Three Enzymes for Trehalose Synthesis in *Bradyrhizobium* Cultured Bacteria and in Bacteroids from Soybean Nodules

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α,α -Trehalose is a disaccharide accumulated by many microorganisms, including rhizobia, and a common role for trehalose is protection of membrane and protein structure during periods of stress, such as desiccation. Cultured *Bradyrhizobium japonicum* and *B. elkanii* were found to have three enzymes for trehalose synthesis: trehalose synthase (TS), maltooligosyltrehalose synthase (MOTS), and trehalose-6-phosphate synthetase. The activity level of the latter enzyme was much higher than those of the other two in cultured bacteria, but the reverse was true in bacteroids from nodules. Although TS was the dominant enzyme in bacteroids, the source of maltose, the substrate for TS, is not clear; i.e., the maltose concentration in nodules was very low and no maltose was formed by bacteroid protein preparations from maltooligosaccharides. Because bacteroid protein preparations contained high trehalase activity, it was imperative to inhibit this enzyme in studies of TS and MOTS in bacteroids. Validamycin A, a commonly used trehalase inhibitor, was found to also inhibit TS and MOTS, and other trehalase inhibitors, such as trehazolin, must be used in studies of these enzymes in nodules. The results of a survey of five other species of rhizobia indicated that most species sampled had only one major mechanism for trehalose synthesis. The presence of three totally independent mechanisms for the synthesis of trehalose by *Bradyrhizobium* species suggests that this disaccharide is important in the function of this organism both in the free-living state and in symbiosis.

Trehalose is a nonreducing disaccharide with an unusual α,α -1,1 linkage between the two glucose molecules. Trehalose is commonly found in fungi, *Saccharomyces cerevisiae*, some bacteria, and insects, and it serves a variety of roles in these organisms. In many organisms it serves to protect membranes and proteins under a variety of stress conditions such as desiccation, cold, and heat (5).

The biosynthesis of trehalose is remarkable in that three completely independent mechanisms for synthesis have been reported. The first involves the formation of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate. This enzyme, trehalose-6-phosphate synthetase (TPS), has been extensively studied in a wide range of organisms, and only a few examples of early publications are provided here (2, 4, 11, 21); more-recent references to TPS are available in the recent review by Elbein et al. (5). The product of TPS (trehalose-6-phosphate) is subsequently dephosphorylated to form trehalose. TPS functions with various nucleotide phosphate derivatives of glucose, and polyanions such as heparin are strong activators of TPS with some NDP-glucose substrates (11, 20). The presence of TPS has previously been reported in studies of *Bradyrhizobium japonicum* and *B. elkanii*, the organisms of interest here, although the levels of activity were low (22).

The second mechanism involves conversion of maltooligosaccharides to maltooligosyl trehalose by intramolecular transglucosylation of the terminal glucose. The trehalose portion of the intermediate is then cleaved by a second enzyme to give

trehalose plus a shorter maltooligosaccharide (12, 17, 18). The enzyme, maltooligosyltrehalose synthase (MOTS), functions with a variety of maltooligosaccharides having numbers of glucose molecules in the range from 4 to 7 (17). MOTS purified from an *Arthrobacter* sp. has no cofactors and an optimum pH of 7.0, and the lowest K_m among the substrates tested was that for maltoheptaose (17). Recently, an interesting thermostable version of the enzyme was purified from *Sulfolobus acidocaldarius*, and this enzyme has a pH optimum of between 5.0 and 5.5. MOTS has been reported to occur in many species of cultured rhizobia, including *Bradyrhizobium* spp., although in many cases enzyme activity was fairly low (27).

The third mechanism, which involves trehalose synthase (TS), catalyzes the conversion of maltose directly to trehalose, again by intramolecular transglucosylation (19). Again, there are no cofactors, and the pH optimum is around 7.5. The enzyme has strict substrate specificity for maltose; i.e., conversion of maltooligosaccharides to trehalose does not occur. The enzyme also catalyzes the reverse reaction, conversion of trehalose to maltose, but the equilibrium is strongly in the direction of trehalose synthesis (19).

Recently, the presence of all three mechanisms of trehalose synthesis was reported for *Mycobacterium bovis* BCG, *M. smegmatis* (3), and *Corynebacterium glutamicum* (28), suggesting that trehalose plays an important role(s) in these organisms. In *C. glutamicum*, TS may function in the reverse direction because of a 1,000-fold-higher concentration of trehalose relative to maltose (28).

