Some Features of Mannitol Metabolism in *Rhizobium japonicum*

By L. D. KUYKENDALL AND G. H. ELKAN

*Department of Microbiology, North Carolina State University,
Raleigh, North Carolina 27607, U.S.A.*

(Received 13 July 1976; revised 20 August 1976)

INTRODUCTION

Little is known about inducible enzyme systems in *Rhizobium japonicum*, although Pedrosa & Zancan (1974) have reported enzymes for arabinose metabolism and Martinez de Drets & Arias (1970) for mannitol, sorbitol, and arabitol metabolism in the fast-growing *Rhizobium meliloti*. The basic metabolic differences between the slow-growing species, such as *R. japonicum*, and the fast-growing rhizobia (Vincent, 1974; Martinez de Drets & Arias, 1972; Elkan, 1971) are not clearly understood but suggest that both physiological and genetical data obtained with either *R. meliloti* or *R. trifolii* might not be applicable to *R. japonicum*.

Previous reports have dealt with the metabolism of glucose and gluconate by *R. japonicum* (Keele, Hamilton & Elkan, 1969, 1970) and indicate that the Entner–Doudoroff glycolytic pathway operates in this organism. The fast-growing rhizobia also possess the pentose phosphate and Embden–Meyerhof pathways (Vincent, 1974; Jordan, 1962; Katznelson & Zagallo, 1957).

This paper reports on the enzymic basis for D-mannitol utilization in clones derived from *R. japonicum* 311b110. These clones differed in symbiotic nitrogen-fixing efficiency and, in their free-living state, exhibited a clear difference in ability to utilize D-mannitol (unpublished observations).

METHODS

Bacterial strains. The strains of *R. japonicum* 1-110, S-110, L1-110, and L2-110 are derivatives which were cloned from *R. japonicum* 311b110 (originally obtained from the United States Department of Agriculture, Beltsville Culture Collection by courtesy of Dr D. F. Weber). The isolation of, and the properties of these strains will be described in detail elsewhere. The strains L-125 and S-125 are derivatives similarly isolated from *R. japonicum* 311b125 (also originally from the U.S.D.A. Culture Collection). *Rhizobium japonicum* ATCC10324 was also used. *Agrobacterium tumefaciens* strain T was from Dr M. A. Cole, University of Illinois, Urbana.

Media. The HM salts composition has been described previously (Cole & Elkan, 1973); this basal salts solution contained, in addition to inorganic salts, 1.3 g *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (Sigma) and 1.1 g 2-(*N*-morpholino)ethanesulphonic acid (Sigma) as buffers. The pH was adjusted to 6.6 with 1 M-NaOH before autoclaving. All carbohydrates were autoclaved separately and added to sterile HM salts with yeast extract (Difco; either 0.25 or 1.00 g l⁻¹). Media contained 0.5 % (w/v) sugar; in the case of mixed carbohydrate media, such as D-mannitol and D-glucose, each carbon source was present at 0.5 % (w/v).

Culture conditions. Stock cultures on agar slants were subcultured at intervals of 6 months,

incubated for 1 week at 28 °C, and then stored at 4 to 5 °C. Primary broth inoculum cultures, after inoculation with material from agar slants, were incubated on a rotary shaker at 28 °C in 10 ml broth in 50 ml Erlenmeyer flasks. Late exponential phase (3- to 5-day-old cultures) were used to inoculate larger, secondary broth cultures which were incubated on a rotary shaker in either 300 ml broth in 1 l Erlenmeyer flasks or in 1 l broth in 2.8 l Fernbach flasks.

Preparation of cell-free extracts. Exponentially growing cells (2.5- to 5.0-day-old cultures) were harvested by centrifuging at 10000 *g* for 10 min, and washed once in HM salts at pH 7.0. The pellets from this washing were resuspended in 0.033 or 0.01 of the original volume of ice-cold 0.9 % (w/v) KCl containing 155 µg cysteine ml⁻¹, adjusted to pH 7.0 with 1 M-NaOH. The bacteria were disrupted by five 30 to 60 s exposures to a Branson Sonifier sonicator with 30 to 60 s rest intervals whilst the probe was chilled with ice-water. The resulting extract was cleared of remaining intact organisms by two successive centrifugings at 10000 *g* for 10 min at 2 to 4 °C. The clear, slightly amber supernatant fluid was kept at about 4 °C and used within 2 h for enzyme assays.

The soluble fraction of extracts of strain LI-110 grown on mannitol was prepared by centrifuging at 4 °C at 100000 *g* for 1 h. It was used to measure the rate of appearance of NADH in enzyme assays containing various concentrations of either D-mannitol or D-arabitol.

