R70-143

APPLIED MICROBIOLOGY, Sept. 1970, p. 492–496
Copyright © 1970 American Society for Microbiology
Vol. 20, No. 3
Printed in U.S.A.

Factors Affecting the Secretion of Staphylococcal Enterotoxin A

Z. H. MARKUS AND G. J. SILVERMAN
Food Laboratory, U.S. Army Natick Laboratories, Natick, Massachusetts 01760

Received for publication 7 May 1970

TECHNICAL LIBRARY
U. S. ARMY
NATICK LABORATORIES
NATICK, MASS. 01760

NATICK

TECHNICAL LIBRARY
U. S. ARMY
NATICK LABORATORIES
NATICK, MASS.

APPLIED MICROBIOLOGY, Sept. 1970, p. 492–496
Copyright © 1970 American Society for Microbiology
Vol. 20, No. 3
Printed in U.S.A.

Factors Affecting the Secretion of Staphylococcal Enterotoxin A

Z. H. MARKUS AND G. J. SILVERMAN

Food Laboratory, U.S. Army Natick Laboratories, Natick, Massachusetts 01760

Received for publication 7 May 1970

The biosynthesis of enterotoxin A by replicating and nonreplicating cells was investigated. Unlike enterotoxin B, a secondary metabolite, enterotoxin A secretion resembled that of a primary metabolite by being secreted during the exponential phase of growth. The amount of toxin produced per unit of growth was not influenced by NaCl, $NaNO_2$, or $NaNO_3$. Several surfactants increased toxin secretion. Toxin secretion by nonreplicating cells was inhibited by chloramphenicol and 2,4-dinitrophenol but not by streptomycin or penicillin G. The optimal *p*H for enterotoxin A production was 6.5 to 7.0. The findings suggest a number of possible reasons for the higher incidence of food poisonings caused by enterotoxin A as compared to enterotoxin B.

In previous investigations (7, 8; Z. Markus, Bacteriol. Proc., p. 81, 1969), nonreplicating cells of *Staphylococcus aureus* were employed. This procedure separates the requirements for the secretion of enterotoxin B from those of growth and replication. As a result of these studies, a mechanism was postulated involving the presence of a toxin precursor (8). The assumption was based on the fact that cells resuspended in a nitrogen-free medium secreted a certain amount of toxin in the presence of chloramphenicol which was not detectable in cell extracts. The amount of toxin precursor present in the cell was dependent upon the rate of aeration and the age of the culture.

Although enterotoxin A has been implicated in a larger number of food poisoning cases than enterotoxin B (1), the conditions necessary for the secretion of enterotoxin A are not well defined. Enterotoxin A is produced in much smaller quantities than enterotoxin B, and there is more uniformity among strains in the amounts of toxin produced (5, 12). Donnelly et al. (2) have shown that the production of enterotoxin A in milk is associated with microbial growth, but the levels of enterotoxin were not quantitated.

This study was undertaken to compare the factors necessary for the secretion of enterotoxin A by replicating and nonreplicating cells with those previously found for enterotoxin B.

MATERIALS AND METHODS

Organism. *S. aureus* 100 and S-6 were obtained from M. Bergdoll, University of Wisconsin, Madison, Wis. Both strains possessed free coagulase and deoxyribonuclease and were bound coagulase-negative.

Media. As in the previous studies with enterotoxin B (7, 8), three media were employed, two utilizing N-Z amine as a source of nitrogen and energy (medium 1 and 2) and the third (medium 3A) lacking a nitrogen source but containing glucose. The latter two media were used in the studies involving toxin secretion by nonreplicating cells.

Medium 1, as described by Rosenswald et al. (13), was the growth medium and contains 4% N-Z amine type A (Sheffield Chemical, Norwich, N.Y.), 0.4% yeast extract (Difco), and 0.1% K_2HPO_4 in distilled water (*p*H 6.6 to 6.8).

Medium 2 contains 4% N-Z amine type A and 0.1% K_2HPO_4.

Medium 3A contains 0.5% glucose and 3.5% K_2HPO_4, the *p*H being adjusted to 7.0. The designation 3A was chosen to avoid confusion with medium 3 described previously (8), which has the same composition but a *p*H of 8.5.

Growth studies. Stock cultures of strain 100 were grown at 37 C for 16 to 18 hr on Trypticase Soy Agar slants (BBL) plus 0.5% yeast extract (Difco) and were stored at 4 C. A loopful of this culture was inoculated into a 500-ml shake-flask, containing 100 ml of medium 1, and incubated at 37 C on a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J., model V) for 18 hr. Repropagation was conducted in the same manner as above but with a 1% inoculum.

