Catabolite Effects on Enzyme Induction and Substrate Utilization in Rhizobium leguminosarum

By M. J. DILWORTH,* I. MCKAY, M. FRANKLIN AND A. R. GLENN
Nitrogen Fixation Research Group, School of Environmental and Life Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

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The catabolism of L-histidine and p-hydroxybenzoate in Rhizobium leguminosarum 3841 is inducible. Glucose or succinate have relatively little effect on the induction of histidase whereas they produce about 50% repression of the p-hydroxybenzoate catabolic system. When grown in the presence of a mixture of carbon sources, e.g. glucose plus histidine, or succinate plus p-hydroxybenzoate, the cells co-utilize both substrates. However, consumption of histidine and p-hydroxybenzoate (which support slower growth rates than glucose or succinate) is substantially reduced in comparison with that of glucose or succinate.

INTRODUCTION

Rhizobia live saprophytically in soil, or engage in a symbiotic relationship with a suitable legume. Although the genus Rhizobium is agronomically important, there is still much about its physiology and biochemistry that is not understood. In both free-living and symbiotic states, rhizobia are likely to be confronted with a mixture of potential carbon sources. Little is currently known about the mechanisms this genus may possess for catabolite control or the selection of substrates from a mixture.

The few studies on the control of substrate selection in Rhizobium suggest differences between species. In Rhizobium japonicum, glucose does not significantly repress induction of mannitol dehydrogenase (Kuykendall & Elkan, 1977), whereas it is repressive in Rhizobium meliloti (Martinez de Drets & Arias, 1970). In Rhizobium trifolii, Ronson & Primrose (1979) have reported repressive effects of sugars on the induction of polyol dehydrogenases. Ucker & Signer (1978) found that succinate, but not glucose, had a marked effect on β-galactosidase induction in R. meliloti. Glucose did not repress β-galactosidase synthesis in R. trifolii, and was utilized simultaneously with lactose (De Hollaender & Stouthamer, 1979). Most of these studies have looked at either enzyme induction or substrate utilization, and there are few data combining both.

To examine catabolite repression in Rhizobium leguminosarum we have chosen two substrates, L-histidine and p-hydroxybenzoate, which have inducible catabolic systems (Glenn & Dilworth, 1981) and which support substantially slower growth rates than glucose or succinate (Dilworth & Glenn, 1981). Utilization of glucose and succinate in R. leguminosarum 3841 has been shown previously to be constitutive (Glenn & Dilworth, 1981). By choosing substrates with inducible catabolic systems, and which support slower growth rates than glucose or succinate, we hoped to maximize the opportunity to observe catabolite repression. This paper reports the effects of glucose and succinate on the induction of enzymes for histidine and p-hydroxybenzoate catabolism and on the utilization of these two substrates.
METHODS

Strains. Rhizobium leguminosarum 3841 is a Str+ derivative of the 300 strain (Johnston & Beringer, 1975) and has been described previously (Glenn et al., 1980); R. leguminosarum WU 235 is a prototrophic strain from the culture collection of the Institute of Agriculture of the University of Western Australia.

Media. Bacteria were grown in batch culture at 28 °C on the liquid minimal medium of Brown & Dilworth (1975) with NH₂₃ (3-8 or 10 mM) as the nitrogen source, and carbon sources at 10 mM (except for p-hydroxybenzoate at 3 mM and histidine at 30 mM). Media were buffered at pH 7.2 with 40 mM-HEPES.

Measurement of bacterial growth. Turbidity of rhizobial cultures was determined at 600 nm in a Varian 635 spectrophotometer (Varian-Techtron, Australia). Cells were diluted in water such that the measured optical density did not exceed 0.3. Since disappearance of substrates from the bacterial cultures was accompanied by substrate utilization rates could be calculated in pmol h⁻¹ O₂ consumed. Area under the optical density-time curve (De Hollaender & Stouthamer, 1979). Linear plots resulted, from which substrate utilization rates could be calculated in μmol h⁻¹ (A₅₆₀⁻¹).

