D-Lactate metabolism and the obligate requirement for CO₂ during growth on nitrite by the facultative lithoautotroph Nitrobacter hamburgensis

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INTRODUCTION

Nitrification is a two-step, biologically mediated process through which ammonia is converted into nitrate (NO₃⁻). Nitrite-oxidizing bacteria (NOB) participate in the second step of nitrification by converting nitrite (NO₂⁻) into NO₃⁻. NOB use NO₂⁻ as their primary energy source to generate reductant, assimilate carbon dioxide (CO₂), and drive oxidative phosphorylation. NO₂⁻ is a relatively poor energy substrate due to the positive midpoint potential of the NO₃⁻−NO₂⁻ couple (Eₒ = +420 mV). Since it is estimated that 85–115 moles NO₂⁻ are required to fix 1 mole CO₂ (Bock et al., 1986, 1991), it is not surprising that some NOB use organic carbon as a carbon and energy source to reduce the burden of relying solely on NO₂⁻ to meet the energy demands of biosynthesis.

Although several phylogenetically distinct genera carry out NO₂⁻ oxidation (Nitrospira, Nitrobacter, Nitrococcus and Nitrospina), most of what is known about the organic carbon metabolism of NOB has been derived from studies of the genus Nitrobacter. Several reports using strains of Nitrobacter winogradskyi have shown that simple organic substrates, such as acetate, pyruvate and glycerol, can support organotrophic growth (Bock, 1976; Delwiche & Feinstein, 1965; Smith & Hoare, 1968; Steinmüller & Bock, 1976, 1977). Nevertheless, the bias of N. winogradskyi towards a lithoautotrophic lifestyle is supported by the fact that organotrophic growth rates are much slower than lithoautotrophic growth rates, and when CO₂ is stripped from cultures containing organic carbon and NO₂⁻ (mixotrophy), these organic carbon sources cannot serve as the sole carbon source (Delwiche & Feinstein, 1965; Ida & Alexander, 1965).

Nitrobacter hamburgensis, a more recent isolate (circa 1983) of the Nitrobacter genus, has been described as having a greater organotrophic potential than N. winogradskyi, as an early description reported (without presenting data) that mixotrophic and organotrophic growth rates were faster than lithoautotrophic growth rates (Bock et al., 1983). Many investigations of N. hamburgensis have been...
conducted with cells grown mixotrophically (Harris et al., 1988; Kirstein et al., 1986; Laanbroek et al., 1994; Spieck et al., 1996), although it is not well understood how this bacterium utilizes, or adapts to, organic carbon. Some evidence of a physiological adaptation to organic carbon by *N. hamburgensis* exists, as cell membranes from mixotrophic and organotrophic cultures have been shown to contain different *b*-type cytochromes (Kirstein et al., 1986). Nevertheless, detailed studies of how organic carbon is processed in *N. hamburgensis* and how its metabolism is influenced by NO$_2$ have not been completed. Furthermore, it is not known whether organic carbon can be used as the sole carbon source in the presence of NO$_2$ (lithoheterotrophy), or whether organic carbon positively or negatively influences the rate of NO$_2$ oxidation in this species.

The recent availability of the *N. hamburgensis* X14 genome sequence prompted a re-examination of mixotrophy and organotrophy in this bacterium, as three genes that could encode lactate dehydrogenases (LDHs) have been identified (Starkenburg et al., 2008). In contrast to the well-studied NAD-dependent LDHs, these genes encode homologues of flavin-dependent LDHs (iLDHs), which oxidize lactate to pyruvate and could provide energy and/or carbon to the cell (Garvie, 1980). In this study, the ability of *N. hamburgensis* to metabolize lactate was examined and its effect on lithoautotrophy was explored.