Experiments in fermentors. Toxin production was studied in 400 ml of medium 1 in a 1-liter vessel at 37 C and aerated at a rate of 2 liters/min; 100 μg of antifoam P-2000 (Dow-Corning, Midland, Mich.) per ml was used to avoid excessive foaming. The *p*H was automatically controlled at 6.9 by the addition of HCl.

Nonreplicating cells. In preliminary experiments, it was found that cells harvested after 3.5 hr of propaga-

ion at 37 C and resuspended in either medium 2 or 3 secreted over 85% of their toxin potential within 4 hr.

For this reason, the cells were propagated as described above and, unless otherwise noted, were harvested by centrifugation at 5,000 × *g* for 10 min, washed twice in 0.01 M phosphate buffer (*p*H 7.0), and adjusted in either medium 2 or 3A to a cell concentration of 5,000 to 6,000 Klett units (6×10^{10} to 8×10^{10} organisms/ml). A 10 ml amount of this suspension was agitated at 240 rev/min in a 100-ml flask at 37 C.

Assay of enterotoxin A. By using purified antigen and antiserum (provided by M. Bergdoll), the single diffusion technique of Weirether et al. (14) developed for enterotoxin B was found to be suitable for the assay of enterotoxin A. The response of the calibration curve for enterotoxin A was linear and sensitive to 0.4 μg/ml if the test was read within 24 hr. Longer incubation increased the sensitivity of the test.

The procedure for the assay of the supernatant was the same as that previously described for enterotoxin B (7, 8), care being taken to compensate for differences in ionic strength.

Chemicals. Aqueous stock solutions of chloramphenicol (Parke, Davis & Co., Detroit, Mich.), potassium penicillin G (Mann Research Laboratories, N.Y.), streptomycin sulfate (NBC, Cleveland, Ohio), and 2,4-dinitrophenol (Eastman, Rochester, N.Y.) were membrane sterilized and appropriately diluted in the final medium.

The surfactants Tweens 20, 40, 60, and 80, polyoxyethylene oleate, Span 60 and 80 (Atlas Chemical, Wilmington, Del.), sodium oleate (Baker Chemical, Phillipsburg, N.J.), Triton X-100 (Rohm and Haas, Philadelphia, Pa.), sucrose monopalmitate, and sucrose monostearate (Colonial Sugars, N.Y.) were autoclaved with medium 1.

Sodium chloride and sodium nitrate were added to medium 1 and sterilized by autoclaving. Sodium nitrite was sterilized by filtration and aseptically added to sterile growth medium 1.

RESULTS

The secretion of enterotoxin A in shake-flask cultures occurred mainly in the exponential phase of growth, although approximately 20% was secreted as the cells entered the early stationary phase of growth (Fig. 1). The cell density reached in these experiments was similar to that previously noted for the production of enterotoxin B by strain S-6 (8).

Growing strain 100 at a controlled *p*H of 6.9 increased the duration of exponential growth and, hence, the total cell crop (Fig. 2). Toxin production also increased during exponential growth, but the concentration produced per Klett unit remained fairly constant (3.4 to 4.6 μg of enterotoxin A per 1,000 Klett units).

Concentrations of NaCl of up to 10% did not essentially alter the ratio of enterotoxin A formation to growth (Fig. 3). Higher NaCl concentrations inhibited bacterial growth (also found for

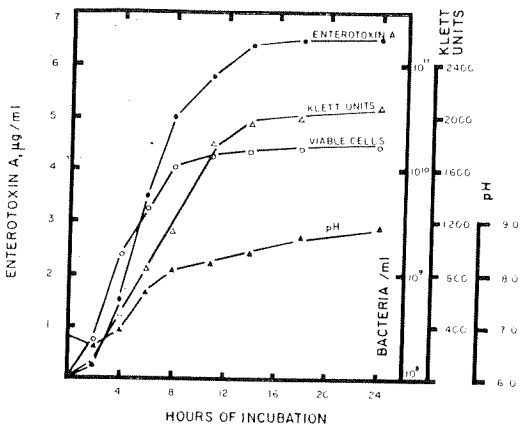

FIG. 1. *Enterotoxin A production by cells grown as shake-flask cultures.*

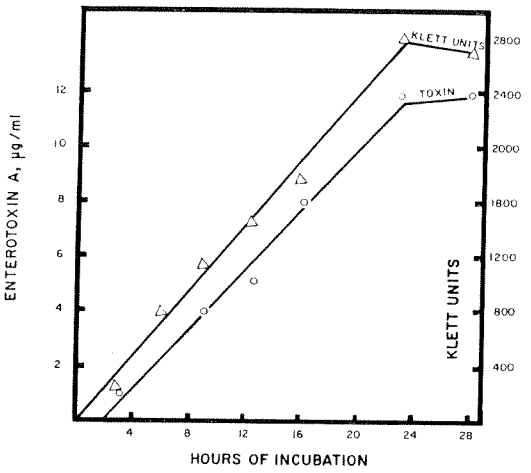

FIG. 2. *Enterotoxin A production by cells grown in a fermentor at a constant pH of 6.9.*

