The Relation Between Glucose Repression of Endo-Polygalacturonate Trans-Eliminase and Adenosine 3′5′-Cyclic Monophosphate Levels in Erwinia carotovora

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ABSTRACT

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The level of intracellular endo-polygalacturonate *trans*-eliminase (PGTE) activity by *Erwinia carotovora* on minimal salts medium was affected by the available carbon source. When cells growing on sodium polypectate were supplemented with glucose, the level of intracellular PGTE activity decreased. Exogenously supplied adenosine 3′,5′-cyclic monophosphate (cAMP) reversed repression of intracellular PGTE activity if the substrate of the enzyme, sodium polypectate, was present. Various analogues of cAMP and other nucleotide derivatives failed to reverse glucose repression of PGTE activity. Measurements of cAMP demonstrated that a decrease in cAMP could be correlated with glucose repression of PGTE activity. As the PGTE activity increased in induced cultures, the specific concentration of cAMP increased.

Additional key words: catabolite repression.

The production of an endo-polygalacturonate *trans*-eliminase (PGTE) by *Erwinia carotovora* plays an integral role in soft rot disease development. Studies employing mutants of *E. carotovora* have implied an essential role for this enzyme in pathogen virulence (4, 8) and purified PGTE preparations cause both tissue maceration and cellular death (16).

Synthesis of PGTE by *E. carotovora* has been reported to be either constitutive, but subject to catabolite repression (15), or inducible (25, 26). Until recently there appeared to be no clear understanding of the mechanism(s) of catabolite or glucose repression of this enzyme as it exists in prokaryotic organisms. The integral role of adenosine 3′,5′-cyclic monophosphate (cAMP) as a regulator in this process has been demonstrated for a number of enzymes (18).

The level of cAMP in *Escherichia coli* cells decreases during catabolite repression of certain enzymes. These changes seem to be correlated with the rate of transcription, but not with translation of the affected operon (17, 22). Exogenously added cAMP has been found effective in overcoming glucose repression of certain catabolic enzymes in *E. coli* (18, 24). Furthermore, mutants defective in adenylate cyclase or a cyclic nucleotide binding protein do not form normal levels of catabolically repressed enzymes (7, 20). Primary evidence concerning site and mode of action of cAMP in bacteria has been obtained from studies with cell-free systems which indicate that cAMP and a cAMP binding protein form a complex at the promoter region of the operon that initiates transcription by facilitating RNA polymerase binding (17, 22).

Although it is clear that synthesis of PGTE in *E. carotovora* is subject to glucose repression and that the bacteria possess adenylate cyclase activity (10), no attempts have been made to determine if levels of cAMP are associated with conditions that lead to repression of pectic enzyme synthesis in this organism. We wish to report on our observations regarding the reversal of glucose repression of intracellular PGTE activity by an exogenous supply of cAMP and our measurements of cAMP levels in association with glucose repression.

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MATERIALS AND METHODS

Culture and growth conditions.—*Erwinia carotovora* (Jones) Holland (isolate EC-14) used in these experiments was obtained from R. Dickey, Cornell University, and maintained on nutrient agar slants. All experiments were carried out in 250-ml, cotton-plugged, side-arm flasks, usually containing no more than 50 ml of liquid media. Flasks were shaken on a rotary shaker at 125 oscillations min^{-1} in an incubator at 30C.

Glucose repression was studied in fully induced cultures growing on a minimal salts medium (25) supplemented with 0.5% (w/v) sodium polypectate (Sigma Chemical Co., Box 14508, St. Louis, MO 63118) initiated with cells obtained from a similar overnight culture. During log phase these cultures were divided in halves; one half was supplemented with glucose to make a final concentration of 0.5%, and the other half was retained as a control. The effect of an exogenous supplement of various nucleotide derivatives during

glucose repression was studied by adding the nucleotide at the time glucose was added to a culture previously growing on sodium polypectate as the sole carbon source. Cyclic AMP, ATP, 8-bromo cAMP, 8-methylthio cAMP, dibutyryl cAMP, 5′ AMP, and adenosine (Sigma Chemical Co.) were filter-sterilized before adding them to the culture to make a final concentration of 5 mM.

