Chemolithooorganotrophic Growth of *Nitrosomonas europaea* on Fructose

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The nitrifying bacterium *Nitrosomonas europaea* can obtain all its carbon for growth from CO₂ and all its energy and reductant for growth from the oxidation of NH₃ and is considered an obligate chemolithoautotroph. Previous studies have shown that *N. europaea* can utilize limited amounts of certain organic compounds, including amino acids, pyruvate, and acetate, although no organic compound has been reported to support the growth of *N. europaea*. The recently completed genomic sequence of *N. europaea* revealed a potential permease for fructose. With this in mind, we tested if *N. europaea* could utilize fructose and other compounds as carbon sources to support growth. Cultures were incubated in the presence of fructose or other organic compounds in sealed bottles purged of CO₂. In these cultures, addition of either fructose or pyruvate as the sole carbon source resulted in a two- to threefold increase in optical density and protein content in 3 to 4 days. Studies with [¹⁴C]fructose showed that >90% of the carbon incorporated by the cells during growth was derived from fructose. Cultures containing mannose, glucose, glycerol, mannitol, citrate, or acetate showed little or no growth. *N. europaea* was not able to grow with fructose as an energy source, although the presence of fructose did provide an energy benefit to the cells. These results show that *N. europaea* can be grown in CO₂-free medium by using fructose and pyruvate as carbon sources and may now be considered a facultative chemolithooorganotroph.

*Nitrosomonas europaea* obtains all of its energy and reductant for growth from the oxidation of ammonia (NH₃) and can obtain all its carbon for biomass from CO₂. Ammonia is oxidized to nitrite (NO₂⁻) and CO₂ is assimilated by ribulose bisphosphate carboxylase and the reductive pentose phosphate cycle (Calvin cycle). Because growth on other carbon or energy sources has not been demonstrated, *N. europaea* has been classified as an obligate chemolithoautotroph.

Obligate lithoautotrophy in *N. europaea* and other bacteria has been investigated for over 100 years, since Winogradsky’s initial characterizations of nitrifiers (reviewed in references 17 and 18). Isotopic labeling studies have provided direct evidence for the incorporation of organic compounds into *N. europaea* and other autotrophs. These compounds include pyruvate (4, 12, 13, 20), acetate (12, 13, 18, 20), α-ketoglutarate and succinate (20), and amino acids (5, 6, 20). The labeling experiments led researchers to consider whether import of these organic compounds affected the growth of *N. europaea* and other nitrifiers. Addition of some amino acids to *N. europaea* cells was found to increase production of NO₂⁻ and production of protein (which are usually correlated with growth) (6). However, addition of formate, acetate, pyruvate, glucose, or peptone had a negligible effect on NO₂⁻ formation in two *Nitrosomonas* strains (12). In another study, addition of pyruvate to *N. europaea* cultures greatly reduced the lag time for resumption of growth when old cells were used as inocula (4). Anaerobically, the protein content of an *N. europaea* culture using pyruvate as an electron donor and nitrate as an electron acceptor increased by 49% over 12 days, although NH₃ was still required (1). In all the studies mentioned above, NH₃ was always required and most of the biomass was obtained from CO₂. In the absence of NH₃, *Nitrosomonas* strains were unable to grow on formate, acetate, pyruvate, glucose, or peptone as an energy source, even when the media were supplemented with yeast extract, Casamino Acids, serine, alanine, or glycine (12). Finally, there has been one report of aerobic heterotrophic growth of *N. europaea* and other obligate chemolithotrophs on media containing 0.2% glucose and 4.7 mM NaHCO₃ but lacking NH₄⁺ (14). This growth required continuous dialysis of the culture media, which the authors suggested was needed to remove inhibitory compounds. However, this finding has not been reproduced, and the results remain inconclusive.

