Effect of temperature on succinate transport by an arctic and a temperate strain of rhizobia[1]

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The effect of temperature on the succinate transport system was studied in the arctic *Rhizobium* strain N31 (isolated from *Astragalus alpinus*) and in the temperate strain SM2 (isolated from *Onobrychis viciifolia*). Only one inducible succinate transport system was found in the two strains as indicated by the linear Eadie–Hofstee plot obtained at 10, 15, and 25°C. The transport of succinate was not affected by arsenate, but was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone, KCN, and iodoacetate, implying an active process, a proton motive force, and essential sulfhydryl groups in the system. At 25°C the apparent K_m and V_{max} values observed were 6.7 and 7.4 μM and 40.8 and 27.9 nmol·min^{-1}·mg protein^{-1} for strains N31 and SM2, respectively. Similar kinetic parameters for succinate transport at 25°C were obtained with the cells of both strains grown at 10 or 25°C. However, when transport was measured at 10°C the K_m and V_{max} values obtained with strain SM2 were higher for cells cultured at 10°C than for those cultured at 25°C, suggesting that this temperate strain might be more affected by low growth temperature than the arctic strain N31. The succinate transport systems in the two strains were affected by temperature in a similar fashion, as indicated by similar Arrhenius plots of V_{max} showing a discontinuity at 20°C and by comparable apparent energy of activation values. These observations suggest that the cold adaptation of strain N31 is not related to a cold adaptation of the succinate carrier.

Key words: arctic, *Rhizobium*, succinate, symbiosis, transport.

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On a étudié l'effet de la température sur le transport du succinate chez la souche arctique de *Rhizobium* N31 (isolée d'*Astragalus alpinus*) et chez la souche tempérée SM2 (isolée d'*Onobrychis viciifolia*). Chez les deux souches une courbe linéaire de Eadie–Hofstee a été obtenue indiquant la présence d'un seul système de transport. Le transport du succinate est induit et il n'est pas affecté par l'arséniate, mais il est inhibé par le carbonyl cyanide *m*-chlorophénylhydrazone, le KCN et l'iodoacétate. Ces résultats indiquent que ce système est actif et qu'il implique un gradient électrochimique de protons ainsi que des groupements -SH. À 25°C les valeurs de K_m et de V_{max} observées étaient respectivement de 6,7 et 7,4 μM et de 40,8 et 27,9 nmol·min^{-1}·mg protéine^{-1} pour les souches N31 et SM2. Lorsque le transport est mesuré à 25°C, la température de croissance des souches (10 ou 25°C) n'a pas influencé les paramètres cinétiques observés. Cependant, lorsque le transport est mesuré à 10°C, les cellules de la souche SM2 cultivées à 10°C ont donné des valeurs de K_m et V_{max} plus élevées que celles obtenues avec les cellules croissant à 25°C. Ceci suggère que la souche tempérée SM2 est plus sensible à une basse température de croissance que la souche N31. Chez les deux souches, la température affecte le transport du succinate de la même manière. Les courbes d'Arrhénius des V_{max} sont semblables, montrant une discontinuité à 20°C et les valeurs apparentes de l'énergie d'activation chez les deux souches sont comparables. Nos résultats suggèrent que l'adaptation au froid de la souche N31 n'est pas reliée à l'adaptation au froid du porteur du succinate.

Mots clés : arctique, *Rhizobium*, succinate, symbiose, transport.

[Traduit par la rédaction]

Introduction

The arctic *Rhizobium* strain N31 was isolated from nodules of *Astragalus alpinus* collected in the Melville peninsula near Sarcpa Lake, Northwest Territories, Canada (Prévost et al. 1987*c*). The optimum growth temperature of the arctic strain N31 is 25°C and that of the temperate strain SM2 isolated from sainfoin (*Onobrychis viciifolia*) is 30°C (Prévost et al. 1987*a*). Strain N31 also forms effective nodules on the temperate legume sainfoin (Prévost et al. 1987*b*), and it is cold adapted. In fact, at 10°C on yeast extract mannitol medium, the doubling time of the arctic strain N31 is 27.3 h whereas that of the temperate strain SM2 is 42.8 h. However, at 25°C the growth rates of the two strains are more comparable (doubling times of 4.5 and 3.6 h for strains N31 and SM2, respectively). Furthermore, at 10°C the

