Regulation of Staphylococcal Enterotoxin B: Effect of Anaerobic Shock

STEPHEN A. MORSE AND ROBERT A. MAH

Department of Microbiology, Harvard School of Public Health, Boston, Massachusetts 02115, and Division of Environmental and Nutritional Sciences, School of Public Health, University of California at Los Angeles, Los Angeles, California 90024

The metabolism of glucose during enterotoxin B synthesis in *Staphylococcus aureus* S-6 was examined under anaerobic conditions in the presence and absence of nitrate. The repression of enterotoxin synthesis which occurs during the oxidative metabolism of glucose was relieved after a shift to anaerobic conditions; glucose was then converted primarily to lactic acid and was metabolized more rapidly, presumably to obtain the equivalent amount of energy available aerobically. A greater proportion of oxidized end products and evidently more energy per glucose molecule was produced in the presence of oxygen. Thus, available energy as judged by a change in the type and proportion of end products appears to be related to the degree of toxin repression. As expected, the addition of nitrate during anaerobic glucose metabolism prevented derepression of toxin synthesis.

The synthesis of staphylococcal enterotoxin B does not begin until the transition from exponential to stationary phase growth (8–10). This is a characteristic property of many extracellular products (15). The kinetics of toxin synthesis are similar for both aerobic and anaerobic growth, although the quantity differs (S. A. Morse and J. N. Baldwin, personal communication). Addition of rapidly metabolizable energy sources, such as glucose or pyruvate, to a casein hydrolysate medium severely repressed the synthesis of enterotoxin B (9). Previous studies (9; S. A. Morse and J. Baldwin, personal communication) demonstrated that this repression is related to the energy state of the cells as reflected by the oxidative decarboxylation of pyruvate. Nutritional alterations of the oxidation of pyruvate by thiamine starvation (9) or by preventing the formation of a functional electron transport system (S. A. Morse and J. N. Baldwin, personal communication) result in a decrease in the repression of toxin synthesis. The present study is concerned with whether the repression of toxin synthesis may be altered by environmental changes which affect the dissimilation of pyruvate.

MATERIALS AND METHODS

Organism and cultural conditions. *Staphylococcus aureus* S-6 was obtained from M. S. Bergdoll (Food Research Institute, Univ. of Wis.). This strain produces large amounts of enterotoxin B and small amounts of enterotoxin A. Storage of the culture, preparation of inocula, and incubation conditions were described previously (7, 10).

Medium. The basal medium consisted of a 1% solution of casein hydrolysate (PHP; Mead Johnson International, Evansville, Ind.) supplemented with thiamine (33 ng/ml) and nicotinic acid (1.2 μg/ml). This solution will be referred to as the PHP medium.

Experimental conditions. A starter culture grown aerobically for 12 h was used as a 1% inoculum in the PHPmedium. After 4 h of growth the culture was chilled and the cells were harvested by centrifugation (20,000 × g for 15 min). Cells were suspended to their original volume in 0.1 M phosphate-buffered PHP (pH 7.7) containing uracil (6.89 μmol/ml) (6) and glucose (14 μmol/ml). Uniformly labeled ^{14}C-leucine (2.5 × 10^{-2} μCi/ml) was added at zero time. After 1 h of incubation, portions of the culture were transferred to separate flasks with and without nitrate (8 μmol/ml) and subjected to anaerobic shock by continuous sparging with a mixture of 95% N_2 and 5% CO_2. Samples were removed at various times and assayed for glucose, enterotoxin, nitrite, and ^{14}C-leucine incorporation.

Determination of end products. Nongaseous end products of glucose metabolism were determined by column chromatography on silicic acid with various concentrations of t-butanol in chloroform as solvents. The technique used for the preparation and packing of the silicic acid and for preparation of solvents was essentially that of Ramsey (13). Addition of sample and elution procedures were those reported by Dobrogosz (5). Glucose-U-^{14}C (0.2 μCi/ml) was added at

the time of anaerobic shock. Incubation was continued for an additional hour before samples were collected.

