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Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI 3 grown in batch culture

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Summary Kinetics of growth and nitrogenase induction in *Frankia* sp. ArI3 were studied in batch culture. Growth on defined medium with NH_4^+ as the N source displayed typical batch culture kinetics; however, a short stationary phase was followed by autolysis. Removal of NH_4^+ arrested growth and initiated vesicle differentiation. Vesicle numbers increased linearly and were paralleled by a rise in nitrogenase (acetylene reduction) activity. Nitrogenase activity (10 n*M* C_2H_4 · mg protein^{-1} · min^{-1}) was sufficient to support growth on N_2 and protein levels rose in parallel with nitrogenase induction. Optimal conditions for vesicle and nitrogenase induction were investigated. Maximum rates of acetylene reduction were obtained with 5 to 10 m*M* K_2HPO_4/KH_2PO_4, 0.1 m*M* $CaCl_2$ and $MgSO_4$. The optimum pH for acetylene reduction and respiration was around 6.7. The amount (5 to 10 μg protein/ml) and stage (exponential) of growth of the ammonium-grown inoculum strongly influenced the subsequent development of nitrogenase activity. Propionate was the most effective carbon source tested for nitrogenase induction. Respiration in propionate-grown cells was stimulated by CO_2 and biotin, suggesting that propionate is metabolized via the propionyl CoA pathway.

Introduction

Until recently, the physiology of nitrogen fixation in actinorhizal root nodules has been severely hampered by lack of understanding of the prokaryotic symbiont. Numerous early attempts to isolate the actinomycete from root nodules were largely unsuccessful (see ref. 6 for review), which has been attributed in part to the slow growth rates of the endophyte. In recent years, numerous isolations of the endophyte belonging to the genus *Frankia* (Frankiaceae, Actinomycetales)[9] have been reported from root nodules of various actinorhizal plants (see ref. [8] for review). Early success in cultivating *Frankia* isolates was achieved using complex media[5,12,13,21,30] often supplemented with lipids[13,28] or lipid extracts[34]. With the successful cultivation of various *Frankia* strains on defined media[11,14,18,19,35,37,39,40] it has become apparent that *Frankia* is more metabolically versatile than previously thought. These studies have shown that a wide variety of sugars, sugar alcohols, lipids and organic acids can serve as the sole carbon source for growth although marked differences in patterns of carbon utilization occur from strain to strain. Inorganic nitrogen sources such as NH_4^+ and nitrate support growth. Many strains are not dependent on exogenous vitamins or cofactors for growth[35].

The onset of nitrogenase activity in developing actinorhizal nodules has been correlated with the differentiation of specialized cells, the vesicles, from terminal swellings of the vegetative filaments of the endophyte[10,31]. The localization of reducing conditions within these specialized cells with tetrazolium dyes[2] and the isolation of vesicle clusters capable of acetylene reduction[46] lead to the proposal that the vesicles are the site of nitrogen fixation. This hypothesis has been substantiated by the correlation of vesicle differentiation with induction of nitrogenase activity in response to nitrogen limitation in aerobic *Frankia* cultures[19,25,40,41]. Since in all of the isolates tested, acetylene reduction occurs at ambient pO_2 levels, protection of the O_2-labile nitrogenase can be provided by the endophyte rather than by the nodules, as is the case in the legume/*Rhizobium* symbiosis[39], and has been attributed to localization of the enzyme in the structurally and presumably biochemically unique vesicle[41,42]. The present study is concerned with optimization of the physico-chemical and nutritional conditions which influence growth and nitrogen fixation in a *Frankia* isolate ArI3.

Materials and methods

Cultivation of organism

The *Frankia* strains used in this study are listed in Table 1. Axenic cultures were maintained at 28°C on a defined medium termed BAP which contained (in m*M*) KH_2PO_4, 7; K_2HPO_4, 3.4; NH_4Cl, 5; NaPropionate, 5; $MgSO_4$, 0.1; $CaCl_2$, 0.07; FeNaEDTA, 10 mg/l; biotin, 450 μg/l; and trace elements according to Tjepkema and coworkers[41]. The pH of the medium was adjusted to 6.7. Phosphate was added after autoclaving. Propionate or other carbon sources used were filter sterilized and added at a final concentration of 5 m*M* for organic acids (sodium salts) and 15 m*M* for sugars and sugar alcohols.

Cells for growth, nitrogenase induction and respiration experiments were grown at 28°C in 1- to 3-l glass cylindrical bottles that were magnetically stirred and sparged (200 to 600 ml/min) with filtered (0.45 micrometer Millipore filters) air dispersed at the bottom of the bottle through a scintered glass tube.

Nitrogenase induction

Nitrogenase induction was initiated by centrifuging an exponential phase culture (8 krpm, 10 min) and washing twice in N-free defined medium (termed induction or B medium). Filter sterilized MOPS (3-N-Morpholinopropanesulfonic acid, pH 6.7) was added to 20 m*M* to control pH. Concentrations of up to 50 m*M* had no inhibitory effect on short-term acetylene reduction or respiratory rates. The washed cells were resuspended in induction media at cell density of between 5 and 10 μg protein/ml and incubated on a rotary shaker (70 rpm) in 1- to 4-l cotton-plugged Erlenmeyer flasks.