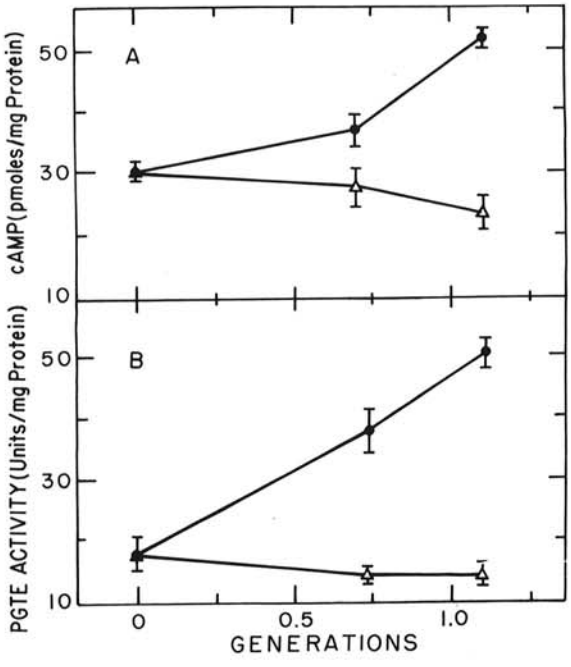

Fig. 1-(A, B). The association of adenosine 3′,5′-cyclic monophosphate (cAMP) levels and endo-polygalacturonate *trans*-eliminase (PGTE) activity during glucose repression in *Erwinia carotovora*. Log phase cultures growing on sodium polypectate were supplemented with glucose at a time designated as zero generation. **A)** Intracellular cAMP concentrations in a bacterial culture incubated on sodium polypectate (•—•) and sodium polypectate plus glucose (Δ—Δ). **B)** Activity of PGTE in cultures growing on sodium polypectate (•—•) and sodium polypectate plus glucose (Δ—Δ). The bars represent the percent deviation from the mean value.

TABLE 1. The effect of an exogenous supply of adenosine 3′,5′-cyclic monophosphate (cAMP) during glucose repression of endo-polygalacturonate *trans*-eliminase (PGTE) activity by *Erwinia carotovora* 1 and 2 hr after treatment[a]

Medium supplemented with:	PGTE at 1 hr		PGTE at 2 hr	
	units/mg protein	% of control	units/mg protein	% of control
Glucose	11.0	55	9.2	33
Glucose + 0.5 mM cAMP	12.6	63	20.7	74
Glucose + 1 mM cAMP	16.8	84	23	83
Glucose + 5 mM cAMP	31.5	148	41.2	147
Control	20	100	28.0	100

[a]Cultures were supplemented with 0.5, 1, or 5.0 mM (final concentration) cAMP simultaneously with the addition of 0.5% (w/v) glucose to cultures preincubated on a sodium polypectate minimal salts medium. Samples were removed for determination of PGTE activity 1 and 2 hr later. Levels of PGTE activity are presented as percentages of the level observed in a culture incubated on sodium polypectate alone (control).

Enzyme extraction and assay.—At various intervals bacterial cell growth was measured by determining the OD at 600 nm in a spectrophotometer. At this time 2-ml samples were removed from cultures and centrifuged at 10,000 *g* for 10 min in a Sorvall RC2-B centrifuge. Pelleted cells were resuspended in 2 ml of 0.5 M Tris-HCl buffer at pH 8.3 and sonicated for 3 min at 30 W in a Bronson ultrasonifier fitted with a microtip. Cell debris was removed by centrifugation as above and soluble endo-polygalacturonate *trans*-eliminase (PGTE) activity was determined by following the increase in absorbance at 235 nm (2). The PGTE activity was measured in a reaction mixture containing 1 ml of enzyme preparation and 1 ml of substrate [0.05% (w/v) polygalacturonic acid (Sigma Chemical Co.) in 0.05 M Tris-HCl buffer at pH 8.3, containing 1×10^{-5} M $CaCl_2$]. One unit of enzyme activity was defined as that amount of enzyme causing an increase in absorbance of 0.1 OD units in 1 min. The specific activity of PGTE varied according to the time during the log phase of the bacterial growth curve when samples were assayed. In a similar manner, the degree of repression of PGTE varied depending upon the time during log phase when glucose was added to the cultures. Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as a standard.

Cyclic nucleotide extraction and assay.—One-ml samples were removed at the same time other samples were removed from cultures for the PGTE assay. These samples also were centrifuged at 10,000 *g* for 10 min but were immediately fixed and extracted for cyclic nucleotides in 1 ml of 7% perchloric acid. Cells were sonicated in perchloric acid for 3 min as above and protein was pelleted by centrifuging at 10,000 *g* for 15 min. The amount of protein in the pellet was determined and the supernatant liquid was neutralized with 6 N KOH and frozen.

Prior to assaying for cAMP, the $KClO_4$ precipitate was pelleted and samples were diluted 1:10 with 0.05 M phosphate buffer at pH 6.2. The concentration of cyclic AMP in the samples were determined by a modified radioimmunossay procedure (9) employing an I^{125} ScAMP antigen and specific antibody, both obtained from Collaborative Research, 1365 Main St., Waltham, MA 02154.