**METHODS**

**Culture conditions.** Stock cultures of *N. hamburgensis* X14 were grown in a chemically defined mineral salts medium at pH 7.5. The base medium contained 750 μM MgSO$_4$, 30 μM FeSO$_4$, 50 μM EDTA, 200 μM CaCl$_2$, and 1 μM CuSO$_4$. A 1 ml volume of a trace metals stock solution (400 μM Na$_2$MoO$_4$, 1 mM MnCl$_2$, 350 μM ZnSO$_4$, 8.4 μM CoCl$_2$) was added per 2 l base medium. After autoclaving, the medium was amended with phosphate buffer stock solution (480 mM KH$_2$PO$_4$, 42 mM NaH$_2$PO$_4$, pH 7.8) at 20 ml l$^{-1}$. NaNO$_2$ (30–45 mM) was added to the phosphate-buffered base medium as the energy and nitrogen source for lithoautotrophic growth. Organotrophic growth medium contained filter-sterilized sodium D-lactate (0.5 mM; Sigma) and 1 mM (NH$_4$)$_2$SO$_4$ as a nitrogen source. Mixotrophic growth medium contained 30–45 mM NaNO$_2$, 2 mM (NH$_4$)$_2$SO$_4$ and 0.5 mM sodium D-lactate. Growth was monitored routinely by determining the OD$_{600}$. Growth experiments were conducted in sterile 160 ml culture bottles prepared with 50 ml culture medium inoculated to initial OD$_{600}$ 0.015. To prevent the influx of atmospheric CO$_2$ during closed-system experiments, the cultures were sealed with Teflon-faced grey butyl rubber stoppers (Supelco) and fastened with aluminium crimp seals. To avoid O$_2$ depletion limiting cell growth, O$_2$ in the headspace was monitored daily and replenished as needed. NO$_2$ was measured spectrophotometrically or colorimetrically, as described elsewhere (Hageman & Hucklesby, 1971). Whole-cell protein content was assessed using a modified Bradford method (Nelson et al., 1982). Cultures were routinely checked for contamination by plating 100 μl samples on Luria agar plates or by inoculating 1/10 nutrient broth (Difco) containing glucose (1 g l$^{-1}$). Culture purity of lactate-containing cultures was additionally verified by visualization of cells at ×25000 magnification with a transmission electron microscope.

**O$_2$-uptake measurements.** *N. hamburgensis* cells were harvested by centrifugation from late-exponential phase cultures, washed, and resuspended in 50 mM potassium phosphate buffer, pH 7.5. Rates of lactate- or NO$_2$-dependent O$_2$ uptake by cell suspensions were measured with a Clark-type O$_2$ electrode (Yellow Springs Instrument) mounted in an all-glass, water-jacketed reaction vessel (1.8 ml volume) held at 30 °C.

**Lactate consumption assay.** Lithotrophically, mixotrophically and organotrophically grown cells of *N. hamburgensis* were harvested by centrifugation from late-exponential phase cultures, washed twice, and resuspended to OD$_{600}$ 0.3–1.0 in sterile phosphate-buffered base medium (pH 7.5). Aliquots (5 ml) of cell suspensions were added to 38 ml culture bottles and sealed with Teflon-faced grey butyl rubber stoppers (Supelco) and fastened with aluminium crimp seals. CO$_2$ in the headspace was measured with a thermal conductivity gas chromatograph (model GC-8A, Shimadzu) equipped with a 90 cm Porapak T column (Waters Associates) with the column temperature set to 150 °C and the detector set to 220 °C. The concentration of D-lactate was measured with a D-lactate assay kit (Megazyme International) according to the manufacturer’s instructions.

**[14C]lactate incorporation.** Resting cells of lithotrophically, mixotrophically and organotrophically grown *N. hamburgensis* were harvested by centrifugation from late-exponential phase cultures and resuspended in sealed 38 ml bottles containing 5 ml phosphate-buffered medium with 2 mM (NH$_4$)$_2$SO$_4$, 1 mM D-lactate and 1.4 μCi (518 M bq) DL-[1-14C]lactate (Sigma-Aldrich). *N. hamburgensis* was inoculated into these sealed bottles to OD$_{600}$ 0.4–0.6. Each culture bottle contained a 1.5 ml plastic tube containing a piece of Whatman filter paper soaked in 100 μl 18 M KOH to trap evolved CO$_2$. At the end of the experiment (t=4 h), the bottles were opened, and the KOH-soaked filter paper was removed and added to a vial with 3.5 ml Ecoluom scintillation fluid (ICN), and $^{14}$CO$_2$ was measured on a Beckman 6500 multi-purpose scintillation counter. A 1 ml aliquot of the cell-free supernatant was added to another sealed vial containing a KOH trap and was acidified with 20 μl 12.1 M hydrochloric acid to measure the residual $^{14}$CO$_2$ dissolved in the incubation medium. This quantity was added to the radiolabel measured in the first KOH trap to determine the total $^{14}$CO$_2$ released from lactate. Cellular incorporation of radioactive $^{14}$C from D-lactate was measured in cells that were harvested by centrifugation, washed twice, and resuspended in 1 ml phosphate-buffered base medium. A 200 μl aliquot of the cell suspension was added to a vial containing 3.5 ml scintillation fluid, and cellular $^{14}$C incorporation was measured as described above.

