INFECTION AND IMMUNITY, Sept. 1977, p. 629-633

Vol. 17, No. 3
Printed in U.S.A.

Repression of Heat-Stable Enterotoxin Synthesis in Enterotoxigenic *Escherichia coli*

JOHN F. ALDERETE AND DONALD C. ROBERTSON

Department of Microbiology, University of Kansas, Lawrence, Kansas 66045

Received for publication 14 March 1977

Five different carbon sources were examined for their ability to control synthesis of heat-stable enterotoxin (ST) by enterotoxigenic (ENT$^+$) *Escherichia coli* grown in either a defined medium containing four amino acids or a minimal salts medium. No ST activity was observed when D-glucose, D-gluconate, and L-arabinose were added separately to the defined medium, whereas glycerol and pyruvate decreased toxin levels. Similar results were obtained using a minimal salts medium, except with pyruvate, which did not support growth. Inhibition of ST synthesis by D-glucose was overcome by the addition of 3×10^{-3} M cyclic adenosine 3',5'-monophosphate. Glucose repression of β-galactosidase synthesis under conditions optimal for inhibition of ST synthesis was also reversed by exogenous cyclic adenosine 3',5'-monophosphate in the presence of the inducer isopropyl-β-D-thiogalactopyranoside. The data suggest that control mechanisms for the synthesis of plasmid gene products of bacterial pathogens are similar to those exerted on the host chromosome.

The repression of inducible enzyme synthesis by glucose is a well-known phenomenon (14, 15). Also, synthesis of several bacterial exotoxins is inhibited by the presence of glucose in the growth medium; for example, synthesis of staphylococcal alpha-toxin and staphylococcal enterotoxins A, B, and C are subject to glucose repression (4, 8). Calandra and Oginsky (2) showed that the synthesis of streptolysin S by *Streptococcus pyogenes* was inhibited by glucose and could not be reversed by addition of exogenous cyclic adenosine 3',5'-monophosphate (AMP) at 4×10^{-7} M. The synthesis of a heat-stable, rapid-acting permeability factor produced by *Salmonella typhimurium* was either decreased or inhibited in a complex medium supplemented with glucose (17). Despite the evidence for catabolite repression of exotoxin synthesis by bacterial pathogens, little evidence exists as to the possible role(s) of cyclic AMP in the regulatory process.

During studies on the nutritional requirements for synthesis of heat-stable enterotoxin (ST) by enterotoxigenic (ENT$^+$) strains of *Escherichia coli*, we observed that glucose inhibited ST production in either a complex medium of Casamino Acids-yeast extract or a defined medium of four amino acids (1). Since it is known that plasmids determine biosynthesis of ST and heat-labile enterotoxin (LT) in ENT$^+$ strains of *E. coli* (6), it was of interest to examine the mechanism of glucose repression and whether it could be reversed by exogenous cyclic AMP. The defined medium that supports ST synthesis at levels equivalent to complex media seemed suitable to study factors involved in regulation.

MATERIALS AND METHODS

Cultures. ENT$^+$ strains of *E. coli* used in this study were supplied by Harley Moon of the National Animal Disease Center, Ames, Iowa. Strain 431 produces only ST, and strain 1362 produces both LT and ST (1). Samples were screened for ST as described below, and LT was determined by the adrenal tumor cell assay (9, 10). Non-enteropathogenic *E. coli* strain O111B4 was used as a control throughout these studies and was kindly provided by Clarence Buller of this department.

Media and growth conditions. The minimal salts medium used in these experiments was prepared as described previously (1) and consisted of basal salts (NaCl, K_2HPO_4, and NH_4Cl), trace salts ($MgSO_4$, $MnCl_2$, and $FeCl_3$), and a metal chelator, tricine. The defined medium contained four amino acids (serine, aspartic acid, proline, and alanine) at concentrations found to be optimal for ST synthesis (1), in addition to components of the minimal salts medium. All sugars were prepared as 25% stock solutions (wt/vol). The minimal salts medium and the amino acids solutions were prepared as two- and tenfold stock solutions, respectively, adjusted to pH 7.5 with 5 N NaOH, and sterilized by autoclaving. Each component was added aseptically to sterile 250-ml Erlenmeyer flasks, with sterile distilled water to a final volume of 50 ml.