Measurement of oxygen consumption. Harvested cells were washed twice in minimal salts solution at pH 7-2 (Brown & Dilworth, 1975) and resuspended at A₅₆₀ = approx. 0.8 in the same salts solution. Substrate-dependent O₂ consumption was measured polarographically at 25 °C using Hanesatech oxygen electrodes (Hansatech, Norfolk, U.K.) and substrates at 1.5 mM final concentration. Results are expressed as nmol O₂ min⁻¹ (mg protein⁻¹).

Histidase. Histidase (L-histidine ammonia-lyase, EC 4.3.1.3) was assayed in toluenized cell suspensions (Dilworth & Glenn, 1982), each assay tube containing 50 μmol Tris/HCl buffer (pH 9), 5 μmol MgCl₂, and 50 μmol L-histidine (pH 9), in a total volume of 0.5 ml. The reaction was started by the addition of 0.5 ml of toluenized cells and terminated after 20 min incubation at 30 °C by the addition of 1.0 ml 2 M-perchloric acid. Samples were centrifuged and the A₅₆₀ of the resulting supernatant determined, enzyme activity was calculated using a molar absorption coefficient of 18800 1 mol⁻¹ cm⁻¹ at 268 nm for urocanic acid (Tabor, 1957).

Uptake of [³H]histidine. Cells were prepared for measurement of [³H]histidine uptake as described by Hudman & Glenn (1980), except that the cells were centrifuged and washed rather than Millipore filtered. L-2,5-[³H]Histidine (1.5-2.2 TBq mmol⁻¹, 40-60 Ci mmol⁻¹) was purchased from Amersham.

Analytical methods. Ammonia was determined by the phenol/hypochlorite method of Fawcett & Scott (1960).

Histidine was assayed in samples of culture supernatants (containing 20 to 100 nmol histidine) by the method of Ray (1967).

Glucose concentrations in culture supernatants (containing 0 to 0.2 μmol glucose) were determined by the glucose oxidase method with 4-aminophenazone as final chromogen (Trinder, 1969). The method using 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was found to be impractical since HEPES catalyses rapid decolorization of the ABTS oxidation product. Such decolorization does not occur with 4-aminophenazone. To measure p-hydroxybenzoate samples of culture supernatant were made 0.1 M in HCl and the A₅₆₀ recorded. Concentrations were calculated using a molar absorption coefficient of 18800 mol⁻¹ cm⁻¹ at 268 nm for urocanic acid (Tabor, 1957).

Succinate was determined as succinohydroxamic acid using a preparation of succinate thiokinase; it could be stored at approx. 0.8 in the same salts solution. Substrate-dependent oxygen consumption was measured polarographically at 26 °C using Hansatech oxygen electrodes (Hansatech, Norfolk, U.K.) and substrates at 1.5 mM final concentration. Results are expressed as nmol O₂ min⁻¹ (mg protein⁻¹).

Protein was determined by the Lowry method using bovine serum albumin as a standard.

Succinate was determined as succincynhydroxamic acid using a preparation of succinate thiokinase [succinate:CoA ligase (ADP-forming); EC 6.2.1.5] partially purified from extracts of succinate-grown Escherichia coli Crookes strain. Cells were grown in 10 l batches on the medium of Bridger et al. (1969), harvested, washed with 0.2-M-potassium phosphate buffer (pH 7.0), and resuspended in 1.0 ml 2 M-perchloric acid. Samples were centrifuged, and the A₅₆₀ of the resulting supernatant determined, enzyme activity was calculated using a molar absorption coefficient of 23000 1 mol⁻¹ cm⁻¹ at 250 nm for succinoyl-CoA (Gibson & Holmes, 1972). To measure p-hydroxybenzoate samples of culture supernatant were made 0.1 M in HCl and the A₅₆₀ recorded. Concentrations were calculated using a molar absorption coefficient of 18800 mol⁻¹ cm⁻¹ at 268 nm for urocanic acid (Tabor, 1957).

