A comparison of carbon source utilization for growth and nitrogenase activity in two *Frankia* isolates

MARY F. LOPEZ
Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, U.S.A. 02139
AND
PATRICIA YOUNG AND JOHN G. TORREY
Cabot Foundation, Harvard University, Petersham, MA, U.S.A. 01366

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The carbon source requirements for the growth and nitrogen fixation of two morphologically distinct *Frankia* isolates were examined. Isolate ArI3 (from *Alnus rubra*) grew well on propionate, malate, acetate, and trehalose, and isolate CcI2 (from *Casuarina cunninghamiana*) grew best on pyruvate, acetate, and propionate. In general, the same carbon sources that supported growth supported both the development of vesicles and nitrogenase activity in long-term induction experiments in both isolates. However, ArI3 cultures induced on proprionate had 7 to 26 times the activity of other carbon sources and ArI3 cultures induced on acetate did not develop any detectable acetylene reduction. In a parallel set of experiments, cultures of both isolates were induced for nitrogenase activity on propionate and the resulting nitrogen fixing cultures were washed free of the organic acid by centrifugation. The washed cultures were incubated in the presence of various carbon sources to determine the ability of a particular substrate to supply energy directly for nitrogen fixation when vesicles and nitrogenase were already present. As was observed in the long-term induction experiments, pyruvate, propionate, and acetate supported the greatest activity in CcI2. Succinate and malate supported the greatest activity in ArI3, and propionate had very little stimulation of acetylene reduction. The reason for the lack of stimulation by propionate for washed cells of ArI3 was unclear but may have been due to toxic concentrations of the organic acid. In an attempt to compare the carbon utilization of ArI3 in pure culture with that in the alder symbiosis, oxygen uptake in the presence of various carbon sources of vesicles clusters isolate from *Alnus rubra* nodules inoculated with ArI3 was compared with the oxygen uptake of nitrogen-fixing pure cultures of ArI3. The oxygen uptake of the isolated vesicle clusters was stimulated by sucrose, trehalose, and glucose, but not by a variety of organic acids. In comparison, nitrogen-fixing pure cultures of ArI3 readily oxidized sugars and organic acids.

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Deux isolats de *Frankia*, distincts sur le plan morphologique, ont servi à l'étude de leurs exigences relativement aux sources de carbone en fonction de leur croissance et de la fixation de l'azote. Une bonne croissance a été réalisée par l'isolat ArI3, prélevé chez *Alnus rubra*, en présence de propionate, de malate, d'acétate et de tréhalose, alors que celle de l'isolat CcI2, prélevé chez *Casuarina cunninghamiana*, fut supérieure en présence de pyruvate, d'acétate et de propionate. Les mêmes sources de carbone qui ont favorisé la croissance ont aussi favorisé, en général, le développement des vésicules et l'activité de la nitrogénase chez les deux isolats dans les expériences d'induction à long terme. Toutefois, l'activité des cultures de l'ArI3 induites sur propionate a été de 7 à 26 fois supérieure à celle des autres sources de carbone. Dans un groupe d'expériences parallèles, l'activité nitrogénase a été induite chez les deux isolats cultivés sur propionate et ces cultures fixatrices d'azote furent délavées et libérées des acides organiques par centrifugation. Les cultures délavées furent incubées en présence de diverses sources de carbone pour déterminer l'aptitude de l'un ou de l'autre des substrats à fournir directement l'énergie requise pour la fixation d'azote, lorsque les vésicules et la nitrogénase étaient déjà présentes. Dans les expériences d'induction à long terme, il fut observé que le pyruvate, le propionate et l'acétate étaient les sources permettant la plus grande activité chez le CcI2. Pour l'ArI3, la plus grande activité s'est manifestée avec le succinate et le malate, alors que le propionate n'a stimulé que très peu la réduction de l'acétylène. La raison de cette absence de stimulation par le propionate chez les cellules délavées d'ArI3 n'est pas claire, mais il semble qu'elle ait été causée par des concentrations toxiques d'acides organiques. Dans une tentative de comparaison entre l'utilisation du carbone par l'ArI3 en culture pure et celle de la symbiose avec l'aulne, l'absorption de l'oxygène par des groupes de vésicules isolées de nodules d'*Alnus rubra* inoculé avec l'ArI3 et en présence de diverses sources de carbone a été comparée avec celle des cultures pures d'ArI3 fixatrices d'azote. L'absorption d'oxygène par les groupes de vésicules a été stimulée par le sucrose, le tréhalose et le glucose, mais elle n'a pas été stimulée par une variété d'acides organiques. Par comparaison, les cultures pures d'ArI3 fixatrices d'azote ont rapidement oxydé les sucres et les acides organiques.