Starter cultures were grown in M-9 minimal medium (13) for 8 h, and each experimental flask was inoculated with the starter culture using a volume to give an initial absorbance of 0.050 (620 nm), which

corresponded to 8.6×10^7 colony-forming units/ml for strain 431 and 1.14×10^8 colony-forming units/ml for strain 1362. The flasks were incubated at 37°C on a New Brunswick rotary shaker (300 rpm). The initial pH was 7.5, and samples were withdrawn at hourly intervals for measurement of optical density at 620 nm, pH, and ST levels. The samples removed did not significantly reduce the total volume. Cells were removed by centrifugation in sterile centrifuge tubes at 23,500 × *g* for 30 min, and culture supernatants were assayed immediately. A bulk inoculation technique was used to obtain a culture of ENT$^+$ *E. coli*, which utilized gluconate as a primary carbon source in a minimal salts medium. A 10% inoculum of ENT$^+$ *E. coli* grown in the defined medium was added to a minimal salts-gluconate (1%) medium and incubated as described above. Samples (3 to 5 drops) were removed every 24 h and inoculated into fresh minimal salts-gluconate medium. After 2 or 3 days of incubation of the bulk inoculated culture, ENT$^+$ *E. coli* were obtained that were able to utilize gluconate as a primary carbon source. The selected organisms retained the ability to synthesize ST when transferred back to the defined medium (1). Alternatively, starter cultures can be prepared using 0.1% D-glucose and 0.5% gluconate with 8 h of incubation.

ST assay. The assay for ST has been described (1). Relative toxin levels were determined by serial twofold dilutions, and the last dilution that gave a gut weight-to-body weight ratio greater than or equal to 0.090 was considered the last positive response. Nonenterotoxigenic *E. coli* strain O111B4 grown under similar conditions consistently gave gut weight-to-body weight ratios of 0.060 ± 0.005. All medium components assayed by using the same concentrations as those used in the defined medium gave negative toxin responses. Each experimental sample was assayed by using three mice, and each experiment was done in triplicate.

β-Galactosidase assay and cyclic AMP addition. Each strain of ENT$^+$ *E. coli* was grown as described above with the exception that cyclic AMP (final concentration, 3×10^{-3} M) was added to duplicate flasks after 4 h of growth. Isopropyl-β-D-thiogalactopyranoside (final concentration, 1.1×10^{-4} M) was also added to one of the experimental flasks. Detectable levels of ST were observed at 4 h when ENT$^+$ *E. coli* were grown in either a defined or complex medium (1). At hourly intervals, β-galactosidase activity was determined using the colorimetric assay based upon the enzymatic hydrolysis of *o*-nitrophenyl-β-galactoside (16). One drop of toluene was added to 1 ml of culture supernatant, followed by vigorous shaking. Two milliliters of *o*-nitrophenyl-β-galactoside (final concentration, 6×10^{-3} M, prepared fresh in 1.0 M Na_2HPO_4, pH 7.0) was added, and the mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 1.0 M Na_2CO_3 (1.0 ml) and centrifuged, and the optical density of the supernatant was measured at 420 nm.

Reagents used. All carbon sources and reagents were purchased from Sigma Chemical Co. except for glycerol, which was obtained from Mallinckrodt.

RESULTS

Growth, pH, and toxin. The effect of several carbon sources on the synthesis of ST was examined either by addition to minimal salts medium (primary carbon source) or by supplementing a defined medium containing four amino acids. When ENT$^+$ *E. coli* strain 431 was grown in the presence of D-glucose, D-gluconate, or L-arabinose, no ST activity was detectable in the supernatant compared with the supernatant of the unsupplemented defined medium (Table 1). Also, no toxin was present when the sugars were used as primary carbon sources in a minimal salts medium. Glycerol, used either as a primary carbon source or to supplement the defined medium, showed decreased toxin levels as compared with the defined medium control (1:2 versus 1:16 dilution of supernatant). No growth was obtained with pyruvate as a primary

TABLE 1. *Effect of carbon sources on ST synthesis by ENT$^+$ E. coli strain 431*