An acetylated derivative of the nucleotide was produced in the samples by adding 14 μliters of a 2:1 (v/v) mixture of triethylamine and acetic anhydride (Eastman Kodak Co., Rochester, NY 14650) to 0.5 ml of each sample as described by Harper and Brooker (9). One hundred microliters of appropriately diluted antibody and I^{125} ScAMP containing 30 mg/ml bovine serum albumin each were added to 100 μliters of the diluted and acetylated sample and the entire reaction mixture was incubated for 20 hr at 4 C. The antibody-antigen complex was precipitated by the addition of 1.6 ml of 95% ethanol and collected by centrifugation at 7,500 *g* for 20 min. The amount of I^{125} bound in each unknown sample was determined by counting in a Searle 1175-Z gamma counter. The concentration of cAMP in each sample was established by comparison with a standard curve in the range of 0 to 200 femtomoles (10^{-15} moles) per sample. In order to verify authenticity of cAMP quantified by

radioimmunoassay procedure, random samples were digested for 30 min at 30 C at pH 6.2 with 0.1 unit beef heart cyclic nucleotide phosphodiesterase (Sigma Chemical Co.). One unit of this enzyme hydrolyzes 1 μmole of cAMP to 5′ AMP in 1 min at 30 C.

RESULTS

Glucose repression of intracellular PGTE activity was studied in samples of cultures growing in log phase on a minimal salts medium supplemented with 0.5% sodium polypectate at 30 C. Under these conditions *E. carotovora* had a generation time of approximately 1.8 hr. The addition of glucose to such a culture previously induced for PGTE production resulted in a decrease in specific activity of PGTE during the next 1.3 generations (Fig. 1).

In order to study the effect of an exogenous supply of cAMP during glucose repression, the nucleotide was supplemented at either 0.5, 1.0, or 5.0 mM final concentration to cultures simultaneously with the addition of glucose. When cAMP was added at these concentrations to cells subjected to glucose repression of PGTE synthesis, the specific activity of PGTE in the cell extracts increased above the specific activity levels in the glucose-repressed control (Table 1). When 5.0 mM cAMP was added during glucose repression, the specific activity of PGTE was well above that observed in nonrepressed controls (Table 1). Analogues of cAMP and other adenine derivatives were ineffective in bringing about reversal of glucose repression (Table 2).

It was observed also that within one generation after cAMP and glucose were added to induced cultures a decrease in growth rate occurred (Fig. 2). This was correlated with both the concentration of exogenous cAMP and the corresponding increase in PGTE activity. Apparently this decreased in growth rate was not a true stationary phase because cells eventually began to divide again after an additional 4 hr of incubation, presumably when cAMP had decreased to lower levels.

Estimation of cyclic AMP levels in repressed and induced cultures by radioimmunoassay of the acetylated derivatives enabled a considerable increase in sensitivity since the derivatives compete more effectively than nonacetylated cAMP with I^{125} ScAMP for the antibody. Intracellular cAMP levels in cell extracts, calculated as a function of protein concentration, showed that the decrease in cAMP concentration in glucose repressed cells was associated with a decrease in PGTE levels (Fig. 1).

Digestion of random samples with beef heart cyclic nucleotide phosphodiesterase verified that close to 100% of the activity measured in the radioimmunoassay was authentic cAMP.

TABLE 2. Effect of an exogenous supply of analogues of adenosine 3′,5′-cyclic monophosphate (cAMP) as well as various other nucleotide derivatives on glucose repression of endo-polygalacturonate *trans*-eliminase (PGTE) activity by *Erwinia carotovora*[a]

Analogue	PGTE activity (units/mg protein)	PGTE activity (% of control)
Control	23	100
Glucose alone	13.3	58
ATP	13.3	58
8-bromo cAMP	14.9	65
8-methylthio cAMP	11.9	52
Dibutyryl cAMP	13.1	57
5′ AMP	7.6	33
Adenosine[b]	6.9	30

[a]Glucose (0.5% w/v) and designated analogues (5 mM) were added simultaneously to a culture growing on sodium polypectate. Two hr (1.1 generations) later, samples were removed and assayed for PGTE specific activity.

[b]Cultures to which adenosine was supplemented ceased growing after 0.75 generations.

DISCUSSION

Only changes in intracellular levels of PGTE are discussed in this report. Although it is known that intra- and extracellular PGTE are similar (14), the various parameters and mechanisms which may be associated with extracellular accumulatiuon of PGTE are as yet poorly defined. Therefore, it seemed logical to study intracellular accumulation of PGTE as this would reflect directly the effects of glucose repression and cAMP concentrations.

