**myo-Inositol catabolism and catabolite regulation in *Rhizobium leguminosarum* bv. *viciae***

Philip S. Poole, Anne Blyth, Colm J. Reid and Kim Walters

On the basis of enzyme assays, myo-inositol appears to be catabolized via 2-keto-myo-inositol and α-2,3-diketo-4-deoxy epi-inositol in *Rhizobium leguminosarum* bv. *viciae*, as occurs in *Klebsiella aerogenes*. The first two enzymes of the pathway, myo-inositol dehydrogenase and 2-keto-myo-inositol dehydratase were increased 7- and 77-fold, respectively, after growth of *R. leguminosarum* on myo-inositol compared to glucose. Cells of *R. leguminosarum* grown on glucose as the carbon source and then resuspended in myo-inositol, increased myo-inositol-dependent O₂ consumption by sixfold in 3 h, confirming this to be an inducible pathway. Succinate, malate and glucose exhibited strong catabolite repression of the myo-inositol catabolic pathway with myo-inositol dehydrogenase and 2-keto-myo-inositol dehydratase being repressed by at least 68% and 80%, respectively, in all cases. A dicarboxylate transport mutant (dctA) was unable to repress either enzyme when grown on myo-inositol and succinate. There was no repression of the first two enzymes of the myo-inositol catabolic pathway in the background of constitutive expression of the dicarboxylate transport system, indicating a dicarboxylate must be present to cause repression. The data imply that dicarboxylates are required intra-cellularly to repress these enzymes. Three transposon mutants were isolated that cannot grow on myo-inositol as the sole carbon source and were unable to induce either myo-inositol dehydrogenase or 2-keto-myo-inositol dehydratase. The mutants were unaffected in the ability to nodulate peas and vetch. Furthermore, vetch plants infected with one mutant (RU360) reduced acetylene at the same rate as plants infected with the wild type. Bacteroids did not oxidize myo-inositol, nor were the catabolic enzymes detected, confirming myo-inositol is not important as an energy source in bacteroids. The possible role of myo-inositol in the rhizosphere is considered.

**Keywords**: *Rhizobium leguminosarum*, myo-inositol, catabolite repression, dct, dicarboxylates

**INTRODUCTION**

*myo*-Inositol is very abundant in both pea and soybean nodules and is the most common compound found inside isolated bacteroids of *Bradyrhizobium japonicum* (Streeter, 1987; Skót & Egsgaard, 1984). Given the presence of large amounts of *myo*-inositol in legume nodules and possibly in the rhizosphere, an important question about carbon flux concerns the regulation of *myo*-inositol utilization. The potential importance of *myo*-inositol catabolism in the rhizosphere is illustrated by *Rhizobium meliloti* strains L5-30 and Rm220-3. In alfalfa nodules, these strains have been shown to produce L-3-O-methylscyillo-inosamine (3-O-MSI) and scyillo-inosamine (sIA) respectively, which belong to the inositol class of compounds called rhizopines (Murphy *et al.*, 1987, 1988, 1993; Saint *et al.*, 1993). In strain L5-30 the genes for synthesis and catabolism of 3-O-MSI are located on the nod–*nif* sym plasmid (Murphy *et al.*, 1987, 1988). Furthermore, the genes for synthesis of 3-O-MSI, but not its catabolism, are symbiotically regulated by *nif*A. It has, therefore, been hypothesized that rhizopines synthesized in the nodule by
bacteroids may be catabolized by free-living bacteria in the rhizosphere. In pea nodules the synthesis of oligonol and O-methyl-stylo-inositol, which also belong to the inositol class of compounds, has been shown to depend on the strain of R. leguminosarum bv. viciae present and a strain of R. leguminosarum bv. viciae has been found that catabolizes 3-O-MSI (Sköt & Egsgaard, 1984; Murphy & Saint, 1992). At present the catabolic pathway for myo-inositol in Rhizobium is uncharacterized, so that it is also unknown whether the products of rhizopine catabolism feed into this pathway.

