Glucose transport by an arctic and a temperate strain of rhizobia[1]

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Glucose transport was studied in two strains of *Rhizobium* species effective on sainfoin (*Onobrychis viciifolia*), the arctic strain N_{31} isolated from *Astragalus alpinus* and the temperate strain SM_2 isolated from sainfoin. The two strains had comparable glucose transport systems with a biphasic kinetics, indicating the presence of a high- and low-affinity transport system. Apparent K_m and V_{max} values for the high- and low-affinity transport systems were, respectively, 4.7 and 53.4 μM and 12.7 and 58.9 nmol · min^{-1} · mg protein^{-1} with N_{31} and 2.6 and 72.6 μM and 10.1 and 64.6 nmol · min^{-1} · mg protein^{-1} with SM_2. Glucose transport systems were inhibited by 2,4-dinitrophenol, KCN, azide, and *N*-ethylmaleimide. NaF did not affect glucose transport, while arsenate showed partial inhibition of the low-affinity transport system with strain N_{31}. These results suggest an active mechanism of transport that is dependent on an energized membrane but does not directly utilize high-energy phosphate compounds. In the two strains, glucose transport is constitutive and repressed by succinate, and it is glucose specific.

Key words: Arctic, glucose, *Rhizobium*, symbiosis, transport.

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Le transport du glucose a été étudié chez deux souches de *Rhizobium* spp. efficaces avec le sainfoin (*Onobrychis viciifolia*), la souche arctique N_{31} isolée d'*Astragalus alpinus* et la souche tempérée SM_2 isolée du sainfoin. Les systèmes de transport du glucose chez les deux souches sont semblables, et ont un profil biphasique de saturation cinétique, ce qui indique la présence de deux systèmes à grande et à faible affinité. Les valeurs de K_m et de V_{max} observées pour les systèmes de grande et de faible affinité étaient, respectivement, de 4,7 et 53,4 μM et 12,7 et 58,9 nmol · min^{-1} · mg protéine^{-1} pour la souche N_{31} et 2,6 et 72,6 μM et 10,1 et 64,6 nmol · min^{-1} · mg protéine^{-1} pour la souche SM_2. Le transport du glucose a été inhibé par le 2,4-dinitrophénol, le KCN, l'azide et le *N*-éthyl-maléimide. Par contre, le NaF n'a pas eu d'effet sur l'absorption du glucose alors que l'arsenate n'a causé qu'une inhibition partielle du système à faible affinité chez la souche N_{31}. Ces résultats suggèrent que le système de transport du glucose chez les deux souches est actif, impliquant une membrane énergisée et qui ne nécessite pas l'utilisation de composés phosphatés riches en énergie. Chez les deux souches le transport du glucose est constitutif, il est réprimé par le succinate, et il est spécifique au glucose.

Mots clés : Arctique, glucose, *Rhizobium*, symbiose, transport.

Introduction

Strains of arctic rhizobia isolated from the roots of *Astragalus alpinus* L., *Oxytropis maydelliana* Trauv., or *Oxytropis arctobia* Bunge are able to nodulate these three arctic legumes, indigenous to the Canadian high arctic and *Onobrychis viciifolia* Scop., the temperate forage legume sainfoin (Prévost *et al.* 1987*c*). Some arctic rhizobia are as effective as strains used in commercial inoculants on sainfoin (Prévost *et al.* 1987*b*). Furthermore, sainfoin nodules formed by the arctic strain N_{31} had a higher nitrogenase activity at low temperatures than those formed by the temperate homologous strain SM_2 (Prévost *et al.* 1987*a*). A growth cold-adaptation mechanism, absent in temperate rhizobia, was also apparent in the free-living cells of arctic rhizobia (Caudry-Reznick *et al.* 1986).

Arctic rhizobia have some characteristics similar to the fast-growing and the slow-growing type of root nodule bacteria (Prévost *et al.* 1987*c*) and on the basis of a DNA homology study, it was suggested that they might constitute a new species (Caudry-Reznick *et al.* 1986).

The fast-growing *Rhizobium* species can be distinguished from the slow-growing *Bradyrhizobium* species according to their carbon metabolism (Jordan 1984; Stowers 1985). Although many reports indicate that dicarboxylic acids are probably the major source of carbon and energy supplied to bacteroids (Emerich *et al.* 1988; McRae *et al.* 1989), studies show, for some strains of rhizobia, that glucose could act as an energy-yielding substrate in functioning nodules (Herrada *et al.* 1989). Therefore, in this study, we have compared the glucose transport system in the arctic strain N_{31} and the temperate strain SM_2 of *Rhizobium* sp. [*Onobrychis*].