**RESULTS**

**Growth response of *N. hamburgensis* to D- or L-lactate**

As both putative D- and L-specific LDH-encoding genes have been identified in its genome, *N. hamburgensis* was cultured in the presence of either D- or L-lactate, with and without NO$_2$$_3$, to assess its mixotrophic and organotrophic potential. When *N. hamburgensis* was grown in medium supplemented with D-lactate and NO$_2$$_3$, the growth rate and final cell yield increased 50 and 60 %, respectively, compared with cultures grown lithoautotrophically on medium containing NO$_2$$_3$ and CO$_2$ (Fig. 1a). Concomitantly, an increase in the total protein content was also observed in *N. hamburgensis* cells grown
mixotrophically with D-lactate and NO\textsubscript{2}/CO\textsubscript{2} (Fig. 1c). Because D-lactate did not affect the gross amount of NO\textsubscript{2} consumed (Fig. 1b), the increased growth rate and cell yield indicated that less NO\textsubscript{2}/CO\textsubscript{2} was consumed per OD\textsubscript{600} unit under mixotrophic conditions. Growth was also observed in N. hamburgensis cultures incubated with D-lactate as the only source of energy (organotrophy) (Fig. 1a). Despite the fact that equal quantities of D-lactate were consumed by both mixotrophic and organotrophic cultures, the cell yield of organotrophically grown cultures was 50\% less than that of mixotrophic cultures containing D-lactate and NO\textsubscript{2}. Similarly, when the amount of protein recovered from these cultures was normalized to OD\textsubscript{600}, organotrophically grown cells contained 30\% less protein per OD\textsubscript{600} unit than lithoautotrophically or mixotrophically grown cells.

In contrast to the growth response to D-lactate, L-lactate had no effect on growth rate or cell yield compared with control cultures growing on NO\textsubscript{2}/CO\textsubscript{2}, and growth was not detected when L-lactate was added in the absence of NO\textsubscript{2}. Furthermore, the optical densities of cultures amended with a combination of L- and D-lactate were not significantly different from those of cultures containing only D-lactate (data not shown), indicating that L-lactate does not inhibit (or enhance) the consumption of D-lactate.

The effect of NO\textsubscript{2} on lactate consumption was measured in resting cells harvested from both lithoautotrophic and mixotrophic cultures (Table 1). Cells harvested from lithoautotrophic cultures readily consumed lactate at 1.17 μmol D-lactate (mg protein)\textsuperscript{-1} h\textsuperscript{-1}. This short-term rate of lactate consumption by lithoautotrophically grown cells was not significantly affected by the addition of NO\textsubscript{2}. Mixotrophically grown cells consumed lactate at a faster rate [1.67 μmol D-lactate (mg protein)\textsuperscript{-1} h\textsuperscript{-1}] than lithoautotrophically grown cells, suggesting that N. hamburgensis had adapted to growth on lactate. In the presence of NO\textsubscript{2}, the short-term rate of lactate consumption by mixotrophically grown cells decreased by 15\%.

**Respiration**

Other evidence of the occurrence of physiological adaptation during growth on lactate was obtained from measurements of respiration (Table 2). D-Lactate supported significantly higher rates of respiration in organotrophically and mixotrophically grown cells compared with cells grown autotrophically. Additionally, NO\textsubscript{2}-dependent respiration was reduced after organotrophic growth on lactate, whereas mixotrophically grown cells maintained high rates of both NO\textsubscript{2} and lactate-dependent O\textsubscript{2} uptake. When the rate of lactate consumption and the rate of lactate-dependent oxygen uptake were compared, mixotrophically grown cells appeared to be more effective at using lactate to support respiration, as lactate-dependent oxygen uptake in mixotrophically grown cells was threefold higher than the rate of lactate-dependent oxygen uptake.
consumption by lithoautotrophically grown cells (Table 2), despite the fact that mixotrophically grown cells consumed only 1.5-fold more lactate than lithoautotrophically grown cells (Table 1).