strain S-6); therefore, toxin concentration was determined after 48 hr of cell growth. As much as 200 μg of $NaNO_2$ per ml and 1,000 μg of $NaNO_3$ per ml did not affect either bacterial growth or toxin formation by strain 100. No synergistic effect between NaCl, $NaNO_2$ and $NaNO_3$ on bacterial growth or on enterotoxin A secretion was found. Strain 100 grown in medium 1 with 10% NaCl, 200 μg of $NaNO_2$ per ml and 1,000 μg of $NaNO_3$ per ml attained a cell concentration of 800 Klett units and produced 2 μg of enterotoxin A per ml.

Surfactants had a varied effect on growth and toxin formation (Table 1). Enterotoxin A secretion was increased by the presence of Tweens 40 and 60, whereas Tweens 20 and 80 had no effect. Span 60 behaved similarly to Tweens 20 and 80;

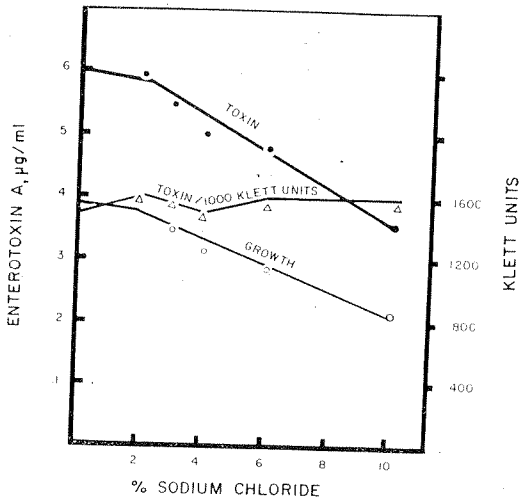

FIG. 3. *Effect of NaCl on growth and enterotoxin A secretion.*

TABLE 1. *Effect of surfactants on growth and toxin formation*

Surfactant[a]	Strain S-6		Strain 100	
	Growth[b]	Enterotoxin B	Growth	Enterotoxin A
		µg/ml		µg/ml
Control	1,450	150	1,500	6.5
Tween 20	1,200	70	1,400	6.8
Tween 40	1,200	150	1,550	8.5
Tween 60	1,600	290	1,550	9.0
Tween 80	1,250	17	1,400	6.5
Span 60	NG[c]	NG	1,500	5.5
Span 80	1,100	8	1,250	ND[d]
Polyoxyethylene oleate	1,200	ND	1,200	4
Sodium oleate	800	ND	300	ND
Triton X-100	180	ND	0	NG
Sucrose monostearate	NG	NG	800	2
Sucrose monopalmitate	550	ND	0	NG

[a] Final concentration used was 0.1% (v/v) in medium 1, except Triton X-100 which was at 0.05%.
[b] Determined from a 16 hr culture at 37 C in medium 1 with a Klett-Summerson colorimeter and a no. 66 filter; values are given in Klett units.
[c] No growth.
[d] Not detectable.

Span 80, although having a minor inhibitory effect on growth, completely inhibited enterotoxin A secretion. The remaining compounds inhibited growth, toxin formation, or both.

Cells harvested during various phases of growth had their toxin-synthesizing abilities evaluated by the non-replicating technique (Table 2). Those cells obtained from the exponential phase (3.5 hr) produced high toxin levels, whereas cells harvested from the stationary phase of growth (16 hr) failed to produce significant amounts of toxin. The presence of N-Z amine in medium 2 doubled the concentration of toxin secreted but did not alter the incapability of cells harvested at 16 hr to synthesize toxin. The effect of *p*H was also important (Table 3). The maximal secretion of toxin occurred at *p*H 7.0, regardless of whether nitrogen was present.

The effect of several metabolic inhibitors on toxin secretion by nonreplicating cells is presented in Table 4. Chloramphenicol and 2,4-dinitrophenol prevented the secretion of enterotoxin A, but normal amounts of toxin were secreted by cells in the presence of streptomycin and penicillin G.

