Coregulation of β -Galactoside Uptake and Hydrolysis by the Hyperthermophilic Bacterium *Thermotoga neapolitana*

MICHAEL Y. GALPERIN, KENNETH M. NOLL,* AND ANTONIO H. ROMANO

Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269-3125

Received 28 October 1996/Accepted 27 December 1996

Regulation of the β -galactoside transport system in response to growth substrates in the extremely thermophilic anaerobic bacterium *Thermotoga neapolitana* was studied with the nonmetabolizable analog methyl- β -D-thiogalactopyranoside (TMG) as the transport substrate. *T. neapolitana* cells grown on galactose or lactose accumulated TMG against a concentration gradient in an intracellular free sugar pool that was exchangeable with external galactose or lactose and showed induced levels of β -galactosidase. Cells grown on glucose, maltose, or galactose plus glucose showed no capacity to accumulate TMG, though these cells carried out active transport of the nonmetabolizable glucose analog 2-deoxy-D-glucose. Glucose neither inhibited TMG uptake nor caused efflux of preaccumulated TMG; rather, glucose promoted TMG uptake by supplying metabolic energy. These data show that β -D-galactosides are taken up by *T. neapolitana* via an active transport system that can be induced by galactose or lactose and repressed by glucose but which is not inhibited by glucose. Thus, the phenomenon of catabolite repression is present in *T. neapolitana* with respect to systems catalyzing both the transport and hydrolysis of β -D-galactosides, but inducer exclusion and inducer expulsion, mechanisms that regulate permease activity, are not present. Regulation is manifest at the level of synthesis of the β -galactoside transport system but not in the activity of the system.

Members of the genus *Thermotoga* are hyperthermophilic strict anaerobes that represent the deepest phylogenetic branch of heterotrophic fermentative bacteria known (*Aquifex* spp. are more deeply branching but are strict autotrophs) (1, 12, 28). Thus, studies of the comparative physiology and biochemistry of carbohydrate transport and metabolism of *Thermotoga* spp. should yield insights concerning the evolution of these processes. *Thermotoga neapolitana* is a particularly advantageous organism for these studies because it is the only hyperthermophile known that is able to grow on defined media with a number of monosaccharides, disaccharides, and polysaccharides as sources of carbon and energy (3, 8).

We have reported that D-glucose is taken up by *T. neapolitana* by an active transport system that appears to be energized by an ion gradient generated by ATP derived from substrate-level phosphorylation (7). We now present information on the β -galactoside transport system of *T. neapolitana*, with an emphasis on its regulation. These studies were undertaken as a sequel to an investigation of the β -galactosidase system of this organism, which demonstrated the inducibility of this enzyme and a cyclic AMP (cAMP)-independent mechanism of catabolite repression (27).

Presented here is evidence that *T. neapolitana* has an active transport system for β -galactoside that is inducible by galactose or lactose and is subject to repression by glucose (catabolite repression). This is in contrast to the glucose transport system, which appears to be constitutive. Glucose did not inhibit the activity of the β -galactoside transport system in induced cells, nor did it provoke efflux of accumulated β -galactoside. Thus, neither inducer exclusion nor inducer expulsion regulates β -galactoside uptake in *T. neapolitana*.

MATERIALS AND METHODS

Organism and growth conditions. *T. neapolitana* NS-E (8) was maintained as described earlier (26). Cells were grown at 77°C under strict anaerobic conditions in TB medium (5), modified by the substitution of 20 mM HEPES for PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]) and the addition of 10 mM $\text{Na}_2\text{S}_2\text{O}_3$ and yeast extract (0.5 g/liter). Sugars were sterilized separately and added to a final concentration of 4 g/liter. Cells were harvested and prepared for transport experiments as described previously (7).

Transport measurements. Transport of β -D-[methyl- ^{14}C]thiogalactopyranoside ([^{14}C]TMG) or 2-deoxy-D-[^{14}C]glucose ([^{14}C]2-DOG) (both from New England Nuclear) was measured in cell suspensions (2 to 4 mg of protein per ml) contained in capped serum bottles kept at 77°C under a continuous flow of nitrogen, as described previously (7, 9). A value for time zero (no uptake) was determined by measuring radioactivity in cell suspensions treated exactly as described above but kept at 25°C instead of 77°C. This value, which represented nonspecific adsorption to cells and the walls of centrifuge tubes, was subtracted from all measurements at 77°C. It did not exceed 0.6 nmol/mg of protein at the [^{14}C]TMG concentration and specific radioactivity used in this study (0.1 mM, 0.5 mCi/mmol). Protein content of cell suspensions was determined by the method of Bradford (4), with bovine serum albumin as a standard. Calculations of intracellular concentrations of [^{14}C]TMG or [^{14}C]2-DOG were based on an intracellular water space of 1.9 μl /mg of protein (7).