[Traduit par le journal]

Introduction

To date, nutritional studies on the nitrogen-fixing symbiotic actinomycete *Frankia* have mostly focused on growth (Blom 1981, 1982; Blom and Harkink 1981; Shipton and Burggraaf 1982; Tisa *et al.* 1983; Lechevalier *et al.* 1983). An examination of these studies clearly shows that *Frankia* strains differ widely in their carbon source requirements. When many *Frankia* strains are placed in a medium lacking NH_4Cl, they differentiate specialized structures termed vesicles and fix nitrogen under aerobic conditions (Tjepkema *et al.* 1980, 1981; Gauthier *et al.* 1981; Burggraaf and Shipton 1983; Murry *et al.* 1984; Lopez *et al.* 1984), but there have been few published studies on the carbon source requirements for vesicle development and nitrogen fixation in *Frankia* (Burggraaf and Shipton 1983; Zhang *et al.* 1986). In this study, we were interested in making a comparison of the carbon utilization patterns for growth and nitrogenase induction in two morphologically distinct *Frankia* isolates. We chose to compare strain ArI3 isolated from *Alnus rubra* nodules (Berry and Torrey 1979) and strain CcI2 isolated from *Casuarina cunninghamiana* nodules

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(Zhang *et al.* 1984). The two strains differ markedly in several respects including belonging to separate cross-inoculation groups, absence or presence of pigmentation, and vesicle production in the presence or absence of NH_4Cl (Berry and Torrey 1979; Zhang *et al.* 1984). ArI3 is an unpigmented strain that differentiates vesicles and develops nitrogenase activity only in the absence of NH_4Cl (Murry *et al.* 1984). CcI2, on the other hand, is a red pigmented strain that produces vesicles in all media tested but nitrogenase activity develops only in the absence of NH_4Cl (M. F. Lopez, unpublished data). Glucose metabolism in these isolates is very similar, however (Lopez and Torrey 1985).

In addition, to try to determine which carbon sources are important in the alder–ArI3 symbiosis, we examined oxygen uptake in the presence of various carbon sources of *Frankia* vesicle clusters isolated from root nodules of plants of *Alnus rubra* which had been inoculated with ArI3.

Materials and methods

Isolates

Frankia HFP013103 isolated from *Alnus rubra* (Berry and Torrey 1979), hereafter referred to as ArI3, and isolate HFP020202 from *Casuarina cunninghamiana* (Zhang *et al.* 1984), hereafter referred to as CcI2, were the isolates used.

Cultural conditions and media

Growth experiments

The medium used for growth of nonnitrogen-fixing cells was the defined BAP medium of Murry *et al.* (1984) which contains 5 m*M* NH_4Cl as an exogenous nitrogen source. Growth experiments were carried out in 25-mm test tubes containing 5 mL of medium and maintained on a shaker at 28°C. All carbon sources were filter sterilized and added separately to autoclaved media in the following concentrations: organic acids, 5 m*M*; sugars and sugar alcohols, 20 m*M*. Approximately 0.025 mL packed cell volume (pcv) of cells per 5 mL medium was used as inoculum and growth experiments were made by subculturing once on the same carbon source after 20–25 days. After subculture, cells were incubated an additional 20–25 days and then harvested. Harvesting was done as follows: duplicate tubes of cells were homogenized by drawing in and out of a 1-mL autopipette several times and duplicate 0.5-mL samples were withdrawn from each tube for protein assays. The remainder of the culture was collected on a preweighed 0.45-μm Millipore (Bedford, MA, U.S.A.) filter using a syringe and a Millipore Swinex filter apparatus. Filters were removed carefully and dried overnight in petri plates at 33°C. The dried filters and cells were then weighed and dry weights were calculated. Parallel growth experiments for both isolates were repeated three times.

Long-term experiments on nitrogenase inductions

The medium used for nitrogenase induction was a modification of the defined B medium of Murry *et al.* (1984) which contained vitamins in the same concentration as BAP medium. Cells were induced following a modification of the methods of Murry *et al.* (1984).