While the probable carbon and energy sources provided by the legume host for nitrogen fixation by Rhizobium are the C4-dicarboxylic acids, nothing is known about myo-inositol catabolism in the nodule. The demonstrated importance of the C4-dicarboxylic acids is based on the inability of bacteroids of Rhizobium to fix nitrogen when mutated in genes encoding either malic enzyme or the dicarboxylate transport protein (detA) (Ronson et al., 1981; Finan et al., 1983; Arwas et al., 1985; Driscoll & Finan, 1993). Succinate is also able to support respiration by isolated bacteroids (Glenal & Dilworth, 1981) and gluconegenic enzymes are induced in nodules of R. leguminosarum bv. viciae, R. meliloti and Rhizobium strain NGR234 (McKay et al., 1985; Finan et al., 1991; Osteras et al., 1991). Furthermore, the determination of phosphoenolpyruvate carboxykinase in the latter two organisms results in either reduced or undetectable rates of nitrogen fixation. This indicates that the levels of sugars in bacteroids must be very low, because even traces of sugars will repress gluconegenic enzymes in R. leguminosarum bv. viciae (McKay et al., 1985). However, these results do not exclude a role for myo-inositol in the bacteroid.

Determining the possible importance of myo-inositol catabolism requires that both the basic metabolic pathway and its regulation are understood. We therefore chose to investigate the regulation of myo-inositol catabolism in R. leguminosarum bv. viciae because it may be relevant to carbon flux in both the legume nodule and rhizosphere.

**METHODS**

**Bacterial strains and growth media.** Strains are described in Table 1. Bacteria were grown at 28 °C on either TY (Beringer, 1974) or the liquid minimal medium of Brown & Dilworth (1975) with potassium phosphate at 0.5 mM, magnesium at 2 mM, nitrogen and carbon sources at 10 mM and buffering provided by 20 mM MOPS, pH 7.0. Unless otherwise stated kanamycin was used at 50 μg ml⁻¹, and streptomycin at 200 μg ml⁻¹.

**Substrate oxidation and membrane transport.** Cultures of R. leguminosarum bv. viciae used for measurement of substrate-dependent oxygen consumption and membrane transport were grown on appropriate carbon sources to the middle of the exponential growth phase (approximately 5 x 10⁸ cells ml⁻¹), harvested by centrifugation, washed and resuspended in minimal salts minus carbon and nitrogen. Substrate-dependent O₂ consumption was measured with a Rank oxygen electrode as described previously, using test substrates at 10 mM (Glenn & Dilworth, 1981). Induction of myo-inositol oxidation was measured in cultures of R. leguminosarum strain 3841 grown to approximately 5 x 10⁸ cells ml⁻¹ on glucose as the sole carbon source, which were then harvested by centrifugation, washed and resuspended in sterile minimal salts containing myo-inositol and NH₄Cl both at 10 mM. Samples of cells were taken immediately and at time intervals over 3 h, harvested and resuspended in minimal salts minus carbon and nitrogen, before measuring the substrate-dependent oxygen consumption.

Transport was measured as described previously (Poole et al., 1985); each assay contained either 18.5 kBq [2-3H]myo-inositol (67.1 GBq mmol⁻¹) or [2,3,4-¹⁴C]succinate (40 GBq mmol⁻¹) at a total concentration of 50 μM.

**Cell isolation and enzyme assay.** Bacteroids were isolated under air by differential centrifugation as described by Glenn et al., 1980). Cultures of R. leguminosarum bv. viciae (500 ml) were grown on various carbon sources, harvested by centrifugation at a cell density of approximately 5 x 10⁸ cells ml⁻¹, washed and resuspended in 40 mM HEPES, pH 7.0, containing 10 mM 2-mercaptoethanol. Cells were disrupted by two passages through a French press at 69,000 kPa. After centrifugation at 3000 g for 20 min the supernatant was used for enzyme assay. myo-Inositol dehydrogenase and 2-keto-my-ino-sitol dehydratase were assayed by procedures modified from Berman & Magasanik (1966a). The dehydrogenase assay contained (in 1 ml): NH₄Cl, 50 μmol; NAD⁺, 0.4 μmol; sodium carbonate, pH 10, 50 μmol; and myo-inositol, 100 μmol. The dehydratase assay contained (in 1 ml): Tris/HCl, pH 8.0, 50 μmol; 2-keto-my-ino-sitol, 1 μmol. Substrate dehydration was monitored continuously at 260 nm and the concentration of the product determined using a molar extinction coefficient of 6000 for the reaction product. myo-Inositol oxygenase was determined as described by Charalampos (1959). Maleate dehydrogenase was assayed by the technique of Saros et al., 1986). β-galactosidase fusions were assayed according to Miller, 1972) except that chloroform permeabilization was replaced by incubation of cells in 0.5 μg lysozyme ml⁻¹ for 5 min followed by incubation in 1 mM EDTA for 15 min. Cells were then lysed by addition of 0.001 % SDS.