The two species of *Bradyrhizobium* are the major microsymbionts of soybean (*Glycine max* [L.] Merr.) in the United States. The occurrence of high concentrations of trehalose in soybean nodules just at the onset of nitrogen fixation was

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reported 25 years ago (23). Subsequent studies showed that all 27 strains of rhizobia tested accumulate some trehalose in culture and that trehalose was by far the major carbohydrate found in cultured *Bradyrhizobium* strains, regardless of the carbon source supplied in the medium (25). Also, it has been reported that, among mono- and disaccharides in nodules, only trehalose is present at higher concentrations in bacteroids than in the nodule cytosol (26). In combination, these results strongly suggest that trehalose is synthesized in soybean nodules by bacteroids.

The main purpose of these studies was to establish which of the three mechanisms for trehalose synthesis occur in *Bradyrhizobium* in culture and, especially, in nodules and to determine which of the mechanisms appear to be dominant. In this report, we show that all three mechanisms occur in both species of *Bradyrhizobium*. Among the three mechanisms, TS has highest activity level and may be most important for trehalose synthesis. Because of the abundance of trehalase in soybean nodules (9, 13, 24), the assay of MOTS and TS in bacteroids was seriously compromised, and inhibition of trehalase became a second emphasis in these studies.

MATERIALS AND METHODS

Studies with cultured bacteria. The two strains of *Bradyrhizobium* that were studied in detail were obtained from the U.S. Department of Agriculture Beltsville Rhizobium Culture Collection (<http://bldg6.arsusda.gov/pberkum/Public/cc1a.html>). Other species and strains used for comparative analysis of cultured bacteria were obtained from a variety of sources (see the footnotes to Table 4). Bacteria were occasionally streaked on agar plates to assure the purity of the cultures. *Bradyrhizobium* species were grown in medium containing 1 g liter⁻¹ each of arabinose and gluconate (AG) as carbon sources, 1 g liter⁻¹ of yeast extract (Difco), and ammonium as the N source (other details are provided in reference 27). In the study of bacterial growth with maltose as a carbon source, 3 mM maltose was used. Other rhizobia were grown in the same medium except for *Sinorhizobium* and *Mesorhizobium* spp., which were grown in tryptone-yeast extract medium (27) (see Table 4). Shake cultures were grown at 30°C in batches of 250 ml at 125 rpm.

Cells were collected by centrifugation at 22,000 × g for 10 min. After the supernatant was discarded, the cells were resuspended in 0.2 M phosphate buffer (pH 7.0) and transferred to a 40 ml centrifuge tube. The mixture was centrifuged again at 22,000 × g for 10 min, and cells were resuspended in 5.0 ml of 10 mM Tris buffer (pH 7.5) containing 40 mM MgCl₂, 10 mM KCl, 3 mM EDTA, and 1.0 mM dithiothreitol. A total of 50 µl of 1% Triton X-100 was added to assist in cell disruption, and cells were sonicated in the centrifuge tubes in an ice bath using a Branson sonifier (model 350), a microtip, and a power setting of 3 in 50% pulse mode for 10 min.

In preliminary studies, relatively high variations in TS activity were noted. Careful investigation of sonication conditions showed that inclusion of Triton slightly increased the rate of TS release. Most of the TS was released in the first 5 min of sonication, but gradual release of TS continued for up to 15 min. The highest specific activity (protein basis) was found after about 10 min of sonication, and this method was adopted as the standard procedure. The main point is that sonication conditions are important and should be consistent across experiments and treatments.

Sonicated mixtures were centrifuged at 48,000 × g for 10 min, and the supernatant (crude protein extract) was transferred to a cold test tube. Crude protein was taken to a cold room and gel filtered through Sephadex G-25, and blue dextran (molecular weight, 3 × 10⁶) was used to mark the position of the protein band. At the time of gel filtration, the crude protein was divided into two portions. The portion for TPS assays was filtered through a column equilibrated with the 10 mM Tris buffer described above. Because an unknown substance in the Tris buffer inhibits TS and MOTS, a second portion of the crude protein was filtered through a column equilibrated with 10 mM phosphate (pH 7.0). This phosphate buffer was also used for elution of protein from the column; thus, the buffer was switched from Tris to phosphate during the filtration process.