Inocula were grown in BAP medium containing propionate, washed twice by centrifugation to remove NH_4Cl in modified B medium lacking a carbon source, and resuspended in 100 mL of modified B medium in a 250-mL Erlenmeyer flask containing the appropriate carbon source to be tested and maintained on a shaker at 28°C. Approximately 0.3 mL pcv inoculum was used per 100 mL of medium. All treatments were done in duplicate. Cells were sampled aseptically on day 3, 5, 7, and 9 for acetylene reduction assays, and cells from day 7 were used to count ArI3 vesicles. Vesicle counts were done on ArI3 only since CcI2 produces vesicles both in media with and without NH_4Cl.

Washing and short-term acetylene reduction experiments

Cells were induced for nitrogenase on propionate medium as described above except that they were cultured in 1-L bottles containing 750 mL of medium sparged with air or in 1-L Erlenmeyer flasks containing approximately 300 mL of medium maintained on a shaker. Cells with nitrogenase activity of at least 300 nmol ethylene·mg protein^{-1}·min^{-1} were harvested by gentle centrifugation (1500 rpm for 10 min) at room temperature and washed twice in modified B medium lacking a carbon source to remove propionate. The cells were then resuspended in modified B medium lacking a carbon source and duplicate 2-mL samples were pipetted into 6 mL acetylene reduction assay vials already containing the appropriate carbon sources to be tested and covered with serum stoppers. Vials were injected with 10% acetylene and assayed for acetylene reduction activity after incubation at 28°C with shaking for 2–3 h.

TABLE 1. Growth of ArI3 and CcI2 on various carbon sources after 20–25 days

Carbon source	Protein, μg·mL^{-1}	Total dry wt., mg
ArI3		
Sucrose	8.2±0	0.7±0
Trehalose	40.1±3.8	3.1±0.2
Maltose	8.2±0	0.1±0
Glucose	3.5±0.8	1.3±0.7
Fructose	3.8±0.5	0.1±0.1
Mannose	3.3±0	0.6±0.1
Sorbitol	1.9±0.3	0.6±0
Mannitol	1.6±0	0.5±0.1
Propionate	9.0±0.8	1.5±0.1
Succinate	35.2±0.2	1.2±0.1
Pyruvate	4.1±0	1.0±0.2
Acetate	14.8±1.6	1.5±0.2
Malate	12.6±1.6	1.6±0.1
Citrate	1.9±0	0.6±0.3
No carbon	2.4±0.2	0.2±0
CcI2		
Sucrose	0.8±0.3	0.8±0.1
Trehalose	1.3±0	0.6±0.3
Maltose	1.3±0.8	1.3±0.1
Glucose	0.6±0.1	1.3±0.1
Fructose	2.2±1.1	0.8±0.5
Mannose	3.4±0.1	0.9±0.1
Sorbitol	2.4±0.2	0.5±0.1
Mannitol	0.5±0	0.4±0.1
Propionate	19.4±3.3	1.3±0.1
Succinate	0.3±0.1	0.3±0.1
Pyruvate	27.7±3.0	2.3±0.5
Acetate	25.5±0.8	1.6±0.1
Malate	2.0±0.1	1.0±0.2
Citrate	0.8±0	0.6±0.1
No carbon	1.1±0	0.6±0.1

NOTE: Cultures were grown as described in Materials and methods. Carbon source concentrations were as follows: organic acids, 5 m*M*; sugars and sugar alcohols, 20 m*M*.

Vesicle counts

Vesicles were counted by the method of Murry *et al.* (1984) using a Petroff–Hausser counting chamber. Quadruplicate samples were counted from each treatment.

Acetylene reduction assays and soluble protein estimations

Acetylene reduction activity and soluble protein concentrations were estimated using previously published methods (Lopez and Torrey 1985).

Isolation of vesicle clusters

The isolation of vesicle clusters was by a modification (Lopez and Torrey 1985) of the techniques of Akkermans *et al.* (1981) and Benson (1982).