**CO2 requirements for mixotrophy**

In response to the observation that *N. hamburgensis* could consume lactate to support growth and respiration, we further assessed if this bacterium could use D-lactate as its sole carbon source in the presence of 35 mM NO$_2^-$ and in the absence of CO$_2$. Growth and NO$_2^-$ consumption were monitored in a closed system (preventing the influx of atmospheric CO$_2$) during treatment with sodium carbonate (Na$_2$CO$_3$) and/or D-lactate as carbon sources (Table 3). In the absence of an added carbon source, growth did not occur in the closed system, despite ~14 mmol NO$_2^-$ being consumed during the experiment (4 days). When 0.5 mM D-lactate was provided as the sole source of carbon, measurable growth did not occur and NO$_2^-$ consumption was similar to that of the negative control cultures without an added carbon source. When a limiting amount of Na$_2$CO$_3$ (0.19 mM) was added, in the presence or absence of D-lactate, similar amounts of growth and NO$_2^-$ consumption were measured (Table 3). When Na$_2$CO$_3$ was provided in excess (0.75 mM), the OD$_{600}$ increased twofold over cultures containing 0.19 mM Na$_2$CO$_3$, and nearly all of the NO$_2^-$ was consumed. In contrast to the lack of stimulation observed when D-lactate was added to CO$_2$-limited cultures, the growth yield of cultures containing both D-lactate and 0.75 mM sodium carbonate increased significantly (45%) compared with cultures containing 0.75 mM Na$_2$CO$_3$ alone. Growth and NO$_2^-$ consumption were not affected when a higher concentration (1.5 mM) of Na$_2$CO$_3$ was added, or if parallel culture bottles were exposed to atmospheric CO$_2$ (data not shown), indicating that 0.75 mM Na$_2$CO$_3$ was saturating lithoautotrophic growth.

**Effect of NO$_2^-$ on lactate and CO$_2$ metabolism**

Since NO$_2^-$ slightly affected lactate consumption in mixotrophically grown cells under CO$_2$-replete conditions (Table 1), it was possible that NO$_2^-$ was affecting lactate consumption and organotrophic growth when CO$_2$ was limiting. Therefore, growth, net CO$_2$ production and lactate consumption were measured in a closed system in cultures containing D-lactate amended with 0, 7.5, 15 or 30 mM NO$_2^-$ initially containing an atmospheric concentration of CO$_2$ (Fig. 2). In cultures amended with NO$_2^-$, the ambient CO$_2$ was quickly fixed and remained undetectable until after the NO$_2^-$ was consumed (Fig. 2c). Although lactate was initially consumed in all treatments, lactate consumption increased in cultures without NO$_2^-$ and as the NO$_2^-$ was depleted in others (Fig. 2b, d). In the presence of 7.5 mM NO$_2^-$, the same amount of lactate was consumed and growth was enhanced by 20% compared with cultures containing lactate alone. In contrast, in cultures amended with higher concentrations of NO$_2^-$ (15 and 30 mM), growth was almost completely inhibited, CO$_2$ remained undetectable, and lactate consumption was suppressed until after depletion of NO$_2^-$. The presence of NO$_2^-$ also negatively affected the rate of lactate consumption. For example, when the lactate consumption values (Fig. 2d) were normalized to changes in OD$_{600}$ (Fig. 2a), the initial rates of lactate consumed per cell in all treatments ranged

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Assay condition†</th>
<th>D-Lactate consumed‡ [µmol lactate (mg protein)$^{-1}$ h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2^-$</td>
<td>D-Lactate</td>
<td>1.17 ± 0.06</td>
</tr>
<tr>
<td>NO$_2^-$ + D-lactate</td>
<td>D-Lactate</td>
<td>1.67 ± 0.07</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>NO$_2^-$ + D-lactate</td>
<td>1.14 ± 0.07</td>
</tr>
<tr>
<td>NO$_2^-$ + D-lactate</td>
<td>NO$_2^-$ + D-lactate</td>
<td>1.43 ± 0.13</td>
</tr>
</tbody>
</table>

* N. hamburgensis cells were harvested from 4-day cultures amended with 45 mM NO$_2^-$, or 45 mM NO$_2^-$ and 0.5 mM D-lactate.
† The initial concentrations of D-lactate and NO$_2^-$ in the assay were 1 and 40 mM, respectively.
‡ D-Lactate was measured periodically over a 3 h incubation. Values represent the mean ± SD of triplicate samples for each assay condition.