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TABLE 1. Regulation of succinate transport in the arctic strain N31 and the temperate strain SM2

Carbon source in growth medium	Uptake (nmol·min^{-1}·mg protein^{-1})	
	N31	SM2
15 mM Succinate	37.2 ± 1.8	26.0 ± 1.3
15 mM α-Ketoglutarate	39.2 ± 1.6	41.7 ± 1.7
15 mM Malate	26.1 ± 1.6	25.7 ± 1.0
15 mM Glutamate	9.4 ± 0.4	5.2 ± 0.1
15 mM Glucose	1.3 ± 0.5	2.4 ± 0.3
10 mM Glucose + 10 mM succinate	21.7 ± 0.1	17.6 ± 0.2

NOTE: The mean values and standard deviations were calculated from duplicate assays of three different experiments.

nitrogenase activity of sainfoin plants nodulated with the arctic strain N31 (35.23 μmol $C_2H_4 \cdot h^{-1} \cdot g$ nodule dry mass^{-1}) is significantly higher than that obtained with strain SM2 (21.36 μmol $C_2H_4 \cdot h^{-1} \cdot g$ nodule dry mass^{-1}; Prévost et al. 1987*a*). Cold-adapted arctic rhizobia also produce more cold shock proteins under freezing conditions than temperate strains (Cloutier et al. 1992).

We have previously described the glucose transport systems in strains N31 and SM2 (Bigwaneza et al. 1991). As four-carbon dicarboxylic acids are probably the major sources of reducing equivalents in bacteroids (Streeter 1991), the object of the present work was to study the effect of temperature on the succinate transport systems in the two strains, to understand the cold adaptation of the arctic strain.

Materials and methods

Bacterial strains

The arctic rhizobial strain N31 was isolated from *Astragalus alpinus* collected in the Sarcpa Lake region, Melville Peninsula, Northwest Territories, Canada (Prévost et al. 1987*c*), and rhizobial strain SM2 was isolated from sainfoin (*Onobrychis viciifolia*) cultivated in Saskatchewan. Both stains are effective on sainfoin (Prévost et al. 1987*b*).

Media

Rhizobia were maintained on yeast succinate agar slants, containing 15 mM succinate (Vincent 1970). A modified 3-(*N*-morpholino)propanesulfonic acid (MOPS) salts solution (Ms; Finan et al. 1981) was prepared as described by Bigwaneza et al. (1991). Carbon sources were added to the media to a final concentration of 15 mM, but when two carbon sources were used simultaneously the final concentration of each was 10 mM.

Preparation of cells for transport assays

Inocula were prepared as previously described, but succinate (0.5% w/v) replaced glucose (Bigwaneza et al. 1991). Cells were grown in 250-mL Erlenmeyer flasks containing 100 mL of Ms-succinate (or other carbon source) inoculated with 1 mL inoculum, on an orbital water bath shaker (Julabo SW-20C; 135 rpm) at 10 or 25°C. Mid log phase cells were harvested and washed twice in phosphate buffered saline (PBS; 3 mM phosphate buffer in 0.7% NaCl, pH 7.0) by centrifugation (10 000 × *g*, 10 min) at their respective growth temperature (10 or 25°C). Cells were resuspended in Ms to yield a final protein concentration of 0.2–0.5 mg/mL and kept at their growth temperature. Transport assays were then completed within 2 h, over which time no loss of activity was observed.

Transport assays

Transport assays were performed as described previously (Bigwaneza et al. 1991). The reaction mixture (1 mL) contained 0.9 mL of cell suspensions, and a mixture (100 μL) of labelled and unlabelled succinate giving a concentration range of 1–100 μM (specific activity 0.02–0.93 GBq·mmol^{-1}; 0.5–25 μCi·μmol^{-1}). The initial rates of transport were calculated from the amount of radioactivity accumulated by the cells in 30 s. Cells 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 with 5 mL PBS and dried under an infrared lamp, and radioactivity was measured as reported previously (Bigwaneza et al. 1991). In experiments with metabolic inhibitors, the cells (0.8 mL) were preincubated for 5 min at 25°C with the inhibitor (0.1 mL) before the addition of ^{14}C-labelled substrate (0.1 mL to give a final concentration of 100 μM labelled succinate).

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 given as the mean of two replications from at least three different experiments.