**Genetic analysis.** Mutagenesis was carried out with the Tn5-lacZ derivative B20, by conjugal transfer of the suicide vector pSUP101: Tn5 from the lionor Escherichia coli strain S17-1 to strain 3841, essentially as previously described (Simon et al., 1989). Approximately 10000 transposon mutants were replicated from TY containing streptomycin and kanamycin onto minimal medium with antibiotics and myo-inositol as the sole carbon source. Colonies unable to grow on myo-inositol were purified twice and tested for enzyme and transport lesions.

Transductions were performed according to Buchanan-Wollaston (1979) using the phage RL38. Transductants were selected for on TY agar containing kanamycin (80 μg ml⁻¹).

Southern analysis was carried out on total genomic DNA that was digested with either EcoRI or BamHI, separated on agarose gels (0.8%) and transferred to Amersham Hybond N+. The probe (pRU75) consisted of an internal fragment from IS50. Hybridization and signal detection were performed using an Amersham ECL kit according to the manufacturer’s instructions.

**Plant assays.** Seeds of Pisum sativum cv. meteor and Vicia sativa were surface-sterilized and germinated in sterile water before adding them to cotton-wool-plugged 250 ml conical flasks containing 100 ml sterile vermiculite and wetted with sterile nitrogen-free rooting solution. This contained 1 mM
Table 1. Strains, bacteriophages and plasmids

<table>
<thead>
<tr>
<th>Strain, bacteriophage or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><em>R. leguminosarum</em> <strong>bv. viciae</strong></td>
<td>Str-resistant derivative of strain 300 <strong>bv. viciae</strong></td>
<td>Johnston &amp; Beringer (1975); Wang et al. (1982)</td>
</tr>
<tr>
<td>CR534</td>
<td>Strain 3855 <strong>bv. viciae dctA</strong>: Tn5</td>
<td>Ronson et al. (1987)</td>
</tr>
<tr>
<td>CR535</td>
<td>Strain 3855 <strong>bv. viciae dctB</strong>: Tn5</td>
<td>Ronson et al. (1987)</td>
</tr>
<tr>
<td>CR538</td>
<td>Strain 3855 <strong>bv. viciae dctD</strong>: Tn5</td>
<td>Ronson et al. (1987)</td>
</tr>
<tr>
<td>RU150</td>
<td>Strain 3841 <strong>dctA</strong>: Tn5, constitutive expression of a functional dicarboxylate transporter</td>
<td>C. J. Reid</td>
</tr>
<tr>
<td>RU360</td>
<td>Strain 3841**: Tn5 defective for growth on myo-inositol**</td>
<td>This work</td>
</tr>
<tr>
<td>RU361</td>
<td>Strain 3841**: Tn5 defective for growth on myo-inositol**</td>
<td>This work</td>
</tr>
<tr>
<td>RU362</td>
<td>Strain 3841**: Tn5 defective for growth on myo-inositol**</td>
<td>This work</td>
</tr>
<tr>
<td>RU437</td>
<td>Strain 3841** dctA**: Tn5</td>
<td>C. J. Reid</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td><strong>pro hidR recA</strong> [RP4-2(Tc::Mu) (Km::Tn7)]; RP4 integrated into its chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S17-1</td>
<td>Generalized transducing phage of <strong>R. leguminosarum</strong></td>
<td>Buchanan-Wollaston (1979)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>Suicide vector carrying Tn5-B20 transposon</td>
<td>Simon et al. (1989)</td>
</tr>
<tr>
<td>pSUP101**: :Tn5-lacZ**</td>
<td>pBluescript SK- carrying 1 kb HpaI-HindIII fragment from IS50</td>
<td>Buchanan-Wollaston (1979)</td>
</tr>
<tr>
<td>pRU75</td>
<td>pLAFR1 cosmids containing strain 3841 genomic DNA that complements RU360 and RU362</td>
<td>This work</td>
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</table>