The recent completion of the *N. europaea* genomic sequence (3) has presented an opportunity to examine the genetic basis for the inability of this organism to grow heterotrophically. In general, while genes for NH₃ catabolism, assimilation of inorganic nutrients, and biosynthesis of organic constituents were found, genes for the catabolism of organic compounds were limited. However, the genes required for catabolism of carbohydrates through the glycolytic pathway to the tricarboxylic acid cycle were present in the genome, suggesting that *N. europaea* might be able to utilize some carbohydrates for growth. A survey of transporter genes in the *N. europaea* genome revealed several genes similar to genes encoding transporters of inorganic compounds and ions and a few genes which appear to encode transporters of amino acids. In addition, genes encoding several components of a potential phosphoenolpyruvate-dependent sugar phosphotransferase (PTS) system which had the highest levels of sequence similarity to fructose or mannose transporters were also identified. These observations raised the possibility that *N. europaea* could utilize fructose or mannose for growth. In this study, we found

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that fructose can indeed support growth of this organism, and we obtained the first evidence of chemolithoorganotrophic growth of *N. europaea*.

**MATERIALS AND METHODS**

**Preparation of cells and analytical methods.** Stock cultures of *N. europaea* were grown as previously described (7) in medium containing (NH₄)₂SO₄ (25 mM) for energy and Na₂CO₃ (3.9 mM) as a carbon source. To prepare inocula for growth experiments, cells from early-stationary-phase cultures grown on NH₄ and Na₂CO₃ were harvested by centrifugation and washed three times in buffer (50 mM Na₂PO₄ [pH 8.0], 2 mM MgSO₄). Sterile 160-ml culture bottles were prepared with 25 ml of *N. europaea* medium containing (NH₄)₂SO₄ (25 mM) but not Na₂CO₃. Filter-sterilized fructose (or another substrate) was added from a stock solution, and the culture bottles were sealed with Teflon-faced gray butyl rubber stoppers (Supelco, Bellefonte, Pa.) fastened with aluminum crimp seals. Carbon dioxide was removed from the headspace and media in the bottles by three cycles of evacuation of the headspace with a vacuum manifold and refilling of the headspace with N₂–O₂ (20%) was added to the bottles as an overpressure. Washed *N. europaea* cell suspensions were injected into the bottles, typically to an initial density at 600 nm of 0.006 (~10⁷ cells/ml). The bottles were incubated with shaking in the dark at 30°C. Growth was monitored by determining the optical density at 600 nm. Nitrite assays (9) and protein assays (biuret) (8) were performed as described previously. The CO₂ in the headspace of the bottles was assayed by thermal conductivity gas chromatography (GC-8A; Shimadzu Scientific Instruments, Columbia, Md.) by using a 3-ft PORAPAK T column (Waters Associates Inc., Framingham, Mass.) run at 155°C with the detector set at 220°C.

To determine if fructose could serve as an energy source, cells were incubated in medium containing fructose (50 mM), (NH₄)₂SO₄ (25 mM), Na₂CO₃ (3.9 mM), and acetylene (1% in the gas phase). As a mechanism-based inactivator of the oxidation of NH₃ by ammonia monooxygenase, acetylene denies cells the ability to oxidize NH₃ for energy. The ratio of the amount of NH₃ oxidized to the amount of C incorporated into cell mass was calculated. The amount of NH₃ oxidized was assumed to be equal to the measured amount of nitrite produced. The amount of carbon incorporated was based on the measured ratio of optical density to carbon content obtained by total carbon analysis as described below.

**¹⁴C labeling.** Bottles were prepared with growth medium (25 ml) lacking carbonate. A 50 mM fructose solution containing ν-[U-¹⁴C]fructose (275 mCi/mmol; DuPont NEN Products, Wilmington, Del.) was added to obtain a final specific activity of 160 μCi/mmol. CO₂ was removed as described above. *N. europaea* cells, prepared as described above, were inoculated into the sealed bottles to obtain an initial optical density at 600 nm of 0.010. The cultures were incubated for 3 days at 30°C with shaking. Each bottle contained an empty glass test tube, and 3 h prior to the end of the labeling experiment, 0.5 ml of 1 M KOH was injected into the test tube to trap the evolved CO₂. At the end of the experiment, the bottles were opened, and the ¹⁴CO₂ content of each KOH solution was measured with a liquid scintillation counter. The cells were sedimented by centrifugation at 12,000 x g, washed three times, and analyzed to determine the optical density, protein content, and ¹⁴C incorporation. Total carbon analysis of the fructose-grown cells with C/N analyzer (Dumas combustion/reduction) linked with mass spectroscopy was performed at the Stable Isotope Research Unit of the Department of Crop and Soil Science, Oregon State University.