Results

Growth of ArI3 and CcI2 on various carbon sources

Table 1 shows the results from a typical experiment on the effect of carbon source on the growth of ArI3 and CcI2. Growth

TABLE 2. Effect of carbon source on vesicle development and induction of nitrogenase activity in ArI3 over a time course of 9 days

Carbon source	C_2H_4, nmol·mg protein^{-1}·h^{-1}	Vesicles·mg protein^{-1}	C_2H_4, nmol·vesicle^{-1}·h^{-1}
Trehalose	11.6±1.3	$2.8\times10^{7} \pm 2.0\times10^{6}$	$4.1\times10^{-7} \pm 7.5\times10^{-8}$
Glucose	NDA	$<10^{5}$*	NDA
Sorbitol	NDA	$<10^{5}$*	NDA
Propionate	242.8±44.1	$1.0\times10^{8} \pm 2.0\times10^{7}$	$2.2\times10^{-6} \pm 8.7\times10^{-9}$
Succinate	31.3±2.8	$8.4\times10^{7} \pm 1.0\times10^{6}$	$1.0\times10^{-7} \pm 2.5\times10^{-8}$
Pyruvate	NDA	$<10^{5}$*	NDA
Acetate	NDA	$<10^{5}$*	NDA
Malate	9.1±0.8	$5.1\times10^{7} \pm 2.0\times10^{6}$	$6.1\times10^{-7} \pm 3.0\times10^{-8}$
Citrate	NDA	$<10^{5}$*	NDA
Gluconate	NDA	$<10^{5}$*	NDA
No carbon	NDA	$<10^{5}$*	NDA

NOTE: Cultures were induced for nitrogenase activity as described in Materials and methods. Concentrations were as follows: organic acids, 5 m*M*; sugars and alcohols, 20 m*M*. Values are the means of four replicates ± standard deviation and acetylene reduction data represent the highest activity observed over an induction period of 9 days. NDA, no detectable activity.

*Less than one per four samples of 0.18 μL = area of counting chamber (see Materials and methods).

was measured by increases in both protein and dry weight. In general, organic acids supported better growth than carbohydrates, both in protein content and dry weight. Propionate, succinate, malate, and acetate were the organic acids that resulted in the greatest growth of ArI3, and pyruvate, acetate, and propionate yielded the most growth (increases in dry weight and protein) of CcI2.

The only carbohydrate tested that supported both protein and dry weight increases in ArI3 was trehalose. When used as sole carbon source for ArI3, it was the substrate that sustained the greatest growth of this isolate under these conditions, nearly twice as much growth as propionate. Trehalose did not, however, support the growth of CcI2. Of the carbon sources that were less optimal for both isolates, organic acids appeared to result in increases in protein but not in dry weight, and carbohydrates resulted in increases in dry weight but not in protein (Table 1). From the data it is clear that protein measurements offered the more sensitive measure of growth.

Effect of carbon source on nitrogenase activity

To test the effect of carbon source on the nitrogenase activity of *Frankia* cultures, we attempted to separate the ability of a carbon source to support vesicle development from the ability of a particular carbon source to provide short-term energy supply to nitrogenase.

Table 2 shows the maximum observed nitrogenase activity and vesicle production over a time course of nitrogenase induction of ArI3 cultures when provided with a variety of carbon sources. Most of the carbon sources tested did not support vesicle development or nitrogen fixation. Propionate, malate, and succinate, organic acids that ArI3 grew well on (Table 1), also sustained vesicle differentiation and eventually nitrogen fixation, whereas cultures on acetate did not. Under the conditions we tested, propionate was by far the best carbon source for nitrogenase activity in the ArI3 induction experiments; the cells utilizing propionate had 7 to 26 times the activity of cells utilizing other substrates. Cultures provided the propionate also differentiated the highest numbers of vesicles, although the cultures utilizing succinate had vesicle numbers that were close to those of the cultures on propionate. Induction on trehalose, which was the carbon source that supported the most growth in ArI3 (Table 1), resulted in cultures that had few vesicles and low nitrogenase activity (Table 2). When nitrogenase activity was calculated on a per vesicle basis, propionate was still the carbon source that sustained the highest rates.

TABLE 3. Effect of carbon source on the induction of nitrogenase activity in CcI2 over a time course of 7 days

Carbon source	C_2H_4, nmol·mg protein^{-1}·h^{-1} Day 5	Day 7
Trehalose	NDA	NDA
Maltose	NDA	NDA
Propionate	104.1±7.5	87.9±4.3
Succinate	NDA	NDA
Pyruvate	205.6±4.7	255.9±44.1
Acetate	78.8±17.4	190.4±7.1
Malate	NDA	NDA
Gluconate	NDA	NDA
No carbon	NDA	NDA

NOTE: Cultures were induced for nitrogenase activity as described in Materials and methods. Concentrations were as follows: organic acids, 5 m*M*; sugars and sugar alcohols, 20 m*M*. Values are the means of two replicates ± standard deviations.

