 to 250 Klett units. All *p*H readings were determined with an Orion digital *p*H meter, model 801 (Orion Research Inc., Cambridge, Mass.).

Isolates were assayed for their egg-yolk reaction on Baird-Parker medium (Difco) and for deoxyribonuclease activity on deoxyribonuclease test agar (Difco).

Samples containing low amounts of enterotoxin were concentrated by a modification of the method of Morse et al. (9). Three milliliters of the supernatant fluid, obtained by harvesting cells at 10,000 rev/min for 10 min, was dialyzed in cellulose tubing for 2 hr at 4 C against tap water for the removal of substances which were found to interfere with the subsequent development of a distinct serological precipitin band. The sample was then dialyzed overnight against 50% polyethylene glycol 20,000 (Fisher Scientific Co., Medford, Mass.) at 4 C and finally against running tap water for 3 to 4 hr at room temperature. The concentrated sample was removed from the dialysis tubing, and the volume was measured and assayed for enterotoxin.

Supernatant fluids were assayed for enterotoxin by the single-gel diffusion technique of Weirether et al. (17). All tubes were incubated at 30 C for 21 hr. Purified enterotoxin B and rabbit antiserum B, supplied by S. J. Silverman, Ft. Detrick, Md., were employed for the preparation of precipitin tubes and standards.

Immunological response on antiserum B agar. Antiserum B agar plates were prepared by the method of Weiss and Robbins (18). One milliliter of rabbit antiserum B, having a standard serum titer of 1:20, was added to 19 ml of their medium per plate to obtain the proper concentration of serum for distinctive zone development. Cultures were spread on prepoured plates and incubated for 24 hr at 37 C and then at room temperature for an additional 48 hr. Colonies were categorized by measuring the diameter of the halo. Four classifications were recognized as follows: zone 1 colonies did not produce a halo, zone 2 colonies produced halos measuring 3.0 to 3.9 mm, zone 3 colonies produced halos measuring 4.0 to 4.9 mm, and zone 4 colonies produced halos measuring greater than 4.9 mm.

Chemicals. Stock solutions of chloramphenicol (Parke, Davis & Co., Detroit, Mich.) were filter sterilized and diluted in sterile distilled water to obtain the desired concentration for addition to the growth medium.

RESULTS

Determination of optimal shaking speeds for enterotoxin B production. At shaking speeds of 250 rev/min or below, toxin secretion by each strain was directly related to final cell turbidity (Table 1). As the shaking speed was increased, an optimum was reached at which enterotoxin B secretion was maximized. Strains which differed greatly in their ability to secrete enterotoxin showed a similar pattern in response to varying rates of shaking. For *S. aureus* strains S-6, 135, and 295, this maximum was found to be 350 rev/min at 37 C, and, for strain 243, it was 375 rev/min. Strain S-6R did not produce enterotoxin B at any shaking speed. Strains 243 and 135 produced small quantities of toxin in the absence of shaking, and no further change was noted in the levels of toxin secretion after 1 week of incubation. No enterotoxin A was detected in any of the supernatant fluids.

Additional experiments utilizing *S. aureus* S-6 were conducted to further characterize the importance of shaking on toxin secretion. Shaking 100 ml of the inoculated basal medium at 250 rev/min in a 1-liter Erlenmeyer flask rather than a 500-ml flask increased the secretion of enterotoxin B from 160 μg/ml to 300 μg/ml. Shaking the medium at 350 rev/min in 500-ml shake flasks covered with cheesecloth allowed a greater gaseous transfer and resulted in the secretion of 100 μg/ml.

Since considerable foaming occurs in flasks shaken at higher speeds, experiments were conducted to determine the effect foaming may have on the secretion of toxin. The addition of 0.2 ml of Antifoam-A (Dow Corning, Midland, Mich.), which eliminated all foaming in cultures shaken at 350 rev/min, resulted in the normal secretion of 300 μg of toxin per ml.