Results and Discussion

Pathway of *myo*-inositol catabolism in *R. leguminosarum* **bv. viciae**

To measure the capacity of *R. leguminosarum* **bv. viciae** to both transport and oxidize various substrates, cells were grown overnight on different substrate combinations and then resuspended in minimal salts without nitrogen and carbon. Substrates were then added to the cell suspensions and the increase in O₂ consumption measured. *myo*-inositol-dependent O₂ consumption by cells of *R. leguminosarum* **bv. viciae** strain 3841 was increased by between 4- and 10-fold by growth on myo-inositol compared to growth on succinate or glucose, respectively (Table 2). By comparison, glucose-dependent O₂ consumption by strain 3841 increased approximately 1·5–2-fold when cells were grown on glucose compared to *myo*-inositol or succinate.
Succinate-dependent O₂ consumption by strain 3841 was increased fivefold by growth of cells on succinate compared to myo-inositol (Table 2). The large increase in myo-inositol-dependent O₂ consumption measured in R. leguminosarum bv. viciae after growth on this compound relative to growth on succinate or glucose suggested that the oxidation of myo-inositol is inducible. To test this, R. leguminosarum bv. viciae was grown to mid-exponential phase on glucose as the carbon source and then harvested, washed and resuspended in minimal medium containing myo-inositol as the sole carbon source. myo-Inositol-dependent O₂ consumption increased sixfold from 10 to 60 nmol min⁻¹ (mg protein)⁻¹ after 3 h incubation, confirming this to be true induction. However, transport of myo-inositol, unlike its oxidation, appeared to be constitutive in strain 3841 with glucose and myo-inositol grown cells transporting myo-inositol at 6·3 and 6·6 nmol min⁻¹ (mg protein)⁻¹, respectively. These rates are low, when compared to rates obtained for primary carbon sources, such as succinate or glucose, of 30–50 nmol min⁻¹ (mg protein)⁻¹ (Glenn et al., 1980). While the apparent rate of myo-inositol transport using the tritiated isotope is low, we have observed a similar low rate of transport of tritiated amino acids where direct comparison could be made to ¹⁴C-labelled compounds (Poole et al., 1985). This isotope effect may be due to either steric hindrance by tritium compared to hydrogen or perhaps loss of the tritium to other volatile components inside the cell.

There are two well documented pathways of myo-inositol catabolism, the first enzyme step being either: (i) a membrane-dependent oxygenase; or (ii) on NAD⁺-dependent dehydrogenase (Charalampous, 1959; Reddy et al., 1981; Berman & Magasanik, 1966a, b; Anderson & Magasanik, 1971a, b). The oxygenase activity occurs in animal tissue, but has not been reported in bacteria, and indeed no orcinol reacting compound, indicative of the oxygenase product, could be detected in R. leguminosarum bv. viciae. However, the specific activity of myo-inositol dehydrogenase in R. leguminosarum was increased sevenfold after growth of cells on myo-inositol relative to glucose (Table 3). This suggests, by analogy to Klebsiella aerogenes, that the NAD⁺-dependent dehydrogenase is the first enzyme in the myo-inositol catabolic pathway in R. leguminosarum bv. viciae. The pH optimum of the dehydrogenase was approximately 10·3 when measured in Tris/HCl and carbonate buffers, with an apparent Kₘ of 4·9 mM.