Assays

Nitrogenase activity was measured under air with the acetylene reduction assay in standardized 6 to 10 ml serum vials. Two to four 2-ml replicate samples were removed aseptically from the culture and incubated with 10% acetylene (v/v) on a rotary shaker (70 rpm) at 28°C. Cells for the CO_2 experiments were buffered with 50 m*M* MOPS (pH 6.7) and sparged with a mixture of argon and 5% O_2 for 15 to 20 min to remove dissolved CO_2. Replicate 2-ml samples were

withdrawn using an argon-flushed syringe fitted with a canula and injected into serum vials containing argon, 20% O_2 and CO_2 at the desired concentration. Ethylene production was measured using a Carle Model 9500 gas chromatograph equipped with a 1.2 m stainless steel column filled with a 25 : 75 (v/v) mixture of Porapak R (80–200 mesh) and N (50–80 mesh). Gas samples (100 µl) were injected directly onto the column. Acetylene was used as an internal standard to minimize injection error. The standard deviation of 2 to 4 replicate assays was generally less than 15% and is shown by the bars in each figure.

Respiration was measured concurrently with acetylene reduction by measuring CO_2 in the gas phase with a Carle Model 8700 thermal conductivity gas chromatograph. A 1.2 m column filled with the Porapak mixture described above was used at 50°C with helium as the carrier gas. The assay remained linear for at least 4 hours. Dissolved CO_2 was assumed to be in equilibrium with CO_2 in the gas phase and was included in calculations of CO_2 production. Overall precision of the assay was about 5%.

Respiration was also measured polarographically with a YSI-model 53 O_2 electrode (Yellow Springs, Ohio) thermostated at 29°C. Cells were concentrated to 40–80 µg protein/ml. For the CO_2 experiments, cells were sparged with N_2 to remove dissolved CO_2 and O_2 was added to air saturation levels. In ammonia-grown cells, rates were linear until very low O_2 tensions were reached. Variation between replicate samples was about 5%. CO_2 was added by bubbling the gas directly into the cell suspension. MOPS (50 m*M*, pH 6.7) was added to control the pH. For the pH experiments, cells were washed twice in BAP medium containing 0.5 m*M* phosphate and adjusted to the desired pH with 50 m*M* concentrations of organic buffer. MES (2-N-Morpholinoethanesulfonic acid) was used below pH 6.5; MOPS from pH 6.5 to 7.2 and TRIS (Tris (hydroxymethyl)aminomethane) above pH 7.2.

Cellular protein was estimated by the Bradford procedure[17] using cells that were sonicated (15 sec at 100 W) with a Braunsonic Model 1510 sonicator and boiled for 10 min in 0.3 *N* NaOH[24].

Vesicles were counted in a Petroff-Hausser chamber at 400X magnification with a phase-contrast microscope. Samples were briefly sonicated (15 s at 100 W) to detach vesicles from the vegetative hyphae. The criteria for mature vesicles were the presence of a refractile cell envelope and an attached 'stalk'.

Results

Growth kinetics

Fig. 1 shows a typical growth curve for batch culture of *Frankia* ArI3 grown in an air-sparged, stirred vessel in defined medium with propionate and NH_4^+ as the sole C and N sources, respectively. A lag period of variable length is normally seen following inoculation which is minimized by use of a large inoculum (10 to 20%, v/v) of log phase cells. An apparent exponential growth phase (as measured by total protein) ensues with a doubling time of less than 48 h. Under these conditions, exponential growth abruptly ceases and is followed by autolysis with soluble and cellular protein declining rapidly. Autolysis was not due to C-limitation since an additional 5 m*M* propionate added on day 5 to one culture did not prevent the decline in protein levels. Microscopic examination of filaments revealed cell wall 'ghosts' apparently devoid of cell contents. Autolysis is a common feature of microbial growth in batch culture and has been attributed to a variety of cultural factors[4,23,37,44]. A similar pattern of apparent autolysis was also observed

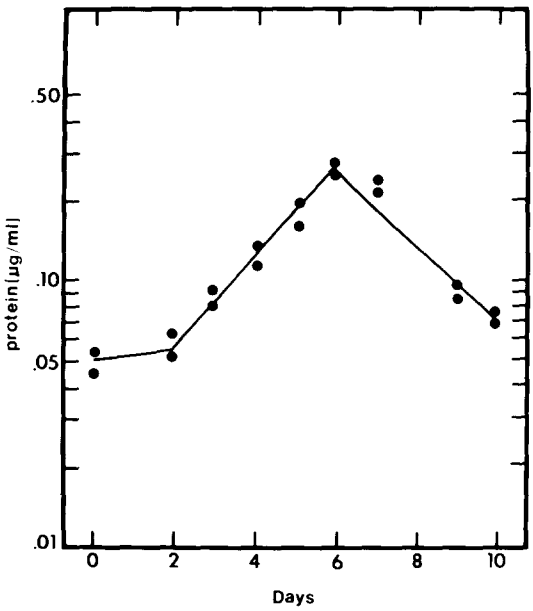

Fig. 1. Growth kinetics of ArI3 in batch culture. Cells were grown in 11 air-sparged cultures in defined medium with 5 m*M* NH_4^+ as the nitrogen source and 5 m*M* propionate as the carbon source. An additional 5 m*M* propionate was added to one culture at day 5.

