Regulation of β-Galactosidase Synthesis in Wild Type and in a Succinate-Resistant Mutant of *Rhizobium meliloti*

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The synthesis of β-galactosidase in *Rhizobium meliloti* WU60 was found to be inducible by lactose and its non-metabolizable analogue, isopropyl-β-D-thiogalactoside (IPTG). In contrast to *Escherichia coli*, galactose and melibiose were very weak inducers of this enzyme in *R. meliloti*. The maximum level of β-galactosidase in this bacterium is 2% of that in fully induced *E. coli*. In addition to glucose, the induced synthesis of this enzyme in *R. meliloti* was repressed by galactose, glycerol, and succinate. In comparison to *E. coli*, addition of cyclic AMP to the growth medium of *R. meliloti* did not alleviate the repressive effect of the above compounds on β-galactosidase synthesis.

High concentrations of sodium succinate (100 mM) were inhibitory to the growth of *R. meliloti*. Spontaneous succinate-resistant mutants could be isolated at low frequency. In contrast to the wild type parent, in a succinate-resistant mutant, the synthesis of β-galactosidase was not repressed by succinate.

Introduction

In the *Rhizobium*-legume symbiosis it has been suggested that the supply of carbon *via* photosynthesis from the host plant may limit nitrogen fixation (for reviews, see refs. [1, 2]). The identity of the carbon sources *in vivo* has not yet been confirmed although sucrose, other sugars, and sugar alcohols are the most abundant forms of photosynthate in soybean nodules [3, 4]. Thus, in both the free-living and symbiotic states, *Rhizobia* are likely to be confronted with a mixture of potential carbon sources. In *Rhizobia* the regulatory mechanism(s) involved in the metabolism of one carbon source in the presence of others has not yet been fully investigated [2].

A number of differences exist in the regulation of enzymes involved in the utilization of carbohydrates in various species of *Rhizobium*. For example, in *Rhizobium japonicum*, glucose does not significantly repress induction of mannitol dehydrogenase [5], whereas it is repressive in *R. meliloti* [6]. In *R. trifolii*, Ronson and Primrose [7] have reported repressive effects of sugars on the induction of polyol dehydrogenase. Ucker and Signer [8] found that succinate, but not glucose, had a marked effect on β-galactosidase induction in *R. meliloti*. Glucose however did not repress β-galactosidase synthesis in *R. trifolii* [9]. Glucose and succinate had relatively little effect on the induction of histidase whereas they repressed the synthesis of *p*-hydroxybenzoate catabolic system in *R. leguminosarum* [10].

In other gram-negative bacteria it is known that the nucleotide cyclic adenosine 3,5-phosphate (cAMP) is involved in the transport and catabolism of various nutrients (for review, see Ref. [11]). A wide variation has been reported on the effect of cAMP in gene regulation of *Rhizobium* species. Lim and Shanmugam [12] have shown that the synthesis of a protein required in hydrogen uptake was stimulated by cAMP in *R. japonicum*, whereas the synthesis of ammonia assimilating enzymes was repressed by cAMP in this strain [13]. Succinate-dependent catabolite repression of β-galactosidase in *R. meliloti*, was not reversed by cAMP.

In this study, we have re-examined the regulation of β-galactosidase synthesis in wild type *R. meliloti* WU60 with respect to its kinetics, inducer specificity, catabolite repression and effect of cAMP. In addition, a detailed investigation was also made of the effect of succinate on induced synthesis of β-galactosidase in wild type *R. meliloti* and its succinate-resistant mutant.

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Materials and Methods

Organism

Rhizobium meliloti WU60 was obtained from Dr. A. R. Glenn, School of Environmental and Life Sciences, Murdoch University, Western Australia, Australia.

Chemicals

O-Nitrophenyl-β-D-galactopyranoside (ONPG), isopropyl-β-D-thiogalactoside (IPTG), chloramphenicol, rifampicin, and tetracycline hydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo. USA. Other chemicals were reagent grade, unless otherwise stated.