Known samples of ^{14}C-labeled ethyl alcohol, -acetate, -pyruvate, -formate, -lactate, and -succinate were chromatographed to determine their elution position. In all cases a consistent elution profile was obtained.

Determination of pyruvate decarboxylation. The rate of pyruvate decarboxylation was estimated from the $^{14}CO_2$ formed during glucose-$3,4$-^{14}C metabolism assuming a functional EMP pathway. The standard experimental protocol was modified as follows. After 1-h incubation, 20-ml samples were added to three 125-ml flasks modified for continuous sparging (flow rate of 44 ml/min of either air or a mixture of 95% N_2 and 5% CO_2). At zero time, glucose-$3,4$-^{14}C (2.82×10^{-2} μCi/ml) was added and $^{14}CO_2$ was trapped in a mixture of monoethanolamine-absolute ethanol (1:2). The trapping solution was replaced at 10-min intervals; after sampling, it was diluted 1:2 with absolute ethanol and mixed, and 1-ml samples were added to scintillation vials. The activity of the CO_2 was determined by liquid scintillation counting after the addition of 15 ml of scintillation fluid (0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl) benzene in toluene).

Enterotoxin measurements. Samples for toxin assay were prepared as previously described (10). Enterotoxin was determined by the single-gel diffusion method of Weirether et al. (16). Reference standards containing 12.5, 25, 50, 100, and 200 μg of toxin per ml were prepared by diluting purified enterotoxin B in phosphate-buffered saline, pH 7.4. A reference curve was plotted for each experiment. The gel-diffusion tubes were incubated in a water bath at 30 C and read at 24 and 48 h.

Miscellaneous measurements. Glucose uptake was measured by determining the amount of glucose remaining in the medium at the indicated times by the glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Nitrate was measured by a modification (4) of the procedure of Nason and Evans (11). Total protein synthesis was measured as previously described (10).

Chemicals. Uniformly labeled leucine (leucine-$U^{14}C$), leucine-$4,5$-^3H, uniformly labeled glucose (glucose-U-^{14}C), glucose-$3,4$-^{14}C, DL-sodium lactate-1-^{14}C, and succinic acid-$1,4$-^{14}C were purchased from New England Nuclear Corp. (Boston, Mass.). Sodium acetate-$1,2$-^{14}C, sodium pyruvate-U-^{14}C, ^{14}C-sodium formate, and ethanol-1-^{14}C, were products of International Chemical and Nuclear Corp., (City of Industry, Calif.). 2,5-Diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl) benzene were products of Nuclear-Chicago Corp. (des Plaines, Ill.). All other reagents were of analytical grade.

RESULTS

Previous results (10) showed that the differential rate of enterotoxin B synthesis by PHP-grown cells of *S. aureus* S-6 may be exponential. However, examination of the initial period

of toxin synthesis gave the appearance of a linear differential rate. The differential rates observed when toxin-producing cells were suspended in buffered medium at pH 7.7 are shown in Fig. 1. At this pH, the addition of glucose decreased the differential rate by 60%. This repression was not due to an inhibition of toxin release but rather to a decrease in the rate of synthesis.

Since glucose repression of toxin synthesis is related to the rapid oxidative decarboxylation of pyruvate (S. A. Morse and R. A. Mah, Bacteriol. Proc., p. 10, 1969), inhibition of this reaction should relax repression. Anaerobic shock inhibited the decarboxylation of pyruvate in *Escherichia coli* (3, 4, 12) and led to the derepression of β-galactosidase synthesis in this organism, apparently by prevention of its rapid dissimilation. The effect of anaerobic shock on the glucose repression of enterotoxin B synthesis is shown in Fig. 2. Anaerobic shock relieved the repression of toxin synthesis and increased the rate by approximately 60% of the rate observed in the absence of glucose. The quantity as well as the differential rate of toxin synthesis increased during anaerobic shock.