Preparation of cell extracts. Cells grown as described above were washed and resuspended in 100 mM Tris-HCl buffer (pH 8.0 at 25°C) containing 5 mM dithiothreitol. Cell extracts were prepared by intermittent sonication of cell suspensions kept in an ice bath under a nitrogen atmosphere (five 1-min bursts with intermittent 1-min cooling periods) followed by centrifugation at 16,000 $\times$ g for 15 min to remove cell debris.

β -Galactosidase and phospho- β -galactosidase assays. A variation of the method of Miller (10) was used. Cells were washed in TB medium, collected by centrifugation, and resuspended in Z buffer (10). The cells were lysed by adding Triton X-100 (0.2% final concentration), and the lysates were cleared by centrifugation. Cell lysates (10 to 20 μl containing 20 to 50 μg of protein) were added to 1 ml of preheated Z buffer and incubated at 77°C for 5 min. The reaction was initiated by adding 0.2 ml of orthonitrophenyl β -D-galactopyranoside (ONPG) or orthonitrophenyl β -D-galactopyranoside-6-phosphate (ONPGP) (both 4 mg/ml; from Sigma Chemical Co.), and the optical density at 420 nm was monitored for 5 to 10 min at 77°C on a Beckman DU-68 spectrophotometer equipped with a thermoregulated cuvette compartment. The control cuvette with no cell extract was used to correct for the nonenzymatic hydrolysis of ONPG or ONPGP at 77°C. Results are expressed as micromoles of ONPG or ONPGP hydrolyzed per minute (1 U) per milligram of protein.

Phosphoenolpyruvate (PEP):sugar phosphotransferase (PTS) assays. These were carried out at 77°C as described previously (7), modified by the omission of sodium dithionite from the suspension buffer and assay mixture. Radiolabelled sugar substrates were [^{14}C]TMG, D-[U- ^{14}C]glucose, D-[U- ^{14}C]lactose (all from

* Corresponding author. Phone: (860) 486-4688. Fax: (860) 486-4331. E-mail: noll@uconnvm.uconn.edu.

FIG. 1. Effect of sugars on the uptake of [^{14}C]TMG by *T. neapolitana*. Galactose-grown cells were incubated at 77°C with 0.1 mM [^{14}C]TMG (0.5 mCi/mmol) with no further addition (●), with 50 mM pyruvate added at -10 min (○), or with 50 mM pyruvate added at -10 min and 10 mM galactose (□), 10 mM lactose (△), or 10 mM glucose (▲) added at -5 min. The dotted line indicates the intracellular concentration of [^{14}C]TMG that is equal to that of the external medium.

New England Nuclear; specific radioactivity adjusted to 0.5 mCi/mmol), or D-[6- ^3H]galactose (ICN Radiochemicals; 1 mCi/mmol).

RESULTS AND DISCUSSION

Effect of sugars on β -galactoside transport and intracellular accumulation. Figure 1 shows that galactose-grown cells were able to accumulate TMG against a concentration gradient in the presence of an energy source such as pyruvate, which generates ATP in *Thermotoga maritima* by the combined actions of pyruvate:ferredoxin oxidoreductase, phosphotransacetylase, and acetate kinase (2, 22). Galactose and lactose, which are metabolizable substrates for this system, inhibited TMG transport by competition for a common carrier. Glucose did not inhibit TMG uptake; rather, it increased the level of intracellular accumulation of TMG to approximately 35-fold that of the external medium, presumably by generating cellular energy from glycolysis. Further indication that galactose, lactose, and TMG have a common carrier and that the TMG taken up was in a mobile free intracellular pool that was readily exchangeable with external substrate is shown in Fig. 2. Addition of excess galactose or lactose to galactose-grown cells that had been allowed to accumulate [^{14}C]TMG for 5 min provoked a rapid efflux of radioactivity. These results establish that TMG is a suitable nonmetabolizable substrate for the β -galactoside transport system.