by Burggraaf and Shipton[19] in several *Frankia* isolates grown under nitrogen-fixing conditions. We have observed autolysis also in the *Frankia* strain $EaNl_{pec}$ but only when grown in defined medium. Autolysis occurs in standing or shaking cultures and with a variety of carbon sources at concentrations up to 20 m*M*. However, in a rich organic medium a long stationary phase is observed and cells remain viable up to a year.

Vesicle and nitrogenase induction

A comparative time-course study of vesicle and nitrogenase induction was conducted on 8 *Frankia* isolates. Acetylene reduction and observations on sporangia formation were measured every 2–3 days after initiating induction by removing NH_4^+. There was considerable variation in maximum acetylene reduction rates among the 8 isolates (Table 1); however, rates correlated well with vesicle numbers in each case. Vesicles and nitrogenase activity were not detected in any of the isolates grown on NH_4^+ (5 m*M*). Sporangia and spores were produced in varying amounts by different isolates. EuIIc, an ineffective symbiont in root nodules of its host, *Elaeagnus umbellata*[7], produced large numbers of active vesicles in culture. ArI3 isolated from its host *Alnus rubra*[12] was chosen for further studies since nitrogenase activity was greatest in this isolate. Furthermore, ArI3 grows homogeneously, thus

Table 1. Comparison of maximum acetylene reduction activity and sporangia formation in cultured *Frankia* strains

Strain	Source (ref. #)	Acetylene reduction* $nmol/C_2H_4 \cdot ml^{-1} \cdot h^{-1}$	Sporangial formation
ArI3	(12)	0.325	+
EuIIc	(7)	0.305	+++
$EaNl_{pec}$	(29)	0.304	0
CaI1	a	0.268	++
EuNf	b	0.116	++
AgII	c	0.106	++++
CpII	(21)	0.103	++++
MpII	a	0.091	+

* rates presented are the maximum rates observed during induction and occurred between days 8 and 10.

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minimizing sampling error, and few spores are produced under these induction conditions.

Effects of nutrients. In a preliminary survey of a variety of sugars, sugar alcohols and organic acids used as the sole C source during induction, maximum acetylene reduction rates in ArI3 were obtained with 5 m*M* propionate. A comparison of specific acetylene reduction rates with vesicle numbers suggested that vesicle differentiation was enhanced by propionate. Sugars supported limited vesicle formation but high rates of acetylene reduction on a per vesicle basis (unpublished data).

Optimum concentration of several inorganic nutrients for nitrogenase induction was studied in time course experiments. Although there was considerable variation in maximum rates of acetylene reduction activities due to other suboptimal conditions in these initial studies, the optimum nutrient levels were consistent in several separate experiments. Maximum acetylene reduction activity occurred at day 10 with potassium phosphate levels between 5 and 10 m*M*. Since the media becomes alkaline when organic acids are utilized, 10 m*M* phosphate was routinely used to increase the buffering capacity of the media. Nitrogenase induction was limited by $CaCl_2$ concentrations less than about 0.05 m*M* while 0.35 m*M* was inhibitory (Fig. 2). A narrow concentration range for $MgSO_4$ was observed (Fig. 2).

Effects of biotin. Growth and induction of nitrogenase in ArI3 did not require exogenous vitamins. However, these processes were strongly

Table 2. Effect of biotin of respiration in ArI3*

Substrate	Presence of biotin		nmol $CO_2 \cdot mg^{-1}$ (protein) $\cdot min^{-1}$
	Growth	Assay	
Propionate	+	−	49.7 ± 0.2
	+	+	47.33 ± 0.7
	−	−	51.75 ± 2.07
	−	+	70.05 ± 1.25
Succinate	+	−	73 ± 9.4
	+	+	59.7
	−	−	78.7 ± 5.8
	−	+	57.47 ± 6.3

* Respiration was measured under air by CO_2 evolution in a 2 h assay. Biotin was added to the growth medium and the assay at a final concentration of 450 μg/l where indicated.

stimulated by exogenous biotin when propionate was used as the sole C source (data not shown). Maximum rates of acetylene reduction were obtained with 450 μg biotin/l in the induction media; higher levels proved inhibitory. Similar results were reported by Shipton and Burggraaf[36] for other *Frankia* strains, however, optimal levels were much higher. Table 2 shows that the short-term respiratory rate of ArI3 grown on propionate without exogenous biotin is stimulated by nearly 40% when biotin is added to the assay system. Biotin had a somewhat inhibitory effect on respiration on succinate-grown cells.