The second enzyme in the myo-inositol degradation pathway in K. aerogenes is a dehydratase which converts 2-keto-myo-inositol to ß-2,3-diketo-4-deoxy εip-inositol. 2-Keto-myo-inositol dehydratase activity was detectable, with an apparent Kₘ of 0·22 mM, in crude extracts of R. leguminosarum bv. viciae. Its specific activity was increased 77-fold by growth of R. leguminosarum bv. viciae on myo-inositol compared to glucose (Table 3). This strongly suggests that at least the first part of the pathway for degradation of myo-inositol is common between K. aerogenes and R. leguminosarum bv. viciae. However, the four subsequent enzyme-mediated steps of the pathway in K. aerogenes were not assayed in R. leguminosarum bv. viciae due to the difficulty of synthesizing the substrates required to measure them.
Isolation of mutants of \textit{R. leguminosarum} \textit{bv. viciae} unable to utilize myo-inositol

Approximately 10000 Tn5–lacZ transposon mutants of \textit{R. leguminosarum} \textit{bv. viciae} strain 3841 were screened and three mutants, RU360, RU361 and RU362, were isolated that were unable to grow on myo-inositol as the sole carbon source. The mutants all showed normal growth on minimal medium containing either pyruvate, arabinose or glucose as the sole source of carbon. None of the mutants had transport rates for myo-inositol that were significantly different from that in strain 3841 (data not shown). Since growth of \textit{R. leguminosarum} \textit{bv. viciae} on pyruvate/myo-inositol did not repress myo-inositol dehydrogenase and only had a small effect on 2-keto-myoinositol dehydratase, mutants with inserts in either gene should express the other gene under these growth conditions. Neither RU360 or RU362 induced myo-inositol dehydrogenase or 2-keto-myoinositol dehydratase when grown on pyruvate/myo-inositol (Table 3). This confirms that these two enzymes are required for the catabolism of myo-inositol in \textit{R. leguminosarum} \textit{bv. viciae}.

Genomic digests of DNA from the Tn5–lacZ-containing mutant strains RU360, RU361 and RU362 were Southern blotted and hybridized with a probe containing an internal fragment of IS50. The probe hybridized to a single EcoRI blotted and hybridized with a probe containing an internal fragment of 5.8 kb and 10 kb, respectively, in both strains RU360 and RU361, indicating they have single transposon insertions (Fig. 1). Due to the similarity in size of the hybridizing bands in strains RU360 and RU361 it is likely these mutants are siblings and for this reason RU361 was not extensively analysed. The genomic digest of strain RU362 has two EcoRI fragments of 4.2 kb and 12 kb which hybridize with the probe containing IS50 (Fig. 1). Considering that the minimum size of an EcoRI fragment containing Tn5–lacZ is slightly less than 5.5 kb, this implies the second band at 4.2 kb may be a copy of IS50 independent from Tn5–lacZ.

The transposons in strains RU360, RU361 and RU362 were transduced, with the bacteriophage RL38, back into the wild-type strain 3841 by selecting for kanamycin resistance. For strain RU360 and strain RU361, 43 transductants of each were tested for growth on myo-inositol and only one transductant from strain RU361 grew on myo-inositol. This confirms the results from Southern analysis that there is a single transposon insertion in strains RU360 and RU361 that is tightly linked to the inability to grow on myo-inositol. Tn5–lacZ did not form an active β-galactosidase fusion in RU360. All 43 transductants of strain RU362 tested grew on myo-inositol. This demonstrates that the complete copy of Tn5–lacZ present in this strain did not cause the inability to grow on myo-inositol. It is possible that the mutation is caused by the copy of IS50 that is independent of the complete transposon or alternatively that a spontaneous mutation has occurred.

Since the pathway of myo-inositol catabolism appears to be inducible, it is not possible to determine whether the transposon insertions are in particular structural genes. They may, for example, be in regulatory genes, mutation of which prevents induction of the myo-inositol catabolic pathway. A genomic cosmid library of strain 3841 was conjugated into strain RU360 and cells able to grow on myo-inositol were isolated. Two overlapping cosmids pRU3068 and pRU3069 were isolated, both of which complemented RU360 and RU362 for growth on myo-inositol. This indicates that both mutations are clustered in the same region.

