Regulation of Endopolygalacturonate Transeliminase in an Adenosine 3′,5′-Cyclic Monophosphate-Deficient Mutant of *Erwinia carotovora*

M. S. Mount, P. M. Berman, R. P. Mortlock, and J. P. Hubbard

Associate professor, research assistant, professor, and graduate research assistant, respectively, Department of Plant Pathology, University of Massachusetts, Amherst, MA 01003. Address of third author: Department of Microbiology, University of Massachusetts, Amherst, MA 01003.

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ABSTRACT

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An adenosine 3′,5′-cyclic monophosphate (cAMP)-deficient mutant of *Erwinia carotovora* was isolated from a nitrosoguanidine-treated β-galactosidase constitutive strain. The mutant was unable to use α-lactose, L-arabinose, D-galactose, L-rhamnose, D-xylose, raffinose, D-cellobiose, glycerol, and sodium polypectate as sole carbon sources unless exogenous cAMP was supplied in the medium. The catabolism of D-ribose, D-mannitol, and D-glucose was not affected in the cAMP-deficient mutant. The synthesis of the inducible enzyme, endopolygalacturonate *trans*eliminase (PGTE), was under the direct control of the cAMP regulatory mechanism. The cAMP-deficient mutant, growing on a casein hydrolysate-minimal salts medium, produced only low levels of PGTE, even in the presence of the inducer, sodium polypectate. When both sodium polypectate and cAMP were added, however, the levels of PGTE were elevated to levels comparable with those of the induced parent strain.

Pectic enzymes are polysaccharide-degrading enzymes that facilitate the ingress and breakdown of plant tissues by many phytopathogens. Endopolygalacturonate *trans*eliminase (EC 4.2.2.2) (PGTE), an extracellular enzyme produced by *Erwinia carotovora*, is reponsible for tissue maceration and development of soft rot disease in vegetables (15). Studies with purified PGTE have demonstrated that this enzyme causes electrolyte loss, maceration, and cellular death in potato and cucumber tissues (15,19). Furthermore, studies with *E. carotovora* mutants indicate that PGTE is the principal enzyme that determines virulence in the pathogen (3,7).

The production of many inducible enzymes by microorganisms is subject to catabolite repression when the organisms are grown in the presence of certain carbohydrates (18). In several strains of *E. carotovora*, the synthesis of PGTE is induced in the presence of pectin substrates and is subject to catabolite repression by glucose (9,14,22,23). It also has been observed that adenosine 3′,5′-cyclic monophosphate (cAMP) prevents this repression and that cellular levels of cAMP are correlated directly with the amount of PGTE activity (9). The importance of cAMP in the regulation of inducible enzyme systems in other prokaryotes is well established (20). Therefore, the levels of cAMP may play a specific role in the synthesis of PGTE in *E. carotovora*, but no genetic data prove that PGTE synthesis is under cAMP regulation. The objective of this investigation was to isolate a cAMP-deficient mutant of *E. carotovora* to clarify the role of cAMP in the synthesis of PGTE.

MATERIALS AND METHODS

Bacterial strains and cultural conditions.—The parent strain used in this investigation was isolate EC14 of *Erwinia carotovora* (Jones) Holland obtained from R. Dickey, Cornell University, and was maintained on nutrient agar slants at 30 C. All derivatives of this strain are described in Table 1. Unless otherwise specified, the bacteria were grown aerobically at 30 C on a minimal salts (MS) medium (0.02% $MgSO_4$, 0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $[NH_4]_2SO_4$) supplemented with 0.02% threonine (T) and 0.2% carbohydrate.

Isolation of mutant strains.—Mutant EC14 *thr* was isolated after treatment of EC14 with N-methyl-N′-nitro-N-nitrosoguanidine (NG) (1) followed by penicillin enrichment (5) and replica plating. This strain was used as the control throughout.