**T-RFLP analysis.** To check the purity of the cultures, terminal restriction fragment length polymorphism (T-RFLP) PCR was performed with total DNA isolated from the cultures by using the 16S rRNA general eubacterial primers 27F-6Fam (5'-6-carboxyfluorescein-AGAGTTTGTATCMTGGCGCAG) (10) (fluorescently labeled) and 338R (5'GCTGTCCTCCGTAAGGAGT) (2) obtained from Invitrogen Corp. (Frederick, Md.). The PCR was carried out for 30 cycles of 97°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The resultant PCR product was digested with restriction endonucleases Adal, HaeIII, and TaqI (Promega, Madison, Wis.) separately, and the resultant fluorescently labeled fragments were analyzed by gel electrophoresis by using the GeneScan Analysis software (Applied Biosystems, Foster City, Calif.).

**RESULTS**

**Growth on fructose.** Analysis of the genome sequence suggested that *N. europaea* might be capable of utilizing fructose for growth. Growth experiments, conducted in bottles purged of CO₂, showed that *N. europaea* cells were indeed able to grow with fructose as the sole carbon source. The optical densities and protein contents of the cultures grown on fructose increased as much as 2.5-fold during the 80 h of incubation (Fig. 1A). The growth rates were higher with higher fructose concentrations tested. Cultures without an added carbon source or with 1 mM fructose did not show significant growth (<0.004-U increase in the optical density at 600 nm over 80 h). Growth on CO₂ (from added Na₂CO₃) resulted in higher
growth rates than growth on fructose (Fig. 1A). Addition of 50 mM fructose to cultures growing on CO2 resulted in slight stimulation of growth (>11%) compared with the growth of cultures growing on CO2 alone (Fig. 1B). No stimulation was observed when 1 mM fructose was added to cultures growing on CO2 (data not shown). The doubling times for the cultures were estimated to be 24 h for growth on 50 mM fructose and 8 h for a culture grown on 3.9 mM carbonate. The doubling time for cultures grown on 50 mM fructose plus 3.9 mM Na2CO3 was estimated to be about 7.4 h. Cells grown on fructose were indistinguishable from cells grown on CO2 by microscopy.

Cultures grown on fructose had lower final cell densities than cultures grown on CO2 (Fig. 1A). To determine if the final densities represented an irreversible cessation of growth, either more fructose (25 mM) or more Na2CO3 (3.9 mM) was added to fructose-grown cultures (50 mM) in the stationary phase. Addition of fructose did not stimulate growth of the cultures, whereas addition of Na2CO3 resulted in an immediate resumption of growth, and the growth rates and final cell densities were similar to those of cultures freshly inoculated into Na2CO3-containing media (data not shown). Addition of fructose or Na2CO3 changed the pHs of the cultures by less than 0.1 U, which was not sufficient to account for the observed results.

14C labeling. To directly demonstrate that N. europaea cells can use fructose as a primary carbon source, we included 14C-labeled fructose in the medium. The amount of fructose incorporated into cell mass was calculated from the amount of 14C label incorporated into the cell pellet. The total amount of carbon that accumulated in the cell mass during growth of the culture was estimated from the increase in the optical density of the culture. The relationship of optical density to carbon content obtained by total carbon analysis was determined to be 125.08 μg of C · ml−1 · optical density unit−1.

For cultures grown with 50 mM fructose and no Na2CO3, [14C]fructose accounted for 91% of the carbon incorporated into cells (Table 1). The percentage dropped to 31.6% for cultures containing 50 mM fructose and 3.9 mM Na2CO3 even though the total amount of carbon assimilated increased 3.7-fold. The percentage dropped further to 2.2% for cultures containing 3.9 mM carbonate and 1 mM fructose. Cultures containing 1 mM fructose and no Na2CO3 did not grow (data not shown). A sodium dodecyl sulfate-polyacrylamide gel of the labeled cell pellet showed that the 14C label appeared to be uniformly incorporated into cell protein (data not shown). These experiments established that when fructose was provided as the sole carbon source, N. europaea cells used sufficient fructose to account for the observed growth of the culture.

