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

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On the basis of enzyme assays, *myo*-inositol appears to be catabolized via 2-keto-*myo*-inositol and D-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; Sköt & 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-methyl-*scyllo*-inosamine (3-O-MSI) and *scyllo*-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 *nifA*. 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 ononitol and *O*-methyl-*scyllo*-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 C_4 -dicarboxylic acids, nothing is known about *myo*-inositol catabolism in the nodule. The demonstrated importance of the C_4 -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 (Glenn & Dilworth, 1981) and gluconeogenic 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, mutation 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 gluconeogenic enzymes in *R. leguminosarum* bv. *viciae* (McKay *et al.*, 1985). However, these results do not preclude 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 40 µg ml⁻¹ and streptomycin at 500 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 5×10^8 cells ml⁻¹), harvested by centrifugation, washed and resuspended in minimal salts minus carbon and nitrogen. Substrate-dependent O_2 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 5×10^8 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 (677.1 GBq mmol⁻¹) or [2,3-¹⁴C]succinate (4.0 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 5×10^8 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 30 000 *g* for 20 min the supernatant was used for enzyme assay. *myo*-Inositol dehydrogenase and 2-keto-*myo*-inositol 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-*myo*-inositol, 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). Malate dehydrogenase was assayed by the technique of Saroso *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 mg 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 donor *Escherichia coli* strain S17-1 to strain 3841, essentially as previously described (Simon *et al.*, 1989). Approximately 10 000 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 *Eco*RI or *Bam*HI, 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

Strain, bacteriophage or plasmid	Description	Source or reference
<i>R. leguminosarum</i> bv. <i>viciae</i>		
3841	Str-resistant derivative of strain 300 bv. <i>viciae</i>	Johnston & Beringer (1975); Wang <i>et al.</i> (1982)
CR534	Strain 3855 bv. <i>viciae</i> <i>dctA</i> ::Tn5	Ronson <i>et al.</i> (1987)
CR535	Strain 3855 bv. <i>viciae</i> <i>dctB</i> ::Tn5	Ronson <i>et al.</i> (1987)
CR538	Strain 3855 bv. <i>viciae</i> <i>dctD</i> ::Tn5	Ronson <i>et al.</i> (1987)
RU150	Strain 3841 <i>dctA</i> ::Tn5, constitutive expression of a functional dicarboxylate transporter	C. J. Reid
RU360	Strain 3841::Tn5 defective for growth on myo-inositol	This work
RU361	Strain 3841::Tn5 defective for growth on myo-inositol	This work
RU362	Strain 3841::Tn5 defective for growth on myo-inositol	This work
RU437	Strain 3841 <i>dctA</i> ::Tn5	C. J. Reid
<i>E. coli</i>		
S17-1	<i>pro hsdR recA</i> [RP4-2(Tc::Mu) (Km::Tn7)]; RP4 integrated into its chromosome	Simon <i>et al.</i> (1983)
Bacteriophage		
RI.38	Generalized transducing phage of <i>R. leguminosarum</i>	Buchanan-Wollaston (1979)
Plasmids		
pSUP101::Tn5- <i>lacZ</i>	Suicide vector carrying Tn5-B20 transposon	Simon <i>et al.</i> (1989)
pRU75	pBluescript SK — carrying 1 kb <i>HpaI</i> – <i>HindIII</i> fragment from IS50	This work
pRU3078–pRU3079	pLAFR1 cosmids containing strain 3841 genomic DNA that complements RU360 and RU362	This work

CaCl₂·2H₂O, 100 µM KCl, 800 µM MgSO₄·7H₂O, 10 µM Fe EDTA, 35 µM H₃BO₃, 9 µM MnCl₂·4H₂O, 0.8 µM ZnCl₂, 0.5 µM Na₂MoO₄·H₂O, 0.3 µM CuSO₄·5H₂O, 7.2 mM KH₂PO₄ and 7.2 mM Na₂HPO₄. The phosphate solutions were autoclaved separately and added to the main stock just prior to use. After 3 d the seeds were inoculated with a bacterial culture and seedlings kept in the dark until the shoot reached the top of the flask, after which the shoot was pulled through, and the flask wrapped with foil to exclude light from the roots. Plants were incubated at 25 °C in a growth room with illumination provided by a Philips Sont-Agro grow light. Four weeks after inoculation, plants were harvested and acetylene reduction carried out on whole plants as described by Trinick *et al.* (1976). Sample nodules were removed and surface-sterilized by immersion in calcium hypochlorite (0.7%) for 10 min. Nodules were then washed three times in sterile distilled water, crushed and bacteria streaked on TY agar. Isolated bacteria were then replica plated and screened for appropriate antibiotic and nutritional markers.