Studies with bacteroids and nodules. Soybean seeds (cv. Flint) were planted in ceramic pots containing about 4 liters of silica sand. Pots were covered with

aluminum foil and autoclaved prior to use. Each seed was irrigated with about 1.5 ml of a liquid culture of the desired bacterial strain at the time of planting, and pots were recovered with aluminum foil in order to conserve moisture and avoid addition of water, which could wash out the inoculum. After seedling emergence, plants were supplied with an N-free nutrient solution (23). Nodules used for isolation of bacteroids were collected without regard to the stage of plant growth, but plants in late reproductive stages and senescence were not used.

Nodules were weighed and ground in a cold mortar with 100 mM phosphate buffer (pH 7.5) containing 150 mM mannitol, 2 mM dithiothreitol, and 1 mM EDTA (22). After filtration through four layers of cheesecloth, the homogenate was centrifuged at 500 × g for 10 min to remove debris. The pellet was discarded, and the supernatant was transferred to a clean centrifuge tube and centrifuged again at 6,000 × g for 10 min to pellet the bacteroids. The bacteroid pellet was suspended in 10 ml of grinding buffer to wash the bacteroids and then centrifuged again at 6,000 × g for 10 min. The bacteroid pellet was suspended in the 10 mM Tris buffer described above under the heading "Studies with cultured bacteria." Sonication, high-speed centrifugation, collection of crude protein, and gel filtration methods were the same as those described for studies with cultured bacteria.

For assays of trehalase in nodule cytosol, nodule homogenate was centrifuged at 48,000 × g for 10 min and a portion of the clear supernatant was gel filtered using 10 mM phosphate (pH 7.0). For analysis of nodule carbohydrates, nodules were ground in 75% ethanol, cytosol was prepared by high-speed centrifugation, and portions were dried for analysis by gas-liquid chromatography (23, 27).

Enzyme assays. Unless otherwise indicated, all substrates and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Validamycin A was purchased from Duchefa Biochemie BV, Haarlem, The Netherlands. Maltotetraose was a gift from Kazuhiko Maruta, Hayashibara Biochemical Laboratories, Okayama, Japan. Trehazolin (no longer available) was a gift from Alan Elbein, University of Arkansas, who originally obtained it from Sankyo Co. in Tokyo, Japan.

Assays for TS and MOTS were very similar except for the substrates used: 8 mM maltose for TS and 2.5 mM maltotetraose for MOTS. The control mixtures contained boiled gel-filtered protein. Reaction mixtures were made up in 1.5 ml microcentrifuge tubes by adding substrates to gel-filtered protein, usually 200 to 300 µl of protein per mixture—generally around 1 mg of protein per assay—in order to keep protein concentrations high. Because of low levels of enzyme activity and relatively long incubation times, two protein concentrations and two incubation times were used (27) for each protein preparation in order to assure approximate linearity with protein and time. The concentrations were roughly 0.5 or 1.0 mg protein per mixture, and the incubation times were 2.5 or 3 h (high protein concentrations) and 5 or 6 h (low and high protein concentrations). Generally, the low protein concentration plus long incubation time and the high protein concentration plus short incubation time mixtures gave the highest rate of product formation; these values were averaged to provide the data reported. The high protein concentration-long incubation time mixture was useful because it allowed at least a rough estimate of low enzyme activity or a confident decision in cases of undetectable activity. Boiled protein mixtures always yielded undetectable trehalose. Mixtures for analysis of trehalase activity (i.e., containing trehalose as the substrate) were included in each set of assays. This is important because of the potential for simultaneous breakdown of the product trehalose.

Reactions were stopped by boiling. For the assay of MOTS, a postincubation with amyloglucosidase was used to degrade the intermediate maltotriose-trehalose and maximize trehalose yield (27). After incubation(s), mixtures were centrifuged at 7,000 × g for 5 min to sediment protein. The entire supernatant was transferred to a gas chromatography vial and dried under a stream of air. Trehalose, maltose, and glucose concentrations were determined by gas-liquid chromatography (23, 27). In assays for TS and MOTS activity in protein preparations from bacteroids, mixtures were preincubated with trehazolin at a final concentration of 80 nM for 30 min at 30°C prior to addition of substrate (1, 10).