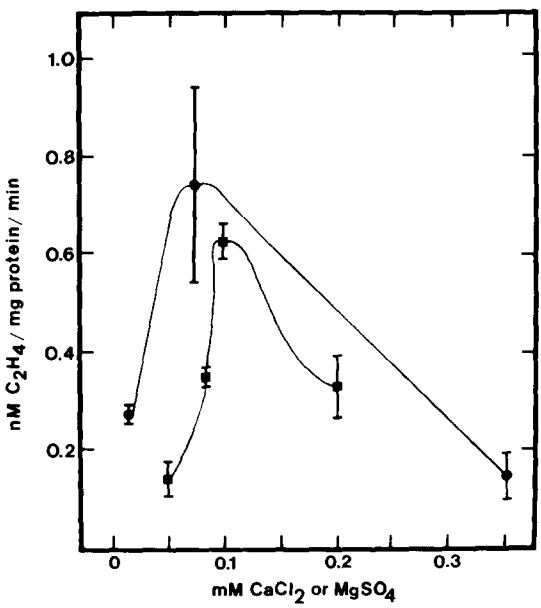

Fig. 2. Maximum rates of acetylene reduction in ArI3 induced with varying levels of $CaCl_2$ (•) and $MgCl_2$ (▪). Acetylene reduction was measured on day 8.

Effects of pH and temperature. The pH of the medium was an important variable affecting induction. The response of acetylene reduction and respiration of ArI3 to various pH values is shown in Fig. 3a and 3b, respectively. The optimum pH for both processes was around 6.7. The range for respiration was broader than that for acetylene reduction. The type of organic buffers used to control pH did not influence the rates of either respiration or acetylene reduction. The rates of both processes varied by less than 10% when assayed at pH 6.5 with MES and MOPS and at pH 7.2 with MOPS and Tris. The pH optimum for these two processes in ArI3 is similar to that reported for growth of *A. glutinosa* isolate LDAgpl[18], and nitrogenase induction of the *Comptonia peregrina* isolate CpII[41].

The optimum temperature for nitrogenase induction in ArI3 was about 25 to 28°C but substantial activity was observed at 20 and at 36°C.

Effect of inoculum, size and growth phase. The size and growth phase of the NH_4^+-grown inoculum strongly influenced the subsequent development of vesicles and nitrogenase activity. Maximum rates of acetylene reduction and the longest duration of nitrogenase activity resulted when exponential phase cells were used as the inoculum

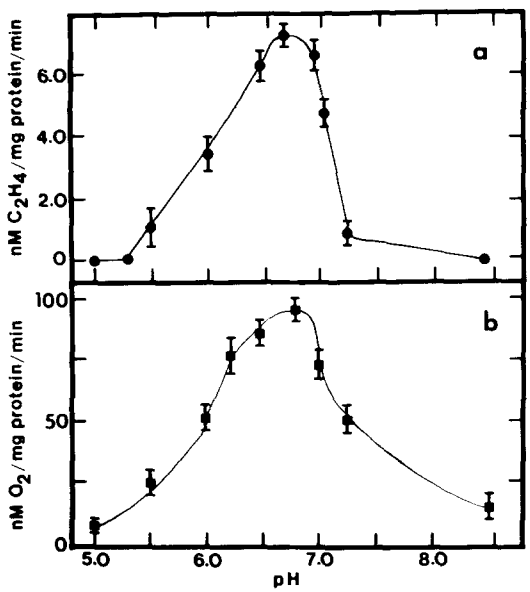

Fig. 3. Response of acetylene reduction activity (a) and O_2 uptake (b) to pH of the assay media. Cells were grown at pH 6.5, and equilibrated at the indicated pH level for at least 1 h. before assaying. Acetylene reduction was measured under atmosphere of Argon:O_2:CO_2 (79:20:1).

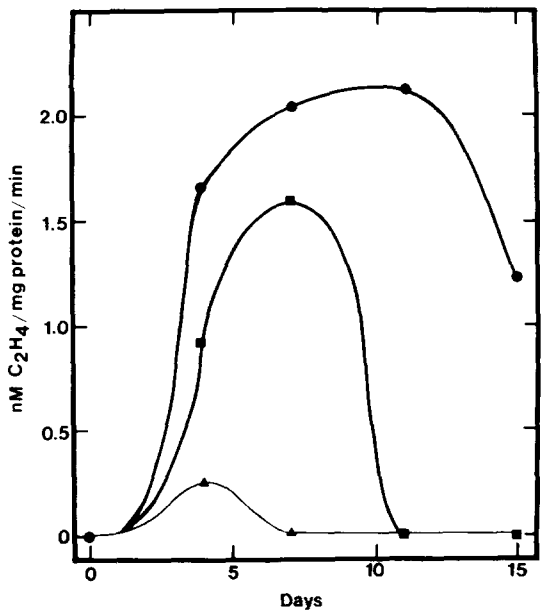

Fig. 4. Effect of growth phase of inoculum on subsequent nitrogenase induction in ArI3. Ammonium-grown cells were harvested in late lag-phase (▪), mid-exponential phase (•) and declining phase (▲) of a batch culture, washed in N-free medium and resuspended at an initial cell density of 7 μg protein/ml. Each point is the mean value of 4 replicate assays from 2 separate experiments.