Media and growth conditions

Cultures were maintained on agar slants of mannitol-salts-yeast extract (MSY) medium [14]. For preparing the inoculum and for enzyme induction experiments described below, the complex medium of Finan *et al.* [15] was used. This medium has the following composition: 6 mM KH_2PO_4, 2 mM K_2HPO_4, 1 mM NaCl, 0.4 mM $MgSO_4 \cdot 7\ H_2O$, 0.2 mM NH_4Cl, 0.1% yeast extract (Oxoid) and 0.1% (w/v) vitamin free caseamino acid (Difco), pH 6.8. Stock solutions of all carbon sources, inducers, and cAMP were filter sterilized.

Liquid cultures were grown with shaking (180 rev. min^{-1}) at 28 °C, starting from a 2% (v/v) inoculum. Growth was followed by measuring the absorbance at 650 nm with a Photo-Chem. colorimeter (MK II, India) as described elsewhere [16].

Kinetics of enzyme induction

The bacteria, in duplicate 200 ml of complex medium [15] containing 0.05% (w/v) L-glutamate, were grown at 28 °C with aeration to a cell density of 5×10^7/ml (0.08 to 0.1 optical density, O.D., unit at 650 nm, Photo-Chem. colorimeter). At this time 0.2% (w/v) lactose was added to the one culture; the other received 5 mM IPTG as inducers. The incubation was continued at 28 °C and growth recorded as above. Five milliliter aliquotes were removed at various times for analysis. Chloramphenicol (100 μg/ml, final concentration) was added to the aliquots to prevent further enzyme synthesis and the samples were then placed in an ice bath. At the end of the experiment, all samples were assayed for enzyme activity as described below.

Effect of carbohydrate on enzyme synthesis

In this experiment cells were grown in duplicate 20 ml aliquots of the above medium in the presence of either arabinose, galactose, glucose, glutamate, glycerol, mannitol, succinate or melibiose as carbon source. As inducers, 5 mM lactose was added to one set of flasks and the other sets received 5 mM IPTG. Cultures grown only in the presence of either IPTG or lactose (as inducers) without further additions of other carbohydrates served as controls. All the cultures were grown as described above until late log phase and then assayed for enzyme activity.

Kinetics of catabolite repression of β-galactosidase

Cells in 400 ml of complex medium lacking a carbon source (carbohydrate) were grown as above to a cell density of 5×10^7/ml. The cultures were then divided aseptically into four equal samples. To one sample, 0.2% (w/v) succinate only was added and out of the remaining 3 samples, one received both lactose (0.2% w/v) and succinate (0.2%) and the remaining two received lactose (0.2%) only in the beginning. Out of these last two samples, to one 0.2% succinate was added at indicated times and the other served as succinate-less control. Similar protocols were set up with IPTG and succinate. The incubation of all the cultures was carried out at 28 °C and 5 ml aliquotes were removed from each culture at various times for analysis as outlined above (see section on enzyme kinetics).

Isolation of a succinate resistant mutant

Spontaneous succinate resistant mutants of *R. meliloti* WU60 were isolated by the method of Glenn and Brewin [17].

Assay of enzyme

At the end of the respective experiments all samples were centrifuged and the cells washed twice with 0.05 M sodium phosphate buffer (pH 7.0) by centrifugation at $10000 \times g$ for 10 min (IEC-25 refregerated centrifuge, India). The cells were resuspended to their original volume in the same buffer but containing 50 μg/ml chloramphenicol. The cells were toluenized and β-galactosidase activity [18] and protein content [19] determined.