Effect of temperature on transport

The effect of temperature on succinate transport was appraised by incubating 0.9 mL of the bacterial cell suspensions for 10 min at the assay temperature, before adding 100 μL of ^{14}C-labelled substrate that was also adjusted to the assay temperature. Cells were collected 30 s or 1 min after the addition of the labelled substrate, at all assay temperatures or at 5°C, respectively.

Chemicals

[2,3-^{14}C]Succinic acid (2.1 GBq·mmol^{-1}) was obtained from NEN Research Products, DuPont Canada Inc., Mississauga, Ont. Other chemicals were obtained from Sigma Chemicals Co., St. Louis, Mo.

Results and discussion

At 25°C, succinate-grown cells of the arctic strain N31 and the temperate strain SM2 had doubling times of 6.5 and 4.5 h, respectively. At 10°C, the observed doubling times were 29.2 for N31 and 36.6 h for SM2. These results confirm that growth of strain N31 is cold adapted, as previously reported with mannitol (Prévost et al. 1987*a*).

Cells of the arctic strain N31 grown on succinate or α-ketoglutarate were able to readily take up [^{14}C]succinate (Table 1). Malate-grown cells of strain N31 showed a slightly lower uptake activity. For the temperate strain SM2, the succinate transport activity observed with α-ketoglutarate-grown cells was almost twice that observed with the succinate- or malate-grown cells (Table 1). For strain N31, the highest succinate uptake activity was also observed with α-ketoglutarate-grown cells, indicating that in both strains this organic acid is the most effective inducer. Very low succinate transport activity was observed when the cells of both strains were grown on glucose as sole carbon source. The addition of 10 mM succinate with 10 mM glucose in the culture medium restored the succinate transport activity. These results suggest that the succinate transport system in the arctic strain N31 and the temperate strain SM2 is inducible. Inducible succinate transport systems were also observed with fast-growing (Glenn et al. 1980; Finan et al. 1981) and slow-growing (McAllister and Lepo 1983) rhizobia. However, constitutive succinate transport systems were also observed in *Rhizobium meliloti* (Hornez et al. 1989), in *Bradyrhizobium japonicum* (San Francisco and Jacobson 1985; Humbeck and Werner 1987), and in cowpea *Rhizobium* (San Francisco and Jacobson 1985).

A linear Eadie–Hofstee plot was obtained when succinate transport was measured with the arctic strain N31 and the