In a similar experiment with CcI2 (Table 3), vesicle numbers were not counted since CcI2 produces vesicles both in the presence and absence of NH_4Cl, but nitrogenase activity does not develop until cultures are placed in media lacking NH_4Cl (unpublished observation). The carbon sources that supported the nitrogen fixation of CcI2 cultures over a time course of nitrogenase induction (Table 3) were essentially the same as those that supported growth in the presence of NH_4Cl (Table 1). Cultures provided with pyruvate, propionate, and acetate all showed acetylene reduction, and the cultures utilizing pyruvate had the highest activities (Table 3).

Figure 1 shows the effect on acetylene reduction of adding various carbon sources to ArI3 and CcI2 cultures which had already been induced for vesicle development and nitrogenase activity on propionate, and were washed free (by centrifugation) of exogenous propionate. The results from these experiments were interesting when viewed in comparison with the long-term induction experiments (Tables 2 and 3). Just washing the cells had a dramatic effect on nitrogenase and resulted in a decrease in activity of 80% (for ArI3) and 43% (for CcI2) when the rates of

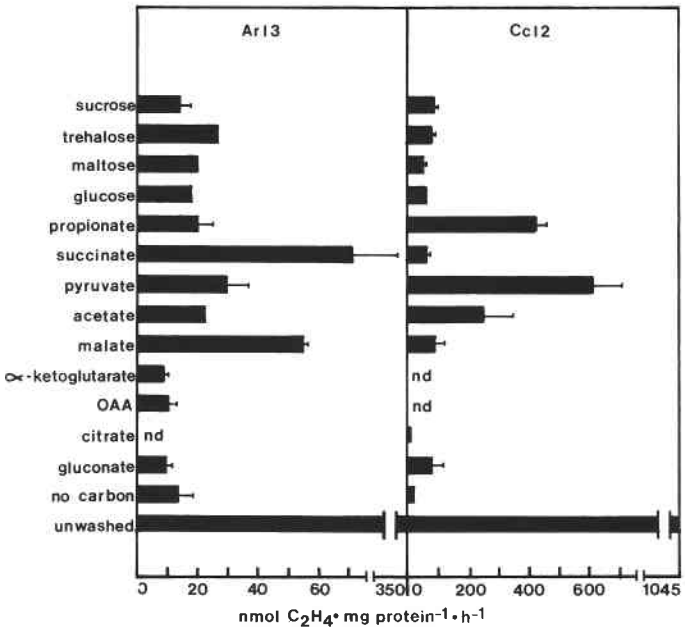

FIG. 1. The effect of carbon source on the nitrogenase activity of ArI3 and CcI2 cultures induced for nitrogenase activity on propionate. Cells were induced for nitrogenase on propionate, washed free of propionate, and prepared as described in Materials and methods. Carbon source concentrations were as follows: organic acids, 5 m*M*; sugars and sugar alcohols, 20 m*M*. Values are the means of two replicates; bar extensions indicate standard deviations. nd, not determined; OAA, oxaloacetic acid.

unwashed cells (Fig. 1) were compared with the highest rates (on any carbon source) observed after washing. It should be noted that the acetylene reduction rates we obtained with CcI2 in various experiments were on the average higher than those obtained with ArI3, but the maximum rates observed were similar for both (unpublished data). The carbon sources that supported the maximum activities after washing in the short-term washing experiments were similar for both isolates to those in the long-term induction experiments, but for ArI3 the relative amounts of activity supported by a particular carbon source were very different.

In the short-term washing experiments with ArI3, succinate supported the highest activity, and malate the second highest (Fig. 1). Pyruvate (which did not yield significant growth (Table 1)), propionate, and trehalose supported lower levels of activity, about 2.8 times less than those of the cells utilizing succinate. The reason why propionate did not stimulate high levels of activity in the washed cells even though propionate was sustaining high rates in the unwashed cells (Fig. 1) is unclear, but may be due perhaps to the sensitivity of ArI3 cells to propionate concentration. Murry *et al.* (1984) showed that the utilization of propionate by ArI3 cells was concentration dependent and that concentrations above 5 m*M* became toxic to nitrogen-fixing cells. If all the propionate in the original medium was not washed out of the cells totally, then adding an additional 5 m*M* of propionate would have resulted in an inhibitory concentration of the organic acid. The concentration of propionate remaining in the cells after washing could not have been very high, however, since the control cells (minus carbon) had very low activity (Fig. 1).