Results and Discussion

1. Kinetics of β-galactosidase induction

The synthesis of β-galactosidase in *R. meliloti* WU60 was induced in the presence of lactose and its non-metabolizable analogue, IPTG (Fig. 1). This finding is at variance with the results of Ucker and Signer [8]. These authors have shown that IPTG or TMG (thiomethyl-β-D-galactoside) did not induce the synthesis of this enzyme in *R. meliloti* strain Rm 2011. It is possible that the conflicting results of the above studies may be related to either the use of different growth media or may arise as a result of strain variations [20].

Although the eventual steady state level of β-galactosidase was the same in the presence of lactose and IPTG, the rate of enzyme synthesis was slightly faster in the presence of lactose than IPTG as inducers. The induction of this enzyme was completely prevented by actinomycin-D, rifampicin, tetracycline, and chloramphenicol (results not shown). These observations tend to suggest that transcription and translation of a lactose operon is necessary during β-galactosidase synthesis in *R. meliloti.*

2. Inducer specificity

The effect of different concentrations of various inducers on the induction of β-galactosidase synthesis is shown in Fig. 2. In contrast to results with *E. coli* [21–25], IPTG compared to lactose was slightly less effective as an inducer of β-galactosidase in *R. meliloti.* Similarly, melibiose was a poor inducer of β-galactosidase in this bacterium (Fig. 2) compared to *E. coli* [25], whereas galactose did not induce this enzyme in the former strain compared to the latter strain. Except lactose, the effect of other inducers on β-galactosidase synthesis in *R. meliloti* and other *Rhizobium* species have not been reported before.

3. Effect of sugars on enzyme synthesis

The effect of different carbon sources on β-galactosidase synthesis is summarised in Table I. In contrast to the findings of Ucker and Signer [8] on *R. meliloti,* and De Hollander and Stouthamer [9] on *R. trifolii,* glucose and galactose were found to

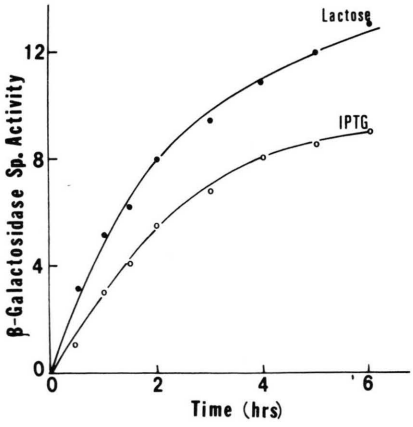

Fig. 1. Kinetics of β-galactosidase synthesis in the presence of lactose or IPTG as inducers in *Rhizobium meliloti* WU60.

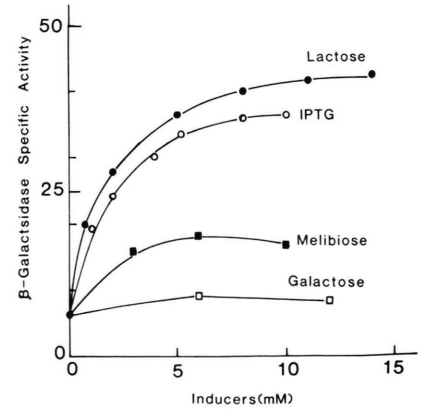

Fig. 2. Effect of various concentrations of different inducers on induction of β-galactosidase synthesis in *Rhizobium meliloti* WU60.

Table I. Effect of various carbon sources on β-galactosidase synthesis in *Rhizobium meliloti* WU60.

Inducers	Carbon source during growth*								
	None	Arabinose	Galactose	Glucose	Glycerol	Mannitol	Melibiose	Succinate	Glutamate
	β-Galactosidase specific activity (nmol/min/mg protein)**								
None	6.7	9.2	5.3	3.5	6.3	8.4	13.4	7.0	11.0
Lactose (5.5 mM)	36.3	25.8	7.5	8.5	10.2	15.4	20.3	18.2	36.5
IPTG (5 mM)	34.0	26.4	10.2	4.3	16.3	27.4	22.5	14.0	39.0

* All carbon sources were used at a final concentration of 0.5% (w/v) except glutamate which was used at a final concentration of 0.05% (w/v).
** The above values are average of three separate experiments.