As we had shown previously for the active transport of 2-DOG by the D-glucose uptake system of *T. neapolitana* (7), TMG uptake required Na^+ ions and was inhibited by sodium arsenate and the ionophore gramicidin D (results not shown). Also, we have extended our findings of an apparent absence of the PEP:sugar PTS (7) by failing to detect PEP-dependent phosphorylation of lactose, galactose, or TMG. Results of assays carried out with cell extracts of galactose-grown cells are shown in Table 1. There was no significant PEP-dependent phosphorylation of lactose, galactose, or TMG, but there was strong ATP-dependent phosphorylation of glucose and galactose. ATP-dependent sugar phosphorylation in the presence of lactose was probably due to phosphorylation of glucose and/or

FIG. 2. Effect of sugars on intracellular concentration of [^{14}C]TMG. Galactose-grown *T. neapolitana* cells were incubated at 77°C in the presence of 0.1 mM [^{14}C]TMG (0.5 mCi/mmol) and 50 mM pyruvate, with no further addition (○) or with the addition of 10 mM galactose (□), 10 mM lactose (△), or 10 mM glucose (▲) at +5 min.

galactose resulting from hydrolysis of lactose catalyzed by β -galactosidase present in the extract (see below).

Figure 2 also shows that addition of glucose did not promote efflux of preaccumulated TMG but again increased the level of intracellular TMG accumulation. Thus, a phenomenon found in certain gram-positive bacteria called inducer expulsion, whereby glucose provokes a rapid expulsion of accumulated β -galactoside (17) or phosphorylated β -galactoside (15), appears to be absent in *T. neapolitana*. Also, the lack of inhibition of β -galactoside uptake by glucose (Fig. 1) indicates that inducer exclusion, a much more widely distributed regulatory mechanism occurring in enteric bacteria and several lactic acid bacteria by which the transport of certain sugars including lactose is inhibited by glucose (for reviews, see references 13, 19, and 24), is also absent in *T. neapolitana*. It is of interest that both these mechanisms are putatively dependent upon specific protein components of the PEP:sugar PTS for their operation: III^{Glc} (also designated as IIAGlc [21]) in the case of inducer exclusion in the gram-negative enterobacteria (13, 19) and HPr in the case of inducer expulsion in gram-positive bacteria (16, 29). The absence of these mechanisms in *T. neapolitana* correlates with an apparent absence of a functional PTS (though we have not rigorously ruled out the possible presence of individual protein PTS components that are not organized into functional phosphorylative sugar translocation systems).

Effect of growth carbon source on the β -galactoside transport system; induction and repression. The β -galactosidase

TABLE 1. PEP- or ATP-dependent phosphorylation of D-galactose, D-glucose, D-lactose, or TMG at 77°C by cell extracts of *T. neapolitana* cells grown on D-galactose

Sugar	Sugar phosphate formed (nmol/min/mg of protein) with phosphoryl donor:		
	None	PEP	ATP
D-Galactose	1.7	3.7	32.0
D-Glucose	2.6	4.2	49.6
D-Lactose	5.4	6.1	17.8
TMG	1.1	1.4	1.1

TABLE 2. Effect of growth carbon source on β-galactosidase and phospho-β-galactosidase activities and the capacity of *T. neapolitana* cells to accumulate [¹⁴C]TMG and [¹⁴C]2-DOG

Carbon source ^a	Sp act (U/mg of protein) ^b		Intracellular accumulation			
			[¹⁴ C]TMG ^c		[¹⁴ C]2-DOG ^c	
	β-Gal	P-β-gal	nmol/mg of protein	[In]/[Out]	nmol/mg of protein	[In]/[Out]
Lactose	0.73	0.02	2.2	12	6.3	17
Galactose	0.83	0.02	4.3	23	10.8	28
Galactose plus glucose	0.07	<0.02	0.3	1.6	6.3	17
Glucose	0.07	<0.02	0.3	1.6	11.0	29
Maltose	0.08	<0.02	0.4	2.1	8.2	22
Cellobiose	0.44	0.02	0.0	<1	4.7	12

^a Four grams per liter in TB medium.^b U = micromoles of ONPG or ONPGP hydrolyzed per minute. β-Gal, β-galactosidase; P-β-gal, phospho-β-galactosidase.^c Shown as nanomoles per milligram of cell protein after 15 min at 77°C. External concentrations of transported substrate were as follows: [¹⁴C]TMG, 0.1 mM (0.5 mCi/mmol); [¹⁴C]2-DOG, 0.2 mM (0.5 mCi/mmol). Energy source was 20 mM sodium pyruvate.

activities and the capacities of *T. neapolitana* cells grown on a variety of carbon sources to carry out concentrative uptake of TMG and the nonmetabolizable D-glucose analog 2-DOG are shown in Table 2. The following observations can be made from these data: (i) cells grown on lactose or galactose showed β-galactosidase activity and the capacity for concentrative uptake of both TMG and 2-DOG; (ii) cells grown on glucose or maltose or galactose plus glucose showed very low β-galactosidase activity and no concentrative uptake of TMG, though all these cells concentrated 2-DOG; cellobiose-grown cells did not actively transport TMG, though they transported 2-DOG and had β-galactosidase activity; (iii) cells induced for β-galactosidase activity had essentially no phospho-β-galactosidase activity; this correlates with the absence of a PEP:lactose PTS, since this latter system is generally accompanied by a phospho-β-galactosidase (25).