Catabolite repression of the pathway for myo-inositol catabolism

Growth of \textit{R. leguminosarum} \textit{bv. viciae} on succinate/myo-inositol reduced the rate of myo-inositol-dependent O2 consumption by 80\%, while it only reduced succinate-dependent O2 consumption by 26\% relative to growth on the respective single substrates (Table 2). Cells of strain 3841 grown on glucose, succinate or malate in combination with myo-inositol had specific activities of myo-inositol dehydrogenase reduced by 85\%, 79\% and 68\%, in the order stated, compared to cells grown on myo-inositol alone (Table 3). Repression of 2-keto-myoinositol dehydratase was even greater in cells grown on myo-inositol plus glucose or succinate or malate with specific activities decreased by 87\%, 85\% and 80\%, in the order stated (Table 3). Growth of strain 3841 on pyruvate/myo-inositol had no effect on myo-inositol dehydrogenase and only reduced the specific activity of 2-keto-myoinositol dehydratase by 46\% relative to cells grown on myo-inositol alone.

One problem in examining catabolite repression in \textit{Rhizobium} is that many of the compounds whose repression has been investigated are only weakly repressed, or have a complex role in both carbon and nitrogen metabolism (De Hollaender & Stouthamer, 1979;
Dilworth et al., 1983; Fitzmaurice & O'Gara, 1991; Mandal & Chakrabarty, 1993; McKay et al., 1989; Ucker & Signer, 1978). C₃-Dicarboxylates have been shown to be a preferred carbon source in *Rhizobium* and organic acids are responsible for catabolite control in *Pseudomonas aeruginosa* (McKay et al., 1989; Wolff et al., 1991; Macgregor et al., 1991). The ability of the C₃-dicarboxylates succinate and malate to repress myo-inositol utilization is consistent with their role as a preferred carbon source. However, glucose is also very effective at repressing myo-inositol utilization, indicating that the repression is not specific for a single class of compound.

**Role of the dct system in the catabolite repression of myo-inositol-degrading enzymes**

It is not clear how catabolite repression is mediated in *Rhizobium*. To try and determine whether catabolite repression of myo-inositol utilization by succinate requires the intracellular accumulation of repressor, the effect of *dct* mutants on myo-inositol catabolism was investigated. The *dct* system consists of three genes in *R. leguminosarum* bv. *viciae* and *R. meliloti*: *dctA* which encodes the putative dicarboxylate transport protein and *dctB-dctD* which encode a two-component sensor and regulator, respectively (Ronson et al., 1984, 1987; Watson, 1990; Jiang et al., 1989; Engelke et al., 1989). These genes are transcribed divergently from *dctA* and activate transcription of *dctA* in response to the presence of dicarboxylates in the environment (Ronson & Astwood, 1985; Ledebur & Nixon, 1992; Wang et al., 1989; Batista et al., 1992; Ledebur et al., 1990; Ronson, 1988; Jording et al., 1992; Yarosh et al., 1989). Most models suggest that in response to the presence of dicarboxylates DctB undergoes auto-phosphorylation and in turn phosphorylates DctD, enabling it to activate transcription (Ronson, 1988; Yarosh et al., 1989; Jording et al., 1992; Ledebur et al., 1990). Strains CR354, CR355 and CR358, which are *dctA, dctB* and *dctD* mutants of *R. leguminosarum* bv. *viciae* 3855, respectively (Ronson et al., 1987), grew as well as the wild type on agar plates with myo-inositol as the sole carbon source. There was also no apparent difference between the growth of all three mutants and the wild type on myo-inositol plus succinate compared to myo-inositol alone. Since the *dct* mutants do not grow on succinate, their rapid growth on succinate plus myo-inositol suggests that myo-inositol utilization is not repressed in any of the *dct* mutants. To investigate this more rigorously, strain RU437, which is a *dctA* mutant of *R. leguminosarum* bv. *viciae* strain 3841 (C. J. Reid, unpublished results), was grown on a variety of carbon sources and the effect on the first two enzymes of the myo-inositol catabolic pathway determined (Table 4). Myo-Inositol dehydrogenase and 2-keto-myo-inositol dehydratase activity were increased 67- and 48-fold, respectively, in strain RU437 by growth on myo-inositol compared to glucose. Cells of strain RU437 grown on glucose and myo-inositol together had activities of myo-inositol dehydrogenase and 2-keto-myo-inositol dehydratase reduced by 47% and 71%, respectively, relative to cells grown on myo-inositol alone (Table 4).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity [μmol min⁻¹ (mg protein)⁻¹]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Myo-Inositol dehydrogenase</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>0.220±0.001</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.034±0.001</td>
</tr>
<tr>
<td>Myo-Inositol/glucose</td>
<td>0.116±0.003</td>
</tr>
<tr>
<td>Myo-Inositol/succinate</td>
<td>0.267±0.007</td>
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</tbody>
</table>

