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Catabolite-Repression-Like Phenomenon in *Rhizobium meliloti*

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We report a phenomenon similar to catabolite repression in *Rhizobium meliloti*. Succinate, which allows the highest observed rate of growth of *R. meliloti*, caused an immediate reduction of β-galactosidase activity when added to cells growing in lactose. A Lac⁻ mutant was unaltered in nodulation and nitrogen fixation capacities, but a pleiotropic mutant deficient in several catabolic properties was unable to produce effective nitrogen-fixing nodules.

A number of reports have described catabolic activities in *Rhizobia* (4, 5). Graham (2) found that a wide range of sugars and tricarboxylic acid cycle intermediates could promote growth in different species of *Rhizobia*, although slow-growing strains had more limited specificities. Elkan's group has interpreted their studies of glucose catabolism in *Rhizobium japonicum* to indicate functional Embden-Meyerhof-Parnas, Entner-Doudoroff, and possibly hexose cycle pathways in addition to the tricarboxylic acid cycle (6, 11). Tuzimura and Meguro (14) showed that both artificially cultured and nodule-extracted *R. japonicum* can oxidize tricarboxylic acid intermediates and fructose-1,6-diphosphate, but that the ability to oxidize glucose or nonphosphorylated saccharides is found only in glucose-grown and not in succinate-grown or symbiotic cells. We report preliminary studies on catabolism in *Rhizobium meliloti*.

Parental *R. meliloti* strain Rm2011 is biotin requiring and streptomycin resistant, and it nodulates alfalfa effectively (9). LB (rich medium) contained 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), 5 g of NaCl, and 40 mg of NaOH per liter. M9A (minimal medium) was, per liter, 5.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl, brought to 1 mM $MgSO_4$, 10 μM $CaCl_2$, and 1 μg of biotin per ml after autoclaving. Carbon source stock solutions were filter sterilized and usually added to a final concentration of 0.2%.

β-Galactosidase was assayed as for *Escherichia coli* (10). α-Glucosidase and β-glucosidase activities were assayed in analogous reactions, using *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-β-D-glucopyranoside, respectively.

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Rm2011 cultured in M9A with lactose plus limiting succinate showed diauxic growth (Fig. 1). Similar experiments with lactose, plus either maltose, sucrose, glucose, or histidine in limiting concentration, showed no diauxie; however, succinate–maltose and succinate–cellobiose diauxies were observed (data not shown). Cells in the second phase of growth had higher levels of β-galactosidase activity than did cells in the first phase.

R. meliloti cells grown in minimal medium had roughly 10-fold more β-galactosidase with lactose than with other carbon sources (Table 1). In contrast to results with *E. coli*, neither isopropyl-β-D-thiogalactopyranoside nor thiomethyl galactopyranoside (not shown) induced β-galactosidase in *R. meliloti* at any concentration between 10^{-6} and 10^{-1} M. The addition of succinate reduced the specific activity of β-galactosidase compared to that with lactose alone. The reduction was not complete, since activity was still significantly above the basal (no lactose) level.

Figure 2 shows the effect of succinate on the differential rate of increase of β-galactosidase. The induced differential rate with lactose was over 10-fold greater than the basal differential rate with succinate, whereas the rate with both lactose and succinate was intermediate, consistent with the diauxie results and with Table 1. In contrast to succinate, neither cellobiose, maltose, sucrose, mannose, arabinose, glucose, nor glycerol lowered the differential rate (not shown). Succinate is the carbon source that allowed the fastest absolute growth rate for this strain (D. S. Ucker, S. B. thesis, Massachusetts Institute of Technology, Cambridge).

Addition of succinate to a culture growing in lactose lowered the differential rate of increase of β-galactosidase activity to nearly a basal rate. After β-galactosidase specific activity was di-