TABLE 2. *Enterotoxin A production by nonreplicating cells harvested at different time intervals during the growth cycle*

Time of growth[a]	Enterotoxin A	
	Medium 2	Medium 3A
hr	µg/ml	µg/ml
3.5	3.5	1.9
6	2.1	1.0
16	ND[b]	ND

[a] At the sampling periods specified, the cells were collected, washed, and adjusted in medium 2 or 3A to a Klett reading of 5,000 to 6,000 units and incubated for 4 hr at 37 C as described in Materials and Methods.
[b] Not detectable.

TABLE 3. *Effect of pH on enterotoxin A production by nonreplicating cells*[a]

*p*H	Enterotoxin A	
	Medium 2[b]	Medium 3A[b]
	µg/ml	µg/ml
5.5	2.2	ND[c]
6.5	3.5	1.0
7.0	4.0	2.0
8.0	2.2	1.0
8.5	1.9	0.5

[a] Cells grown for 3.5 hr in medium 1 were harvested, washed, resuspended at the appropriate *p*H, and incubated at 37 C for 4 hr by the procedure described in Materials and Methods.
[b] 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.
[c] Not detectable.

TABLE 4. *Effectiveness of various compounds to inhibit enterotoxin A production by nonreplicating cells*[a]

Inhibitor	Concn/ml	Enterotoxin A
		μg/ml
Control		3.0
Chloramphenicol	100 μg	ND[b]
2,4-Dinitrophenol	2 μmoles	ND
Streptomycin sulfate	0.007 μmoles	2.6
Penicillin G	100 units	2.9

[a] Harvested after 3.5 hr of growth, the standardized cell suspension was incubated for 4 hr at 37 C in medium 2 supplemented with the above compounds.

[b] Not detectable.

Cells propagated at 20 C attained the stationary phase of growth after 3 days. These cells were resuspended in medium 2 or 3A at 37 C by the nonreplicating technique. After an incubation period of 4 hr, 3.5 μg of enterotoxin A per ml was found in medium 2 and none in 3A. For cells grown at 37 C for 3.5 hr and resuspended at 20 C in medium 2 or 3A, only traces of enterotoxin A were subsequently found by the nonreplicating technique.

DISCUSSION

Enterotoxins A and B appear to be quite similar in their chemical structure and in their clinical symptoms of intoxication. Either toxin can be produced by nonreplicating cells in a nitrogen-free medium. This effect is increased by glucose, and air must be present. Toxin synthesis is also stimulated by the presence of protein hydrolysates. However, there are important differences in the biosynthesis of enterotoxins A and B.

It was previously established (7, 8) that enterotoxin B was produced by cells entering the stationary phase of growth and that, for a given medium, the secretion of enterotoxin B could be varied by altering cultural conditions. The optimal *p*H for the secretion of enterotoxin B in a nitrogen-free medium was 8.0 to 8.5 and 7.0 to 7.5 in the presence of protein hydrolysates. A certain amount of secretion occurred at 37 C with nonreplicating cells grown at 37 C in the presence of chloramphenicol. Cells propagated at 20 C and resuspended under nonreplicating conditions at 37 C demonstrated a lag of several hours before toxin secretion occurred.

In contrast, the secretion of enterotoxin A by strain 100 was found in the study to occur mainly during the exponential phase of growth, was directly related to cell numbers, and was influenced to a much lesser extent by cultural conditions. The optimal *p*H for the formation of enterotoxin A, in either the presence or absence of protein hydrolysates, was 6.5 to 7.0. Chloramphenicol completely inhibited toxin secretion by nonreplicating cells of strain 100. These results indicated differences in biosynthesis of enterotoxins A and B and the absence of any appreciable concentration of a partial or completely formed toxin precursor in strain 100.

In many cases, the effect of a given surfactant on toxin production by strains S-6 or 100 differed. However, these observations are not unique to this system because Tween 80, oleic acid, and other surfactants have been found by Friedman (3) to differ in their effect on two enterotoxin B producing strains. The value of surfactants for increasing enzyme yields was emphasized by Reese and Maguire (11), who assumed that these compounds affect cell permeability. An alternate explanation for their activity could be that they affect oxygen transfer in liquid media (4).

McLean et al. (6), using ATCC strain 14458, found that NaCl inhibited enterotoxin B secretion to a greater extent than growth. Our results with strain S-6 (*unpublished data*) confirm this observation. However, in the case of enterotoxin A, the amount of toxin secreted in the presence of NaCl was directly related to growth.