The protein concentration in the crude enzyme preparations was estimated by the absorption method of Warburg & Christian as described by Layne (1957).

Enzyme induction. To study induced and constitutive levels of D-mannitol dehydrogenase, organisms were grown in media containing either 0.5 % glycerol or 0.5 % glucose as the sole carbon source, or these same sugars plus 0.18 % D-mannitol. The bacteria were harvested in the mid to late exponential phase of growth since the enzymes of interest had optimal specific activities during this period. The bacteria were then used to prepare crude extracts which were assayed for the enzymes under study.

The kinetics of induction of D-mannitol dehydrogenase in cultures of *R. japonicum* were studied with extracts prepared from cultures grown in HM salts containing 0.5 % glucose and 0.025 % yeast extract for 2 to 5 days. The specific activity of this enzyme was estimated at various times after adding 0.18 % D-mannitol to the experimental flasks.

Assay of polyol dehydrogenase(s). The method described by Martinez de Drets & Arias (1970) was followed. The reaction mixtures contained (in a volume of 1.1 ml): 60 µmol Na₂CO₃/NaHCO₃ buffer, pH 9.7; 60 µmol of either D-mannitol or D-sorbitol or D-arabitol; 0.75 µmol NAD; and crude cell-free extract to give 100 to 500 µg protein ml⁻¹ in the assay. The rate of the NADH formation was followed by the increase in *E*₃₄₀ after adding substrate.

The substrate specificity of the inducible polyol dehydrogenase(s) of *R. japonicum* was studied using assays containing pairs of the substrates D-mannitol, D-arabitol and D-sorbitol with each substrate at half the standard assay concentration.

Chemicals. NAD, NADP and D-arabitol were from Sigma; D-mannitol (bacteriological grade) was from Difco. Before use in enzyme assays, the mannitol was twice recrystallized from 20 to 25 % (w/v) aqueous solutions.

RESULTS AND DISCUSSION

Rhizobium japonicum clones, which differed in their ability to fix nitrogen symbiotically and which, in the free-living state, also differed in their ability to utilize D-mannitol, showed a direct

Table 1. Comparison of D-mannitol dehydrogenase activities in cultures of different *R. japonicum* strains grown with glycerol in the presence of D-mannitol (10 mM) or in its absence

Strain	Specific activity of NAD-dependent D-mannitol dehydrogenase [nmol min ⁻¹ (mg protein) ⁻¹]	
	Mannitol absent	Mannitol present
I-110	0.9	1.4
S-110	0.0	0.4
LI-110	9.7	128.5
L2-110	5.6	42.0
S-125	< 1.0	4.3
L-125	7.4	60.9

Table 2. The influence of the carbon source in the medium on the specific activity of D-mannitol dehydrogenase in *R. japonicum* strain LI-110

Carbon source	Specific activity [nmol NADH formed min ⁻¹ (mg protein ⁻¹)]		Ratio of activities (D-mannitol:D-arabitol)
	D-Mannitol as substrate	D-Arabitol as substrate	
D-Mannitol	155.3	73.1	2.13
D-Glucose	9.2	4.8	1.92
D-Mannitol and D-glucose	112.2	52.2	2.15
D-Arabitol	236.5	109.6	2.16
D-Fructose	39.6	20.5	1.93
D-Gluconate	10.3	5.1	2.02
D-Sorbitol	159.4	89.7	1.78
Ribitol	17.9	8.4	2.13
Xylitol	37.3	20.1	1.87
Glycerol	10.7	5.1	2.10

correlation between the presence of an inducible NAD-dependent D-mannitol dehydrogenase (EC 1.1.1.67) and their ability to metabolize D-mannitol (Table 1). The very low activity [< 5 nmol NADH produced min⁻¹ (mg protein)⁻¹] in strains I-110, S-110 and S-125, grown in either the presence or absence of D-mannitol, may not be significant. Strains LI-110, L2-110 and L-125 had relatively high constitutive levels of D-mannitol dehydrogenase which were increased at least sevenfold by the presence of D-mannitol in the growth medium.

The kinetics of D-mannitol dehydrogenase synthesis were studied in a culture of strain LI-110 growing on D-glucose. In the presence of D-mannitol, after 1 h, the specific activity of the enzyme increased linearly with time, and doubled within 2.0 to 2.5 h. After 5 h the initial specific activity had increased fourfold under these conditions. The doubling time of strain LI-110 growing in this medium was about 31 h. Organisms in the mid-exponential and early stationary phases (not shown) have higher specific activities of this enzyme when grown in a D-mannitol and D-glucose medium than do similar cultures grown in a medium containing only D-mannitol as carbon source.