(Fig. 4). Lower, but still substantial acetylene reduction rates were obtained when late, lag-phase cells were used as inoculum. However, when cells from the declining phase were used, little or no nitrogenase activity or vesicles developed.

Maximum specific acetylene reduction rates were obtained with an initial cell density of between 5 and 10 μg protein/ml. Higher and lower cell densities inhibited the induction process.

Kinetics of nitrogenase induction

The time course of vesicle differentiation and nitrogenase induction in ArI3 cultured in the medium developed in this study is shown in Fig. 5. Vesicles were extremely rare and acetylene-reduction could not be detected in ammonium-grown filaments. Within 4 days after initiating induction by removing NH_4^+ from the medium, mature vesicles developed and increased rapidly in number. Induction of nitrogenase activity closely paralleled vesicle differentiation. In this experiment, propionate (initially 5 m*M*) became limiting by day 8 and nitrogenase activity and vesicle numbers decreased. Addition of 5 m*M* propionate at this time resulted in a linear increase in both vesicle numbers and specific nitrogenase activity until day 13. Propionate may

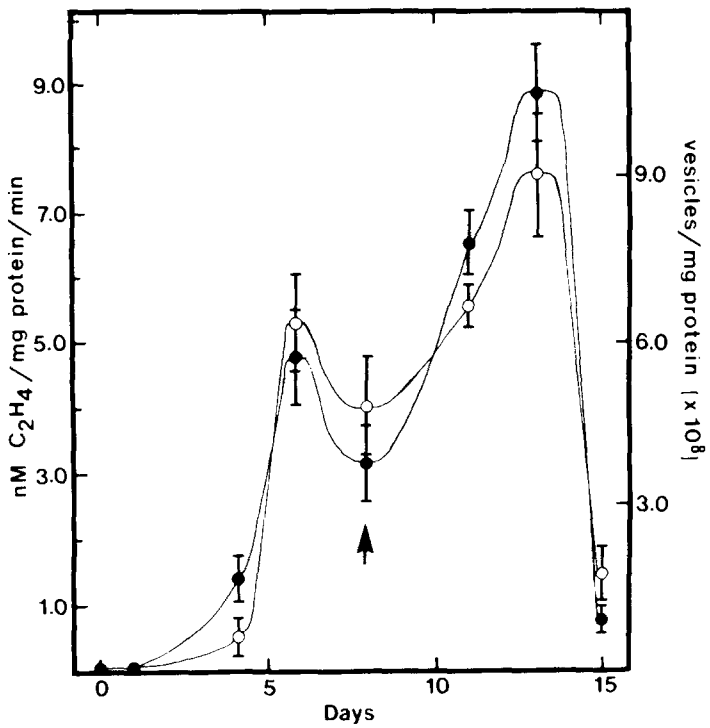

Fig. 5. Kinetics of vesicle differentiation (○) and nitrogenase activity (●) in ArI3. An additional 5 m*M* propionate was added at day 8.

have become limiting at this point and nitrogenase activity was nearly abolished. Autolysis apparently occurs since protein and vesicles decrease in parallel with nitrogenase activity.

Growth on N_2

Induction of nitrogenase resulted in resumption of growth at the expense of atmospheric N_2 (Fig 6). A substantial (10 to 40%) increase in protein was consistently seen within 24 to 48 h. after removing NH_4^+. This protein synthesis was supported by residual NH_4^+ or nitrogenous reserves, rather than by nitrogen fixation since acetylene reduction could not be detected at this point and since growth occurred under a N_2-free (argon) atmosphere (data not shown). However, as vesicles matured, acetylene reduction increased exponentially and cellular protein followed in parallel. Assuming a 3 to 4.5:1 ratio of acetylene:N_2, and that 6.25% of protein in N^{20}, then the rates of acetylene reduction between days 8 and 13 are more than sufficient to support the observed rise in protein.