It was postulated (6, 8; Z. Markus, Bacteriol. Proc., p. 81, 1969) that enterotoxin B synthesis was characteristic of a secondary metabolite because its secretion was initiated during the late exponential and early stationary phase of growth. In contrast, it is apparent that enterotoxin A synthesis follows the pattern of a primary metabolite since it was synthesized continuously by cells during exponential growth. Cells of strain 100 harvested during the stationary phase did not produce significant amounts of enterotoxin A. However, the enterotoxins secreted by strains 100 and S-6 appear to satisfy the definition of Raynaud and Alouf (10) for true protein exotoxins. Neither strain secretes toxin during the late stationary phase of growth, and no intracellular toxin is detectable.

The incidence of food poisoning caused by enterotoxin A is higher than that caused by enterotoxin B (1). Our findings suggest a number of possible reasons for this observation. Being associated with growth, enterotoxin A is more likely to appear earlier than would enterotoxin B and at a *p*H range more commonly associated with food (*p*H 6 to 7). In certain situations, growth of strain S-6 occurred without enterotoxin B secretion. This is less likely in the case of enterotoxin A. Strain 100, an enterotoxin A producer, propagated at lower temperatures will begin to secrete toxin much sooner at 37 C than strain S-6 (8).

It is also conceivable that the higher incidence may be due to a greater frequency of enterotoxin A producing strains and to the fact that humans are more susceptible to this toxin than to enterotoxin B (9).

ACKNOWLEDGMENTS

We thank Charles L. Cooney, Department of Nutrition and Food Science, Massachusetts Institute of Technology, for performing the experiments on toxin production at constant *p*H in the fermentor system.

Part of this work was performed by the senior author while a research associate at the Department of Nutrition and Food Science, Massachusetts Institute of Technology, supported by Public Health Service grant UI 00678-07 from the National Center for Urban and Industrial Health. The author gratefully acknowledges the Visiting Scientist Award received from the National Research Council, National Academy of Sciences, Washington, D.C., in connection with the present studies.

LITERATURE CITED

1. Bergdoll, M. S. 1967. The staphylococcal enterotoxins, p. 1–25. *In* R. I. Mateles and G. N. Wogan (ed.), Biochemistry of some foodborne microbial toxins. M.I.T. Press, Cambridge, Mass.
2. Donnelly, C. B., J. E. Leslie, and L. A. Black. 1968. Production of enterotoxin A in milk. Appl. Microbiol. **16**:917–924.
3. Friedman, M. E. 1968. Inhibition of staphylococcal enterotoxin B formation by cell wall blocking agents and other compounds. J. Bacteriol. **95**:1051–1055.
4. Hulme, M. A., and D. W. Stranks. 1970. Induction and the regulation of production of cellulase by fungi. Nature (London) **226**:469–470.
5. Kato, E., M. Khan, L. Kujovich, and M. S. Bergdoll. 1966. Production of enterotoxin A. Appl. Microbiol. **14**:966–972.
6. McLean, R. A., H. D. Lilly, and J. A. Alford. 1968. Effects of meat-curing salts and temperature on production of staphylococcal enterotoxin B. J. Bacteriol. **95**:1207–1211.
7. Markus, Z., and G. J. Silverman. 1968. Enterotoxin B production by nongrowing cells of *Staphylococcus aureus*. J. Bacteriol. **96**:1446–1447.
8. Markus, Z., and G. J. Silverman. 1969. Enterotoxin B synthesis by replicating and nonreplicating cells of *Staphylococcus aureus*. J. Bacteriol. **97**:506–512.
9. Raj, H. D., and M. S. Bergdoll. 1969. Effect of enterotoxin B on human volunteers. J. Bacteriol. **98**:833–834.
10. Raynaud, M., and J. A. Alouf. 1970. Intracellular versus extracellular toxins, p. 67–117. *In* S. J. Ajl, S. Kadis, and T. C. Montie (ed.), Microbiological toxins, vol. 1. Academic Press Inc., New York.
11. Reese, E. T., and A. Maguire. 1969. Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol. **17**:242–245.
12. Reiser, R. F., and K. F. Weiss. 1969. Production of staphylococcal enterotoxins A, B, and C in various media. Appl. Microbiol. **18**:1041–1043.
13. Rosenwald, A. C., and R. E. Lincoln. 1966. Streptomycin inhibition of elaboration of staphylococcal enterotoxin protein. J. Bacteriol. **92**:279–280.
14. Weirether, F. J., E. E. Lewis, A. J. Rosenwald, and R. E. Lincoln. 1966. Rapid quantitative serological assay of staphylococcal enterotoxin protein. Appl. Microbiol. **14**: 284–291.