Optimal temperature for enterotoxin production. The optimal temperature for entero-

TABLE 1. *Effect of shaking rate on bacterial density, pH, and enterotoxin secretion by S. aureus strains after 24 hr of incubation at 37 C*

Rev/min	Strain S-6			Strain 243			Strain 135			Strain 295			Strain S-6 R		
	pH	Klett units	Toxin (μg/ml)	pH	Klett units	Toxin (μg/ml)	pH	Klett units	Toxin (μg/ml)	pH	Klett units	Toxin (μg/ml)	pH	Klett units	Toxin (μg/ml)
0	7.0	200	ND[a]	7.7	510	20	7.0	210	10	7.3	270	ND	7.2	205	ND
100	7.5	910	50	8.1	850	35	8.0	660	85	8.1	955	ND	8.1	660	ND
200	7.9	1,255	100	8.1	1,315	75	8.2	1,160	125	8.1	1,470	3	8.1	1,105	ND
250	8.1	1,500	160	8.2	1,760	135	8.2	1,350	180	8.2	1,700	6	8.2	1,370	ND
300	8.4	2,075	190	8.3	1,970	150	8.4	2,220	230	8.3	2,380	8	8.4	1,830	ND
325	8.4	2,090	280	8.5	2,100	180	8.3	1,820	300	8.4	2,400	15			
350	8.6	2,100	320	8.4	2,140	200	8.5	1,800	470	8.5	2,260	20	8.4	1,380	ND
375	8.3	2,000	200	8.4	2,100	325	8.7	1,900	200	8.3	2,110	16			
400	8.4	2,040	140	8.5	2,150	215	8.3	1,500	200	8.3	1,960	10	8.3	1,350	ND

[a] Not detectable.

toxin B secretion by *S. aureus* S-6 shaken at 350 rev/min is 37 C (Table 2). The final pH and enterotoxin secretion showed a progressive increase from 25 to 37 C as the temperature was increased, although a similar progression was not observed in bacterial density. Neither growth nor toxin was detected in cultures incubated at 45 C.

Relation of enterotoxin production to cell growth. When *S. aureus* S-6 was shaken at 250 rev/min, 14 μg of toxin per ml was secreted during the exponential phase of growth (0 to 4 hr), and an additional 69 μg of toxin per ml was produced by 10 hr. The remainder of the toxin secreted, 57 μg, for a total of 140 μg/ml, was secreted during the subsequent stationary phase and phase of declining viable CFU.

Shaking at 350 rev/min (Fig. 1) results in the secretion of 26 μg of toxin per ml during the exponential growth phase (0 to 4 hr) and an additional 164 μg/ml during the next 6 hr. At 16 hr, a total of 300 μg of enterotoxin per ml was secreted. Comparable results were obtained with strain 135, although higher toxin concentrations were secreted by this strain.

The relationship for strains S-6 and 135 between growth, as measured by CFU/ml or Klett units, and enterotoxin B concentration is illustrated in Fig. 2. Toxin secretion was detected at 2 hr, and secretion continued even though CFU/ml decreased after 10 to 12 hr of growth. In contrast, Klett units continued to increase for 16 hr. Microscopic measurement did not reveal any appreciable change in clumping but did indicate the presence of appreciable numbers of dead cells.

The productivity of enterotoxin secretion by *S. aureus* was calculated on the basis of either CFU or Klett units during the growth cycle (Table 3).

TABLE 2. *Effect of growth temperature on enterotoxin B synthesis by S. aureus* S-6[a]

Temp (C)	Final pH	Klett units	Enterotoxin B (μg/ml)
20	7.4	1,140	5
20[b]	8.2	1,250	10
25	8.3	2,460	70
30	8.3	2,400	115
35	8.4	1,980	180
37	8.5	2,100	320
40	8.5	1,930	120
45	7.1	20[c]	ND[d]

[a] Cultures grown for 24 hr at 350 rev/min.
[b] Culture was shaken for 48 hr.
[c] Initial inoculum.
[d] Not detectable.

DISCUSSION

Differences in enterotoxin yields were influenced by shaking rates, flask sizes, the type of flask closure, and ratios of media volume to flask volume (factors which influence interfacial areas for oxygen transfer).