Materials and methods

Bacterial strains

The arctic strain of rhizobia N_{31} was isolated from *Astragalus alpinus* collected in the Sarcpa Lake region, Melville Peninsula, Northwest Territories, Canada (Prévost *et al.* 1987*c*), and strain SM_2 was isolated from sainfoin cultivated in Saskatchewan. Both strains are effective on sainfoin (Prévost *et al.* 1987*b*).

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Media

Rhizobia were maintained on yeast glucose (YG) agar slants (Vincent 1970). MOPS salts solution (Ms) was prepared as described by Finan *et al.* (1981), but phosphate concentration was increased to 2.4 mM K_2HPO_4 and 0.8 mM KH_2PO_4 and trace elements and vitamins were added as follows (milligrams per litre): ferric citrate, 5; $CoCl_2 \cdot 6H_2O$, 4×10^{-3}; $CuSO_4 \cdot 5H_2O$, 8×10^{-3}; H_3BO_3, 2.86; $H_2MoO_4 \cdot H_2O$, 9×10^{-2}; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; biotin, 0.1; thiamine-HCl, 1; and calcium pantothenate, 2. Carbon sources were added to media to a final concentration of 15 mM. Carbon sources, MOPS-KOH, and $MgSO_4$ were filter sterilized separately and added to the autoclaved salts.

Cell growth

Inocula were prepared by cultivating rhizobia for 4–5 days, in 250-mL Erlenmeyer flasks containing 50 mL of YG broth, at 25°C on a rotary shaker (160 rpm). Cells were collected, washed twice, and resuspended in phosphate buffered saline (PBS; 3 mM phosphate buffer in 0.7% NaCl, pH 7.0).

For transport experiments, 50 mL Ms-glucose (or other carbon source) was inoculated with 0.5 mL inoculum and incubated as described earlier. Cell growth was followed by measuring the absorbance increase at 600 nm in a Bausch & Lomb Spectronic 20. Cells were harvested after 24 and 48 h incubation for strain SM_2 and N_{31}, respectively, at optical density values at 600 nm of 0.6 to 0.8 (mid-log phase).

Preparation of cells for transport experiments

Mid log phase cells were harvested and washed twice in PBS, by centrifugation (10 000 × *g*, 10 min, 4°C). Cells were resuspended in Ms to yield a final protein concentration of 0.2–0.5 mg/mL and stored at 4°C. Transport assays were then completed within 4 h, over which time no loss of activity was observed. Cells were incubated on a rotary shaker (125 rpm) at 25°C for 30 min before being used in transport experiments.

Transport assays

Assays were carried out at 25°C by using a modification of the procedure of Hudman and Glenn (1980). Transport assays performed in a final volume of 2 mL with a concentration of glucose of 0.1 mM (1.85 kBq) were initiated by adding 100 μL of a mixture of nonradioactive glucose and D-[U-^{14}C]glucose to 1.9 mL cell suspension. At various time intervals the cells of 250-μL samples were collected on nitrocellulose filters (0.2-μm pore size, 2.5 cm diam., Sartorius) by means of a manifold connected to a vacuum line. The filters were washed immediately, using 10 mL PBS, dried under an infrared lamp, and placed in scintillation vials containing 8 mL of Beckman Ready Safe cocktail. Radioactivity was counted using a LKB Wallac Rackbeta 1217 liquid-scintillation counter (80–87% efficiency).

For measurements of uptake rates the final reaction volume was 1 mL. Labelled and unlabelled glucose were added to 0.9 mL cell suspensions to give a concentration range of 1 to 100 μM and final specific activities of 7.4 MBq mmol^{-1}. The initial rates of transport were calculated from the amount of radioactivity accumulated by the cells in 30 s. Appropriate competitors (1 mM) or inhibitors and [^{14}C]glucose (5 or 100 μM for the high- and low-affinity transport systems) were also added to cell suspensions to give a final volume of 1 mL. Competitors and inhibitors were added 30 s prior to the addition of radioactive solute and glucose transport was measured for 5 min.

Nonspecific binding of ^{14}C-labelled substrate to the bacterial cells was appraised as described by Finan *et al.* (1981), by using cells treated for 30 min with toluene before assay. Data are means of three replications from at least two experiments.