Effect of carbon source on the oxygen uptake of pure cultures and vesicle clusters isolated from nodules

To try to determine the carbon sources that are important in the *Frankia* symbiosis, we compared the oxygen uptake in the presence of various carbon sources of *Frankia* vesicle clusters isolated from *Alnus rubra* nodules inoculated with ArI3 with that of pure cultures of ArI3 that had been induced for nitrogenase on propionate and then washed free of the organic acid by centrifugation. The vesicle clusters were isolated as described in Materials and methods. Isolated *Frankia* vesicle clusters contain numerous plant mitochondria embedded within them (Akkermans *et al.* 1983; Lopez and Torrey 1985), and in addition, it may be inferred that vesicle clusters sustain some damage during isolation since acetylene reduction activity is abolished under aerobic conditions (A. D. L. Akkermans, J. van Straten, and W. Roelofsen. 1977. Recent Dev. Nitrogen Fixation (Proc. Int. Symp. 2nd. 1976). pp. 591–603). The results from the oxygen uptake experiments are shown in Table 4. Organic acids did not stimulate the oxygen uptake of ArI3 vesicle clusters, but a variety of sugars did. Trehalose resulted in the greatest stimulation with an oxygen uptake rate roughly 50% above that of the control. Sucrose and glucose stimulated the oxygen uptake of vesicle clusters 24–29% above the control rate. The oxygen uptake of the washed, pure cultures of ArI3 was stimulated by a variety of substrates including both sugars and organic acids. Propionate showed the highest stimulation, 92% above the control. Pyruvate, malate, sucrose, trehalose, and glucose all caused a 40–50% stimulation of oxygen uptake and succinate and fructose stimulated 18–28%.

Discussion

The comparison of carbon source utilization by two morphologically distinct frankiae in this study indicates that *Frankia* isolates are restricted in substrate preference for growth and nitrogenase activity and that isolates from different host-compatibility groups may be very different in some aspects of carbon metabolism. Other workers have reported similar results (Blom 1982; Shipton and Burggraaf 1982; Tisa *et al.* 1983) for the growth of various strains of *Frankia*.

In this study, we found evidence that in most cases the carbon sources which could support the growth of *Frankia* strains on media supplemented with NH_4Cl also supported the development of nitrogenase activity. It was also evident that some carbon sources that did not sustain growth or nitrogenase activity were respired by these isolates (i.e., they stimulated oxygen uptake), and therefore the lack of growth or nitrogen fixation was not due to an inability of these isolates to take up the substrates. Similar results have been observed with pure cultures of *Parasponia–Rhizobium* (Mohaptra and Gresshoff 1984). In that study, a number of sugars supported increases of dry weight but not protein in the rhizobia cultures and incubation in the presence of those sugars did not result in nitrogen fixation activity.

The utilization of trehalose by ArI3 was interesting in several respects. Trehalose under the conditions of this study was the carbon source that resulted in the greatest growth of ArI3, but it supported only small amounts of vesicle differentiation and nitrogen fixation. These results may have been due to differences in oxygen tension between the two types of experiments. Lopez *et al.* (1986) found that lowered oxygen tensions (5–10% in the gas phase) were necessary for growth and nitrogen fixation of ArI3 utilizing trehalose. Even though both treatments were maintained on a shaker in this study, it is likely that the oxygen tension was lower in the growth experiments than in the nitrogenase induction experiments because of a lower surface area to volume ratio in the test tubes (used for the growth studies) than in the flasks (used for the induction experiments).