The assay of trehalase-6-phosphate synthase was performed to detect glucose-6-phosphate-dependent formation of UDP from UDP glucose (3, 22). Substrates were added to gel-filtered protein to maintain a high protein concentration; heparin was included at a final concentration of 40 µg per ml. Duplicate mixtures were incubated for 60 min, and reactions were stopped by boiling for 3.0 min. After centrifugation at 7,000 × g for 5 min, supernatants were transferred to a clean tube for analysis of UDP concentrations (3).

Protein concentrations in gel-filtered preparations were determined using a modified Lowry procedure, reagents from Bio-Rad Laboratories (Hercules, CA), and bovine serum albumin as a standard.

TABLE 1. Preliminary studies of two enzymes of trehalose metabolism in *B. japonicum* (USDA 110) and *B. elkanii* (USDA 324) and the effect of trehalase inhibitors^a

Source of cells	Strain	Trehalase inhibitor	Concn (μ M)	Enzyme activity (nmol mg ⁻¹ protein h ⁻¹)	
				Trehalase synthase	MOTS
Nodules	USDA 110	None		<0.5 ^b	<0.5
Nodules	USDA 324	None		<0.5	<0.5
Nodules	USDA 110	Validamycin A	100	<0.5	<0.5
Nodules	USDA 324	Validamycin A	100	<0.5	<0.5
Cultured bacteria	USDA 110	None		17.5	~1 ^c
Cultured bacteria	USDA 110	Validamycin A	100	0.7	<0.5
Cultured bacteria	USDA 110	Trehazolin	0.06	22.8	~1
Nodules	USDA 110	Trehazolin	0.08	53.0	27.8

^a Data represent the averages of the results obtained with two or three reaction mixtures and one or two protein preparations. Trehalase 6-phosphate synthase was not assayed in these preparations.

^b No trehalose peak on any chromatogram.

^c Very small but definite trehalose peak on chromatogram.

RESULTS

After several failures to detect TS in various protein preparations from *Rhizobium* sp. strain NGR 234, activity was observed in protein extracts from *B. japonicum* USDA 110 and *B. elkanii* USDA 324 (Table 1). We also confirmed that these species have low levels of MOTS activity (27). When these assays were extended to include bacteroid soluble protein preparations, we were surprised to find undetectable activity (Table 1). However, high trehalase activity (Fig. 1) in these preparations was noted and it was apparent that trehalase would have to be inhibited for the presence or absence TS and MOTS to be validated.

The trehalase inhibitor validamycin A has been used in several studies of trehalose metabolism in nodules (14, 15). We confirmed that validamycin A inhibits soybean nodule trehalase activity (Fig. 1A), although the concentrations required were rather high. However, addition of validamycin A to assays of bacteroid soluble protein still did not provide evidence for the presence of TS and MOTS (Table 1). In tests of protein preparations from cultured USDA 110, we discovered that validamycin A also strongly inhibits TS and MOTS at concentrations required to inhibit trehalase (Table 1).

We then tested the efficacy of trehazolin as a trehalase inhibitor and found strong inhibition of trehalase activity from nodule cytosol at very low trehazolin concentrations (Fig. 1B). Inhibition of trehalase activity was 98.7% at a trehazolin concentration of 14 nM and about 99.7% at a trehazolin concentration of 56 nM. A final concentration of 80 nM trehazolin was used in subsequent assays of TS and MOTS in protein preparations from bacteroids.

A comparison of the three enzymes in cultured bacteria and bacteroids of the two species is shown in Table 2. There may be some differences between *Bradyrhizobium* species with respect to enzyme levels, but these differences, if real, were small relative to differences between bacteria and bacteroids. Although all three enzymes were present in bacteroids and bacteria, the dominant enzyme in cultured bacteria was TPS, with moderate levels of TS and low levels of MOTS. In contrast, TS

was the dominant enzyme in nodules followed by MOTS at moderate levels and TPS at low levels. Thus, in the transition from cultured bacteria to bacteroids, TPS was significantly down-regulated and TS and MOTS were significantly up-regulated.

It has been reported that the trehalose concentration in soybean nodules varies according to the strain of *Bradyrhizobium* involved (25), with *B. elkanii* USDA 324 inducing a high concentration and *B. japonicum* USDA 110 a low concentration. This was confirmed here in analyses of nodules from different plant growth stages (Table 3). Although the totals of all mono- and disaccharides were similar in the two types of nodules, the amount of trehalose as a proportion of total carbohydrate was nearly three times greater in strain USDA 324-formed nodules than in strain USDA 110-formed nodules. Comparison of these results to those in Table 2 shows that trehalose accumulation in nodules was unrelated to the level of

FIG. 1. (A) Effect of validamycin A on trehalase activity from the cytosol of soybean nodules. Note that the scale for validamycin A concentration is not linear. (B) Effect of trehazolin concentration on trehalase activity from the cytosol of soybean nodules. Note that the scale for enzyme activity is logarithmic and that the scale for trehazolin concentration is not linear. Enzyme activity with a trehazolin concentration of 280 nM was undetectable.