**Table 1. D-Lactate consumption rates by lithoautotrophic and mixotrophic cells of N. hamburgensis**

<table>
<thead>
<tr>
<th>Growth condition*</th>
<th>Assay condition†</th>
<th>D-Lactate consumed‡ [µmol lactate (mg protein)$^{-1}$ h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2^-$</td>
<td>D-Lactate</td>
<td>1.17 ± 0.06</td>
</tr>
<tr>
<td>NO$_2^-$ + D-lactate</td>
<td>D-Lactate</td>
<td>1.67 ± 0.07</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>NO$_2^-$ + D-lactate</td>
<td>1.14 ± 0.07</td>
</tr>
<tr>
<td>NO$_2^-$ + D-lactate</td>
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<td>1.43 ± 0.13</td>
</tr>
</tbody>
</table>

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† The initial concentrations of D-lactate and NO$_2^-$ in the assay were 1 and 40 mM, respectively.
‡ D-Lactate was measured periodically over a 3 h incubation. Values represent the mean ± SD of triplicate samples for each assay condition.

**Table 2. Respiration rates of lithoauto-, mixo- and organotrophically grown N. hamburgensis**

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>O$_2$ uptake* (nmol min$^{-1}$ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2^-$</td>
<td>630.9 ± 17.3</td>
</tr>
<tr>
<td>NO$_2^-$ + D-lactate</td>
<td>685.9 ± 31.6</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>367.7 ± 10.3</td>
</tr>
</tbody>
</table>

* Endogenous rates of O$_2$ uptake were subtracted from all reported values. Respiration rates were measured after a single passage for each respective growth condition. Values indicate the mean ± SD of triplicate samples for each growth condition.
from 39 to 130 μg lactate-C (OD600 unit)⁻¹ h⁻¹. Lactate consumption increased to a maximum rate of 284 μg lactate-C (OD600 unit)⁻¹ h⁻¹ by 66 h in cultures not amended with NO₂⁻. Yet, in cultures amended with 15 mM NO₂⁻, a low rate of lactate consumption [69 μg lactate-C (OD600 unit)⁻¹ h⁻¹] was sustained until after the NO₂⁻ was depleted, reaching a maximum rate [182 μg lactate-C (OD600 unit)⁻¹ h⁻¹] by 118 h. Taken together, these results suggest that NO₂⁻ is suppressing lactate consumption and that CO₂ fixation continues to occur in the presence of NO₂⁻ and lactate. Nevertheless, because lactate was consumed constitutively at a low rate, once the NO₂⁻ was depleted (or the NO₂⁻-induced CO₂ limitation was relieved) growth was able to occur with all treatments.

To further assess the effect of NO₂⁻ on lactate and CO₂ assimilation, the consumption and distribution of [1-¹⁴C]D-lactate were measured in cells harvested from lithoautotrophically, mixotrophically or organotrophically grown cultures of *N. hamburgensis* (Table 4). Regardless of how *N. hamburgensis* was grown, when cells were incubated with lactate in the absence of NO₂⁻, most ¹⁴C from lactate was retrieved as CO₂, and only 31–42 % of the ¹⁴C was incorporated into cells. In contrast, in the presence of

### Table 3. Growth and NO₂⁻ consumption by *N. hamburgensis* in response to the presence of carbonate and D-lactate

<table>
<thead>
<tr>
<th>CO₂⁻ concn (mM)</th>
<th>D-Lactate concn (mM)</th>
<th>OD₆₀₀⁺</th>
<th>NO₂⁻ consumed (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.016 ± 0.002</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td>0.19</td>
<td>0</td>
<td>0.023 ± 0.001</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td>0.75</td>
<td>0</td>
<td>0.047 ± 0.008</td>
<td>31.4 ± 2.4</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.016 ± 0.001</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>0.19</td>
<td>0.5</td>
<td>0.027 ± 0.004</td>
<td>25.2 ± 0.3</td>
</tr>
<tr>
<td>0.75</td>
<td>0.5</td>
<td>0.068 ± 0.005†</td>
<td>33.0 ± 0.3</td>
</tr>
</tbody>
</table>

*OD₆₀₀ and NO₂⁻ consumed, after 72 h of growth. The initial OD₆₀₀ and concentration of NO₂⁻ were 0.015 and 35 mM, respectively.
†Statistically significantly difference (P≤0.005) from cultures containing carbonate without lactate.