14CO2 was found in the headspaces of the culture bottles following acidification of the media at the end of the experiment. Cultures containing 50 mM fructose with and without Na2CO3 had 14CO2 levels in the headspace representing 11 and 13%, respectively, of the amount of 14C assimilated into the cell pellets. In media containing 1 mM fructose and 3.9 mM carbonate, the amount of 14CO2 in the headspace was only about 1.4% of the amount of 14C incorporated into the pellet. Thus, some of the fructose was converted to CO2, but the amount was small compared to the amount that was assimilated into biomass.

Energy from fructose. The results described above indicate that fructose can serve as a carbon source for growth of N. europaea. To determine if fructose could also serve as an energy source, cells were incubated in medium containing fructose, NH4+ , Na2CO3, and acetylene (which prevents the use of NH3 as an energy source). These cultures did not grow, indicating that N. europaea cells cannot derive energy from the catabolism of fructose to support growth. On the other hand, cells assimilating fructose (in the absence of acetylene) oxidized less NH3 to produce a similar amount of biomass (Table 1). In N. europaea, NO3− production is the result of NH3 oxidation and is therefore representative of the energy demands of cells during growth. In medium containing 3.9 mM Na2CO3 alone the organism oxidized 27.5 mol of NH3 per mol of C fixed (data not shown). In medium containing Na2CO3 and 1 mM fructose, 27 mol of NH3 was oxidized per mol of C fixed. In medium containing Na2CO3 and 50 mM fructose, only 22 mol of NH3 was oxidized per mol of C, a reduction of about 20%. In cultures containing 50 mM fructose alone, 15 mol of NH3 was oxidized per mol of C fixed, a reduction of 45%. Thus, the availability of fructose clearly reduced the requirement for NH3.

Culture purity and CO2 leakage. The possibility that the observed growth could have been due to contamination of the cultures was eliminated by various means. First, plates with rich media contained no contaminants. Second, the same growth results were obtained with N. europaea strains containing an antibiotic marker (Kan) (11) grown in the presence of kanamycin (20 μg/ml). Third, total genomic DNA isolated from fructose-grown cultures and digested with the restriction endonuclease EcoRI showed the same digestion pattern on agarose gels as N. europaea control DNA (data not shown). And finally, T-RFLP PCR performed with 16S rRNA gene
universal eubacterial primers was used to detect possible bacterial contaminants. The PCR produced a DNA fragment whose length (341 bp) was the same as the length of the 16S rRNA sequence found in the genomic sequence of *N. europaea*. The 341-bp PCR fragment, digested with the restriction endonucleases *Alu*, *Hae*III, and *Taq*I separately, produced DNA fragments consistent with the *N. europaea* sequence and did not produce other fragments indicative of DNA from other bacteria. Therefore, the cells growing in the fructose cultures in these experiments were *N. europaea* cells.

The possibility that the observed growth might have been due to CO$_2$ leakage into the bottles or carbonate contamination in the growth media was also considered. However, when gas chromatography was used to monitor the CO$_2$ contents in control bottles without cells, there was no indication of CO$_2$ leakage. To test for carbonate contamination in the growth medium, bottles were purged of CO$_2$ under a vacuum, and the leakage. To test for carbonate contamination in the growth media was also considered. However, when carbonate to CO$_2$. Gas chromatography of the headspace gases showed that the amounts of carbonate in the media were <100 nmol, a level too low to support growth of the cultures.

**Growth on pyruvate and other substrates.** Since *N. europaea* was found to grow on fructose, other organic substrates, which had previously been shown to be utilized by the cells, were tested to determine their abilities to support growth. These experiments were performed like the experiments with fructose. It was found that *N. europaea* cells could grow by using pyruvate as the sole carbon source (Fig. 2A). Growth was observed with pyruvate concentrations ranging from 1 to 50 mM. Unlike high fructose concentrations, a high pyruvate concentration (50 mM) was inhibitory after an initial period of growth. The doubling time for growth on 10 mM pyruvate was 20 h. No bacterial contaminants were detected in the cultures when they were tested on rich medium plates, by microscopic examination, or by comparing the restriction enzyme digestion patterns of genomic DNA, as was done with fructose cultures. When *N. europaea* cells were cultured with both fructose (50 mM) and pyruvate (10 mM), the growth rates were higher than the growth rates with either substrate alone (Fig. 2B).