Protein was determined by the Lowry method using bovine serum albumin as a standard.
Catabolite effects in Rhizobium

RESULTS

Histidase induction

Cells of *R. leguminosarum* 3841 were grown on glucose or glycerol minimal salts media to mid-exponential phase ($A_{600}^{\text{nm}} = \text{approx.} 0.5$), centrifuged, washed once in HEPES-buffered minimal salts and resuspended at the same cell density in minimal salts media containing L-histidine (3 mM) or glucose or glycerol or succinate (all at 10 mM). The cultures were shaken at 28 °C and samples taken at intervals for $A_{600}^{\text{nm}}$ and histidase assay. Histidase, the first enzyme in L-histidine catabolism, was induced only in the presence of L-histidine (Fig. 1), consistent with previous results on L-histidine-dependent $O_2$ consumption in this strain (Glenn & Dilworth, 1981). The attainment of fully induced levels of enzyme took place relatively slowly. Similar results were obtained with *R. leguminosarum* WU 235, except that the specific activity of the enzyme was 50% higher than in strain 3841. Strain WU 235 grew more rapidly on L-histidine (mean generation time 5.5 to 6.0 h) than strain 3841 (mean generation time 7.0 to 7.5 h).

Since strain 3841 grows rapidly on glucose/ or succinate/minimal salts (mean generation times of 3.5 to 4.0 h) the effect of substrates such as glucose or succinate on the induction of histidase was investigated. Induction experiments similar to those described previously were carried out, except that cells were resuspended in minimal salts containing L-histidine (3 mM), either alone, or supplemented with glucose (10 mM) or succinate (10 mM). Samples were taken at intervals for measurement of turbidity and histidase activity. The effect of these additional carbon sources varied somewhat between experiments; occasionally there was some repression of histidase activity (Dilworth & Glenn, 1981), but usually there was little or no effect (Fig. 1). This contrasts with the situation in *Pseudomonas fluorescens* where the presence of glucose causes a 40% reduction in histidase synthesis during growth (Jacoby, 1964).

These data suggest that in these particular mixtures *R. leguminosarum* develops a substantial capacity to utilize histidine, even in the presence of substrates supporting more rapid growth. Furthermore, they suggest that, unless some other physiological control mechanism operates to inhibit enzyme activity, or that other enzymes in the pathway behave differently, there should be simultaneous utilization of both substrates.
Co-utilization of substrates by inducing cells

To see if there was simultaneous substrate utilization, the rates of disappearance of substrates were measured. Cells were grown in glucose minimal salts to mid-exponential phase, centrifuged, washed once in buffered minimal salts and resuspended to $A_{600nm} = \text{approx.} \ 0.45$ in buffered minimal salts containing L-histidine (3 mM) or glucose (10 mM) or L-histidine (3 mM) plus glucose (10 mM). Although the rate of synthesis of histidase in the two cultures was not markedly different, the steady-state consumption of L-histidine was reduced from 0.60 μmol h$^{-1}$ (A$_{600nm}$)$^{-1}$ in the control to 0.20 μmol h$^{-1}$ (A$_{600nm}$)$^{-1}$ in the culture containing glucose (Fig. 2). This may be due to some other enzyme in the pathway being limiting, to some direct effect of glucose or other intracellular metabolite on histidase activity, or to some other form of metabolic control. The addition of glucose, or succinate, to histidase assay mixtures showed that these metabolites per se had no effect on histidase activity. However, this does not exclude the possibility that some other metabolite, either derived from, or whose concentration is influenced by glucose or succinate, has an effect on histidase activity.