TABLE 2. Activity of three enzymes of trehalose synthesis in *B. japonicum* (USDA 110) and *B. elkanii* (USDA 324) cultured bacteria and bacteroids^a

Species and source of cells	Enzyme activity (nmol mg ⁻¹ protein h ⁻¹ [mean ± SE]) (n) ^b		
	Trehalose synthase	MOTS	Trehalose 6-P synthetase
<i>B. japonicum</i>			
Culture	22.6 ± 1.8 (8)	2.3 ± 0.3 (8)	36.1 ± 11.7 (6)
Nodules	59.7 ± 3.2 (6)	27.6 ± 3.4 (6)	2.1 ± 0.9 (7)
<i>B. elkanii</i>			
Culture	16.5 ± 0.6 (4)	1.7 ± 0.2 (3)	50.5 ± 9.0 (8)
Nodules	45.4 ± 20.3 (4)	14.8 ± 6.2 (4)	3.9 ± 1.9 (6)

^a Trehalase activity in cultured bacteria was <0.25 nmol mg⁻¹ protein h⁻¹. Trehalase activity in bacteroid protein preparations was substantial (Fig. 1) but was inhibited by preincubation of protein with 80 nM trehazolin. Because of the very small supply of trehazolin available, it was not used in assays of bacterial soluble protein.

^b n, number of independent protein preparations; results for each protein preparation were calculated as the averages for two reaction mixtures (see text).

enzyme activity and that other mechanisms must be involved in the regulation of trehalose accumulation.

Another motive for the analysis of nodule carbohydrates was to assess the concentrations of maltose, the substrate for TS, which had the highest level of activity of the three trehalose synthesis enzymes in bacteroids. The results showed that maltose was a very small component of nodule carbohydrates (Table 3). Inasmuch as the maltose concentration in bacteroids was reported to be only 27% of the total nodule maltose concentration (26), this result suggests that the maltose concentration is very low in bacteroids and that the maltose concentration is probably unrelated to TS activity. It should also be noted that in analyses of carbohydrates from cultured bacteria, maltose was never detected (25). In spite of the relatively high activity of TS in cultured *Bradyrhizobium* spp., we have confirmed in these studies that maltose is undetectable in extracts from cultured bacteria (data not shown).

It is also curious that maltose was a poor or very poor carbon source for growth of these *Bradyrhizobium* strains (Fig. 2). That *B. elkanii* grows slightly faster than *B. japonicum* in liquid cultures is consistent with anecdotal observations with a variety of media (data not shown). So perhaps it is not surprising that there was better growth of *B. elkanii* with maltose as the sole carbon source. Nevertheless, it is clear that maltose is not a good source of carbon for either species.

TABLE 3. Concentrations of trehalose and maltose in extracts from whole nodules formed by *B. japonicum* (USDA 110) or *B. elkanii* (USDA 324)^a

Species	Concn (mg g ⁻¹ fresh weight)		
	Total for all carbohydrates ^b	Trehalose (% of total)	Maltose
<i>B. japonicum</i>	12.0	0.80 (6.7)	0.086
<i>B. elkanii</i>	12.4	2.35 (19.0)	0.135

^a Data represent the averages of results for two nodule extracts for each species of rhizobia.

^b Other carbohydrates in these extracts, in decreasing order of concentration, were sucrose > D-chiro-inositol > D-pinitol ≅ glucose > myo-inositol. Fructose was not detected in these extracts. In *B. elkanii* nodules, trehalose ranked second in concentration level after sucrose.

FIG. 2. Growth of *B. japonicum* USDA 110 and *B. elkanii* USDA 324 in medium with AG as carbon sources or maltose as the sole carbon source.

The effect of adding 3 mM maltose to the AG medium on the level of TS and MOTS activity was also tested. An effect of maltose addition on TS level was expected, at least for *B. elkanii*, but there was no significant effect of the presence of maltose on TS or MOTS activity in either *B. japonicum* USDA 110 or *B. elkanii* USDA 324 (data not shown).