The β-galactosidase constitutive mutants were obtained after treatment of EC14 *thr*, growing on MST medium containing 0.2% lactose, with NG (1) and selection on media containing MST, 0.2% D-glucose, and 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described by Miller (13). When X-gal is hydrolyzed by β-galactosidase (EC 3.2.1.23), a deep blue dye is released. In the absence of the inducer, isopropyl-β-D-thiogalactoside (IPTG), a dark blue colony would indicate constitutive levels of enzyme. One such deep blue colony was cloned and exhibited high levels of β-galactosidase activity in the presence or absence of IPTG (see Results, Tables 2 and 3). To obtain cAMP-deficient

TABLE 1. Derivative strains of *Erwinia carotovora* isolate EC14 used to study endopolygalacturonate *trans*eliminase regulation in an adenosine 3′,5′-cyclic monophosphate-deficient mutant

Strain	Parent	Properties of derivatives
EC14 *thr*	EC14[a]	Threonine auxotroph Inducible endopolygalacturonate *trans*eliminase (PGTE) synthesis Inducible β-galactosidase synthesis
EC14 β-gal C	EC14 *thr*	Threonine auxotroph Inducible PGTE synthesis Constitutive β-galactosidase synthesis
EC1491	EC14 β-gal C	Threonine auxotroph 3′,5′-cAMP deficient Inducible PGTE and constitutive β-galactosidase synthesis only in presence of 3′,5′-cAMP

[a]Isolated from *Calla palustris* in 1946 by P. A. Ark, University of California, Berkeley.

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mutants, this β-galactosidase constitutive mutant was grown on MST medium with 0.2% D-glucose and treated with NG as described previously. Colonies that had lost the ability to produce a blue color on X-gal medium with no IPTG were selected and cloned. Mutants obtained by the latter method were plated on media containing MST and 0.2% selected carbohydrates, either in absence or presence of 0.5 mM cAMP. These carbohydrates included α-lactose, L-arabinose, D-galactose, D-mannitol, L-rhamnose, D-xylose, raffinose, D-cellobiose, glycerol, D-ribose, and sodium polypectate (NaPP). The mutant EC1491 demonstrated a positive growth response to the addition of cAMP on many of these carbon sources and was selected for further experimentation.

Enzyme assays.—β-Galactosidase activity was determined from cultures that were grown overnight at 30 C on media containing MST, and either 0.2% D-ribose or D-glucose. Some of the cultures also contained 0.5 mM cAMP or, in some experiments, 1 mM IPTG. A sample of each culture was added to identical fresh medium, and cell density was measured with a Bausch and Lomb Spectronic 20 colorimeter until an OD_{600nm} of 0.3–1.0 was obtained. Enzyme units were calculated by measuring the formation of *o*-nitrophenol from hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) by β-galactosidase as described by Miller (13).

To determine PGTE activity, the bacterial cultures were grown overnight at 30 C in a medium containing MST, and 0.5% casein hydrolysate, which previously had been eluted through a Dowex 1X-2, 400 column to remove any contaminating cyclic nucleotides. A sample of each culture was transferred to fresh media. When the OD_{600nm} of a culture reached approximately 0.2, the culture was dispensed into several flasks and NaPP or cAMP or both were added to a final concentration of 0.5% and 0.5 mM, respectively. Glass distilled water was added to the control flasks. The cultures then were aerated at 30 C, and at different intervals, the cell density was determined at OD_{600nm}. Samples (2 ml) were removed from each flask and the cells were collected by centrifugation at 10,000 × *g* for 10 min. The supernatant fluid was assayed for extracellular levels of PGTE by the periodate-thiobarbituric acid (TBA) method (17). The pelleted cells were resuspended in 2 ml of 0.05 M Tris-HCl buffer at pH 8.0 and disrupted by sonification in a Branson ultrasonifier for 3 min at 30 W. Cell debris was removed by centrifugation and the supernatant fluid was assayed for PGTE by TBA. The reaction mixture contained 0.1 ml of 0.6% (w/v) sodium polypectate in 0.05 M Tris-HCl buffer (pH 8.0), plus 10^{-4} M $CaCl_2$ and 0.01 ml of enzyme, and was incubated for 1 hr at 30 C. One unit of enzyme activity was defined as that amount of enzyme in 1 ml of cell sonicate or cell-free culture fluid that released 0.01 μmole of unsaturated oligouronides from NaPP per hour. This corresponded to an increase in OD_{548nm} of 0.3. Specific activity was defined as units as enzyme activity per milligram of dry weight of bacterial cell mass in 1 ml of culture.