The maximum amount of enterotoxin B secreted by strain S-6 in this study, 320 μg/ml, was comparable to that obtained by Reiser and Weiss (12), although they obtained less enterotoxin with strain 243 (133 μg/ml) than the levels achieved in this study (325 μg/ml). A reevaluation of the experiments of Segalove and Dack (14), but at optimal shaking speeds, indicates that 37 C is the optimal temperature. Tatini et al. (Bacteriol. Proc., p. 17, 1971) reported that ratios between enterotoxin secretion or deoxyribonuclease activity and optical density indicated stimulative effects of higher temperatures on the production of both deoxyribonuclease and enterotoxins; the highest ratio occurred at 45 C followed by 40 C and 37 C. Contrary to the above finding, and in agree-

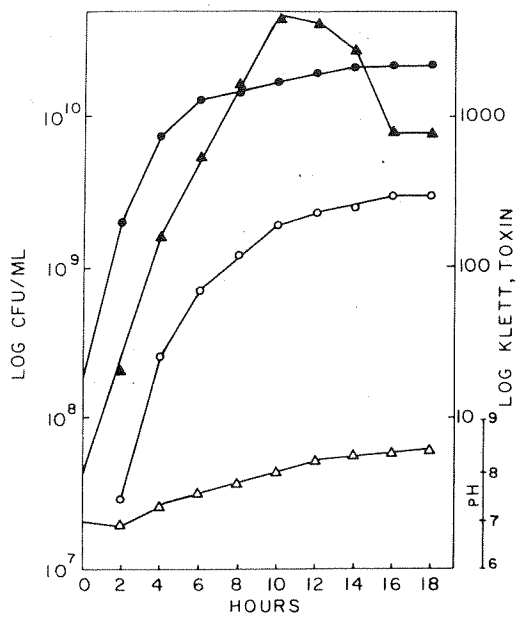

FIG. 1. *Enterotoxin B secretion by S. aureus S-6 shaken at 350 rev/min at 37 C. Symbols: ●, Kletts; ▲, total plate count; ○, enterotoxin B; △, pH.*

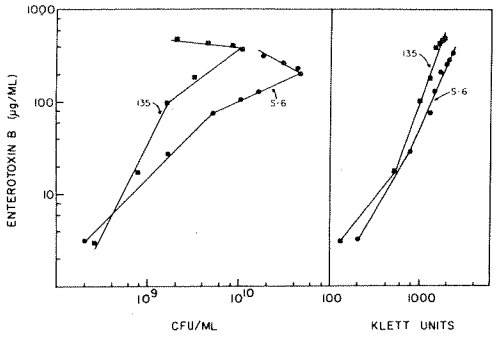

FIG. 2. *Relationship of CFU per milliliter and Klett units to enterotoxin secretion. Symbols: ●, strain S-6; ■, strain 135. Shaking speed was 350 rev/min. Initial value was taken at 2 hr, and each succeeding value is at 2-hr intervals.*

ment with Scheusner and Harmon (Bacteriol. Proc., p. 18, 1971), neither growth nor toxin secretion was observed in cultures incubated at 45 C.

Weiss and Robbins (18) noted that the synthesis in vitro of enterotoxin is a reflection of variability of individual cells within a population to produce enterotoxin. Sugiyama et al. (15) found that colonies derived from individual cells within a population of strain S-6 differ in the size of halos formed on antiserum agar and that the size of the precipitin zone was correlated with the ability of the culture to produce toxin. This technique of using antiserum agar does not appear to explain why populations shaken at 350 rev/min produced more toxin than those shaken at 250 rev/min. When based on CFU, productivity values were higher for strain 135 than S-6 after the initial 2 hr of growth. The maximal values for strain S-6 occurred prior to 4 hr of growth, whereas that for strain 135 was around 6 hr. The possibility that the secretion of enterotoxin during the stationary phase was due to the presence of a toxin precusor pool (6) was investigated. Penicillin (100 µg/ml), added to prevent growth, and chloramphenicol (100 µg/ml), found to indicate the presence of a precusor pool (6), were each added to 10-hr growth flasks shaken at 350 rev/min. No further increase in enterotoxin B secretion was detected in either case after continued incubation for an additional 8 hr. Control flask cultures without these antibiotics continued to secrete enterotoxin B (a total increase in 170 µg/ml) during the additional 8 hr of incubation.