If oxidative metabolism, with oxygen as the major terminal electron acceptor, was involved in the regulation process, addition of a suitable alternate electron acceptor might reverse the derepression resulting from anaerobic shock. Since *S. aureus* possesses a nitrate reductase, nitrate (8 μmol/ml) was added as a substitute electron acceptor for oxygen at the time of anaerobic shock. The addition of nitrate prevented the derepression of enterotoxin synthesis (Fig. 2). The continued repression was not due to nitrate itself, since the addition of nitrate (8 μmol/ml) to aerobic cultures had no appreciable effect on the differential rate of synthesis. Rather, nitrate served as an electron acceptor, and nitrite (Fig. 2) was produced.

In every experiment a lag in toxin synthesis occurred before derepression (Fig. 2) and lasted approximately 15 min. An examination of the rate of total protein synthesis (Fig. 3a) indicated that, during this lag, protein synthesis changed to a new rate characteristic of the growth conditions. This shift is further evident (Fig. 3b) upon examination of the differential rate of glucose uptake. The increase in the differential rate of glucose uptake in anaerobic cultures with and without nitrate indicated that the cells metabolized more glucose, presumably to obtain the equivalent amount of energy available under aerobic conditions.

The effect of anaerobic shock was further investigated by determining changes in the

nongaseous end products of glucose metabolism. The results are shown in Table 1. Under aerobic conditions, glucose was dissimilated mainly to pyruvate, acetate, and lactate. Under anaerobic conditions, glucose was dissimilated to lactate, with smaller amounts of acetate, formate, ethanol, and diacetyl produced. The presence of a high concentration of lactate indicated that pyruvate was serving as the terminal electron acceptor. In the presence of nitrate, the concentration of lactate de-
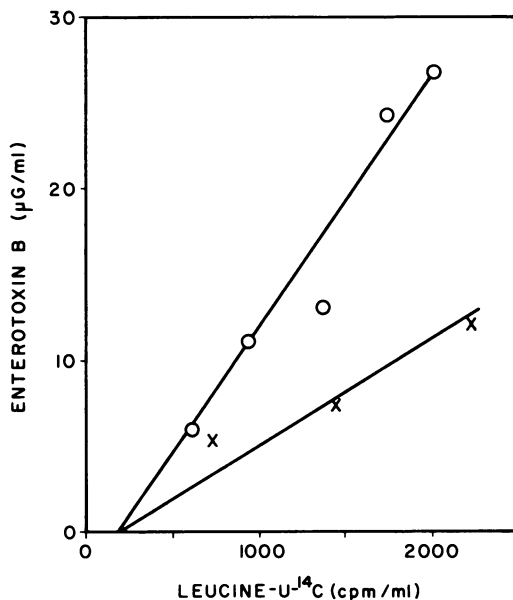
FIG. 1. *Effect of glucose on the differential rate of enterotoxin B synthesis. Cells were suspended in 0.1 M phosphate-buffer PHP (pH 7.7). Glucose (14 μmol/ml) was added at zero time. Symbols: O, without glucose; ×, with glucose.*
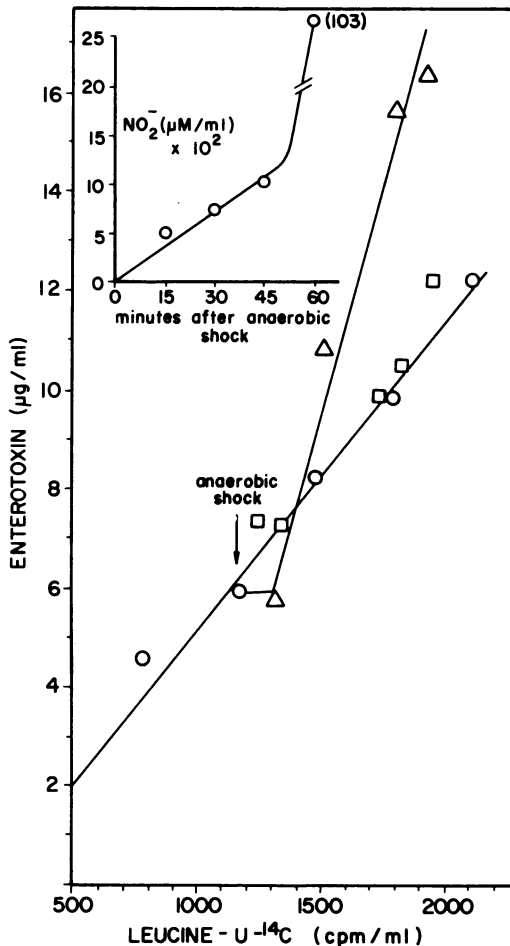
FIG. 2. *Effect of anaerobic shock on toxin synthesis. Symbols: O, aerobic; Δ, anaerobic; □, anaerobic + NO_3^- (8 μmol/ml). The arrow indicates the time of NO_3^- addition as well as anaerobic shock.*
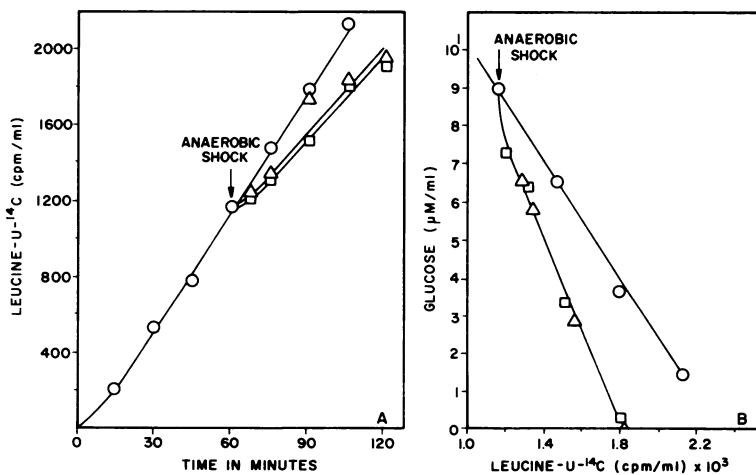
FIG. 3. *Effect of anaerobic shock on (A) total protein synthesis and (B) differential rate of glucose uptake. Symbols: O, aerobic; Δ, anaerobic; □, anaerobic + NO_3^- (8 μmol/ml).*