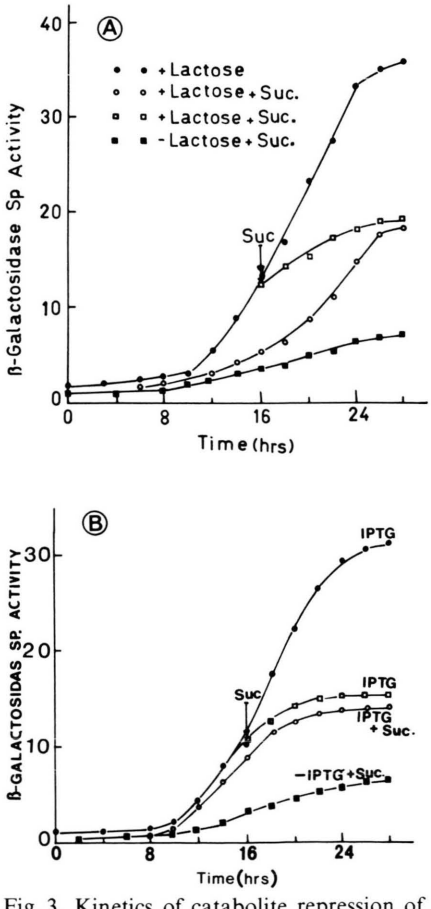

Fig. 3. Kinetics of catabolite repression of β-galactosidase by succinate (*suc*) in the presence of either lactose (A) or IPTG (B) as inducers in *Rhizobium meliloti* WU60.

be the most potent repressors of β-galactosidase synthesis in *R. meliloti* WU60. Arabinose, mannitol, and melibiose showed very weak repression, whereas glycerol and succinate produced intermediate effect. The contradictory findings of the above studies may be due to a difference in culture media or due to strain variations. For example, the induction of mannitol dehydrogenase in *R. meliloti* [6] and polyol dehydrogenase in general in *R. trifolii* [7] was repressed by glucose, whereas the synthesis of mannitol dehydrogenase in *R. japonicum* [5] was repressed by succinate but not by glucose. The higher levels of β-galactosidase activity on glutamate plus lactose or IPTG as compared to that when succinate was used as a carbon source, may be related to a differential levels of endogenous cAMP [12].

4. Kinetics of catabolite repression by succinate

The kinetics of catabolite repression of β-galactosidase synthesis by succinate was similar to that reported by others [8]. Lactose as well as IPTG induced synthesis of this enzyme in *R. meliloti* WU60 was repressed by succinate (Figs. 3 A and B). The order of addition of succinate had no effect on the repression of enzyme synthesis. For example, similar results were obtained when succinate was added at the time of inoculum (zero time) or during the exponential phase of growth (Fig. 3 A and B). The kinetics of succinate dependent repression of enzyme

synthesis were slightly different in the presence of lactose or IPTG when succinate was added at zero time, but were similar when succinate additions were made during exponential phase of growth.

5. Effect of succinate on β-galactosidase synthesis in wild type and in a succinate resistant mutant

Like *R. leguminosarum* [17], high concentrations of sodium succinate (100 mM) were also inhibitory to the growth of *R. meliloti* WU60. Spontaneous succinate resistant mutants of *R. meliloti* were isolated by the method of Glenn and Brewin [17]. Out of several putative succinate resistant mutant, only one such mutant was examined with respect to the effect of succinate on β-galactosidase synthesis. The results of such a study are shown in Fig. 4. Succinate at a concentration of 10 mM inhibited the synthesis of β-galactosidase by 48% and by 2% in wild type and in a succinate resistant mutant, respectively. At higher concentrations, a progressive inhibition of enzyme synthesis occurred in both wild type and its succinate resistant mutant. However, the extent of inhibition was still greater in wild type compared to mutant. For example, 60 mM succinate produced 100% and 65% inhibition in wild type and in mutant strain, respectively.