We found great diversity in the enzymes of trehalose synthesis in a few other rhizobia tested (Table 4). Thus, even if the genes for TS and/or MOTS were present, there was an undetectable level of catalytic activity in some of these bacteria. MOTS was previously reported to have been detected in a

TABLE 4. Enzymes of trehalose synthesis in other genera and species of rhizobia from liquid cultures^a

Species and strain	Enzyme activity (nmol mg ⁻¹ protein h ⁻¹ [mean ± SE]) (no. of observations) ^b		
	Trehalose synthase	MOTS	Trehalose 6-P synthetase
<i>Rhizobium</i> sp. strain NGR 234 ^c	<0.5 ^g (8)	59.0 ± 3.7 (8)	<1 ^h (2)
<i>R. leguminosarum</i> bv. viciae LS 2202 ^d	<0.5 (3)	47.7 ± 8.4 (3)	<1 (3)
<i>R. tropici</i> 9030 ^e	<0.5 (2)	<0.5 (2)	ND ⁱ (2)
<i>S. meliloti</i> GR4 ^f	<0.5 (3)	<0.5 (3)	29.5 ± 0.8 (3)
<i>M. loti</i> R7-A ^f	<0.5 (3)	<0.5 (3)	27.7 ± 0.7 (3)

^a For all species, the activity for trehalase was <0.25 nmol mg⁻¹ protein h⁻¹ and trehazolin was not used in assays.

^b For each entry, the number of observations represents the number of protein preparations.

^c Source, William Broughton, Universite de Geneve, Geneva, Switzerland.

^d Source, Leif Skot, University of Wales, United Kingdom.

^e Source, Esperanza Martinez, Centro De Investigacion Sobre Fijacion De Nitrogeno, Cuernavaca, Mexico.

^f Source, José Olivares, Departamento de Microbiologia del Suelo y Sistemas Simbióticos, Estación Experimental de Zaidin (CSIC), Granada, Spain.

^g No trehalose peak on any chromatogram.

^h The treatment-control difference in UDP was positive but very small.

ⁱ ND, not detectable.

TABLE 5. Effect of NaCl added to culture media on enzymes in trehalose synthesis in *B. japonicum*, *S. meliloti*, and *M. loti*^a

Bacterium	Enzyme activity (nmol mg ⁻¹ protein h ⁻¹) (SE)					
	Trehalose synthase		MOTS		Trehalose-P synthetase	
	Control	Salt ^b	Control	Salt ^b	Control	Salt ^b
<i>B. japonicum</i>	32.9 (9.0)	46.9 (12.1)	1.2 (0.6)	2.2 (0.5)	19.7 (1.6)	31.1 (3.4)
<i>S. meliloti</i>	NA ^c	NA	NA	NA	29.5 (0.8)	10.9 (0.1)
<i>M. loti</i>	NA	NA	NA	NA	27.7 (0.7)	11.7 (1.6)

^a The strain of *B. japonicum* was USDA 110; other strains were those shown in Table 4. The results represent the means and standard errors of the results obtained with two or (generally) three protein preparations. In all cases, control and salt cultures were grown and compared side by side.

^b For *B. japonicum*, the salt concentration was 40 mM; for the other rhizobia, the salt concentration was 100 mM.

^c NA, not assayed.

different strain of *Sinorhizobium meliloti* (27), so there may be a strain-specific variation within this species. *R. tropici* is an enigma, because we were unable to detect any of the three enzymes for trehalose synthesis in two protein preparations from the type strain of this species.

Trehalose serves as an osmoprotectant in many organisms, including *S. meliloti*, when they are exposed to various stresses (5, 6). Thus it was of interest to determine the possible impact of salt stress on the various enzymes of trehalose synthesis. This experiment was done by the addition of NaCl to the standard liquid media. For *B. japonicum*, 40 mM salt was used, and for *S. meliloti*, 100 mM salt was used. (Higher concentrations than these inhibit growth of bacteria; data not shown.) In *B. japonicum*, the activity of all three enzymes was increased after growth in media containing salt (Table 5). However, MOTS activity remained quite low and the increases for the other two enzymes were only modest. Also note that, for TS, error variation was rather high in these trials, making firm conclusions difficult. For the other two species tested, TPS activity levels were actually depressed when cells were grown in the presence of salt; the numbers of CFU per milliliter were similar in control and salt cultures, so this difference cannot be attributed to lower cell numbers (data not shown). Studies of soybean plants grown in sand culture and irrigated with 40 mM salt daily actually showed a decline in trehalose accumulation in response to the salt treatment (data not shown).