Carbon source[a]	Defined medium[b]			Minimal salts medium[c]		
	OD_{620}[d]	Final pH	Toxin activity[e]	OD_{620}	Final pH	Toxin activity
No addition	3.5	7.9	1:16	NG[f]	NG	NG
D-Glucose	5.5	5.2	ND[g]	3.5	4.8	ND
D-Gluconate	5.5	6.5	ND	5.5	6.6	ND
L-Arabinose	3.0	5.2	ND	3.0	5.0	ND
Glycerol	3.2	7.0	1:2	3.0	6.2	1:2
Pyruvate	3.0	7.9	1:8	NG	NG	NG

[a] All carbon sources were used at a 1.0% final concentration.

[b] Defined medium was as described in the text and contained four amino acids at a concentration equivalent to 2.0% Casamino Acids.

[c] Minimal salts medium was as described in the text and contained basal salts, trace salts, and tricine.

[d] OD_{620}, Optical density at 620 nm.

[e] Toxin assay after 8 h of incubation; last dilution to give a gut weight-to-body weight ratio greater than or equal to 0.090.

[f] NG, No growth.

[g] ND, No toxin detectable in the supernatant.

carbon source, and addition to the defined medium decreased toxin levels. Synthesis of ST by *E. coli* strain 1362, which produces both ST and LT, was also inhibited by addition of various carbon sources (Table 1) to the defined medium. Furthermore, growth and pH changes in the defined medium and M-9 minimal salts medium were similar for both strains, with slightly lower ST levels produced by strain 1362.

The decreased pH observed with D-glucose, D-gluconate, and L-arabinose, but not pyruvate, suggested that pH may be important in elaboration of ST. The use of morpholinopropane sulfonate to control the pH (1, 13) or manual adjustment of pH did not result in any detectable ST with the suckling mouse assay; consequently, a pH effect as observed for *Vibrio cholerae* (3) did not seem to play a role. Decreased ST activity appeared to be due to repression of ST synthesis when the various carbon sources were added to the defined medium or when used as the primary carbon source in a minimal salts medium.

Effect of exogenous cyclic AMP on ST synthesis. The relative toxin levels of ENT$^+$ *E. coli* strain 431, which produces only ST, and strain 1362, which produces both ST and LT, grown in a defined medium, in the presence and absence of 0.2% glucose are shown in Table 2. Addition of 0.2% glucose to the defined medium or minimal salts medium resulted in detectable toxin only in the undiluted supernatant compared with the unsupplemented defined medium, where 1:16 and 1:8 dilutions of supernatant were the last dilutions to give a positive response for strains 431 and 1362, respectively. Exogenous cyclic AMP (3×10^{-3} M) added at 4 h of growth (Fig. 1) resulted in derepression of toxin synthesis when assayed at 8 and 12 h of growth. Enterotoxin levels were similar when 0.2% glucose was used as the primary carbon source or when it was added to the defined medium in the presence of exogenous cyclic AMP. It is important to note that when toxin was observed after derepression with exogenous cyclic AMP, the pH was not adjusted with glucose as the primary carbon source (Fig. 1). Also, much to our surprise, the presence of cyclic AMP in the defined medium increased ST levels at 8 h. High levels of exogenous cyclic AMP, up to 15×10^{-3} M, did not cause fluid accumulation in the mouse gut, thereby eliminating a possible false positive response. Supernatants from non-enterotoxigenic *E. coli* strain O111B4 did not elicit fluid accumulation under any condition optimal for ST synthesis.

TABLE 2. *Effect of exogenous cyclic AMP on ST synthesis by ENT$^+$ E. coli grown in different media*

Medium	Addition of 3×10^{-3} M cyclic AMP	Toxin activity[a]	
		Strain 431	Strain 1362
Defined[b]	−	1:16	1:8
	+	1:64	1:32
Defined + 0.2% D-glucose	−	Undiluted[c]	Undiluted
	+	1:8	1:8
Minimal salts + 0.2% D-glucose	−	Undiluted	Undiluted
	+	1:16	1:8

[a] As described in Table 1.

[b] Contains four amino acids as described in the text at concentrations equivalent to 2.0% Casamino Acids.

[c] Undiluted, Toxin detected in the undiluted supernatant only.