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**Fig. 2.** Effect of NO₂⁻ on growth, and on CO₂ and lactate consumption. *N. hamburgensis* cultures were amended with 0 (●), 7.5 (∆), 15 (■) and 30 mM NO₂⁻ (○). Growth (a), concentration of NO₂⁻ (b), CO₂ (c) and lactate (d) were monitored over the course of the experiment. The experiment was conducted in sealed bottles that initially contained atmospheric amounts of CO₂ (~0.038 %). Data points indicate the mean of triplicate cultures of each treatment.
**Table 4. Fate of [1-14C]lactate**

Values shown are in μmol lactate (mg protein)⁻¹. Each value represents the mean ± SD of three biological replicates.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Assay condition</th>
<th>Cells (%)</th>
<th>CO₂ (%)</th>
<th>Total consumed* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂⁻</td>
<td>Lactate</td>
<td>0.34 ± 0.01</td>
<td>31</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Lactate + NO₂⁻</td>
<td>0.73 ± 0.01</td>
<td>61</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>NO₂⁻ + D-lactate</td>
<td>Lactate</td>
<td>0.49 ± 0.02</td>
<td>42</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Lactate + NO₂⁻</td>
<td>0.66 ± 0.07</td>
<td>68</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>Lactate</td>
<td>0.67 ± 0.05</td>
<td>37</td>
<td>1.12 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Lactate + NO₂⁻</td>
<td>0.85 ± 0.05</td>
<td>63</td>
<td>0.50 ± 0.08</td>
</tr>
</tbody>
</table>

*Total amount of D-lactate consumed after 4 h of incubation.

NO₂⁻, a greater percentage of lactate-C was assimilated (61–68 %) and the percentage of ¹⁴C retrieved as CO₂ decreased significantly. More [¹⁴C]lactate was consumed by organotrophically grown cells than by autotrophic and mixotrophically grown cells, further suggesting a physiological adaptation to growth on lactate as a sole energy source. In the presence of NO₂⁻, more ¹⁴C was incorporated into cell material, regardless of how cells were grown, yet the proportional distribution and amount of ¹⁴C in cellular material versus CO₂ did not shift as drastically in cells that had been exposed to lactate previously. Furthermore, the total amount of [¹⁴C]lactate consumed by lithoautotrophically grown cells was unaffected by the presence of NO₂⁻, whereas lactate consumption decreased 18 and 25 % in mixotrophically and organotrophically grown *N. hamburgensis*, respectively.

Because (1) the amount and rate of lactate consumption correlated with changes in both NO₂⁻ and CO₂, and (2) CO₂ fixation appeared to be induced by NO₂⁻, we wanted to determine whether the suppressive effect of NO₂⁻ on lactate metabolism was caused indirectly by a CO₂ limitation. *N. hamburgensis* was grown organotrophically on lactate in sealed culture bottles with and without a CO₂ trap. In organotrophic cultures without a CO₂ trap, almost 90 % of the lactate (0.5 mM) was consumed by 96 h (Fig. 3b). When CO₂ was stripped from replicate cultures, the initial rates of growth and lactate consumption were not significantly altered. By 72 h, the rate of growth and lactate consumption slowed, leading to a 20 % decrease in growth yield (Fig. 3a, b). In contrast, when NO₂⁻ was added in lieu of a CO₂ trap, *N. hamburgensis* did not grow at all and 75 % of the initial concentration of lactate was not metabolized.