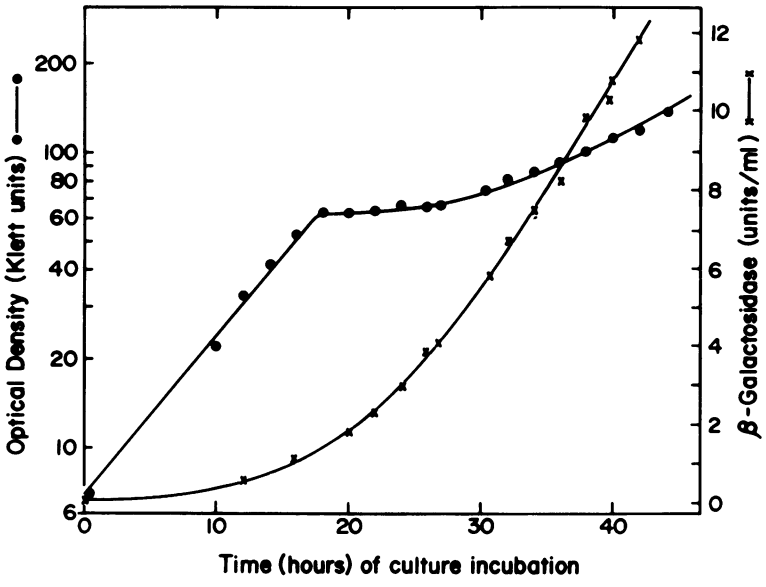

FIG. 1. *Diauxic growth of R. meliloti. Rm2011 was cultured in M9A with 0.1% succinate and 0.1% lactose. Cell density was measured turbidimetrically, and β-galactosidase activity was assayed as for E. coli (10). 1 Klett unit = 6 × 10⁶ viable cells/ml.*

TABLE 1. *β-Galactosidase specific activity*[a]

Carbon source[b]	*R. meliloti*			*E. coli*
	Rm2011	Rm1826	Rm2620	LS519
Lactose	7.2	ND[c]	ND	250
Succinate	0.8	ND	ND	
Mannose	0.8	0.07	0.07	
Mannose + IPTG 10^{-2} M	0.5	0.07	ND	
Lactose + succinate	2.2	ND	ND	
Lactose + mannose	6.9	0.2	0.07	
Lactose + succinate + mannose	2.5	0.1	0.08	
Root nodule[d]	<0.5	ND	ND	

[a] β-Galactosidase specific activity was determined as for *E. coli* (10); units are nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per optical density at 600 nm unit of culture turbidity; correspondence between optical density values of cultured and nodule cells has not been determined, and hence the value indicated for root nodule cells should be regarded as only approximate.

[b] In liquid cultures of medium M9A. IPTG, Isopropyl-β-D-thiogalactopyranoside.

[c] Not determined.

[d] Cell assay directly after isolation from root nodule squash.

luted to the repressed level, activity again increased, but now at normal succinate-mediated repressed rate (Fig. 2). This phenomenon resembles transient catabolite repression in *E. coli* (8).

Similar succinate-mediated repression of α-glucosidase and β-glucosidase activities has also been observed (not shown).

β-Hydroxybutyrate, a compound related to intermediates of the tricarboxylic acid and glyoxylate cycles, accumulates as a polymer at high concentration within bacteroids in nodules (13). Although it is an apparent candidate for a carbon source for the bacteroid, β-hydroxybutyrate does not serve as carbon source for cultured *R. meliloti*, and its addition to a culture growing with lactose did not affect the differential rate of increase of β-galactosidase activity (not shown). We do not know whether β-hydroxybutyrate is able to enter growing cells.

The addition of cyclic AMP (cAMP) to Rm2011 grown in lactose plus succinate did not affect the differential rate (not shown). This is true even for cells permeabilized with ethylenediaminetetraacetic acid, according to Lieve (7). That permeabilization is effective was shown by the fact that the cells normally resistant to actinomycin D became sensitive to the drug after ethylenediaminetetraacetic acid treatment. We conclude that cAMP had no effect on the phenomenon we were studying, although it is conceivable that enzyme synthesis cannot be increased after the permeabilization treatment. However, we wish to note that cAMP at 5 mM or greater always reduced the growth rate of Rm2011 by 50% relative to equivalent conditions without cAMP, regardless of carbon source.

Rm2011 was mutagenized with UV light (10^8 cells/ml, 300 ergs/mm^2, 0.2% survival, 250-fold