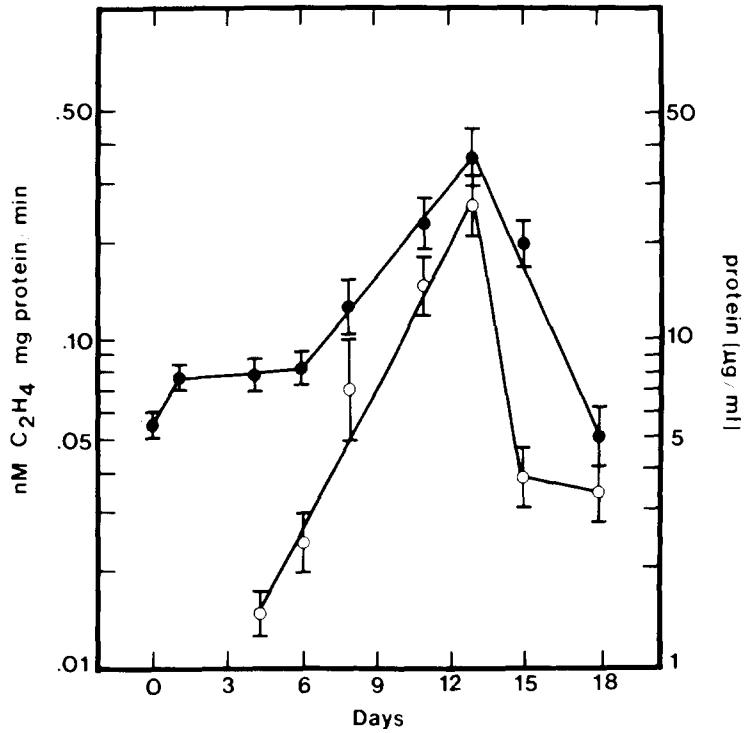

Fig. 6. Kinetics of nitrogenase induction (○) and growth (protein) (●) in ArI3.

Propionate metabolism

Our initial attempts to utilize propionic acid as a C source for growth and incubation of ArI3 were unsuccessful, which, as became apparent, was due to the restricted concentration range for oxidation of this organic acid. Fig. 7a shows the response to propionate concentration of respiratory rates in cells of ArI3 which were induced to form vesicles in 5 m*M* propionate, washed in C-free media and incubated for 18 h until endogenous respiration was lost. Maximum respiratory rates were supported by 0.5 to 5.5 m*M* propionate. Respiratory activity was inhibited by higher levels and abolished at 12.5 m*M* propionate. A similar trend was observed in ammonia grown cells (Fig. 7b) that were washed 3 times in C-free medium to remove exogenous propionate and assayed immediately. Under these conditions a substantial rate (nearly 60% of maximum) of O_2-uptake occurred without exogenous propionate indicating a high endogenous respiratory capacity. High propionate levels did not inhibit the endogenous rate which indicates that the inhibitory effect of propionate involves an effect of propionate uptake or subsequent metabolism rather than by uncoupling respiration.

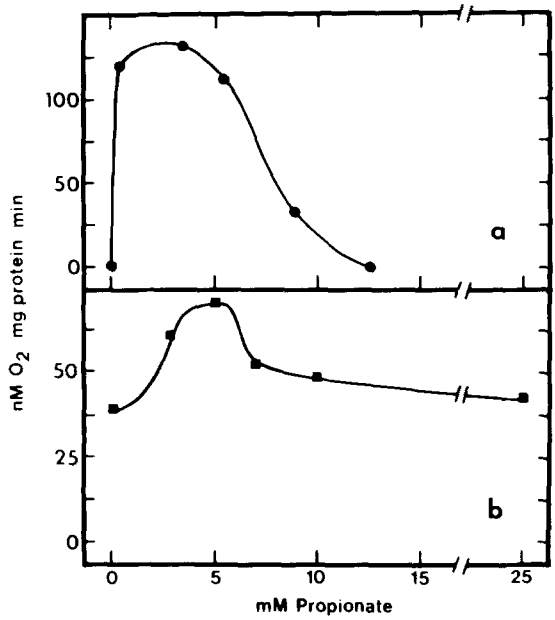

Fig. 7. Response of respiration (O_2 uptake) to exogenous propionate concentration. 7a. Actively-fixing cells were washed and starved for C 18 hours prior to assay. 7b. Ammonia-grown cells were washed 3 times to remove exogenous propionate and assayed within 1 h.

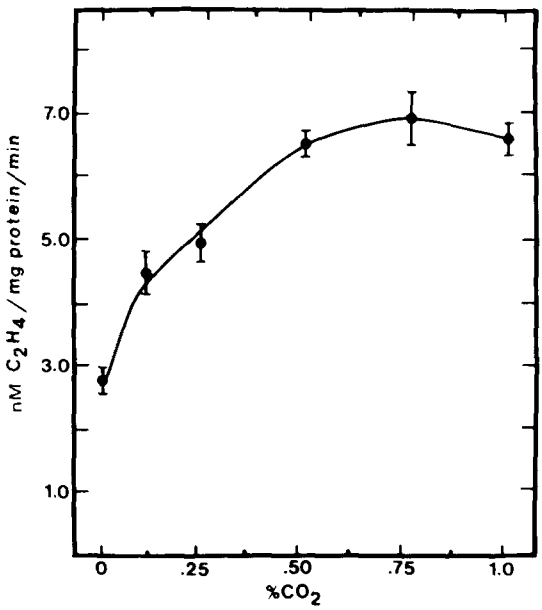

Fig. 8. Effect of exogenous CO_2 on acetylene reduction rates (2-h assay) of *Frankia* sp. ArI3 induced on propionate as the sole carbon source.