These results demonstrate that in a *dctA* mutant glucose is able to significantly reduce the induction of the first two enzymes of myo-inositol catabolism, as occurs in the wild type (Tables 3 and 4). However, cells of strain RU437 grown on succinate plus myo-inositol had activities of the first two enzymes of myo-inositol catabolism that were equal to or greater than when grown on myo-inositol alone (Table 4). This clearly demonstrates that succinate is unable to repress myo-inositol catabolism in a *dct* mutant. There are two reasons why this may occur. Firstly, the *dct* system, which is a sensor for dicarboxylates in the environment, may be involved in signal transduction for catabolite repression by dicarboxylates. Secondly, if intracellular dicarboxylates cause catabolite repression, then a mutation in their principal transport system would prevent access to the cytoplasm. To distinguish between these possibilities we examined repression of 2-keto-myo-inositol dehydratase in strain RU150, which constitutively expresses the *dct* system due to a Tn5 insertion 9 bp upstream of the *dctA* ribosome binding site (C. J. Reid, unpublished results). The constitutive expression of the dicarboxylate transport system was demonstrated by measuring succinate uptake. Strain RU150 grown on glucose as the sole carbon source transported succinate at 46±1 (SEM, three replicates) nmol min⁻¹ (mg protein)⁻¹. This can be compared to the parental strain 3841 which transports succinate at 50±5 and 37±2 (SEM, three replicates) nmol min⁻¹ (mg protein)⁻¹ when grown on succinate and malate, respectively, but only at 2±0.1 (SEM, three replicates) nmol min⁻¹ (mg protein)⁻¹ when grown on glucose.

Cells of strain RU150 grown on myo-inositol (10 mM) as the sole carbon source had a specific activity of myo-inositol dehydratase of 0.622±0.06 (SEM, three replicates) mmol min⁻¹ (mg protein)⁻¹. When grown on myo-inositol (10 mM) plus malate (10 mM) the specific activity of myo-inositol dehydratase was 0.101±0.04 (SEM, three replicates) mmol min⁻¹ (mg protein)⁻¹. These data demonstrate that the constitutive expression of the *dct* system alone is insufficient to repress 2-keto-myo-inositol de-
hydratase. A dicarboxylate is still required, suggesting that it causes catabolite repression after it has reached the cytoplasm. The role of the DCT system would simply be to deliver the dicarboxylate to the cytoplasm. The alternative that the DCT system is directly involved in the signalling pathway for catabolite repression only when a dicarboxylate is bound to the transport system is unlikely. This is because dctA mutants of Rhizobium have been shown to constitutively express dctA, and this is dependent on the normal DctB/DctD phosphorylation pathway (Ronson, 1988; Yarosh et al., 1989). Thus, the signalling pathway for expression of the dicarboxylate transport protein is fully active in such mutants, yet they are unable to repress myo-inositol dehydrogenase and 2-keto-myoinositol dehydratase.

Sucinate is known to be a repressor of β-galactosidase in R. meliloti (Ucker & Signer, 1978) and it has been shown recently that this requires a functional DCT system (Jelesko & Leigh, 1994). lacZ as well as lacW and lacX, which form a lac regulon, were all repressed by succinate. It was concluded that the mechanism does not appear to be solely due to inducer exclusion but presumably requires catabolite repression (Jelesko & Leigh, 1994).

