

# D-Lactate metabolism and the obligate requirement for CO<sub>2</sub> during growth on nitrite by the facultative lithoautotroph *Nitrobacter hamburgensis*

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*Nitrobacter hamburgensis* X14 is a facultative lithoautotroph that conserves energy from the oxidation of nitrite (NO<sub>2</sub><sup>-</sup>) and fixes carbon dioxide (CO<sub>2</sub>) as its sole source of carbon. The availability of the *N. hamburgensis* X14 genome sequence initiated a re-examination of its mixotrophic and organotrophic potential, as genes encoding three flavin-dependent oxidases were identified that may function to oxidize lactate, providing energy and carbon for growth. The response of *N. hamburgensis* to D- and L-lactate in the presence (mixotrophy) and absence (organotrophy) of NO<sub>2</sub><sup>-</sup> was examined. L-Lactate did not support organotrophic growth or stimulate mixotrophic growth. In contrast, D-lactate enhanced the growth rate and yield of *N. hamburgensis* in the presence of NO<sub>2</sub><sup>-</sup>, and served as the sole carbon and energy source for growth in the absence of NO<sub>2</sub><sup>-</sup> with ammonium as the sole nitrogen source. Lithoautotrophically grown cells immediately consumed D-lactate, suggesting that a lactate metabolic pathway is constitutively expressed. Nevertheless, a physiological adaptation to lactate occurred, as D-lactate-grown cells consumed and assimilated lactate at a faster rate than NO<sub>2</sub><sup>-</sup>-grown cells, and the D-lactate-dependent O<sub>2</sub> uptake rate was significantly greater in cells grown either organotrophically or mixotrophically compared with cells grown lithoautotrophically. Although D-lactate was assimilated and metabolized to CO<sub>2</sub> in the presence or absence of NO<sub>2</sub><sup>-</sup>, exposure to atmospheric CO<sub>2</sub> or the addition of 0.75 mM sodium carbonate was required for mixotrophic growth and for optimum organotrophic growth on D-lactate.

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## INTRODUCTION

Nitrification is a two-step, biologically mediated process through which ammonia is converted into nitrate (NO<sub>3</sub><sup>-</sup>). Nitrite-oxidizing bacteria (NOB) participate in the second step of nitrification by converting nitrite (NO<sub>2</sub><sup>-</sup>) into NO<sub>3</sub><sup>-</sup>. NOB use NO<sub>2</sub><sup>-</sup> as their primary energy source to generate reductant, assimilate carbon dioxide (CO<sub>2</sub>), and drive oxidative phosphorylation. NO<sub>2</sub><sup>-</sup> is a relatively poor energy substrate due to the positive midpoint potential of the NO<sub>3</sub><sup>-</sup>–NO<sub>2</sub><sup>-</sup> couple ( $E_0^{\prime} = +420$  mV). Since it is estimated that 85–115 moles NO<sub>2</sub><sup>-</sup> are required to fix 1 mole CO<sub>2</sub> (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 NO<sub>2</sub><sup>-</sup> to meet the energy demands of biosynthesis.

Although several phylogenetically distinct genera carry out NO<sub>2</sub><sup>-</sup> 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; Steinmuller & 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 CO<sub>2</sub> is stripped from cultures containing organic carbon and NO<sub>2</sub><sup>-</sup> (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

Abbreviations: iLDH, flavin-dependent LDH; LDH, lactate dehydrogenase; NOB, nitrite-oxidizing bacteria.

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  $\text{NO}_2^-$  have not been completed. Furthermore, it is not known whether organic carbon can be used as the sole carbon source in the presence of  $\text{NO}_2^-$  (lithoheterotrophy), or whether organic carbon positively or negatively influences the rate of  $\text{NO}_2^-$  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 affect 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  $\mu\text{M}$   $\text{MgSO}_4$ , 30  $\mu\text{M}$   $\text{FeSO}_4$ , 50  $\mu\text{M}$  EDTA, 200  $\mu\text{M}$   $\text{CaCl}_2$  and 1  $\mu\text{M}$   $\text{CuSO}_4$ . A 1 ml volume of a trace metals stock solution (400  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 1 mM  $\text{MnCl}_2$ , 350  $\mu\text{M}$   $\text{ZnSO}_4$ , 8.4  $\mu\text{M}$   $\text{CoCl}_2$ ) was added per 2 l base medium. After autoclaving, the medium was amended with phosphate buffer stock solution (480 mM  $\text{KH}_2\text{PO}_4$ , 42 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.8) at 20 ml  $\text{l}^{-1}$ .  $\text{NaNO}_2$  (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  $(\text{NH}_4)_2\text{SO}_4$  as a nitrogen source. Mixotrophic growth medium contained 30–45 mM  $\text{NaNO}_2$ , 2 mM  $(\text{NH}_4)_2\text{SO}_4$  and 0.5 mM sodium D-lactate. Growth was monitored routinely by determining the  $\text{OD}_{600}$ . Growth experiments were conducted in sterile 160 ml culture bottles prepared with 50 ml culture medium inoculated to initial  $\text{OD}_{600}$  0.015. To prevent the influx of atmospheric  $\text{CO}_2$  during closed-system experiments, the cultures were sealed with Teflon-faced grey butyl rubber stoppers (Supelco) and fastened with aluminium crimp seals. To avoid  $\text{O}_2$  depletion limiting cell growth,  $\text{O}_2$  in the headspace was monitored daily and replenished as needed.  $\text{NO}_2^-$  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  $\mu\text{l}$  samples on Luria agar plates or by inoculating 1/10 nutrient broth (Difco) containing glucose (1 g  $\text{l}^{-1}$ ). Culture purity of lactate-containing cultures was additionally verified by visualization of cells at  $\times 25\,000$  magnification with a transmission electron microscope.