The consumption of glucose in the culture containing glucose alone was 0.73 μmol h$^{-1}$ (A$_{600nm}$)$^{-1}$, whereas in the culture containing L-histidine and glucose the consumption rate was 0.61 μmol h$^{-1}$ (A$_{600nm}$)$^{-1}$. This difference, although small, suggests a sparing effect of L-histidine on glucose consumption. The growth rate on glucose plus L-histidine (mean generation time 3 h) is faster than on glucose plus NH$_4^+$ (mean generation time 4 h).

The effect of glucose and succinate on histidine consumption was investigated further in experiments using fully induced cells. Cells of strain 3841 were grown on L-histidine (30 mM) to mid-exponential phase ($A_{600nm} = \text{approx.} \ 0.8$) and then resuspended into L-histidine (3 mM) alone, into L-histidine plus glucose or succinate (10 mM) or into glucose or succinate alone (10 mM). Samples were taken at 0.5 h intervals for measurement of turbidity and substrate concentrations. The addition of either glucose or succinate markedly lowered the rate of histidine catabolism (Table 1). There was a simultaneous sparing effect of histidine on consumption of either glucose or succinate. There was no evidence for an initial faster rate of L-histidine catabolism, which then decreased if other substrates were present (Fig. 3).

Similar experiments on histidine-grown cells resuspended in equimolar concentrations of glucose and histidine, or an excess of histidine, gave very similar results.

Effect of glucose and succinate on L-[3H]histidine uptake

One possible explanation for the effects of glucose and succinate on L-histidine metabolism is that they directly affect L-histidine uptake. However, glucose or succinate (10 mM) had no effect on L-[3H]histidine uptake whether added to the cells 1 min prior to measuring uptake, or after the cells were exposed to glucose or succinate for 1, 2 or 3 h (results not shown).

These experiments provide evidence for the co-utilization of L-histidine and glucose or L-histidine and succinate. A complicating factor involved in using L-histidine as a carbon source is that it can also serve as a nitrogen source, and it might be argued that utilization occurs because L-histidine could be a better source of nitrogen than NH$_4^+$. However, the amounts of L-histidine catabolized are well in excess of those required simply to supply nitrogen, and actually result in NH$_4^+$ accumulation in the medium (results not shown), implying that histidine represents a genuine energy supply to the organism.

Induction of p-hydroxybenzoate catabolism

p-Hydroxybenzoate-dependent O$_2$ consumption has been used to assay for the induction of p-hydroxybenzoate catabolism. Previous work (Glenn & Dilworth, 1981) has shown that this system is inducible only in the presence of p-hydroxybenzoate, and that growth on p-hydroxybenzoate is relatively slow (mean generation time 9.0 to 9.5 h).

Effect of glucose and succinate on the induction of p-hydroxybenzoate catabolism

*Rhizobium leguminosarum* 3841 was grown on glucose/ or succinate/minimal salts medium at 28 °C, centrifuged, washed twice in buffered minimal salts and resuspended in fresh media
Table 1. Consumption of glucose, histidine and succinate by Rhizobium leguminosarum 3841

Cells were grown in histidine (30 mM) to mid-exponential phase ($A_{660}^{\text{um}} = \text{approx. 0.8}$) and then resuspended in histidine (3 mM) alone, histidine (3 mM) plus glucose (10 mM), glucose (10 mM) alone, histidine (3 mM) plus succinate (10 mM), or succinate (10 mM) alone.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Consumption rate [$\mu$mol h$^{-1}$ ($A_{660}^{\text{um}}$)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>0.67</td>
</tr>
<tr>
<td>Histidine/glucose</td>
<td>0.44 1.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.31</td>
</tr>
<tr>
<td>Histidine/succinate</td>
<td>0.29 1.25</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of glucose or succinate on L-histidine consumption. Cells were grown on L-histidine and then resuspended in L-histidine (O), L-histidine plus glucose (●), or L-histidine plus succinate (▲) and the rate of histidine utilization determined.