Enzyme assays

Phosphoenolpyruvate:glucose phosphotransferase activity and glucokinase activity were measured as described by Romano *et al.* (1979).

Measurement of O_2 consumption

Substrate-dependent O_2 consumption was measured by using a biological oxygen monitor (Yellow Spring Instrument, Ohio) as previously described (Lafrenière *et al.* 1987).

TABLE 1. Substrate-dependent O_2 consumption (nmol $O_2 \cdot min^{-1} \cdot mg$ protein^{-1}) by free-living cells of the arctic *Rhizobium* strain N_{31} and the temperate strain SM_2

Carbon source in growth medium (15 mM)	Strain	Rate of O_2 consumption in the presence of: Glucose (10 mM)	Succinate (10 mM)	Malate (10 mM)
Glucose	N_{31}	182.0	64.3	21.4
	SM_2	234.9	55.2	81.0
Succinate	N_{31}	0.0	208.0	181.0
	SM_2	0.0	225.6	197.2

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Chemicals

D-[U-^{14}C]Glucose (136.9 MBq mmol^{-1}) was purchased from NEN Research Products, DuPont Canada Inc., Mississauga, Ont. Other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Results and discussion

Succinate- and malate-dependent O_2 consumption by glucose-grown cells of the arctic strain of *Rhizobium* N_{31} and the temperate strain SM_2 were much lower than those observed with the succinate-grown cells (Table 1). These data suggest, as previously observed with other *Rhizobium* strains (Finan *et al.* 1981; Lafrenière *et al.* 1987; Lafontaine *et al.* 1989; McAllister and Lepo 1983), that the C_4-dicarboxylic acid transport system in the two strains is probably inducible. Both strains oxidized glucose, succinate, and malate in a similar fashion. When grown on succinate, cells of strains N_{31} and SM_2 were unable to oxidize glucose (Table 1). This could be the results of the presence of an inducible glucose transport system in the two strains, or the inhibition of a constitutive transport system by succinate, or the presence of a catabolite repression-like phenomenon mediated by succinate or malate (Ucker and Signer 1978).

Glucose- and arabinose-grown cells of both the arctic strain N_{31} and the temperate strain SM_2 were able to take up [^{14}C]glucose immediately (Fig. 1), indicating that glucose transport systems in the two strains are probably constitutive. Cells grown on minimal salts medium with glutamate or pyruvate as sole carbon source were also able to take up glucose without any lag phase, and the presence of these carbon sources with glucose in the culture medium had no effect on glucose transport (results not shown). Cells grown on succinate or succinate and glucose had very low uptake rates, implying that the glucose uptake in these strains is repressed by succinate. Similar observations were previously made with the fast-growing *Rhizobium leguminosarum* (Hudman and Glenn 1980) and the slow-growing *Bradyrhizobium* spp. (San Francisco and Jacobson 1986; Stowers and Elkan 1983).

A nonlinear Eadie–Hofstee plot was obtained when glucose transport was measured with strain N_{31} and strain SM_2 (Fig. 2). This revealed the presence of at least two glucose transport systems in the two strains studied. The transport of glucose was