The succinate resistant mutants of *R. leguminosarum* were found to fall into two classes. One class of mutants (*suc* I) appeared to metabolize succinate at an enhanced rate. The other class (*suc* II) showed decreased rates of succinate uptake and metabolism [17]. The lack of inhibition of β-galactosidase synthesis at lower concentration (10 mM) of succinate in the mutant strain compared to wild type parent seems to suggest that our succinate resistant mutant of *R. meliloti* WU60 may belong to class II of Glenn and Brewin [17]. However, further studies on succinate transport and metabolism in mutant strain *vs.* wild type are needed to resolve such classification of the mutant. These studies are in progress in our laboratory.

6. Effect of cAMP

Consistent with the findings of Ucker and Signer [8], cAMP was unable to reverse the repressive effect of sugar on β-galactosidase synthesis in *R. meliloti* WU60 (Table II). The inability of cAMP to overcome the catabolite repression of β-galactosidase by glucose was not due to impermeability of the cells to this compound. This was demonstrated by its inhibitory effect on the growth of cells (Table II). Like *R. japonicum* [13], cAMP inhibited the growth of *R. meliloti* in a concentration dependent manner (Table II). In contrast to cAMP, other adenine compounds like 5′-AMP, ADP and ATP had no effect on the growth of *R. meliloti* (results not shown), an observation, again consistent with the results of Upchurech and Elkan [13] on *R. japonicum.*

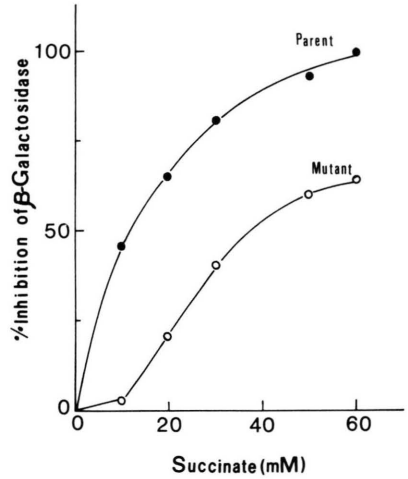

Fig. 4. Effect of different concentrations of succinate on β-galactosidase synthesis in wild type parent and its succinate resistant mutant of *Rhizobium meliloti* WU60. Lactose (0.5%) was used as a inducer.

Table II. Effect of cyclic AMP on growth and β-galactosidase synthesis in *Rhizobium meliloti* WU60.

Additions	Maximum growth (O.D. 650 nm)	β-Galactosidase activity (nmol/min/mg protein)
IPTG only	0.67	34.0
IPTG plus Glucose	0.78	3.0
IPTG plus Glucose plus cAMP [mM]		
1	0.57	3.2
2	0.53	3.5
3	0.47	4.2
4	0.45	4.7
5	0.40	5.8
8	0.32	7.0
10	0.25	8.2

Glucose and IPTG were used at a final concentration of 0.5% (w/v) and 5 mM, respectively.

Kiely and O'Gara [26], from their gene cloning studies, have pointed out that in contrast to *E. coli, R. meliloti* displayed an altered pattern of catabolite repression control by cAMP. These authors have further shown that when *E. coli* gene coding for adenyl cyclase was cloned into *R. meliloti,* it was not fully expressed by *R. meliloti.* In contrast to this finding, it was observed in another study [24] that the lactose (*lac*) operon of *E. coli,* when cloned into *R. meliloti* was fully expressed at the level of *E. coli* by *R. meliloti*; while this bacterium is unable to express its own *lac* operon fully, and the reason for this, is at present not clear.

Despite the presence of adenyl cyclase and cAMP [26] in *R. meliloti,* the reason for the inability of cAMP to alleviate catabolite repression of β-galactosidase caused by sugars in this bacterium is still unknown and this needs further investigation.

Acknowledgements

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