containing glucose (10 mM) or succinate (10 mM), glucose (10 mM) plus p-hydroxybenzoate (1.5 mM), succinate (10 mM) plus p-hydroxybenzoate (1.5 mM), or p-hydroxybenzoate alone (1-5 mM). Samples were taken at intervals for $A_{660}^{\text{um}}$ determination, a portion centrifuged and the supernatant retained for determination of p-hydroxybenzoate, glucose and succinate concentrations. The pellets were washed twice in minimal salts at pH 7.2 and used to measure p-hydroxybenzoate-dependent O$_2$ consumption. There was no induction of the catabolic enzymes in the absence of p-hydroxybenzoate (Glenn & Dilworth, 1981). Cells initially grown on glucose or succinate induced the system to the same specific activity. The presence of glucose or succinate depressed the p-hydroxybenzoate-dependent O$_2$ consumption to 40–50% of the value in control flasks (Fig. 4). However, the cells still developed a substantial capacity to utilize the poorer growth substrate.

After 8 h the cells were centrifuged and resuspended in fresh media identical to those in which they had been suspended initially. At intervals samples were taken, the turbidity measured and culture supernatants used to assay substrate concentrations. The pellets were washed twice in minimal salts (pH 7.2) and assayed for p-hydroxybenzoate-dependent O$_2$ consumption. The final levels of enzyme induction were 250 nmol O$_2$ min$^{-1}$ (mg protein)$^{-1}$ in the cultures containing only p-hydroxybenzoate, whereas in the cultures containing glucose or succinate plus p-hydroxybenzoate the induction reached only 100 nmol O$_2$ min$^{-1}$ (mg protein)$^{-1}$.

Either glucose or succinate decreased the rate of p-hydroxybenzoate catabolism, but did not eliminate it (Table 2). Glucose and succinate consumption rates were lowered when p-hydroxybenzoate was being catabolized in their presence.
Fig. 4. Effect of glucose and succinate on the induction of enzymes for \(p\)-hydroxybenzoate catabolism by *Rhizobium leguminosarum* 3841. Cells grown on glucose/minimal salts were resuspended in \(p\)-hydroxybenzoate alone (○) or in \(p\)-hydroxybenzoate plus glucose (▲). Cells grown on succinate were resuspended in \(p\)-hydroxybenzoate alone (curve essentially identical to that for glucose cells (data not shown)) or \(p\)-hydroxybenzoate plus succinate (●).

Fig. 5. Effect of glucose and succinate on \(p\)-hydroxybenzoate utilization by cells of *Rhizobium leguminosarum* grown on \(p\)-hydroxybenzoate and resuspended in media containing \(p\)-hydroxybenzoate (○), \(p\)-hydroxybenzoate plus glucose (●), or \(p\)-hydroxybenzoate plus succinate (▲).

Table 2. Consumption of glucose, \(p\)-hydroxybenzoate and succinate by *Rhizobium leguminosarum* 3841

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Consumption rate (\mu\text{mol h}^{-1} (A_{600}^{\text{cm}})^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p)-Hydroxybenzoate</td>
<td>0.68</td>
</tr>
<tr>
<td>(p)-Hydroxybenzoate/glucose</td>
<td>0.20</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.12</td>
</tr>
<tr>
<td>(p)-Hydroxybenzoate/succinate</td>
<td>0.14</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>1.82</td>
</tr>
</tbody>
</table>