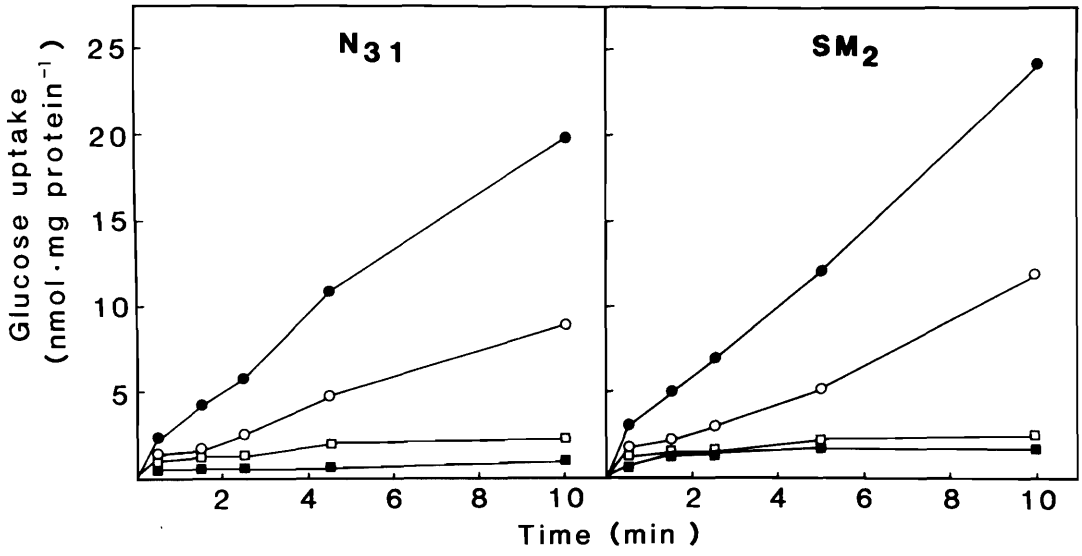

FIG. 1. The transport of [^{14}C]glucose by the arctic strain N_{31} and the temperate strain SM_2 grown on glucose (●), arabinose (○), succinate (■), and glucose + succinate (□).

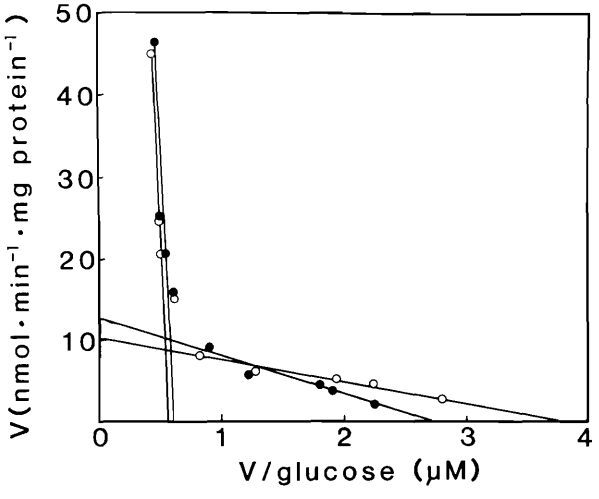

FIG. 2. Kinetics of glucose transport in cultured cells of the arctic strain N_{31} (●) and the temperate strain SM_2 (○). Data are plotted according to the Eadie–Hofstee method.

a saturable function of substrate concentration for both strains. The apparent K_m and V_{max} values determined by measuring the initial transport rate for the high-affinity glucose system (substrate concentration 1–10 μM) in the arctic strain N_{31} were higher than those obtained with the temperate strain SM_2 (Table 2). However, for the low-affinity transport systems (substrate concentration 10–100 μM), higher apparent K_m and V_{max} values were obtained with strain SM_2 (Table 2). Two uptake systems have also been reported for glucose, in *R. leguminosarun* (de Vries *et al.* 1982) and *Bradyrhizobium* spp. (San Francisco and Jacobson 1986), and for succinate in *B. japonicum* (Humbeck and Werner 1987). Adaptation to carbon-substrate limitation can result in the elaboration of high-affinity uptake systems (Harder and Dijkhuizen 1983). Recently, Cornish *et al.* (1988) have described the production by *Agrobacterium radiobacter* of two distinct periplasmic binding proteins which are involved in glucose transport and are maximally derepressed during growth under glucose limitation.

TABLE 2. Kinetic parameters of the high- and low-affinity glucose transport systems in strains N_{31} and SM_2

	High		Low	
Strain	K_m (μM)	V_{max}	K_m (μM)	V_{max}
N_{31}	4.7	12.7	53.4	58.9
SM_2	2.6	10.1	72.6	64.6

NOTE: V_{max} is expressed in nmol·min^{-1}·mg protein^{-1}. Kinetic values were for a substrate range between 1 and 100 μM.

The high- and low-affinity glucose transport systems were inhibited by more than 70% by the protonophore 2,4-dinitrophenol and by KCN (Table 3). Azide, like KCN, interferes with the last step in oxidative phosphorylation, the transfer of electrons to O_2.

The low-affinity glucose transport systems in the two strains studied were also inhibited by azide, by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), another proton ionophore, and by an inhibitor of ATPase, *N,N'*-dicyclohexylcarbodiimide (DCCD). These results indicate that glucose transport systems in the arctic strain N_{31} and the temperate strain SM_2 are active and depend on a highly energized membrane and that a proton-motive force is probably associated with these processes. Arsenate, acting as an organic-phosphate analogue, had no effect on glucose transport in strain SM_2 and caused 6 and 60% inhibition of the high- and low-affinity systems in strain N_{31}, respectively. The fact that NaF had no effect on glucose transport and the absence of significant activities of phosphoenolpyruvate:glucose phosphotransferase and glucokinase in the toluene-treated cells of the two strains (0.3 and <0.06 nmol·min^{-1}·mg protein^{-1} for PEP phosphotransferase and glucokinase respectively), confirm that a proton-motive force is implicated in glucose transport and that the direct use of high-energy phosphate compounds such as phosphoenolpyruvate by the glucose transport mechanism is unlikely. Absence of the PEP-dependent phosphotransferase mechanism of glucose transport