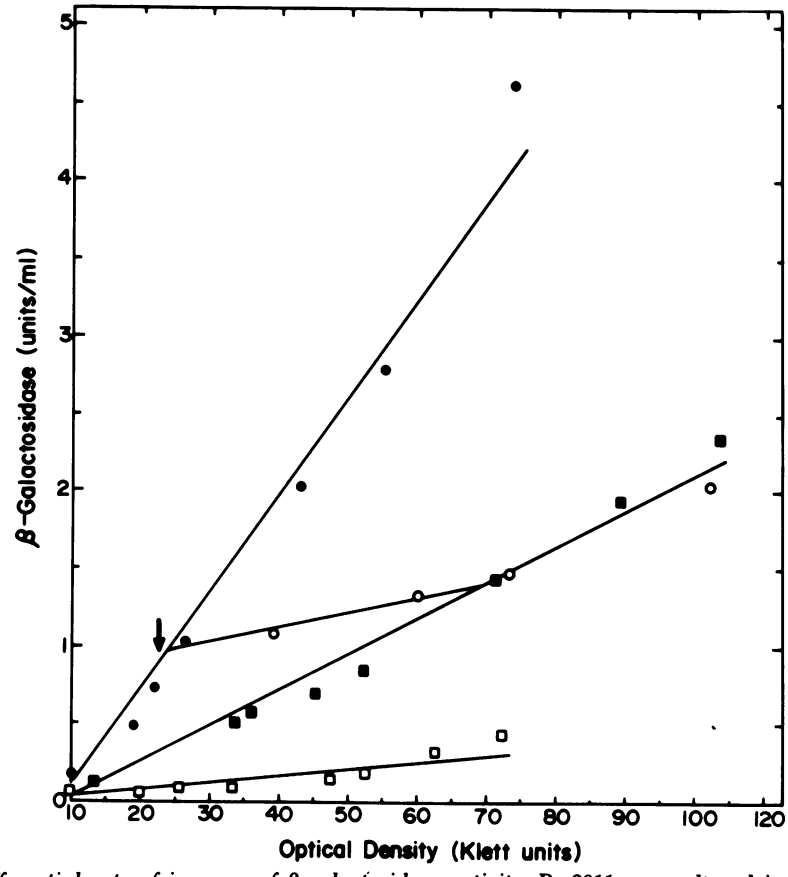

FIG. 2. *Differential rate of increase of β-galactosidase activity. Rm2011 was cultured in supplemented M9A; cell density and β-galactosidase activity were measured as for E. coli (10). Carbon source supplements were: lactose, 0.2% (●); lactose, 0.2% plus addition of succinate to 0.2% at arrow (○); succinate, 0.2% and lactose, 0.2% (■); succinate, 0.2% (□). 1 Klett unit = 6 × 10⁶ viable cells/ml.*

increase in rifampin-resistant colonies). Enrichment by carbenicillin treatment (9) gave approximately 1 to 2% catabolic mutants and 1 to 4% auxotrophs among the survivors.

A Lac⁻ mutant, Rm1826, was isolated after carbenicillin treatment in lactose. It failed to grow on lactose but did grow on maltose, melibose, and cellobiose. The mutant had less than 1/40 of the wild-type β-galactosidase activity, and Lac⁺ revertants regained wild-type activity. The residual activity was less inducible than wild-type activity (Table 1), although thermal activity and thermostability profiles of wild and mutant β-galactosidase were identical, with a temperature optimum at 30°C. The mutation has been localized betwen *pyr* and *leu* on the *R. meliloti* genetic map (9).

Schwartz and Beckwith (1) and Perlman and Pastan (12) selected mutants of *E. coli* deficient in catabolite repression as unable to grow on more than one sugar. We isolated Rm2620 after carbenicillin treatment of mutagenized Rm1826 cells growing in M9A as unable to grow on a mixture of cellobiose and maltose. (We were unable to isolate such mutants from Rm2011 with combinations of cellobiose, maltose, and lactose.) β-Galactosidase activity in Rm2620 was uninducible by lactose and remained at the basal level of its parent, Rm1826 (Table 1). α-Glucosidase and β-glucosidase activities were also at wild-type basal levels and uninducible by maltose or cellobiose (respectively) at concentrations as high as 2% (not shown). The pleiotropic mutation reverted at high frequency ($>10^{-5}$); no Lac⁺ revertants were isolated from among 10^{10} cells of Rm2620, although the same Lac⁻ mutation in its parent Rm1826 reverted at 1.6×10^{-6}.

Nodulation of alfalfa was tested according to Vincent (15), and nitrogenase activity of nodules was measured by acetylene reduction (3). Rm1826 was as effective as wild type. However, Rm2620 appeared to make very few nodules and not to fix nitrogen, although revertants regained both capacities (Table 2).

TABLE 2. *Acetylene reduction by plants nodulated with different strains of R. meliloti*

Bacterial strains[a]	Ethylene production (nmol/h per plant)[b]	No. of nodules per plant
Rm2011	80	>10
Rm1826	81	>10
Rm2620	0	5–8
Rm2620 revertants	78	>10

[a] A homogeneous culture of the indicated strain was used to inoculate several plants, as described in the text. Nodules were squashed, and the recovered bacteria were tested for revertants. Values for acetylene reduction were averaged over several plants; only plants with revertant-free (<1%) nodules were considered.

[b] Acetylene reduction was measured by the production of ethylene, as described in (3).

In summary, succinate, the preferred carbon source for Rm2011, exerted a catabolite-repression-like effect on β-galactosidase and other activities. A Lac⁻ mutant, Rm1826, seemed similar to *E. coli* Lac⁻ mutants. However, a pleiotropic mutant, Rm2620, lost several catabolic capacities simultaneously, although cAMP did not seem to be involved. Further work will be needed to establish the connection between the lesion in Rm2620 and the diauxie seen in growth on succinate plus lactose. Most intriguing are the deficiencies in nodulation and nitrogen fixation in Rm2620, which suggest a connection between regulation of these phenomena and of catabolic physiology.

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