CO_2 stimulation of respiration and acetylene reduction

In our early studies, non-linear rates of acetylene reduction were often observed in propionate-induced cells particularly at low cell densities (less than 5 μg protein/ml) and in cells assayed under defined gas mixtures. This may have been due to limiting dissolved CO_2. The stimulatory effect of exogenous CO_2 on short term acetylene reduction activity in ArI3 that was sparged with a mixture of argon and O_2 to remove dissolved CO_2, is seen in Fig. 8. Increasing exogenous CO_2 stimulated acetylene reduction until the effect was saturated between 0.75 to 1% CO_2. Significant acetylene reduction rates were observed without exogenous CO_2 in the assay vessel. Although less than 1 nmol CO_2/ml was detected in the gas phase when the assay was initiated, CO_2 increased rapidly during the course of the assay and acetylene reduction rate accelerated (Fig. 9). In contrast, cells incubated with saturating CO_2 showed a linear rate of acetylene reduction for more than 2 h.

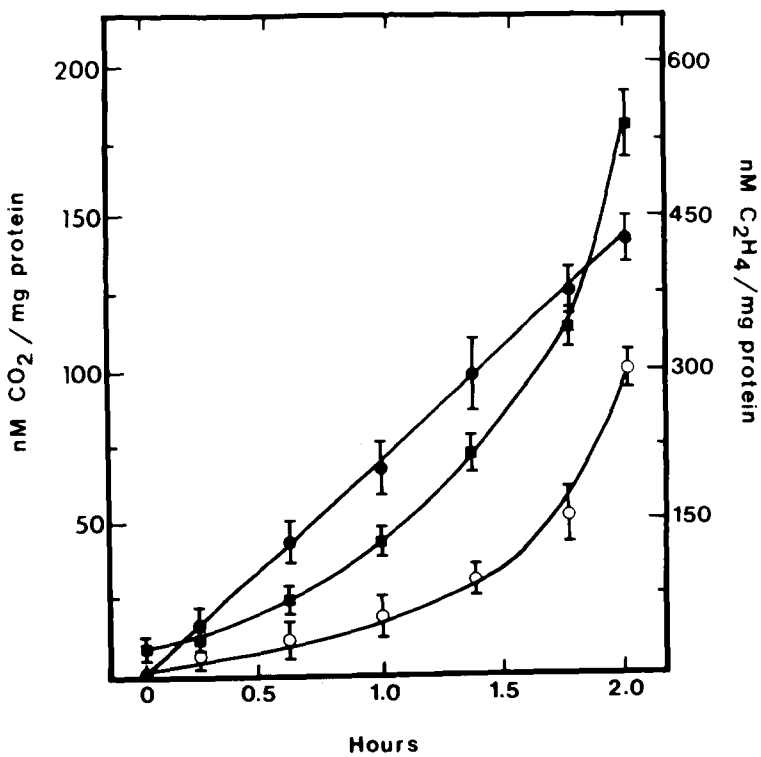

Fig. 9. Time course of acetylene reduction and CO_2 evoulution by *Frankia* sp. ArI3. Acetylene reduction under Argon + 20% O_2 in the absence of exogenous CO_2 (○) and with 1% CO_2 (●) in the gas phase. CO_2 evolution (■).

Table 3. Effect of exogenous CO_2 on respiration of ArI3 grown on various carbon sources

Carbon source	nmol $O_2 \cdot mg^{-1}$ (protein) $\cdot min^{-1}$	% CO_2 stimulation
Propionate	49.75 ± 2.1	64
Pyruvate	79.8 ± 2.85	22.5
Succinate	54.65	0
Acetate	51.77 ± 0.46	0
Malate	90.35 ± 3.5	0
Trehalose	63.55	0
Cellobiose	51.5 ± 0.7	0
Maltose	58.35 ± 4.6	0

A variable, but substantial (20 to 40%) CO_2 stimulation of respiration was also demonstrated in ammonia-grown cells that were sparged with N_2 to remove endogenous CO_2. This effect was not due to a change in pH of the medium since with the high buffer levels used (50 m*M*), pH values were maintained to within 0.1 pH unit after addition of exogenous CO_2. However, CO_2 stimulation of both acetylene reduction and respiration was seen only within the optimum pH range for these processes (between 6.3 and 6.9). This result suggests that at sub-optimal pH, factors other than CO_2 limit respiration and acetylene reduction. A specific requirement for one of the hydration products of CO_2, either H_2CO_3 or HCO_3^-, which predominate in this pH range, cannot, however, be discounted.

Respiratory rates of ArI3 grown with a variety of carbon sources are summarized in Table 3. A CO_2 stimulation of respiration was seen only when propionate or pyruvate was used as the sole carbon source for growth. The magnitude of CO_2 stimulation was variable. This was apparently due to variation in endogenous CO_2 levels in the assay. The high cell densities used for polarographic measurement of O_2 result in rapid CO_2 buildup. The CO_2-assimilation reaction may be quickly saturated and the stimulatory effect of CO_2 on respiration masked. However, the CO_2 response was substantial when either propionate or pyruvate was used as substrate. We could not detect a CO_2 stimulation of respiration even after repeated trials, when other organic acids or disaccharides were used as the sole C source.