Cyclic nucleotide extraction and assay.—Cultures were grown overnight in media containing MST, 0.1% D-ribose, and 0.5% casein hydrolysate that had been eluted through Dowex as described previously. A sample of each culture was transferred to fresh medium and when growth reached early log phase (about OD_{600nm} 0.20–0.22), samples were taken and each culture was divided into two flasks, one containing 0.5% sodium polypectate. Three 1.0-ml samples were collected from each flask at each doubling of the OD_{600nm}. One milliliter of 7% perchloric acid was added immediately to each 1-ml sample and the mixture was sonified for 3 min as described previously. Cell debris was removed by centrifugation and the supernatant fluid was assayed for total concentration (intracellular and extracellular) of cAMP. Radioimmunoassays were performed as described by Harper and Brooker (8) and modified by Hubbard et al (9). Specific activity was defined as picomoles of cAMP per milligram dry weight of bacterial cell mass in 1 ml of culture.

Chemicals.—Unless otherwise noted, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO 63118). Nutrient agar was from Difco Laboratories (Detroit, MI 48232). Thiobarbituric acid, D-galactose, triethylamine, and raffinose were from Eastman Organic Chemicals (Rochester, NY 14650). Glycerol and acetic anhydride were from Fisher (Pittsburgh, PA 15219), nitrosoguanidine from Aldrich Chemical Co. (Milwaukee, WI 53233), and

TABLE 2. Activities of β-galactosidase in *Erwinia carotovora* mutants grown on D-glucose[a]

Strain	Media	Units[b]
EC14 *thr*	Glucose MST	19
EC14 *thr*	Glucose MST + IPTG	188
EC14 β-gal C	Glucose MST	150
EC14 β-gal C	Glucose MST + IPTG	105

[a]All strains were grown on 0.2% D-glucose, 0.02% $MgSO_4$, 0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, and 0.02% threonine (glucose MST), with or without the presence of 1 mM isopropyl-β-D-thiogalactoside (IPTG) and 0.1- or 0.5-ml samples were tested during exponential growth.

[b]Units were defined by J. H. Miller, pages 48–55, 352–355 *in* Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 466 p.

TABLE 3. Activities of β-galactosidase in *Erwinia carotovora* mutants grown on D-ribose[a]

Strain	Media	Units[b]
EC14 *thr*	Ribose MST	12
EC14 *thr*	Ribose MST + cAMP	25
EC14 β-gal C	Ribose MST	1,450
EC14 β-gal C	Ribose MST + cAMP	1,500
EC1491	Ribose MST	19
EC1491	Ribose MST + cAMP	1,175

[a]All strains were grown on 0.2% D-ribose, 0.02% $MgSO_4$, 0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, and 0.02% threonine (ribose MST), with or without 0.5 mM adenosine 3′,5′-cyclic monophosphate (cAMP) and 0.1- or 0.5-ml samples tested during exponential growth.

[b]Units were defined by J. H. Miller, pages 48–55, 352–355 *in* Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 466 p.

TABLE 4. Specific activity of cAMP in *Erwinia carotovora* mutants

Strain and media[a]	Generation[b]	cAMP-specific activity[c]
EC14 *thr*	0	391.8
	1	382.9
	2	464.0
EC14 *thr* + NaPP[d]	0	391.8
	1	484.1
	2	766.8
EC1491	0	8.8
	1	7.6
	2	8.4
EC1491 + NaPP[d]	0	8.8
	1	8.0
	2	8.8

[a]All strains were grown on 0.1% ribose, 0.5% casein hydrolysate, 0.02% threonine, and 0.02% $MgSO_4$, 0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$.