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.

Table 2. Substrate-dependent oxygen uptake by *R. leguminosarum* bv. *viciae* strain 3841 grown on various substrates

All results are the mean of two independent extracts. Bacteroids are from one plant harvest and the SEM is of three replicate assays. ND, not determined; UD, undetectable.

Substrate	Oxygen uptake [nmol O ₂ min ⁻¹ (mg protein) ⁻¹] on:		
	<i>myo</i> -Inositol	Glucose	Succinate
<i>myo</i> -Inositol	129	67	16
Succinate	30	45	79
Glucose	13	101	ND
Succinate/ <i>myo</i> -Inositol	26	59	58
Bacteroids	UD	ND	18 ± 1.3

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 results 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

Table 3. Specific activities of enzymes of the *myo*-inositol pathway in *R. leguminosarum* bv. *viciae*

Values are the mean of three or more independent extracts ± SEM. Bacteroids are from one plant harvest and the SEM is of three replicate assays.

Strain	Substrate	Specific activity [μmol min ⁻¹ (mg protein) ⁻¹]	
		<i>myo</i> -Inositol dehydrogenase	2-Keto- <i>myo</i> - inositol dehydratase
3841	<i>myo</i> -Inositol	0.254 ± 0.034	0.85 ± 0.129
	Glucose	0.038 ± 0.005	0.011 ± 0.003
	Succinate	0.053 ± 0.009	0.012 ± 0.003
	Malate	0.081 ± 0.020	0.017 ± 0.005
	Pyruvate	0.045 ± 0.017	0.019 ± 0.009
	Glucose/ <i>myo</i> -inositol	0.082 ± 0.034	0.143 ± 0.074
	Succinate/ <i>myo</i> -inositol	0.137 ± 0.034	0.107 ± 0.052
	Malate/ <i>myo</i> -inositol	0.121 ± 0.051	0.092 ± 0.039
	Pyruvate/ <i>myo</i> -inositol	0.277 ± 0.103	0.462 ± 0.09
RU360	Pyruvate/ <i>myo</i> -inositol	0.08 ± 0.017	0.009 ± 0.005
RU362	Pyruvate/ <i>myo</i> -inositol	0.072 ± 0.036	0.033 ± 0.008
Bacteroids		0.011 ± 0.001	0.029 ± 0.001

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 *K_m* in crude extract 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 D-2,3-diketo-4-deoxy *epi*-inositol. 2-Keto-*myo*-inositol dehydratase activity was detectable, with an apparent *K_m* 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.

Fig. 1. Southern blot analysis of Tn5-*lacZ* mutants. Lanes; 1, 3 and 5, *Eco*RI digests of RU360, RU361 and RU362 chromosomal DNA, respectively; 2, 4 and 6, *Bam*HI digests of RU360, RU361 and RU362 chromosomal DNA, respectively. Positions and sizes (in kb) of marker DNA molecules are shown to the left.

Isolation of mutants of *R. leguminosarum* bv. *viciae* unable to utilize *myo*-inositol

Approximately 10000 Tn5-*lacZ* transposon mutants of *R. leguminosarum* 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 *R. leguminosarum* bv. *viciae* on pyruvate/*myo*-inositol did not repress *myo*-inositol dehydrogenase and only had a small effect on 2-keto-*myo*-inositol 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-*myo*-inositol dehydratase when grown on pyruvate/*myo*-inositol (Table 3). This confirms that these two enzymes are required for the catabolism of *myo*-inositol in *R. leguminosarum* 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 *Eco*RI and *Bam*HI 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 *Eco*RI fragments of 4.2 kb and 12 kb which hybridize with the probe containing IS50 (Fig. 1). Considering that the minimum size of an *Eco*RI

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 *R. leguminosarum* 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-*myo*-inositol 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-*myo*-inositol dehydratase by 46% relative to cells grown on *myo*-inositol alone.