TABLE 1. *Composition of nongaseous products from glucose*

Product	Growth condition					
	Aerobic		Anaerobic + NO_3^-		Anaerobic	
	dpm/ml	%	dpm/ml	%	dpm/ml	%
Diacetyl ..	360	2.9	90	0.5	580	2.1
Ethanol ..	180	1.4	1,070	6.1	900	3.3
Acetate ...	3,620	29.4	3,980	22.6	1,940	7.1
Pyruvate .	1,770	14.4	0	0.0	0	0.0
Formate ..	0	0.0	2,100	11.9	1,090	4.0
Lactate ...	5,620	45.6	9,480	53.8	21,800	79.7

creased and was accompanied by an increase in acetate, formate, and ethanol. No pyruvate was detected under anaerobic conditions in the presence or absence of nitrate. Thus, although nitrate is reduced to nitrite, it apparently does not completely replace oxygen as a terminal electron acceptor under these conditions, since the end products differ.

The differential rate of pyruvate decarboxylation was estimated from the $^{14}CO_2$ formed from the metabolism of glucose-$3,4$-^{14}C which is presumably converted first to pyruvate-1-^{14}C. The results (Fig. 4) indicated that the $^{14}CO_2$ was formed at the same differential rate regardless of growth conditions. However, the total amount of $^{14}CO_2$ formed aerobically was considerably greater than under anaerobic conditions with or without nitrate. The amount of lactate and formate produced under anaerobic conditions can account for the lower amount of $^{14}CO_2$ formed from pyruvate-1-^{14}C. These results do not permit discrimination between the direct decarboxylation of pyruvate, e.g., pyruvate decarboxylase, and the CO_2 formed from formate.