[b]Sample from overnight culture of each strain was added to fresh media and monitored until early log phase was reached (OD_{600nm}, about 0.22). At this 0 point, samples were taken and cultures were divided into two flasks, one without and one with sodium polypectate. Samples were taken at each subsequent generation (doubling time of the cell mass, about 2 hr). OD_{600nm} of all flasks at each sample point was comparable.

[c]Specific activity is defined as picomoles of cAMP per milligram dry weight of bacterial cell mass and represents both exogenous and intracellular activity.

[d]At generation 0, sodium polypectate (NaPP) was added to a final concentration of 0.5%.

^{125}I ScAMP antigen and specific antibody from Collaborative Research (Waltham, MA 02154).

RESULTS

Cyclic AMP-deficient mutants.—The standard methods to detect cAMP-deficient mutants of *Escherichia coli* on indicator plates, such as eosin-methylene blue (10,11) and tetrazolium (4,12), were not suitable for EC14 *thr* because this mutant failed to produce the proper color. Therefore, a different approach had to be devised to screen for cAMP-deficient mutants.

A β-galactosidase constitutive mutant was isolated after mutagenesis of EC14 *thr*. This mutant, in the presence or absence of the inducer IPTG, exhibited activities of β-galactosidase comparable to those of the induced control EC14 *thr*, but the non-induced control showed significantly less activity (Table 2). The constitutive levels of enzyme were much higher when the mutant was grown on D-ribose (Table 3) than on D-glucose, which appeared to repress the synthesis of β-galactosidase.

The β-galactosidase constitutive mutant was treated with NG and yielded mutant EC1491. The latter mutant did not produce constitutive levels of β-galactosidase unless cAMP was supplied to the medium (Table 3). The levels of cAMP produced by the mutant were extremely low compared with that of the control strain (Table 4). This low level was constant, regardless of the carbon source available or stage of growth. This small amount of cAMP was not adequate to support growth of EC1491 on several carbohydrates that are known to be metabolized under cAMP regulation in other prokaryotes (16). Exogenously supplied cAMP was observed to be necessary for EC1491 to grow on media containing D-galactose, D-xylose, L-rhamnose, D-raffinose, L-arabinose, α-lactose, glycerol, D-cellobiose, or NaPP as substrate. The addition of cAMP analogues and various nucleotide derivatives such as 8-bromoadenosine 3′,5′-cyclic monophosphate, N^6, O^2 dibutyryl adenosine 3′,5′-cyclic monophosphate, adenosine 2′,3′-cyclic monophosphate, adenosine 5′-monophosphate, adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine, or guanosine 3′,5′-cyclic monophosphate did not replace the cAMP requirement for growth on media with the above-mentioned carbohydrates. Cyclic AMP was not a requirement for growth of EC1491 on D-ribose or D-mannitol.

To determine if the synthesis of PGTE was affected by the cAMP deficiency of EC1491, total enzyme activities, both intracellular and extracellular, were quantitated (Fig. 1). Cultures of EC1491 and EC14 *thr* growing logarithmically on a casein hydrolysate medium were supplemented with the inducer, NaPP, or cAMP or a combination of these; then enzyme levels were measured at hourly intervals. Mutants EC14 *thr* and EC1491 exhibited low levels of PGTE activity when the inducer was absent from the medium. If only NaPP was present, EC14 *thr* showed induced levels of PGTE, but enzyme activity in EC1491 remained low. When both cAMP and NaPP were present in the medium, however, the enzyme activities of the two strains were increased significantly. These data support the contention that cAMP is a regulator of PGTE synthesis in *E. carotovora*.