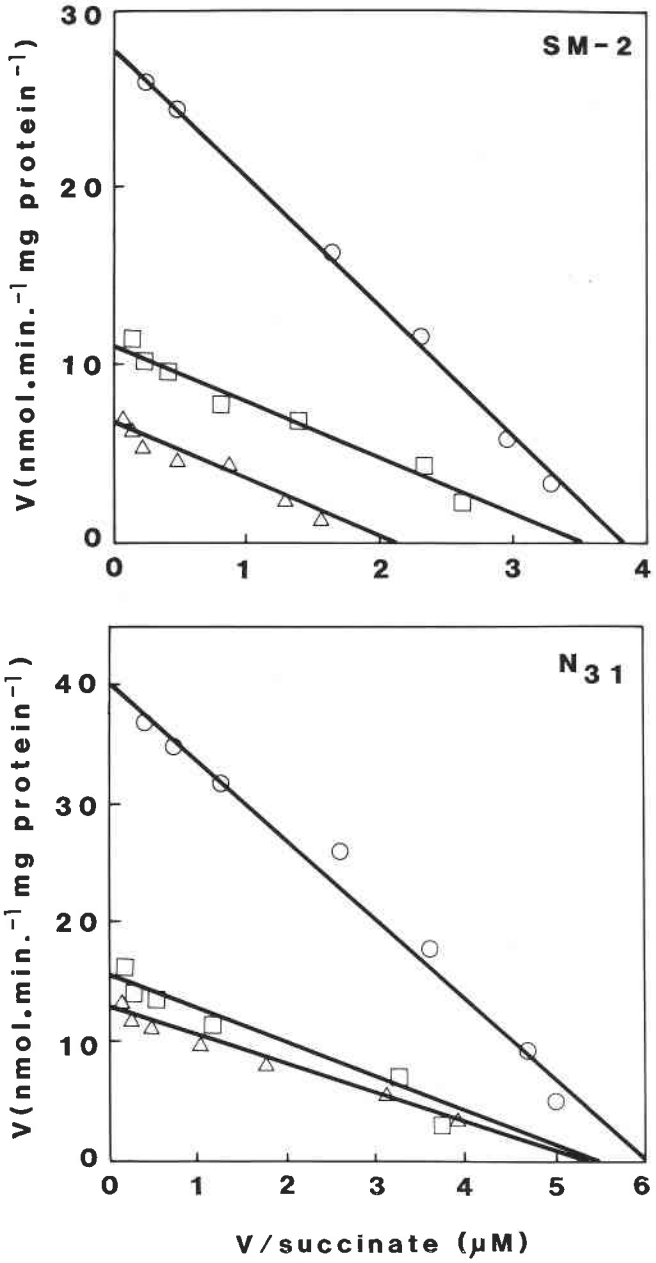

FIG. 1. Kinetics of succinate transport in cultured cells of the arctic strain N31 and the temperate strain SM2 at 10°C (△), 15°C (□), and 25°C (○). Data are plotted according to the Eadie–Hofstee method.

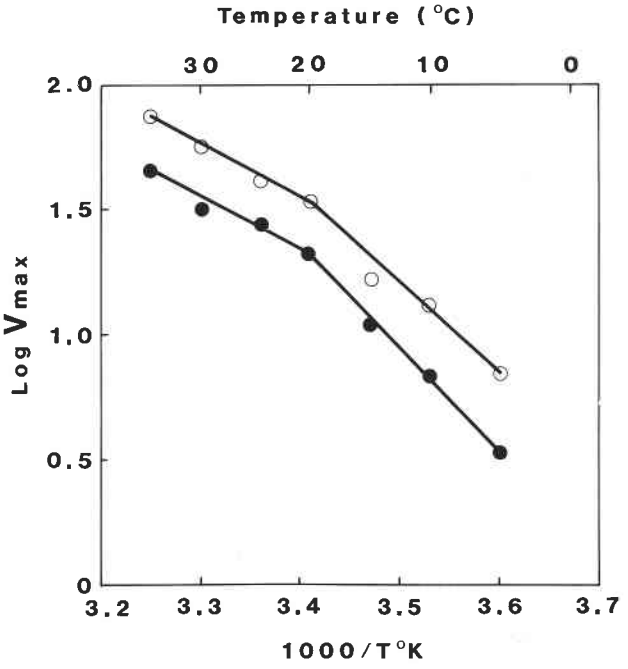

FIG. 2. Arrhenius plot of V_{max} of succinate transport in the arctic strain N31 (○) and the temperate strain SM2 (●). 1000/T°K, 1000/(temperature in °K).

TABLE 2. Effect of growth and assay temperatures on the kinetic parameters of the succinate transport system in the arctic *Rhizobium* strain N31 and the temperate strain SM2

Strain	Growth temperature (°C)	Assay temperature (°C) 25 K_m (μM)	25 V_{max}	10 K_m (μM)	10 V_{max}
N31	25	6.7 ± 0.7	40.8 ± 2.4	2.4 ± 0.1	13.0 ± 1.2
	10	6.9 ± 1.1	39.8 ± 0.9	2.5 ± 0.3	14.3 ± 0.2
SM2	25	7.4 ± 0.5	27.9 ± 2.6	3.5 ± 0.2	7.0 ± 0.2
	10	8.2 ± 0.8	26.4 ± 1.8	5.0 ± 0.3	9.3 ± 1.4

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 mean values and standard deviations were calculated from duplicate assays of three different experiments.

temperate strain SM2 at 10, 15, or 25°C (Fig. 1). This indicates that there is only one succinate transport system in the two strains studied, as previously observed with fast- and slow-growing rhizobia (Glenn et al. 1980; Finan et al. 1981; McAllister and Lepo 1983; San Francisco and Jacobson 1985; Hornez et al. 1989). However, Humbeck and Werner (1987) have observed the presence of two succinate uptake systems in a strain of *B. japonicum*, and we have previously found that the two strains studied have both high- and low-affinity glucose transport systems (Bigwaneza et al. 1991). The transport of succinate was a saturable function of substrate concentration for both strains. In general, strain N31 absorbed succinate better than strain SM2, as indicated by higher uptake (Table 1) and higher V_{max} values (Table 2). When succinate transport was assayed at 25°C, the growth temperature (10 or 25°C) did not affect the apparent K_m and V_{max} values obtained with strain N31 or SM2 (Table 2). Similar results were noted with the arctic strain N31 at an assay temperature of 10°C. Comparable results previously showed that the uptake of 2-deoxyglucose and L-leucine in Gram-positive psychrotrophic bacteria was essentially unaffected by growth at low temperatures (Wilkins 1973).