Anaerobic conditions, with or without nitrate, did not alter the assimilation of glucose-U-^{14}C into various cell fractions when compared to the aerobic control (unpublished data).

DISCUSSION

The synthesis of enterotoxin B by *S. aureus* is repressed by the addition of glucose. This repression is similar in several respects to the catabolite repression β-galactosidase in *E. coli;* reactions associated with pyruvate decarboxylation are of particular importance in influencing the catabolite repression of this enzyme (12). Inhibition of pyruvate decarboxylation by anaerobic shock eliminated this repression (4, 12). However, the anaerobic reversal of catabo-

lite repression could be eliminated if nitrate or nitrite was used as the terminal electron acceptor (4). In *S. aureus*, the repression of enterotoxin B synthesis was also reversed by anaerobic shock and was accompanied by an increase in the amount of toxin formed.

S. aureus possesses a nitrate reductase which is inducible by nitrate in the absence of oxygen (2). The presence of this enzyme was confirmed in strain S-6 by following the formation of nitrite from nitrate under anaerobic conditions. The derepression of enterotoxin synthesis was prevented by the presence of nitrate and was similar in this respect to its effect on the derepression of β-galactosidase synthesis in *E. coli* (12).

Since the aerobic to anaerobic shift affects the expression of toxin synthesis, it is conceivable that the repressive mechanism is associated with the energy state of the cell. The observed effect of nitrate on the regulatory functions involved in enterotoxin synthesis during anaerobic shock supports this concept. Since nitrate is not directly involved in carbon metabolism, its influence on toxin repression is most likely related to its capacity to serve as an acceptor for anaerobically generated reduced nicotinamide adenine dinucleotide. Such activity has been reported in S. aureus (1).

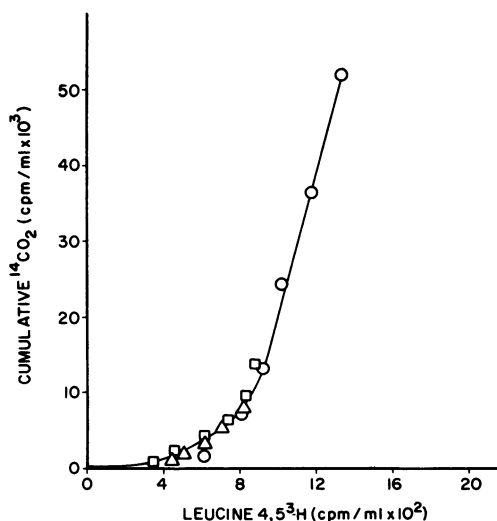Anaerobic nitrate reduction altered the balance of fermentation end products in *E. coli*

FIG. 4. *Effect of anaerobic shock on the differential rate of* $^{14}CO_2$ *formation from glucose-$3,4$-^{14}C. Total protein synthesis was determined by addition of leucine-$4,5$-^3H (0.2 $\mu Ci/ml$) at zero time. Samples were assayed as previously described (8). Symbols: O, aerobic; Δ, anaerobic; $\square$, anaerobic + NO_3^- (8 $\mu mol/ml$).*

(5). During the reduction of nitrate by *S. aureus* S-6, the balance of end products was also altered, apparently in favor of the dismutation of pyruvate to acetate and formate. This observation has been previously reported during anaerobic growth (1). The proportion of lactic acid in the aerobic cultures and anaerobic cultures with nitrate was 26 to 35% less than anaerobic cultures without nitrate. This fact, coupled with the increased proportion of oxidized products in the aerobic and nitrate cultures, suggests that a more efficient utilization of glucose probably occurred and that repression probably was exerted in the presence of more available energy than was present under anaerobic conditions. Evidently, the amount of nitrate reductase necessary for repression under anaerobic conditions is not very great, since enterotoxin remains repressed when nitrate is added to the culture at the time of anaerobic shock (which should cause derepression).