In subsequent experiments (similar to those carried out previously with histidine), cells were grown on \(p\)-hydroxybenzoate (3 mM) to mid-exponential phase \(A_{600}^{\text{nm}} = \text{approx. 0.8}\) and then resuspended in \(p\)-hydroxybenzoate (1.5 mM) alone, \(p\)-hydroxybenzoate (1.5 mM) plus glucose (10 mM), glucose (10 mM) alone, \(p\)-hydroxybenzoate (1.5 mM) plus succinate (10 mM), or succinate (10 mM) alone. Samples were taken at 0.5 h intervals for measurement of \(A_{600}^{\text{cm}}\) and culture supernatants retained for assay of substrate concentrations. There was always a reduction in the rate of utilization of \(p\)-hydroxybenzoate if glucose or succinate was present. During the first 1.5 to 2.0 h the rate of \(p\)-hydroxybenzoate consumption was essentially constant whatever the composition of the medium. In the experiments shown in Fig. 5 the rates of \(p\)-hydroxybenzoate disappearance in \(p\)-hydroxybenzoate alone, \(p\)-hydroxybenzoate plus glucose, or \(p\)-hydroxybenzoate plus succinate were 0.83, 0.85 and 0.80 \(\mu\text{mol h}^{-1} (A_{600}^{\text{cm}})^{-1}\), respectively. However, after 2 h there was a decrease in the rate of \(p\)-hydroxybenzoate utilization in the presence of glucose [to 0.58 \(\mu\text{mol h}^{-1} (A_{600}^{\text{cm}})^{-1}\)] or succinate [to 0.42 \(\mu\text{mol h}^{-1} (A_{600}^{\text{cm}})^{-1}\)].
DISCUSSION

The results show that catabolic enzyme systems for substrates which support slow growth of *R. leguminosarum* 3841 (‘poor’ substrates) are induced, even in the presence of substrates like glucose or succinate which support more rapid growth (‘good’ substrates). Furthermore, the poorer substrates are utilized, even when glucose or succinate is present in considerable excess. However, the rates of consumption of the ‘poor’ substrates are reduced to a much greater extent (45–80%) than those of the ‘good’ substrates (20%), suggesting that there is a preference for the latter. Reber & Kaiser (1981) have also reported co-utilization of glucose and aromatic substrates, in *Pseudomonas putida*.

We propose that in the two strains of *R. leguminosarum* we studied there are two regulatory mechanisms acting on catabolism. The first acts on enzyme synthesis and/or degradation. Thus, cells grown in the presence of *p*-hydroxybenzoate and glucose or *p*-hydroxybenzoate and succinate develop only 40–50% of the capacity for *p*-hydroxybenzoate oxidation of cultures grown on the aromatic substrate alone. The second appears to act as a physiological control on existing enzyme systems. For example, cells fully induced for L-histidine catabolism show an immediate decrease in their rate of L-histidine utilization upon the addition of glucose or succinate. This is not a direct effect of glucose or succinate on L-histidine uptake, or histidase activity, though the effects of metabolites derived from them cannot be excluded.

The delayed effects of glucose and succinate on the rate of *p*-hydroxybenzoate utilization by cells grown on the aromatic substrate appear to be different from their immediate effects on the rate of L-histidine consumption. Changes in the synthesis, or turnover, of the enzymes of *p*-hydroxybenzoate catabolism appear the most likely explanation. The effects of succinate reported here are similar to those described by Mandelstam & Jacoby (1965) for the mandelate catabolic pathway in *Pseudomonas fluorescens*. Because the final level of induction of the oxidative pathway for *p*-hydroxybenzoate in the presence of succinate is 40% of the control with *p*-hydroxybenzoate alone, while the utilization rate for *p*-hydroxybenzoate is only 20% of the control, some form of physiological regulation is indicated.

The extent to which we can generalize about rhizobia from our results is limited, even though the two genetically unrelated strains of *R. leguminosarum* used (3841 and WU 235) responded in essentially the same way. Our results are similar to those reported by De Hollaender & Stouthamer (1979) for co-utilization of glucose and lactose mixtures by *R. trifolii*.

Taken together, these experiments support our earlier prediction (Dilworth & Glenn, 1981) that when confronted with a binary mixture of utilizable carbon substrates free-living rhizobia are likely to use both. Unless the control processes in the bacteroid are modified by the differentiation process, the physical environment of the nodule, or the complexity of the substrate mixture, simultaneous utilization of substrates would also be expected.

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REFERENCES