An exogenous supply of cAMP at 0.5, 1.0, or 5.0 mM was effective to various degrees in reversing glucose repression of PGTE synthesis. Reversal of catabolite repression by cAMP, as noted here (Table 1), has been reported in association with other enzyme systems (18, 24). For example, cAMP effects a reversal of catabolite repressed β-galactosidase synthesis in *E. coli* (19).

In agreement with previous investigations with other organisms (18) it was found that only 3′,5′-cAMP was effective in causing a reversal of glucose repression

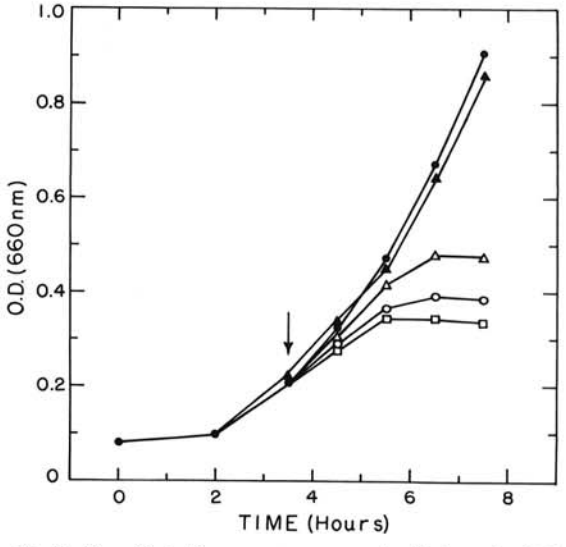

Fig. 2. The effect of an exogenous supply of adenosine 3′,5′-cyclic monophosphate (cAMP) on growth rate of *Erwinia carotovora* during glucose repression. Cyclic AMP and glucose (0.5%) were added (arrow) to log phase cultures growing on sodium polypectate. The final concentrations of supplemented cAMP were 5.0 mM (□—□), 1 mM (*o*—*o*) and 0.5 mM (Δ—Δ). No deviations in growth rates were observed between cultures supplemented with glucose (▲—▲) or sodium polypectate alone (•—•).

indicating that levels of cAMP may play a specific role during glucose repression in *E. carotovora* as well.

Growth inhibition by cAMP during glucose repression (Fig. 2) is associated with both the concentration of cAMP and changes in specific activity of PGTE. The mechanism which leads to inhibition of growth is not clear. One possibility is that a rapid breakdown of cAMP occurs with a subsequent accumulation of adenosine, thus causing an imbalance in the nucleotide pool. As indicated in Table 2, supplemental adenosine brought about a cessation of growth of cultures within 0.75 generations. Alternately growth inhibition may be due to some form of aberrant catabolism (1, 6).

Rapid changes in intracellular levels of cAMP have been associated with both transient and permanent forms of catabolite repression in *E. coli* (17). Data presented here indicate that catabolite repression of intracellular PGTE activity is associated with a decrease in intracellular levels of cAMP in *E. carotovora* as well. This is of interest in that catabolite repression may not always be associated with cellular cAMP levels. For example, catabolite repression of glucose-6-phosphate dehydrogenase in *Pseudomonas aeruginosa*, an anaerobe, may not be associated with changes in intracellular levels of cAMP (23), although the organism does possess adenylate cyclase and phosphodiesterase enzymes.

Although dynamics of cAMP accumulation have been studied extensively in *E. coli* under various conditions resulting in catabolite repression, no fully reasonable hypothesis concerning factors mediating intracellular accumulation of the nucleotide have been proposed. Regulation of intracellular levels of cAMP may be achieved by modulation of adenylate cyclase activity. Although no effect upon adenylate cyclase activity by glucose can be observed when the enzyme is extracted from cells, a sugar repression of cyclase activity could be demonstrated in an in vivo assay (21).

Glucose repression of polysaccharide-degrading enzymes has been discussed in relation to a number of phytopathogens (3) but little is known regarding the physiology of this form of repression in this class of organisms. Regulation of sugar levels in potato tissues has been correlated with a decrease in tissue breakdown, presumably as a result of sugar repression of the pectic enzymes of *E. carotovora* (5). Furthermore, latency of soft rot disease development following inoculation of healthy cucumber tissues with *E. carotovora* may be associated with a repression of pectic enzyme synthesis by some factor(s) present in the host tissues (13). Although it was demonstrated that *E. carotovora* contains a cAMP generating system, there is no conclusive evidence that cAMP exists in higher plants (11) and thereby contributes to the overall regulation of the pathogen interaction with its host. Data presented in this report demonstrate a correlation between glucose repression of intracellular levels of PGTE and cellular levels of cAMP in *E. carotovora*.

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