One problem in examining catabolite repression in *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). C₄-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 C₄-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, which binds to the tandem upstream activator sites of *dctA* enabling it to activate transcription (Ronson, 1988; Yarosh *et al.*, 1989; Jording *et al.*, 1992; Ledebur *et al.*, 1990). Strains CR534, CR535 and CR538, 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 6- 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 4. Catabolite repression of *myo*-inositol-degrading enzymes in the *dctA* mutant *R. leguminosarum* bv. *viciae* strain RU437

Values are the mean of six determinations from two independent extracts $\pm$ SEM.

Substrate	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	
	<i>myo</i> -Inositol dehydrogenase	2-Keto- <i>myo</i> - inositol dehydratase
<i>myo</i> -Inositol	0.220 $\pm$ 0.001	0.905 $\pm$ 0.022
Glucose	0.034 $\pm$ 0.001	0.019 $\pm$ 0.003
<i>myo</i> -Inositol/glucose	0.116 $\pm$ 0.003	0.263 $\pm$ 0.006
<i>myo</i> -Inositol/succinate	0.267 $\pm$ 0.007	1.012 $\pm$ 0.037

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) $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. This can be compared to the parental strain 3841 which transports succinate at 50 ± 5 and 37 ± 2 (SEM, three replicates) $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ when grown on succinate and malate, respectively, but only at 2 ± 0.1 (SEM, three replicates) $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ 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) $\text{mmol min}^{-1} (\text{mg protein})^{-1}$. 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) $\text{mmol min}^{-1} (\text{mg protein})^{-1}$. 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-*myo*-inositol dehydratase.

Succinate 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* (Wolff *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-*myo*-inositol 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) $\mu\text{mol min}^{-1}$ (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 $\mu\text{mol h}^{-1}$ 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

- Anderson, W. A. & Magasanik, B. (1971a). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* identification of the intermediate 2-deoxy-5-keto-D-gluconic acid. *J Biol Chem* **246**, 5653–5661.
- Anderson, W. A. & Magasanik, B. (1971b). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* conversion of 2-deoxy-5-keto-D-gluconic acid to glycolytic intermediates. *J Biol Chem* **246**, 5662–5675.