Since both dicarboxylates and glucose are strong repressors of the myo-inositol catabolic pathway, any consideration of the nature of the intracellular signalling pathway for catabolite repression in Rhizobium must account for the lack of specificity with regard to the repressor. The lack of a direct role in signalling for the DCT system is consistent with there being an intracellular mechanism perhaps dependent on catabolism of the compound that leads to repression. There are numerous possible intracellular signalling intermediates including either a direct product of dicarboxylate metabolism or a central factor such as energy charge or redox potential. It is apparent that to further define the signalling pathway there is a need to isolate specific catabolic control mutants as has been done with P. aeruginosa (Wolf et al., 1991; Macgregor et al., 1991).

Properties on plants of mutant strains unable to utilize myo-inositol

While dicarboxylates are probably the principal carbon and energy sources for bacteroid metabolism (Ronson et al., 1981; Finan et al., 1983; Arwas et al., 1985; Glenn et al., 1984; Glenn & Dilworth, 1981), it was important to determine if myo-inositol has a direct role in fuelling nitrogen fixation. Isolated bacteroids did not exhibit any myo-inositol-dependent O₂ consumption even though they showed succinate-dependent O₂ consumption (Table 2). While this implies that myo-inositol is not important as an energy source in the bacteroid, the possibility of bacteroid damage cannot be excluded, particularly as these bacteroids were isolated under air. To ensure that the absence of myo-inositol-dependent O₂ consumption was not due to bacteroid damage during isolation, the activities of myo-inositol dehydrogenase and 2-keto-myoinositol dehydratase were measured in isolated bacteroids.

The two enzymes were present at very low levels compared to that seen in free-living cultures grown on myo-inositol (Table 3). Malate dehydrogenase was assayed as a control and the activity was 9.2 ± 0.326 (SEM, three replicates) μmol min⁻¹ (mg protein)⁻¹.

To further examine whether the inability to catabolize myo-inositol would alter nitrogen fixation in bacteroids, the mutant strains RU360, RU361 and RU362 were inoculated onto peas and strain RU360 also onto vetch. Pea plants inoculated with these strains formed pink nodules indistinguishable from those formed by the parent strain 3841. In addition, vetch inoculated with strain RU360 had the same shoot dry weight and reduced acetylene at a similar rate to plants inoculated with strain 3841 (0.33 versus 0.38 μmol h⁻¹ per plant for RU360 and 3841, respectively). Bacteria extracted from 15 nodules of peas inoculated with strain RU360, 14 from strain RU361 and 22 from strain RU362 were first grown on TY, then replica-plated. All bacteria were kanamycin-resistant and failed to grow on myo-inositol as the sole carbon source. Thus, the data for O₂ consumption, enzyme activity and mutant analysis indicate that substantial catabolism of myo-inositol does not occur in bacteroids of pea nodules.

Given the ubiquitous presence of myo-inositol in legume nodules and the demonstrated ability of some strains of bacteroids to synthesize rhizopines, which belong to the inositol class of compounds, catabolism of myo-inositol may be particularly important to survival and competition of Rhizobium in the rhizosphere. Now that mutants of the myo-inositol catabolic pathway are available, it should be possible to determine whether the immediate breakdown products of rhizopines from the inositol family feed into the same catabolic pathway as that used by myo-inositol. If they do then myo-inositol-utilizing mutants will also be blocked in rhizopine utilization. The lack of myo-inositol degradation in the nodule is consistent with the model that some bacteroids may synthesize rhizopines in the nodule for catabolism by free-living bacteria in the rhizosphere (Murphy et al., 1987, 1988, 1993; Saint et al., 1993). If rhizopines are present in the rhizosphere and the products of their degradation feed into the myo-inositol degrading pathway then we would predict that the enzymes of myo-inositol degradation will be induced in the rhizosphere and may be important in competition and survival. There is also the possibility that myo-inositol itself is present in the rhizosphere and that rhizopine synthesis is an evolutionary step to sequester it for those organisms capable of its synthesis and degradation. This is currently under investigation.

REFERENCES


dicarboxylate transport mutants of Rhizobium trifolii form ineffective nodules on Trifolium repens. Proc Natl Acad Sci USA 78, 4284–4288.