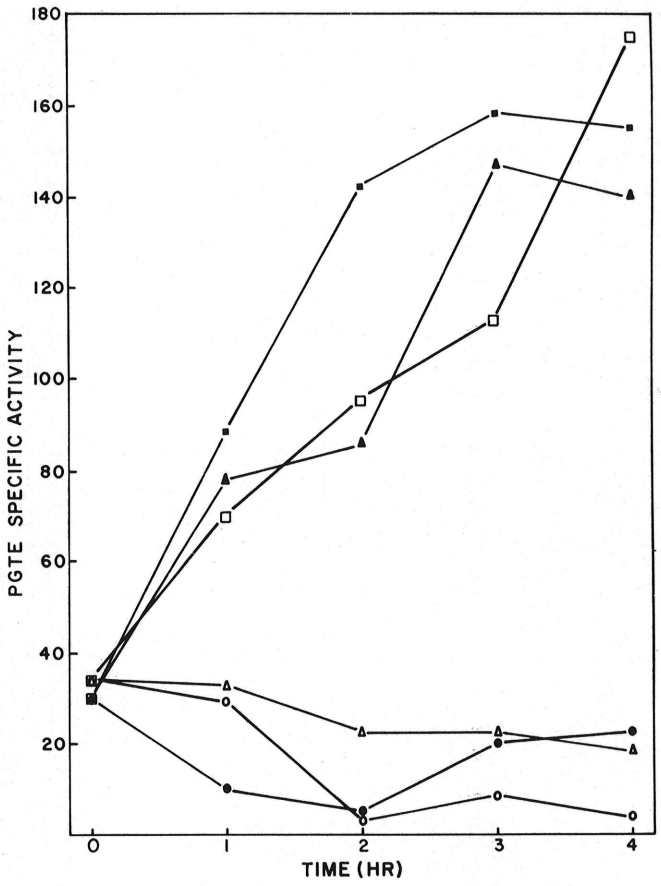

Fig.1. Endopolygalacturonate *trans*eliminase (PGTE) activities, both intracellular and extracellular, during growth of EC14 *thr* and EC1491 on 0.02% $MgSO_4$, 0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, 0.5% casein hydrolysate, 0.02% threonine, and supplemented, in some cases, with 0.5% sodium polypectate (NaPP) or 0.5 mM 3′,5′-cAMP or both. Early log phase cultures in casein were supplemented at time 0. (●) = EC14 *thr* on casein only, (▲) = EC14 *thr* supplemented with NaPP, (■) = EC14 *thr* supplemented with NaPP and cAMP, (o) = EC1491 on casein only, (Δ) = EC1491 supplemented with NaPP, and (□) = EC1491 supplemented with NaPP and cAMP. Specific activity is in micromoles of product per hour per milligram of dry cell weight.

DISCUSSION

The present knowledge of genetics of *Erwinia* spp. does not afford the vast information that has been formulated about *E. coli*. In studies with *E. coli*, two classes of mutants that have lost their ability to use a number of carbon sources have been isolated (6,16,21): (i) adenylate cyclase mutants (*cya*), which can overcome catabolite repression with the addition of cAMP to the medium, and (ii) catabolite-repressible protein mutants (*crp*), which do not respond to exogenous cAMP. The data presented here suggest that EC1491 could be a *cya* mutant, since it cannot produce adequate levels of cAMP and therefore must have an exogenous source of cAMP to utilize various carbon sources. Unlike *E. coli* (16), EC1491 does not require cAMP for the use of mannitol. Several of the sugars under cAMP control in EC1491, such as L-arabinose, L-rhamnose, D-xylose, and D-galactose, are neutral sugar components of the primary cell walls of plants. A tempting speculation is that the rate of use of these sugars by *E. carotovora* would influence the growth rate of the bacterium and subsequent pathogenesis directly.

The current concept is that pectic enzymes released by phytopathogens are the key factors involved in enzymatic hydrolysis of plant cell walls. These enzymes serve as wall-modifying enzymes, since their action renders other polysaccharide components in cell walls more susceptible to hydrolysis (2). An understanding of the regulation of these enzymes is essential to an understanding of the mechanisms of host-parasite interaction in the disease cycle. Experimental evidence suggests the importance of cAMP in the synthesis of PGTE (9). This study supports the concept of a cAMP-regulated pectic enzyme "operon" in *E. carotovora*.

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