Discussion

The growth kinetics of ArI3 in batch culture resemble those of other bacteria and filamentous fungi. An apparent exponential phase is observed with growth on NH_4^+ and N_2 which in mycelial organisms is determined by the balance between apical growth of the individual hyphae and the branching rate as described earlier[27]. A striking feature of growth in batch culture of ArI3 and other *Frankia* strains[19] in

defined (but not organic) media, is the absence of a stationary phase; after exponential growth, hyphae progressively lyse. Addition of propionate reverses the autolytic process only at low cell densities (Fig. 5). Autolysis in response to carbon limitation has been described in other bacteria[23,34,37]. However, other factors, perhaps induction of an activating factor upon reaching a critical cell mass[4], must be involved, since autolysis in inorganic media was also observed using higher carbon levels (Fig. 1) and ArI3 can be washed free of carbon and starved for long periods without lysis (see details of cell preparation in Fig. 7a). Autolysis may be an important feature in symbiosis since hyphal disintegration is observed in aging nodules[32].

Nitrogenase induction was influenced by several physical and nutritional factors. The most important was the removal of NH_4^+ which at 5 m*M* completely repressed vesicle formation and acetylene reduction in the 8 strains tested. Removal of NH_4^+ resulted in the parallel induction of vesicles and nitrogenase activity which was followed by growth apparently at the expense of the N_2. Apparent growth on N_2 of several *Frankia* strains was shown by the data of Burggraaf and Shipton[19]. Thus, unlike some *Rhizobium* strains in culture[33,45] which excrete a majority of the N_2 fixed into the medium as NH_4^+, at least some strains of *Frankia* in culture are capable of assimilating the NH_4^+ fixed and using it for growth. The occurrence of glutamine synthetase and glutamate dehydrogenase in the plant but not endophyte fractions of *Alnus* nodule homogenates[15], indicated that, like the *Rhizobium*/legume symbiosis[16], NH_4^+ is assimilated by the host rather than the symbiont in the nodule.

The concentration of several salts in the medium was critical for nitrogenase induction. Low levels of $CaCl_2$, $MgSO_4$ and K_2HPO_4/KH_2PO_4 limited development of nitrogenase activity; higher levels of the Mg and Ca salts were inhibitory. Inhibition was probably due to precipitation with other essential nutrients rather than a direct inhibitory effect on cellular metabolism and may be related to the earlier observation[41] that the chelating agent EDTA was essential for vesicle formation under similar nutritional conditions.

Propionate supports rapid growth when used as the sole carbon source in all but one (see 38) *Frankia* isolate reported to date[10,11,14,36]. The enzymes required for its utilization appear to be constitutive in some isolates[38]. A substantial CO_2 stimulation of propionate-supported growth in *Frankia* sp. AvcII was reported by Akkermans and coworkers[3]. Although CO_2 is an essential metabolic intermediate required for bacterial growth, endogenous production of respiratory CO_2 normally masks this requirement. In ArI3 the CO_2 stimulation of

acetylene induction and respiration apparently exceeds the basal CO_2 requirement for growth since relatively high levels of exogenous CO_2 (about 0.75% for acetylene reduction) were required to saturate the reaction(s).

A CO_2 requirement for expression of nitrogenase activity was also demonstrated on free-living *Rhizobium* which was distinct from its role in growth[1]. It is unclear whether the effect of CO_2 on acetylene reduction in *Frankia* ArI3 was specific or a consequence of its positive effect on respiration. The latter view seems more plausible since a differential effect of CO_2 on the two processes was not observed. Furthermore, CO_2 stimulation of respiration was dependent on the carbon source; of the substrates tested, only propionate and pyruvate oxidation was stimulated by CO_2 suggesting that oxidation of these substrates requires a carboxylation reaction.

Propionate utilization in *Rhizobium* occurs by conversion to succinate via propionyl CoA carboxylase and subsequent oxidation by the Krebs cycle[22] rather than by the β-oxidation pathway described in plant tissue[26]. Biotin stimulation of propionate oxidation *in vivo* suggests operation of the propionyl CoA pathway in *Frankia* sp. ArI3. Both propionyl CoA carboxylase and pyruvate carboxylase contain biotin as the prosthetic group. Although biotin is apparently synthesized by this organism, since growth of ArI3 does not require an exogenous source[36] respiration was stimulated by an exogenous supply of this vitamin.

The large scale culturing technique developed and described here for nitrogenase derepression in rapidly agitated liquid culture provides more precise gaseous and nutritional control and allows for less variability in sampling than has been obtained previously. The rates of acetylene reduction obtained (more than 10 nmol/mg/min) are sufficient to support growth on N_2 and are comparable to rates in other free-living nitrogen fixing organism. Expression of nitrogenase in free-living *Frankia* will allow investigation of the regulatory mechanisms controlling vesicle differentiation and nitrogenase synthesis and activity by key factors like O_2 and combined N and C sources which will be critical in understanding the symbiotic associations.

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