DISCUSSION

The most important finding reported here is that the two species of *Bradyrhizobium* have three independent mechanisms for trehalose synthesis. Obviously, this does not prove the importance of this disaccharide in these organisms, but it is difficult to explain why an organism would retain three different mechanisms for synthesis of a compound unless it plays an important role. Trehalose is known to be of critical importance in many microorganisms (3, 5, 28), and the presence of three trehalose synthesis mechanisms in *Bradyrhizobium* spp. supports the inclusion of this genus in this group. However, some of other rhizobia analyzed did not have all three mechanisms (Table 4). In fact, some of the species sampled appeared to have only one mechanism for trehalose synthesis, and no mechanism for trehalose synthesis was found in *R. tropici*,

suggesting that trehalose may not be equally important for all of the members of the *Rhizobiaceae*.

It is also interesting that certain enzymes of trehalose synthesis were up-regulated while others were down-regulated when the *Bradyrhizobium* bacteria entered a symbiotic relationship with the soybean plant (Table 2). We were unable to determine why TS and MOTS are dominant in bacteroids whereas TPS activity levels are much lower in bacteroids than in cultured bacteria. It would appear that the higher osmotic potential of the nodule environment was not the reason for these differences, assuming that salt stress can mimic the osmotic conditions in the nodule. Thus, the reason for the shifts in activity of enzymes of trehalose synthesis in nodules remains to be explained.

The dominance of TS in bacteroids is curious, because the maltose concentrations in nodules and, especially, in bacteroids are very low (26) (Table 3). Also, bradyrhizobia grow only poorly when maltose is used as a carbon source (Fig. 2) and supplying maltose to bradyrhizobia in culture did not increase the level of TS activity. Bacteroids do accumulate glycogen, so maltose released from this glucose polysaccharide may be the source of substrate for TS. Results of our assays of MOTS indicate that the second enzyme, maltooligosyltrehalose hydrolase, is also present; i.e., in the absence of the follow-up incubation with amyloglucosidase, some trehalose is formed (data not shown). However, it is unlikely that this hydrolytic activity could be responsible for maltose formation because, in MOTS assays using maltoheptaose, maltose formation was never observed. Thus, how maltose is formed to provide substrate for TS in bacteroids remains unknown; some linkage of an enzyme for maltose formation directly to TS is an interesting possibility.

Various experimental approaches over many years have documented the presence of high-level trehalase activity in legume root nodules (9, 13, 16, 24). The consensus is that trehalase is a host plant enzyme probably localized outside of the symbiosome membrane. However, it appears that some of the enzyme is bound to bacteroids during the nodule maceration and centrifugation steps and is released during sonication with detergent. Regardless of the explanation for high trehalase activity in bacteroid protein preparations, this enzyme is a significant deterrent to the study of TS and MOTS activity in bacteroids because of hydrolysis of trehalose, the product. The most commonly used trehalase inhibitor, validamycin A, was found to be a potent inhibitor of both TS and MOTS, and use of this inhibitor should be avoided in studies of trehalose metabolism in nodules unless there is clear demonstration that these enzymes are not functional. Trehazolin provided excellent control of unwanted trehalase activity and permitted analysis of TS and MOTS in bacteroids (see also reference 8).

Although only low concentrations of trehalose in some legume nodules have been reported previously (16), accumulation of substantial quantities of trehalose in soybean nodules is well documented (23, 25, 26) (Table 3). The patterns for enzymes of trehalose synthesis only demonstrate the versatility of trehalose synthesis in nodules and do not explain the reasons for trehalose accumulation. The lack of a major response of enzymes to salt stress in cultured bradyrhizobia and the lack of salt effects on trehalose accumulation in nodules suggest that osmotic stress may not be an important determinant of treha-

lose accumulation. Trehalose accumulation in cultured rhizobia is markedly stimulated by low oxygen concentrations (7), and this is in agreement with the observation that trehalose accumulation in nodules occurs just at the onset of nitrogen fixation (23). However, exactly how low free-oxygen concentrations in the nodule would control trehalose accumulation remains unknown.

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