Other potential growth substrates for *N. europaea* were tested, including glucose (25 and 50 mM), glycerol (20 mM), mannose (5, 10, 25, and 50 mM), mannitol (5, 10, and 50 mM), citrate (25 mM), and sodium acetate (10 and 25 mM). These growth experiments were performed like the experiments with fructose. The cultures did not grow on these organic substrates. In addition to not supporting growth, mannose at higher concentrations (>10 mM) inhibited the growth of cultures even in the presence of carbonate (data not shown). Thus, the ability of *N. europaea* to utilize organic compounds for growth appears to be limited to a few substrates.

**DISCUSSION**

The growth and labeling experiments showed that *N. europaea* cells can grow by using either fructose or pyruvate as the sole carbon source. These findings may be unexpected in light of the traditional view that *N. europaea* is an obligate chemo-lithoautotroph which grows only in the presence of NH$_3$ and CO$_2$. However, we do not believe that these results directly contradict the results of previous studies. To our knowledge, investigators have not specifically looked for growth of *N. europaea* on CO$_2$ as the sole carbon source. In the case of pyruvate, previous work clearly showed that this compound is imported and affects the growth of *N. europaea*. However, the previous studies were conducted in the presence of carbonate or CO$_2$, and none of the workers reported trying to grow the cells with pyruvate as the sole carbon source. Therefore, in light of our results, it may be more accurate to describe *N. europaea* as a facultative chemolithoorganotroph.

Chemolithotrophs meet most of their needs for cell carbon through assimilation of C$_1$ compounds, such as CO$_2$, despite the fact that many chemolithotrophs have been shown to assimilate organic carbon compounds (18). Our experiments confirmed the preference of *N. europaea* cells for CO$_2$ as the carbon source, as shown by the higher growth rates and higher final cell densities observed when cells were grown on CO$_2$ and by the lower proportion of [H]fructose-derived carbon incorporated into cell mass when the cells were grown in the presence of carbonate. The preference for CO$_2$ is noteworthy in light of the high energetic cost of fixing CO$_2$ and the potential energy savings that using reduced carbon substrates would represent.

The lower growth rates and lower cell densities observed
during growth on either fructose or pyruvate as the sole C source show that there are significant limitations to using these substrates. These limitations could include either transport or metabolic inefficiencies or both. It has long been suggested that the reason that autotrophs are unable to use organic compounds as growth substrates may be that the transporters for organic compounds are either absent or rate limiting (19). Alternatively, metabolic limitations could include low substrate turnover rates, low substrate affinities of catabolic enzymes, or feedback inhibition. The high concentrations of fructose required to maximize growth of *N. europaea* could be consistent with either transport or metabolic limitations, with the exception of feedback inhibition. Growth of *N. europaea* cells on pyruvate resulted in higher growth rates and higher cell densities than growth on fructose, although the values were still less than those obtained with carbonate or CO₂. Since pyruvate should be readily metabolized in the cell, the slower growth with pyruvate than with CO₂ might reflect transport limitations for this compound.