TABLE 3. Effect of inhibitors on the glucose transport system in the arctic strain N_{31} and the temperate strain SM_2

Inhibitor[a]	Concn. (mM)	% inhibition of transport: Low affinity N_{31}	Low affinity SM_2	High affinity N_{31}	High affinity SM_2
Arsenate	4	60	4	6	3
KCN	4	83	85	78	89
2,4-DNP	0.1	78	86	86	74
CCCP	0.05	79	50		
DCCD	0.5	88	64		
Azide	4	81	90		
NEM	1	79	85		
NaF	4	19	7		

NOTE: Control rates were 0.87 and 2.05 with strain N_{31} and 0.67 and 2.45 nmol·min^{-1}·mg protein^{-1} with strain SM_2 for the high-affinity (5 μM glucose) and low-affinity (100 μM) uptake systems, respectively.

[a]2,4-DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide.

TABLE 4. Specificity of the glucose transport systems in the arctic strain N_{31} and the temperate strain SM_2

Competitor[a] (1 mM)	% inhibition of transport: Low affinity N_{31}	Low affinity SM_2	High affinity N_{31}	High affinity SM_2
D-Glucose	89	89	95	90
α-MG	14	38	15	25
3-*O*-MG	9	29	12	20
D-Fructose	6	32	13	15
D-Galactose	8	39	9	11
D-Mannose	25	6	2	9
Sorbitol	8	29	10	3
D-Xylose	82	39	54	30
Succinate	7	19	10	15
Malate	15	21	9	18
Glutamate	9	36	8	21

NOTE: Control rates were 0.83 and 3.34 with strain N_{31} and 0.70 and 3.13 nmol·min^{-1}·mg protein^{-1} with strain SM_2 for the high-affinity (5 μM glucose) and low-affinity (100 μM) uptake systems, respectively. All data are from triplicate assays of two different experiments.

[a]α-MG, α-methyl-D-glucopyranoside; 3-*O*-MG, 3-*O*-methyl-D-glucopyranoside.

has also been reported for *R. leguminosarum* (de Vries *et al.* 1982) and *Bradyrhizobium* spp. (San Francisco and Jacobson 1986). In the two strains studied glucose transport was also inhibited by *N*-ethylmaleimide (NEM), suggesting the presence of essential -SH groups in the carrier system. A requirement of functional sulfhydryl groups for glucose transport was also observed in *R. leguminosarum* (de Vries *et al.* 1982), *Rhizobium meliloti* (Theodoropoulos *et al.* 1985), and in cowpea rhizobia (Stowers and Elkan 1983).

The specificity of the glucose transport system in the arctic strain N_{31} and the temperate strain SM_2 was studied by preincubating cells with various compounds (at 10- and 200-fold the glucose concentration) 30 s prior to the addition of [^{14}C]glucose. Unlabelled glucose was the most effective competitor for the high- and low-affinity systems. Glucose uptake was not significantly inhibited by the structural analogues of glucose, α-methyl-D-glucopyranoside (α-MG) and 3-*O*-methyl-D-glucopyranoside (3-*O*-MG), indicating that these compounds and glucose do not have identical affinity for the carriers. Fructose, galactose, mannose, sorbitol, succinate, malate, and glutamate did not show any substantial inhibition of the two transport systems (Table 4). With strain N_{31}, xylose inhibited the low-affinity transport system, but the high-affinity system was only partially inhibited. These results indicate, as previously observed with other rhizobia (de Vries *et al.* 1982; Stowers and Elkan 1983), that the transport of glucose in the arctic strain N_{31} and the temperate strain SM_2 is glucose specific.

Conclusions

This work indicates that a similar process of glucose transport is present in the arctic strain N_{31}, isolated from *Astragalus alpinus* and effective with sainfoin, and in the effective temperate strain SM_2, isolated from sainfoin. In the two strains, glucose transport has a biphasic kinetics, indicating the presence of a high- and a low-affinity system. It is probably a constitutive and active process depending on a highly energized membrane and implicating a proton-motive force. Glucose transport is repressed by succinate and malate.

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