TABLE 4. Effect of carbon source on the oxygen uptake of vesicle clusters isolated from *A. rubra* versus nitrogen-fixing cells of ArI3 induced for nitrogenase on propionate and then washed free of the organic acid. Data are presented as nanomoles of O_2 per milligram protein per minute and as calculated percentages of the control (no carbon)

Carbon source	Oxygen uptake of vesicle clusters	% control	Oxygen uptake of washed pure cultures	% control
Sucrose	172.0	129.0	79.0	158.0
Trehalose	200.0	150.0	70.0	140.0
Maltose	155.0	116.6	nd	—
Fructose	150.0	112.5	59.0	118.0
Glucose	166.6	124.9	72.0	144.0
Propionate	133.3	100	96.0	192.0
Succinate	133.3	100	64.0	128.0
Pyruvate	133.3	100	77.0	154.0
Acetate	133.3	100	nd	—
Malate	133.3	100	76.0	152.0
No carbon	133.3	100	50.0	100

NOTE: Vesicle clusters were isolated as described in Materials and methods. ArI3 cells were harvested from a 1-L culture induced for nitrogenase on propionate, washed free of propionate, and prepared as described in Materials and methods.

Lowered oxygen tensions in the test tubes would favor growth on trehalose, whereas higher, more aerobic oxygen tensions would inhibit the growth and development of nitrogenase activity in the flasks. Lopez *et al.* (1986) found that lowered oxygen tensions (5–10% in the gas phase) did not inhibit the growth of ArI3 on propionate.

With the exception of trehalose, both ArI3 and CcI2 were able to utilize only organic acids for substantial growth and nitrogen fixation in this study. These results were interesting when contrasted with the results from the experiments on the oxygen uptake of vesicle clusters and the stimulation of the acetylene reduction of nodule slices. The isolated vesicle clusters showed a stimulation of oxygen uptake or acetylene reduction only when incubated with sugars and not organic acids. Akkermans *et al.* (1983) and Lopez and Torrey (1985) showed that plant mitochondria were intermingled and trapped in isolated vesicle clusters. The mitochondria were often in very close association with the *Frankia* vesicles. It is also possible that some soluble glycolytic enzymes from the plant cytosol could be associated with the vesicle clusters and not totally washed away in the isolation. Whether or not the oxidation of sugars in the oxygen uptake experiments in this study was occurring within the *Frankia* vesicles or was due to associated plant enzymes and mitochondrial respiration is unknown. However, ArI3 vesicle clusters isolated from nodules have been shown to contain the same glycolytic enzymes as pure cultures of *Frankia* (Lopez and Torrey 1985). That trehalose stimulated the oxygen uptake of the vesicle clusters is interesting since trehalose is a form of carbon stored in pure *Frankia* cultures (Lopez *et al.* 1983, 1984) and is also found in isolated vesicle clusters (unpublished data). Akkermans *et al.* (1981, 1983) and Huss-Dannell *et al.* (1982) found that the respiration of isolated vesicle clusters from *Alnus glutinosa* could only be stimulated by the addition of NADH or a mixture of malate, glutamate, and NAD. No stimulation was observed with a variety of organic acids and sugars alone. Differences in *Frankia* strains or techniques of vesicle cluster isolation may explain these discrepancies.

From the data on carbon utilization in two *Frankia* isolates obtained in this study, a model for carbon flow in the *Frankia* symbiosis might be proposed. Since the pure cultures of ArI3 did not grow well or fix nitrogen utilizing sugars (even though these substrates were readily respired), it is likely that at least one of the carbon sources that is translocated to *Frankia* vesicles in symbiosis is an organic acid. This assumption presupposes that pure nitrogen-fixing cultures of ArI3 have a carbon metabolism that approximates that found in the symbiosis. In fact, most of the enzymes assayed to date in pure cultures of *Frankia* have also been found in isolated vesicle clusters, with the exception of the glyoxylate shunt enzymes (Akkermans *et al.* 1981; Akkermans *et al.* 1983; Huss-Danell *et al.* 1982; Lopez and Torrey 1985). The observation that the isolated vesicle clusters in this study had oxygen uptake that was stimulated only by sugars and not by organic acids suggests several possibilities.

Frankia vesicles in the symbiotic state, or more accurately, when observed as isolated vesicle clusters, may not be able to take up organic acids directly as can cells in pure culture. It is not unlikely that the organic acid uptake system of the endophyte is damaged during vesicle cluster isolation. The oxidation of sugars by the vesicle clusters in this study may or may not have been occurring in *Frankia* vesicles, but it seems likely that the hydrolysis of trehalose was at least, since trehalose and trehalase (the enzyme that catalyzes its hydrolysis) are not commonly found in higher vascular plants (Elbein 1974). A possible conclusion, then, would be that both sugars and organic acids are necessary to support the growth and nitrogen fixation of *Frankia* in symbiosis.

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