**DISCUSSION**

The ability of *N. hamburgensis* to metabolize lactate (a previously unknown organic carbon source for this organism) was predicted from the genome; however, the selective capacity of *N. hamburgensis* to grow on D- but not L-lactate was surprising given that both D- and L-ILDHs have been annotated (Starkenburg et al., 2008). The reason for the lack of growth on L-lactate is unclear as the putative L-isomer-specific iLDH (EC 1.1.2.3, Nham_1112) shares 78 % protein sequence identity with an iLDH in the close alphaproteobacterial relative of *N. hamburgensis*, *Rhodopseudomonas*, which can metabolize and grow on L-lactate (Horikiri et al., 2004; Markwell & Lascelles, 1978). Candidate genes for D-lactate metabolism include Nham_4010, a gene which encodes an FAD/FMN-dependent oxidase (EC 1.1.2.4) with an unspecified substrate range, and a putative glycolate oxidase (Nham_3202-4), which has been shown to turn over D-lactate in *Escherichia coli* (Lord, 1972). Transport may be a limiting factor for metabolism of both D- and L-lactate, since the only identified lactate permease gene (Nham_2174) is likely a pseudogene, given that a translated protein product would span two reading frames. Other candidate lactate transporters include a putative tellurite-resistance/dicarboxylate transporter (TDT-family transporter gene located in the operon that encodes the NO₂⁻ oxidoreductase. Similar TDT-family members have been shown to transport dicarboxylic acids (Grobler et al., 1995). Further investigation is required to determine which genes encode the products that are responsible for both the uptake and the initial metabolism of D-lactate.

Our results for the metabolism of organic carbon by *N. hamburgensis* appear to differ from published literature regarding organotrophy in both *N. winogradskyi* and *N. hamburgensis*. First, although NO₂⁻ stimulates acetate assimilation by both lithoautotrophically and organotrophically grown cells of *N. winogradskyi* (Smith & Hoare, 1968), NO₂⁻ did not affect lactate consumption by lithoautotrophically grown cells of *N. hamburgensis*, and NO₂⁻ even slightly reduced the rate of lactate consumption in mixotrophically and organotrophically grown cells. Second, although organic compounds in general positively affect the rate of NO₂⁻ oxidation in other *Nitrobacter* species (Steinmuller & Bock, 1976; Tandon & Mishra, 1968), our data suggest that lactate does not increase the
rate of NO\textsubscript{2} oxidation in \textit{N. hamburgensis}, but instead reduces the NO\textsubscript{2} requirement of the cell, as evidenced by a faster growth rate and an increase in growth yield in mixotrophic versus lithoautotrophic conditions. Third, an early description of the growth phenotype of \textit{N. hamburgensis} indicated that organotrophic growth rates on pyruvate, acetate or glycerol were faster than lithoautotrophic growth (Bock \textit{et al.}, 1983). In our laboratory, the generation time of \textit{D}-lactate-grown cells was threefold slower than that of lithoautotrophic cultures, and growth rates on pyruvate or acetate were also consistently slower than either lithoautotrophic or mixotrophic growth (our unpublished results). The discrepancy in organotrophic growth rates between our studies and other reports may be explained by the fact that a chemically defined minimal medium was used in our experiments, whereas in earlier investigations, \textit{N. hamburgensis} was grown in an undefined, complex medium containing yeast extract and peptone (Bock \textit{et al.}, 1983; Harris \textit{et al.}, 1988; Kirstein & Bock, 1993; Sundermeyer-Klinger \textit{et al.}, 1984). We have observed that ammonium amendment of NO\textsubscript{2}-containing cultures enhances the growth rate and cell yield of lithoautotrophically grown \textit{N. hamburgensis} (our unpublished results), implying that reduced forms of organic nitrogen could have stimulated the faster mixotrophic and organotrophic growth rates of \textit{N. hamburgensis} reported in the earlier studies.

Although a lactate metabolic pathway in \textit{N. hamburgensis} appears to be constitutively expressed, our results indicate that the physiology of \textit{N. hamburgensis} changes in response to lactate, in both the presence and absence of NO\textsubscript{2}. The rates of \textit{D}-lactate consumption and \textit{D}-lactate-dependent O\textsubscript{2} uptake were faster in organotrophically compared to lithoautotrophically grown cells, and others have shown that a shift occurs in the cytochrome and FMN content of organotrophically grown \textit{N. hamburgensis} (Bock \textit{et al.}, 1986; Kirstein \textit{et al.}, 1986). These adaptive changes also complement our observation that lactate-dependent O\textsubscript{2} uptake rates were threefold higher in organotrophically and mixotrophically grown cells compared with cells harvested from lithoautotrophic cultures.