Conditions which favor the rapid decarboxylation of pyruvate tended to promote the catabolite repression of β-galactosidase synthesis by *E. coli* (5). A similar finding was observed for the repression of enterotoxin B synthesis (9). Although the addition of nitrate did not increase pyruvate decarboxylation, it appeared to increase the energy state of the cell as judged by the change in the type and proportion of end products formed.

Although some similarities exist, it is entirely possible that the repression of enterotoxin B synthesis by rapidly metabolizable energy sources, such as glucose and pyruvate, is not the result of catabolite repression. Tanaka and Iuchi (14) recently demonstrated that the glucose repression of amylase in *Vibrio parahaemolyticus* was reversed by the addition of cyclic 3',5'-adenosine monophosphate. In contrast, the glucose repression of an extracellular proteinase in the same strain was insensitive to cyclic 3',5'-adenosine monophosphate. The authors concluded that the repression of the extracellular proteinase was distinct from catabolite repression. Our preliminary studies (not shown) revealed that cyclic 3',5'-adeno-

sine monophosphate did not reverse the glucose repression of enterotoxin B. However, additional studies are needed.

LITERATURE CITED

1. Barron, E. S. G., and C. M Lyman. 1939. Studies on biological oxidation. CI. The metabolism of pyruvic acid by animal tissues and bacteria. J. Biol. Chem. 127:143–161.
2. Chang, J. P., and J. Lascelles. 1963. Nitrate reductase in cell-free extracts of a haemin-requiring stain of *Staphylococcus aureus*. J. Gen. Microbiol. 89:503–510.
3. Cohn, M., and K. Horibata. 1959. Physiology of the inhibition by glucose of the induced synthesis of the β-galactosidase-enzyme system of *Escherichia coli*. J. Bacteriol. 78:624–635.
4. Dobrogosz, W. J. 1965. The influence of nitrate and nitrite reduction on catabolite repression in *Escherichia coli*. Biochim. Biophys. Acta 100:533–566.
5. Dobrogosz, W. J. 1966. Altered end-product patterns and catabolite repression in *Escherichia coli*. J. Bacteriol. 91:2263–2269.
6. Gardner, J. F., and J. Lascelles. 1962. The requirement for acetate of a streptomycin-resistant strain of *Staphylococcus aureus*. J. Gen. Microbiol. 29:157–164.
7. Mah, R. A., D. Y. C. Fung, and S. A. Morse. 1967. Nutritional requirements of *Staphylococcus aureus* S-6. Appl. Microbiol. 15:886–870.
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. Morse, S. A., and J. N. Baldwin. 1971. Regulation of staphylococcal enterotoxin B: effect of thiamine starvation. Appl. Microbiol. 22:242–249.
10. Morse, S. A., R. A. Mah, and W. J. Dobrogosz. 1969. Regulation of staphylococcal enterotoxin B. J. Bacteriol. 98:4–9.
11. Nason, A., and J. H. Evans. 1955. Nitrate reductase from *Neurospora*, p. 411–415. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
12. Okinaka, R. T., and W. J. Dobrogosz. 1967. Catabolite repression and pyruvate metabolism in *Escherichia coli*. J. Bacteriol. 93:1644–1650.
13. Ramsey, H. A. 1963. Separation of organic acids in blood by partition chromatography. J. Dairy Sci. 46:480–483.
14. Tanaka, S., and S. Iuchi. 1971. Induction and repression of an extracellular proteinase of *Vibrio parahaemolyticus*. Biken J. 14:81–96.
15. Weinberg, E. D. 1970. Biosynthesis of secondary metabolites: role of trace metals, p. 1–44. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 4, Academic Press Inc., New York.
16. Weirether, F. J., E. E. Lewis, A. J. Rosenwald, and R. E. Lincoln. 1966. Rapid quantitative serological assay of staphylococcal enterotoxin B. Appl. Microbiol. 14:284–291.