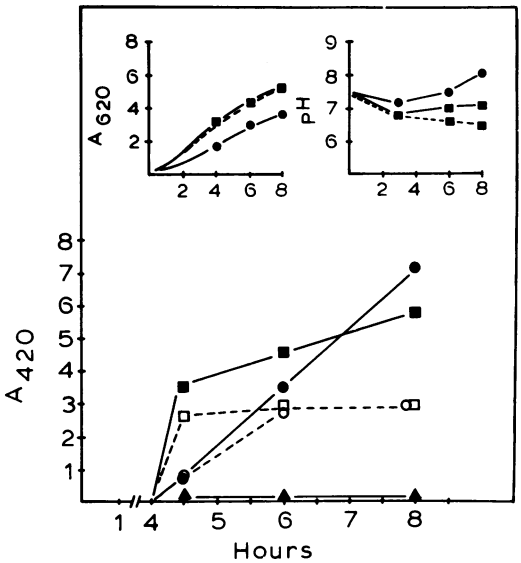

FIG. 1. *β-Galactosidase activity, growth (optical density at 620 nm), and pH in various media. Symbols: β-Galactosidase activity in defined medium and minimal salts medium in the presence of 0.2% D-glucose (▲); β-galactosidase activity in defined medium + isopropyl-β-D-thiogalactopyranoside (IPTG) (○); β-galactosidase activity in defined medium and minimal salts medium with 0.2% D-glucose + IPTG (□); β-galactosidase activity in defined medium + IPTG + cyclic AMP (●); β-galactosidase activity in defined medium and minimal salts medium with 0.2% D-glucose + IPTG + cyclic AMP (■). Inserts show growth (optical density at 620 nm) and change in pH in defined medium (●——●), defined medium with 0.2% D-glucose (■——■), and minimal salts medium with 0.2% D-glucose (■·····■).*

The induction of β-galactosidase as a control under the same conditions was examined to give an indication of the regulatory mechanisms on chromosomal versus extrachromosomal deoxyribonucleic acid. β-Galactosidase was not detectable unless an inducer, isopropyl-β-D-thiogalactopyranoside, was added, and levels were further increased by addition of exogenous

cyclic AMP (Fig. 1). The presence of inducer, with or without exogenous cyclic AMP, appeared to give higher rates of enzyme synthesis for ENT$^+$ *E. coli* grown with glucose compared with growth in a defined medium without glucose. It is important to note that although previous investigators (14, 15) have not reported derepression of inducible enzymes after 30 min, it was necessary to assay β-galactosidase levels up to 4 h after addition of isopropyl-β-D-thiogalactopyranoside and cyclic AMP for comparison with ST synthesis. As might be expected, the synthesis of β-galactosidase continued with only addition of inducer, and increased levels were observed with subsequent addition of cyclic AMP when measured at hourly intervals up to 8 h of incubation.

DISCUSSION

Recently it was shown that regulation of plasmid-coded lactose genes is similar to that observed on the host chromosome (5). The enzymes for lactose utilization were induced by different galactosides and sensitive to catabolite repression. Since control mechanisms on chromosomal and extrachromosomal deoxyribonucleic acid may be similar, it was of interest to examine the effect of exogenous cyclic AMP on ST synthesis when glucose was present in the growth medium. Addition of exogenous cyclic AMP yielded ST levels similar to those observed in a defined medium in the absence of glucose (1). The derepression of β-galactosidase synthesis with inducer and cyclic AMP under conditions optimal for inhibition of ST synthesis suggested that similar control mechanisms are involved. Furthermore, the data reported in this paper suggest that synthesis of ST by ENT$^+$ *E. coli* is subject to the same regulatory mechanisms as are many inducible enzymes (β-galactosidase, tryptophanase, D-serine deaminase, and β-glucuronidase) (14, 15).