Despite these physiological adaptations, \textit{N. hamburgensis} could only use \textit{D}-lactate as the sole carbon source in the absence, but not the presence, of NO\textsubscript{2}, suggesting (but not proving conclusively) that lithoheterotrophic growth is not possible. The apparent inability of \textit{N. hamburgensis} to grow on NO\textsubscript{2} and \textit{D}-lactate (a C3 molecule) as the sole energy and carbon source, respectively, could be the result of an insufficient supply of the CO\textsubscript{2} required for biosynthetic carboxylations. However, this hypothesis is not consistent with findings from an earlier study in which various C4–C6 compounds did not rescue growth of \textit{N. winogradskyi} in the absence of CO\textsubscript{2} (Delwiche & Feinstein, 1965). Alternatively, the lack of growth and the reduced rate of lactate metabolism in the presence of NO\textsubscript{2} during CO\textsubscript{2} limitation may be explained by a redox imbalance. Since reductant for biosynthesis is thought to be generated via an energetically unfavourable reverse flow of electrons from NO\textsubscript{2} to complex I (Sewell & Aleem, 1969), continuous consumption of reductant by CO\textsubscript{2} fixation (and the concomitant maintenance of an oxidized pool of electron carriers) may be required to sustain reverse electron flow from NO\textsubscript{2}. Use of CO\textsubscript{2} fixation as a ‘reductant sink’ is not unprecedented, as other alphaproteobacteria maintain redox balance by fixing CO\textsubscript{2} to regenerate oxidized electron.

Fig. 3. Effect of CO\textsubscript{2} limitation on organotrophic growth of \textit{N. hamburgensis} on \textit{D}-lactate. Turbidometric growth (a), and \textit{D}-lactate (b) and NO\textsubscript{2} (c) consumption were monitored under different types of CO\textsubscript{2} limitation. Cultures (50 ml) in sealed bottles were inoculated with NO\textsubscript{2}-grown cells to an initial OD\textsubscript{600} of 0.015 into media containing 0.5 mM \textit{D}-lactate (○), 0.5 mM \textit{D}-lactate+KOH trap (□), or 35 mM NO\textsubscript{2} and 0.5 mM \textit{D}-lactate (▲). An additional supplement of 20 mM NO\textsubscript{2} was added [indicated by an arrow in (c)] to the NO\textsubscript{2}-containing culture at 72 h to avoid NO\textsubscript{2} limitation.
carriers during photoheterotrophic growth (Dubbs & Tabita, 2004; Shively et al., 1998). Similarly, if oxidized electron carriers cannot be regenerated efficiently by *N. hamburgensis* in the absence of CO₂, they would be unavailable to serve as electron acceptors to oxidize lactate. It is possible that *N. hamburgensis* is able to overcome this redox imbalance after a more prolonged adaptation to organotrophic growth, since reduced electron carriers generated from lactate oxidation must be driving a forward flow of electrons to oxygen to generate ATP. When other facultative lithoautotrophs encounter organic carbon, in many cases a complete repression of autotrophic CO₂ fixation occurs (Shively et al., 1998; Tabita, 1988). On the other hand, in both *Thiobacillus intermedius* and *Ralstonia eutropha*, RuBisCO and other Calvin cycle enzymes are only partially repressed by the presence of some organic carbon sources and fully repressed by others (Shively et al., 1998; Tabita, 1988). At least some repression of autotrophy occurs in *Nitrobacter*, because RuBisCO activity has been reported to be suppressed 50–99 % in organotrophically grown cells of *N. winogradskyi* strain ‘agilis’ (Smith & Hoare, 1968; Steinmuller & Bock, 1977) and our results indicate that NO₃⁻-dependent O₂ uptake was also suppressed by ~40 % after growth solely on lactate. Undoubtedly, further experimentation is required to determine the details of how the redox state of a NO₃⁻-driven autotrophic metabolism in *N. hamburgensis* is affected by D-lactate.

In summary, our data suggest that *Nitrobacter* can take full advantage of D-lactate when it is the sole source of energy, yet if NO₃⁻ is present, the organism’s heterotrophic and mixotrophic potential is hampered by an inability to stop fixing CO₂ and efficiently switch to an organic carbon source. Further investigations of the obligate requirement of *Nitrobacter* for CO₂ during growth on NO₃⁻ may help elucidate the physiological constraints of utilizing relatively poor inorganic energy sources, and aid in understanding how *N. hamburgensis* and other facultative lithoautotrophs regulate the metabolism of mixed energy and carbon sources.

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