- Arwas, R., McKay, I. A., Rowney, F. R. P., Dilworth, M. J. & Glenn, A. R. (1985). Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. *J Gen Microbiol* **131**, 2059–2066.
- Batista, S., Castro, S., Aguilar, O. M. & Martinezdrets, G. (1992). Induction of C(4)-dicarboxylate transport genes by external stimuli in *Rhizobium meliloti*. *Can J Microbiol* **38**, 51–55.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.
- Berman, T. & Magasanik, B. (1966a). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* dehydrogenation and dehydration. *J Biol Chem* **241**, 800–806.
- Berman, T. & Magasanik, B. (1966b). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* ring scission. *J Biol Chem* **241**, 807–813.
- Brown, C. M. & Dilworth, M. J. (1975). Ammonia assimilation by *Rhizobium* cultures and bacteroids. *J Gen Microbiol* **86**, 39–48.
- Buchanan-Wollaston, V. (1979). Generalized transduction in *Rhizobium leguminosarum*. *J Gen Microbiol* **112**, 135–142.
- Cheralampous, F. C. (1959). Biochemical studies on inositol. *J Biol Chem* **234**, 220–227.
- De Hollaender, J. A. & Stouthamer, J. A. (1979). Multicarbon substrate growth of *Rhizobium trifolii*. *FEMS Microbiol Lett* **6**, 57–59.
- Dilworth, M. J., McKay, I. A., Franklin, M. & Glenn, A. R. (1983). Catabolite effects on enzyme induction and substrate utilization in *Rhizobium leguminosarum*. *J Gen Microbiol* **129**, 359–366.
- Driscoll, B. T. & Finan, T. M. (1993). NAD⁺-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Mol Microbiol* **7**, 865–873.
- Engelke, T., Jording, D., Kapp, D. & Pühler, A. (1989). Identification and sequence analysis of the *Rhizobium meliloti* *dctA* gene encoding the C₄-dicarboxylate carrier. *J Bacteriol* **171**, 5551–5560.
- Finan, T. M., Wood, J. M. & Jordan, D. C. (1983). Symbiotic properties of C₄-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J Bacteriol* **154**, 1403–1413.
- Finan, T. M., Mcwhinnie, E., Driscoll, B. & Watson, R. J. (1991). Complex symbiotic phenotypes result from gluconeogenic mutations in *Rhizobium meliloti*. *Mol Plant-Microbe Interact* **4**, 386–392.
- Fitzmaurice, A. M. & O'Gara, F. (1991). Glutamate catabolism in *Rhizobium meliloti*. *Arch Microbiol* **155**, 422–427.
- Glenn, A. R. & Dilworth, M. J. (1981). Oxidation of substrates by isolated bacteroids and free-living cells of *Rhizobium leguminosarum* 3841. *J Gen Microbiol* **126**, 243–247.
- Glenn, A. R., Poole, P. S. & Hudman, J. F. (1980). Succinate uptake by free-living and bacteroid forms of *Rhizobium leguminosarum*. *J Gen Microbiol* **199**, 267–271.
- Glenn, A. R., McKay, I. A., Arwas, R. & Dilworth, M. J. (1984). Sugar metabolism and the symbiotic properties of carbohydrate mutants of *Rhizobium leguminosarum*. *J Gen Microbiol* **130**, 239–245.
- Jelesko, J. G. & Leigh, J. A. (1994). Genetic characterisation of a *Rhizobium meliloti* lactose utilization locus. *Mol Microbiol* **11**, 165–173.
- Jiang, J., Gu, B., Albright, L. M. & Nixon, B. T. (1989). Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum* *dct* genes. *J Bacteriol* **171**, 5244–5253.
- Johnston, A. W. B. & Beringer, J. E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *J Gen Microbiol* **87**, 343–350.
- Jording, D., Sharma, P. K., Schmidt, R., Engelke, T., Uhde, C. & Pühler, A. (1992). Regulatory aspects of the C₄-dicarboxylate transport in *Rhizobium meliloti* – transcriptional activation and dependence on effective symbiosis. *J Plant Physiol* **141**, 18–27.
- Ledebur, H. & Nixon, B. T. (1992). Tandem *DctD*-binding sites of the *Rhizobium meliloti* *dctA* upstream activating sequence are essential for optimal function despite a 50-fold to 100-fold difference in affinity for DctD. *Mol Microbiol* **6**, 3479–3492.
- Ledebur, H., Gu, B., Sojda, J. I. & Nixon, B. T. (1990). *Rhizobium meliloti* and *Rhizobium leguminosarum* *dctD* gene products bind to tandem sites in an activation sequence located upstream of σ^{54} -dependent *dctA* promoters. *J Bacteriol* **172**, 3888–3897.
- Macgregor, C. H., Wolff, J. A., Arora, S. K. & Phibbs, P. V. (1991). Cloning of a catabolite repression control (*cre*) gene from *Pseudomonas aeruginosa*, expression of the gene in *Escherichia coli*, and identification of the gene product in *Pseudomonas aeruginosa*. *J Bacteriol* **173**, 7204–7212.
- McKay, I., Glenn, A. & Dilworth, M. J. (1985). Gluconeogenesis in *Rhizobium leguminosarum* MNF3841. *J Gen Microbiol* **131**, 2067–2073.
- McKay, I. A., Dilworth, M. J. & Glenn, A. R. (1989). Carbon catabolism in continuous cultures and bacteroids of *Rhizobium leguminosarum* MNF3841. *Arch Microbiol* **152**, 606–610.
- Mandal, N. C. & Chakrabarty, P. K. (1993). Succinate-mediated catabolite repression of enzymes of glucose metabolism in root-nodule bacteria. *Curr Microbiol* **26**, 247–251.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Murphy, P. J. & Saint, C. P. (1992). Rhizopines in the legume-rhizobium symbiosis. In *Molecular Signals in Plant-Microbe Communications*, pp. 377–390. Edited by D. P. S. Verma. Boca Raton: CRC Press.
- Murphy, P. J., Heycke, N., Banfalvi, Z., Tate, M. E., Debruijn, F., Kondorosi, A., Tempe, J. & Schell, J. (1987). Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. *Proc Natl Acad Sci USA* **84**, 493–497.
- Murphy, P. J., Heycke, N., Trenz, S. P., Ratet, P., De-Bruijn, F. & Schell, J. (1988). Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. *Proc Natl Acad Sci USA* **85**, 9133–9137.
- Murphy, P. J., Trenz, S. P., Grzemski, W., Debruijn, F. J. & Schell, J. (1993). The *Rhizobium meliloti* rhizopine *mos* locus is a mosaic structure facilitating its symbiotic regulation. *J Bacteriol* **175**, 5193–5204.
- Osteras, M., Finan, T. M. & Stanley, J. (1991). Site-directed mutagenesis and DNA sequence of *pckA* of *Rhizobium* NGR234, encoding phosphoenolpyruvate carboxykinase – gluconeogenesis and host-dependent symbiotic phenotype. *Mol & Gen Genet* **230**, 257–269.
- Poole, P. S., Franklin, M., Glenn, A. R. & Dilworth, M. J. (1985). The transport of L-glutamate by *Rhizobium leguminosarum* involves a common amino acid carrier. *J Gen Microbiol* **131**, 1441–1488.
- Reddy, C. C., Pierzchala, P. A. & Hamilton, G. A. (1981). *myo*-Inositol oxygenase from hog kidney. *J Biol Chem* **256**, 8519–8524.
- Ronson, C. W. (1988). Genetic regulation of C₄-dicarboxylate transport in rhizobia. In *Nitrogen Fixation: Hundred Years After*, pp. 547–551. Edited by H. Bothe, F. J. Bruijn & W. E. Newton. New York: Gustav Fischer.
- Ronson, C. W. & Astwood, P. M. (1985). Genes involved in the carbon metabolism of bacteroids. In *Nitrogen Fixation Research Progress*, pp. 201–207. Edited by E. D. Evans, P. J. Bottomley & W. E. Newton. Dordrecht: Martinus Nijhoff.
- Ronson, C. W., Lyttleton, P. & Robertson, J. G. (1981). C₄-

- dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proc Natl Acad Sci USA* **78**, 4284–4288.
- Ronson, C. W., Astwood, P. M. & Downie, J. A. (1984). Molecular cloning and genetic organization of C₄-dicarboxylate transport genes from *Rhizobium leguminosarum*. *J Bacteriol* **160**, 903–909.
- Ronson, C. W., Astwood, P. M., Nixon, B. T. & Ausubel, F. M. (1987). Deduced products of C₄-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. *Nucleic Acids Res* **15**, 7921–7934.
- Saint, C. P., Wexler, M., Murphy, P. J., Tempe, J. & Tate, M. E. (1993). Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. *J Bacteriol* **175**, 5205–5215.
- Saroso, S., Dilworth, M. J. & Glenn, A. R. (1986). The use of activities of carbon catabolic enzymes as a probe for the carbon nutrition of snakebean nodule bacteroids. *J Gen Microbiol* **132**, 243–249.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis of Gram-negative bacteria. *Biotechnology* **1**, 784–791.
- Simon, R., Quandt, J. & Klipp, W. (1989). New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene* **80**, 161–169.
- Sköt, L. & Egsgaard, H. (1984). Identification of ononitol and O-methyl-scyllo-inositol in pea root nodules. *Planta* **161**, 32–36.
- Streeter, J. G. (1987). Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol* **85**, 768–773.
- Trinick, M. J., Dilworth, M. J. & Grounds, M. (1976). Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. *New Phytol* **77**, 359–370.
- Ucker, D. S. & Signer, E. R. (1978). Catabolite-repression-like phenomenon in *Rhizobium meliloti*. *J Bacteriol* **136**, 1197–1200.
- Wang, T. L., Wood, E. A. & Brewin, N. J. (1982). Growth regulators, *Rhizobium* and nodulation in peas. *Planta* **155**, 350–355.
- Wang, Y.-P., Birkenhead, K., Boesten, B., Manian, S. & O'Gara, F. (1989). Genetic analysis and regulation of the *Rhizobium meliloti* genes controlling C₄-dicarboxylic acid transport. *Gene* **85**, 135–144.
- Watson, R. J. (1990). Analysis of the C₄-dicarboxylate transport genes of *Rhizobium meliloti*: nucleotide sequence and deduced products of *dctA*, *dctB* and *dctD*. *Mol Plant-Microbe Interact* **3**, 174–181.
- Wolff, J. A., Macgregor, C. H., Eisenberg, R. C. & Phibbs, P. V. (1991). Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. *J Bacteriol* **173**, 4700–4706.
- Yarosh, O. K., Charles, T. C. & Finan, T. M. (1989). Analysis of C₄-dicarboxylate transport genes in *Rhizobium meliloti*. *Mol Microbiol* **3**, 813–823.

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