The percentages of toxin-producing colonies derived from cells present during growth at suboptimal and optimal shaking rates are similar during the first 8 hr and differ at 16 and 20 hr (Table 4). For cultures shaken at 250 rev/min, the population of cells at 12 hr yielded nearly equal percentages of zone 3 and 4 colonies (48 and 52%, respectively), followed by a reduction to 3% of zone 4 colonies by 20 hr. In the 12th hr of growth of a culture shaken at 350 rev/min, 6% of its population yielded zone 1 and 2 colonies; by the 20th hr, the per-

TABLE 3. *Productivity[a] of enterotoxin B secretion by S. aureus*

Time (hr)	Productivity			
	Strain S-6		Strain 135	
	CFU	Klett units	CFU	Klett units
0–2	1.9	1.7	1.3	3.3
2–4	1.6	4.2	2.6	3.6
4–6	0.8	8.3	9.5	17.7
6–8	0.5	25.0	5.0	29.7
8–10	0.3	32.0	2.3	77.1
10–12		13.3		26.7
12–14		13.0		33.3
14–16		62.0		44.4

[a] Productivity was calculated, by the following formulae. CFU: Δ toxin/Δ CFU/Δ time expressed as micrograms of toxin/10^8 CFU/2 hr. Klett: Δ toxin/Δ Klett/Δ time expressed as micrograms of toxin/Klett (1/100)/2 hr. Shaking rate was 350 rev/min.

TABLE 4. *Distribution of zone development by enterotoxin B producing colonies of S. aureus S-6 on antiserum agar*[a]

Hr	Toxin (μg/ml)	No. colonies measured	Percentage of colonies producing precipitin zones			
			Zone 1	Zone 2	Zone 3	Zone 4
(250 rev/min)						
0	ND[b]	124	3	24	63	10
4	12	37	0	28	67	5
8	68	25	0	20	60	20
12	110	27	0	0	48	52
16	180	53	0	31	58	11
20	180	64	3	33	61	3
(350 rev/min)						
0	ND	124	3	24	63	10
4	16	27	4	26	60	10
8	120	33	0	46	46	8
12	210	71	1	5	68	26
16	320	133	0	69	29	2
20	320	94	3	76	19	2

[a] Cultures were grown at 37 C in the basal medium and transferred to immune serum plates. All colonies were egg-yolk and deoxyribonucleic positive.

[b] Not detectable.

centage of zone 1 and 2 colonies increased to 79%. All colonies were found to be both egg-yolk and deoxyribonuclease positive. In fact, after 8 hr of shaking, the culture grown at 350 rev/min possessed a higher percentage of cells producing smaller zones (see Table 4). One possible explanation for this inability to reflect differences in toxin secretion is that this technique may not measure those important environmental factors which actually exist in the flask during growth and which regulate toxin synthesis.

Appropriate strains of *S. aureus* release enterotoxin B into the medium throughout the 16-hr incubation period. Viable cell populations, as measured by CFU per milliliter, tended to remain the same or to decrease after 10 hr of shaking at 350 rev/min, whereas Klett units continued to increase. This suggests that there is a certain amount of reproduction occurring even though net CFU per milliliter decreases and that this "growth" may be responsible for toxin synthesis in the culture after 10 hr of shaking. This suggestion is supported by the ability of either chloramphenicol or penicillin to prevent further toxin secretion in 10-hr cultures.

Calculations of "productivity" indicate that the culture is actively synthesizing toxin within 2 hr. In the case of strain S-6, there is a subsequent decrease in productivity during growth. Strain 135 optimizes its productivity during the initial 4 to 8 hr of shaking.

Enterotoxin B appears to be a secondary product as defined by Demain (1), i.e., a metabolite having "no general function in the life processes although they may be important to the particular organism." It does not appear to fit the more restrictive definition suggested by Weinberg (16). Similarly, although enterotoxin B is an extracellular toxin, it does not appear to fit the definition of Raynaud and Alouf (11) for this class of proteins.

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