The specific activities in extracts of *R. japonicum* LI-110 (Table 2) showed that, regardless of the carbon source in the medium, the ratio of the activity obtained with D-mannitol as substrate to that obtained with D-arabitol as substrate was about 2.0. After adding D-mannitol to cultures growing on glucose, the activity with D-mannitol or D-arabitol as substrate

Fig. 1. The influence of substrate concentration on the rate of appearance of NADH. The soluble fraction of a cell-free extract of strain LI-110, grown on D-mannitol, was used. The final protein concentration in the reaction mixtures was 70 to 80 $\mu\text{g ml}^{-1}$. The standard assay system was used, except that the substrate concentration was varied as indicated. ●, D-mannitol; ○, D-arabitol.

showed the same rate of increase. These observations suggest that the same enzyme is involved in the oxidation of D-mannitol and D-arabitol.

The substrate specificity of the enzyme was studied by comparing reaction velocities obtained with pairs of polyols with those obtained with individual polyols (Martinez de Drets & Arias, 1970). The activities found with either D-mannitol or D-arabitol combined with D-sorbitol were additive. In contrast, the combined reaction rates with D-mannitol and D-arabitol were less than, or equal to, the reaction velocity obtained with D-mannitol alone. In some experiments, the combined reaction rate was an average of the velocities obtained when D-mannitol and D-arabitol were assayed separately. This is consistent with a single enzyme being involved in the oxidation of these substrates since additive rates would have been expected if two distinct enzymes were involved.

The dependence of the reaction rate on substrate concentration (Fig. 1) showed that at saturating substrate levels, the reaction velocity obtained with D-mannitol was about twice that with D-arabitol, and for this reason we have called the enzyme a D-mannitol dehydrogenase. However, the approximate substrate concentration at which the reaction attains half maximal velocity was lower for D-arabitol than for D-mannitol (Fig. 1). Lineweaver-Burk treatment of these data gave apparent K_m values of 6.5 mM and 11.8 mM for D-arabitol and D-mannitol, respectively.

The D-mannitol dehydrogenase of *R. japonicum* appears to be specific for NAD as cofactor with either D-mannitol or D-arabitol as the substrate since NADP did not substitute for NAD at 0.7 mM or 2.8 mM when assayed at pH 9.7 or pH 8.0.

In *R. japonicum* and the faster-growing species *R. meliloti* (Martinez de Drets & Arias, 1970) the ability to utilize either mannitol or arabitol is determined by the presence of an inducible NAD-specific dehydrogenase which is capable of utilizing either D-mannitol or D-arabitol as substrate.

In *R. meliloti* the activity of the dehydrogenase in cell-free extracts was two or three times higher than in *R. japonicum*, and the ratio of D-mannitol:D-arabitol activities ranged from 0.2 to 0.3 compared with a value of 2.0 for *R. japonicum*. The apparent K_m values for the

R. japonicum enzyme with D-mannitol and D-arabitol were similar to those found for the *R. meliloti* enzyme (Martinez de Drets & Arias, 1970), with a lower apparent K_m obtained with D-arabitol than with D-mannitol in each case. In both organisms D-mannitol is apparently directly oxidized as the free hexitol rather than via a phosphorylated intermediate, since extracts of mannitol-grown cells showed dehydrogenase activity with D-mannitol but not with D-mannitol 1-phosphate.

The constitutive and induced levels of D-mannitol dehydrogenase in extracts of *R. japonicum* ATCC10324 were similar to those obtained with strain LI-110. The levels of polyol dehydrogenase in *Agrobacterium tumefaciens* and the ratios of substrate activity were more similar to those of *R. meliloti* (Martinez de Drets & Arias, 1970) than those we have found in *R. japonicum* and those reported for the faster-growing species *R. meliloti* (Martinez de Drets & Arias, 1970).

Strains LI-110 and L2-110 which possess the inducible D-mannitol dehydrogenase are considerably less efficient in symbiotic nitrogen fixation, as measured by acetylene reduction, than strains (I-110 and S-110) which lack this inducible enzyme (unpublished observations). However, this may be peculiar to the genetic background of strains LI-110 and L2-110 since strains S-125 and L-125 do not show this correlation.

The NAD-linked 6-phosphogluconate dehydrogenase of *R. japonicum* is inhibited allosterically by D-mannitol (K. Mulongoy & G. H. Elkan, unpublished observation). We suggest that in strains LI-110 and L2-110 the presence of intracellular D-mannitol dehydrogenase results in a reduced efficiency of D-glucose utilization and consequently in a diminished symbiotic dinitrogen-fixing ability.

The work was supported in part by the National Soybean Crop Improvement Council and the United States Department of Agriculture, Agricultural Research Service. This is paper no. 4987 of the journal series of the North Carolina Agriculture Experiment Station, Raleigh, North Carolina, U.S.A.

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