The growth of *N. europaea* on fructose or pyruvate demonstrates that the cells are metabolically competent to use these compounds for growth. Pyruvate can be directly oxidized to acetyl coenzyme A. In the case of fructose, the metabolic fate of the compound depends in part on the mechanism by which it enters the cell. PTS transport systems characteristically phosphorylate the carbohydrate as it enters the cell. If fructose enters as fructose 6-phosphate, it should be readily metabolized via the glycolytic and gluconeogenesis pathways. However, if fructose enters as fructose 1-phosphate (as is common in other bacteria), it would have to either be phosphorylated to fructose 1,6-bisphosphate by 1-phosphofructokinase or be converted to fructose 6-phosphate by a phosphohexose mutase. However, genes encoding these enzymes were not identified in the genome. The other potential growth substrates for *N. europaea* that were tested, glucose, glycerol, mannose, mannitol, citrate, and sodium acetate, were unable to support growth. Previous studies involving the assimilation of organic substrates by *N. europaea* have generally been concerned with either (i) establishing the ability to take up labeled compounds in the presence of NH₃ and Na₂CO₃ or (ii) attempting to replace NH₃ with an organic substrate as an energy source. While pyruvate, acetate, and glucose assimilation was examined in these previous studies, none of the studies looked for growth when the organic compound was used as a sole carbon source. Thus, as with the results for fructose and pyruvate, the growth results which we observed with the compounds listed above did not contradict the results of previous work. The lack of growth on acetate may be due in part to the low rate of acetate uptake by *N. europaea* observed previously (18). The range of organic substrates able to support growth of *N. europaea* appears to be limited to a few specific compounds.

The NH₃-oxidizing bacteria are unique among the diverse groups of chemolithotrophic bacteria in that there have been no NH₃ oxidizers identified to date that are capable of heterotrophic growth (i.e., growth in which organic compounds are utilized for energy) (18). We nevertheless considered the possibility that fructose could be used as an energy source for the cells, as well as a carbon source. However, neither fructose nor the other organic compounds tested could replace NH₃ as a sole energy source for growth. On the other hand, the observation that lower levels of NO₂⁻ were produced in the presence of fructose relative to the amount of carbon fixed indicates that the assimilation of fructose by cells provides the cells with some energy benefit. This benefit may be due to a lower requirement for energetically expensive CO₂ fixation by the Calvin cycle.

Mannose was included in our experiments as a potential growth substrate since analysis of the *N. europaea* genomic sequence indicated that the potential PTS carbohydrate transporter exhibits sequence similarity to both fructose and mannose transporters. Mannose did not support growth in our experiments and in fact inhibited growth at concentrations above 10 mM when cells were growing on Na₂CO₃. The ability of mannose to inhibit growth may suggest that the sugar indeed entered the cell and interfered with metabolism. Many PTS transporters transport alternative substrates, although with reduced affinity (15). Thus, it is possible that fructose and mannose may be taken up by the same transporter.

A closer examination of the PTS genes found in the *N. europaea* genome failed to identify the cognate substrate for the putative transporter. A typical PTS complex contains two phosphoproteins, EI and HPR, which are common to all PTS systems, as well as three additional carbohydrate-specific domains (IIA, IIB, and IIC), which may occur as separate proteins or with two or three domains combined into a single protein (16). In the *N. europaea* genome, the genes encoding IIA, HPR, and EI are located together. A separate, second gene for IIA was also found. However, BLAST searches of the genome failed to locate strong candidates for genes encoding IIB and IIC. Perhaps *N. europaea* does possess IIB and IIC homologs but the sequences have diverged too much to be recognized. While the fructose PTS system in some bacteria uses a unique protein, FPr, instead of HPr, no gene encoding FPr was found in *N. europaea*. The absence of a gene encoding FPr, as well as the genes encoding the enzymes needed to bring fructose 1-phosphate into central metabolism (see above), suggests that fructose may be imported via a non-fructose-type PTS system. The primary substrate for the putative PTS transporter remains unclear. It must also be noted that while it seems reasonable to consider a role for a PTS transporter for growth on fructose, the results presented here do not directly establish that fructose assimilation occurs via the identified PTS transporter. A transporter for pyruvate was not identified in the genomic sequence. Although the mechanisms by which these compounds are imported remain enigmatic, the uncertainties do not diminish the primary observation of this study, namely, that *N. europaea* is able to grow on fructose or pyruvate as a carbon source.

Given that the genomic sequence of *N. europaea* encodes so few transporters for organic substrates, the finding that fructose transport can occur seems unusual. At this point it is unclear what role assimilation of fructose or pyruvate might play for *N. europaea* in the environment when its preferred carbon source, CO₂, should be generally available. However, since *N. europaea* is often limited by the amount of NH₃ and therefore by the amount of energy available, these organic compounds might provide benefits both as sources of metabolic intermediates and by reducing the biosynthetic energy needs of the cells.
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