These observations indicate that the glucose transport system is constitutive, while the β-galactoside transport system is inducible by growth on lactose and galactose and is subject to repression by glucose. This pattern of induction and repression is similar to that found for β-galactosidase, both in this study and in a previous one (27). Both the β-galactoside transport system (permease) and β-galactosidase of *T. neapolitana* are inducible by lactose or galactose and are subject to repression by glucose (catabolite repression). Thus, these two systems show an overall appearance of coordinate regulation, but there is no evidence yet for an organized operon or regulon. It will be of great interest to determine at the genetic level the regulatory mechanism(s) that is operative in this phylogenetically deep-rooted bacterium. The β-galactosidase (*lacZ*) gene of *T. maritima* has been sequenced elsewhere (11) and shown to have significant homology to *lacZ* of *Escherichia coli* and the β-galactosidase genes of eight other bacteria and a yeast. An open reading frame 5' to that gene had sequence homology with *Bacillus lac* genes, but unlike the *lacZ* homolog, no β-galactosidase activity resulted from the cloned gene in *E. coli*. An adjacent partial open reading frame had sequence identity with the ATP-binding domain of an ATP-dependent family of oligopeptide transporters. No identifiable carbohydrate permease or repressor genes were found up to 900 bp downstream of the *T. maritima lacZ* gene. Clearly, an understanding of the regulation of β-galactosidase transport and catabolism in *Thermotoga* spp. will require more study at the genetic level.

Curiously, the β-glucoside cellobiose did not induce the β-galactoside transport system, though it did induce β-galactosidase, as had been noted previously (27). Another atypical

feature of this cellobiose-induced β-galactosidase noted in this previous study was its lack of repression by glucose. This β-galactosidase was electrophoretically distinct from a β-glucosidase that was also induced by cellobiose but indistinguishable from lactose-induced β-galactosidase (27). The possibility of there being more than one β-galactosidase is being examined.

Vargas and Noll (27) found very low levels of cAMP in *T. neapolitana* that did not vary significantly with different carbon sources and concluded that cAMP was not involved in the observed catabolite repression of β-galactosidase in this bacterium. It can be similarly inferred that the induction and repression of the β-galactoside transport system described here are also independent of cAMP. Moreover, these regulatory mechanisms appear to operate independently of the PEP:sugar PTS, whose protein components also are considered to play important roles in catabolite repression. In gram-negative enteric bacteria, III^{Glc} is involved in the regulation of adenylate cyclase (13, 19) and thus cellular cAMP levels. HPr has been implicated in catabolite repression in *Bacillus subtilis* (6, 14).

In summary, *T. neapolitana* has a mechanism of catabolite repression of β-galactoside transport and metabolism that is simpler than that of more modern bacteria and lacks a mechanism for regulation at the level of activity of the transport system (inducer exclusion or expulsion). A tentative hypothesis might be that inducer exclusion, inducer expulsion, and PTS- and cAMP-dependent catabolite repression evolved with the PTS proteins in organisms that branched from the evolutionary tree later than *T. neapolitana* (18). It will be of interest to determine whether the catabolite repressor/activator (Cra) protein system (20) is present and functional in *Thermotoga* species. One of the metabolic activators of this latter system, fructose-1,6-bisphosphate, is a metabolite of the Embden-Meyerhof-Parnas pathway that has been found in *T. maritima* (23).

ACKNOWLEDGMENTS

This work was supported by research grants DE-FG02-93ER20122 from the U.S. Department of Energy and MCB-9418197 from the National Science Foundation.

REFERENCES

1. Achenbach-Richter, L., R. Gupta, K. O. Stetter, and C. R. Woese. 1987. Were the original eubacteria thermophiles? Syst. Appl. Microbiol. 9:34-39.
2. Adams, M. W. W. 1994. Biochemical diversity among sulfur-dependent, hyperthermophilic microorganisms. FEMS Microbiol. Rev. 15:261-277.