It is noteworthy that derepression of ST synthesis was observed when glucose was used as the primary carbon source in a minimal salts medium. It was not necessary to adjust the pH due to glucose catabolism, supporting the observation that ST is not subject to a pH effect, as is observed for release of cholera toxin when *V. cholerae* is grown in the presence of glucose (3). Furthermore, it was not necessary to either osmotically shock the ENT$^+$ *E. coli* by the procedure of Nossal and Heppel (7) or release lipopolysaccharide by ethylenediaminetetraacetic acid treatment by the procedure of Leive (11, 12) to observe toxin activity, which supports previous observations that ST is not kept within the periplasmic space or bound to the outer membrane of ENT$^+$ *E. coli* (1). In a minimal salts medium, glucose can support ST synthesis in the presence of exogenous cyclic AMP; thus, the amino acids found to give toxin levels equivalent to the complex medium may serve both as a carbon source for ST synthesis and to produce optimal cyclic AMP levels for derepression.

Since the synthesis of ST appears to be subject to catabolite repression by various carbon sources while maximal levels of LT are found in the presence of glucose (data not shown), the data suggest that ST and LT are distinct species, each subject to different control mechanisms on their respective plasmids. Furthermore, it would appear that ST is not a subunit of LT, as had been previously suggested (6, 18). A better understanding of the factors that govern the synthesis of each enterotoxin will aid in our understanding of their contribution and role(s) in the disease process.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-12357 under the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases. John F. Alderete is the recipient of a Ford Foundation Fellowship.

LITERATURE CITED

1. **Alderete, J. F., and D. C. Robertson.** 1977. Nutrition and enterotoxin synthesis by enterotoxigenic strains of *Escherichia coli*: defined medium for production of heat-stable enterotoxin. Infect. Immun. **15**:781-788.
2. **Calandra, G. B., and E. L. Oginsky.** 1975. Cellular streptolysin S-related hemolysins of group A streptococcus C203S. Infect. Immun. **12**:13-28.
3. **Callahan, L. T., III, and S. H. Richardson.** 1973. Biochemistry of *Vibrio cholerae* virulence. Nutritional requirements for toxin production and the effects of pH on toxin elaboration in chemically defined medium. Infect. Immun. **7**:567-572.
4. **Duncan, J. L., and G. J. Cho.** 1972. Production of staphylococcal alpha-toxin. II. Glucose repression of toxin formation. Infect. Immun. **6**:689-694.
5. **Guiso, N., and A. Ullmann.** 1976. Expression and regulation of lactose genes carried by plasmids. J. Bacteriol. **127**:691-697.
6. **Gyles, C., M. So, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**:40-49.
7. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055-3062.
8. **Jarvis, A. W., and R. C. Lawrence.** 1975. Glucose repression of enterotoxins A, B, and C and other extracellular proteins in staphylococci in batch and continuous culture. J. Gen. Microbiol. **86**:75-87.
9. **Kwan, C. N., and R. M. Wishnow.** 1974. Adenosine-3',5'-cyclic monophosphate and adrenal tumor cell morphology. In Vitro **10**:268-273.
10. **Kwan, C. N., and R. M. Wishnow.** 1974. Cholera enterotoxin stimulation of cAMP in cultured adrenal tumor cells. Exp. Cell Res. **87**:436-438.
11. **Leive, L.** 1965. A nonspecific increase in permeability in *Escherichia coli* produced by EDTA. Proc. Natl. Acad. Sci. U.S.A. **53**:745-750.

12. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. **21**:290–296.
13. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119**:736–747.
14. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine-5'-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
15. **Perlman, R., and I. Pastan.** 1968. Cyclic 3',5'-AMP: stimulation of β-galactosidase and tryptophanase induction in *E. coli*. Biochem. Biophys. Res. Commun. **30**:656–664.
16. **Revel, H. R., S. E. Luria, and B. Rotman.** 1961. Biosynthesis of beta-D-galactosidase controlled by phage-carried genes. I. Induced beta-D-galactosidase biosynthesis after transduction of gene Z+ by phage. Proc. Natl. Acad. Sci. U.S.A. **47**:1956–1967.
17. **Sandefur, P. D., and J. W. Peterson.** 1976. Isolation of skin permeability factors from culture filtrates of *Salmonella typhimurium*. Infect. Immun. **14**:671–679.
18. **Santos, D. S., S. Palchaudhuri, and W. K. Maas.** 1975. Genetic and physical characteristics of an enterotoxin plasmid. J. Bacteriol. **124**:1240–1247.