The kinetic parameters of succinate transport in strains N31 and SM2 grown at 25°C were affected by lower assay temperatures in a similar fashion (Table 3). In fact, when the assay temperature increased, the K_m and V_{max} values increased in both strains, and the Q_{10} values (the number of times by which an enzymatic activity is increased by a 10°C rise in temperature) calculated from V_{max} were 2.3 and 3.3 between 5 and 15°C, and 2.6 and 3.0 between 10 and 20°C, for strains N31 and SM2, respectively. In contrast, between 5 and 15°C, the Q_{10} calculated from nitrogenase activity was 3.3 for strain N31 and 23.5 for strain SM2, indicating that temperature had a more marked effect

TABLE 3. Effect of temperature on the kinetic parameters of the succinate transport systems in the arctic *Rhizobium* strain N31 and the temperate strain SM2

Temperature (°C)	Strain N31 K_m (μM)	Strain N31 V_{max}	Strain SM2 K_m (μM)	Strain SM2 V_{max}
5	2.0	7.1	1.5	3.4
10	2.4	13.0	3.5	7.0
15	3.4	16.3	3.1	11.1
20	5.8	33.9	5.6	21.3
25	6.7	40.8	7.4	27.9
30	9.6	57.8	8.1	32.2
35	13.1	74.9	11.6	45.1

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 mean values and standard deviations were calculated from duplicate assays of three different experiments.

TABLE 4. Effect of inhibitors on the succinate transport system in the arctic strain N31 and the temperate strain SM2

Inhibitor	Concn. (mM)	% inhibition of transport N31	% inhibition of transport SM2
Arsenate	4	3	0
CCCP	0.1	95	94
KCN	4	93	87
Iodoacetate	4	83	89

NOTE: Control rates were 37.04 and 26.02 nmol·min^{-1}·mg protein^{-1} with strains N31 and SM2, respectively. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

on the symbiotic system formed by the latter strain (Prévost et al. 1987*a*). Furthermore, Arrhenius plots of V_{max} of succinate transport showed a discontinuity at 20°C in both strains (Fig. 2). The apparent activation energies derived from V_{max} calculated as previously described (McKinley and Vestal 1984) were 15.5 and 18.8 kcal·mol^{-1} below 20°C and 10.1 and 8.8 kcal·mol^{-1} above 20°C for strains N31 and SM2, respectively. These results indicate that the succinate transport system is probably not implicated in the cold adaptation of arctic strain N31, as reflected by bacterial growth and nitrogenase activity in symbiosis with sainfoin (Prévost et al. 1987*a*). In fact, to date there are no convincing data to show that individual carrier protein molecules are cold labile (Herbert 1986). Further studies with bacteroids are necessary to confirm this observation, and future investigations should concentrate on the effect of temperature on membrane lipid composition, which can affect active transport processes (Eze and McElhaney 1987).

The succinate transport systems in the two strains studied were not affected by arsenate, which acts as an organic phosphate analogue, but were strongly inhibited by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, by KCN (which interferes in the last step of oxidative phosphorylation), and by the sulfhydryl reagent iodoacetate (Table 4). These results indicate that the succinate transport systems in the arctic strain N31 and the temperate strain SM2 are active and that a proton motive force is probably associated with these processes. Since succinate transport was insensitive to arsenate, direct utilization of ATP by the succinate transport mechanisms in the two strains studied is unlikely. In the two carrier systems, essential sulfhydryl groups are also present. The present data are consistent with previous studies on succinate transport by fast- and slow-growing rhizobia (Glenn et al. 1980; Finan et al. 1981; McAllister and Lepo 1983; San Francisco and Jacobson 1985; Hornez et al. 1989).

Conclusions

The present work shows that the arctic *Rhizobium* strain N31 and the temperate strain SM2 have a similar process of succinate transport. The two strains have only one inducible and active succinate transport system. In the two strains a proton motive force is implicated and both carriers have essential sulfhydryl groups. As succinate transport in both strains is affected by low temperature in a similar fashion, succinate transport probably does not play an important role in the cold adaptation of the arctic strain N31.

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