3. Belkin, S., C. O. Wirsén, and H. W. Jannasch. 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. *Appl. Environ. Microbiol.* **51**:1180–1185.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
5. Childers, S. E., M. Vargas, and K. M. Noll. 1992. Improved methods for cultivation of the extremely thermophilic bacterium *Thermotoga neapolitana*. *Appl. Environ. Microbiol.* **58**:3949–3953.
6. Deutscher, J., E. Küster, U. Bergstedt, V. Charrier, and W. Hillen. 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to catabolite repression in Gram-positive bacteria. *Mol. Microbiol.* **15**:1049–1053.
7. Galperin, M. Y., K. M. Noll, and A. H. Romano. 1996. The glucose transport system of the hyperthermophilic anaerobic bacterium *Thermotoga neapolitana*. *Appl. Environ. Microbiol.* **62**:2915–2918.
8. Jannasch, H. W., R. Huber, S. Belkin, and K. O. Stetter. 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic eubacterial genus *Thermotoga*. *Arch. Microbiol.* **150**:103–104.
9. Kashket, E. R. 1981. Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. *J. Bacteriol.* **146**:369–376.
10. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Moore, J. B., P. Markiewicz, and J. H. Miller. 1994. Identification and sequencing of the *Thermotoga maritima* *lacZ* gene, part of a divergently transcribed operon. *Gene* **147**:101–106.
12. Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol.* **176**:1–6.
13. Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev.* **57**:543–594.
14. Reizer, J., U. Bergstedt, A. Galinier, E. Küster, M. H. Saier, Jr., W. Hillen, M. Steinmetz, and J. Deutscher. 1996. Catabolite repression resistance of *gnt* operon expression in *Bacillus subtilis* conferred by mutation of His-15, the site of phosphoenolpyruvate-dependent phosphorylation of the phosphocarrier protein HPr. *J. Bacteriol.* **178**:5480–5486.
15. Reizer, J., M. J. Novotny, C. Panos, and M. H. Saier, Jr. 1983. Mechanism of inducer expulsion in *Streptococcus pyogenes*: a two-step process activated by ATP. *J. Bacteriol.* **156**:354–361.
16. Reizer, J., A. H. Romano, and J. Deutscher. 1993. The role of HPr, a phosphocarrier protein of the phosphotransferase system, in the regulation of carbon metabolism in Gram positive bacteria. *J. Cell. Biochem.* **51**:19–24.
17. Romano, A. H., G. Brino, A. Peterkofsky, and J. Reizer. 1987. Regulation of β -galactoside transport and accumulation in heterofermentative lactic acid bacteria. *J. Bacteriol.* **169**:5589–5596.
18. Romano, A. H., and M. H. Saier, Jr. 1992. Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. I. Physiologic and organismic considerations, p. 143–170. *In* R. P. Mortlock (ed.), *The evolution of metabolic function*. CRC Press, Inc., Boca Raton, Fla.
19. Saier, M. H., Jr. 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *Microbiol. Rev.* **53**:109–120.
20. Saier, M. H., Jr., and T. M. Ramseier. 1996. The catabolite repressor/activator (Cra) protein of enteric bacteria. *J. Bacteriol.* **178**:3411–3417.
21. Saier, M. H., Jr., and J. Reizer. 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *J. Bacteriol.* **174**:1433–1438.
22. Schäfer, T., M. Selig, and P. Schönheit. 1993. Acetyl-CoA synthase (ADP-forming) in archaea, a novel enzyme involved in acetate formation and ATP synthesis. *Arch. Microbiol.* **159**:72–83.
23. Schröder, C., M. Selig, and P. Schönheit. 1994. Glucose fermentation to acetate, CO₂ and H₂ in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*. *Arch. Microbiol.* **161**:460–470.
24. Thompson, J. 1987. Regulation of sugar transport and metabolism in lactic acid bacteria. *FEMS Microbiol. Rev.* **46**:221–231.
25. Thompson, J. 1987. Sugar transport in the lactic acid bacteria, p. 1–38. *In* J. Reizer and A. Peterkofsky (ed.), *Sugar transport and metabolism in Gram-positive bacteria*. Ellis Horwood, Chichester, United Kingdom.
26. Vargas, M., and K. M. Noll. 1994. Isolation of auxotrophic and antimetabolite-resistant mutants of the hyperthermophilic bacterium *Thermotoga neapolitana*. *Arch. Microbiol.* **162**:357–361.
27. Vargas, M., and K. M. Noll. 1996. Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is independent of cAMP. *Microbiology* **142**:139–144.
28. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.
29. Ye, J. J., J. W. Neal, X. Cui, and M. H. Saier, Jr. 1994. Regulation of the glucose:H⁺ symporter by metabolite-activated ATP-dependent phosphorylation of HPr in *Lactobacillus brevis*. *J. Bacteriol.* **176**:3484–3492.