**$\text{O}_2$ -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  $\text{NO}_2^-$ -dependent  $\text{O}_2$  uptake by cell suspensions were measured with a Clark-type  $\text{O}_2$  electrode (Yellow Springs Instrument) mounted in an all-glass, water-jacketed reaction vessel (1.8 ml volume) held at 30 °C.

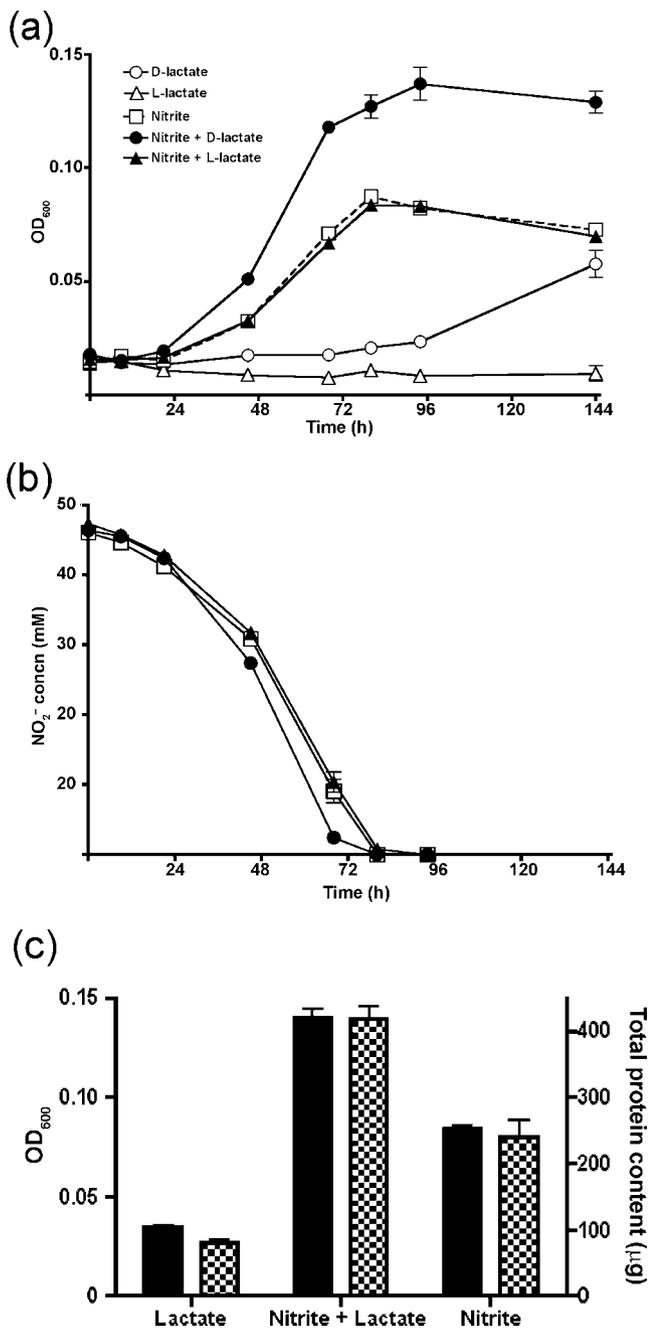
**Lactate consumption assay.** Lithotrophically, mixotrophically and organotrophically grown cells of *N. hamburgensis* were harvested by centrifugation from late-exponential phase cultures, washed twice, and resuspended to  $\text{OD}_{600}$  0.3–1.0 in sterile phosphate-buffered base medium (pH 7.5). 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.  $\text{CO}_2$  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.

**[ $^{14}\text{C}$ ]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  $(\text{NH}_4)_2\text{SO}_4$ , 1 mM D-lactate and 1.4  $\mu\text{Ci}$  (518 Mbq) DL-[1- $^{14}\text{C}$ ]lactate (Sigma-Aldrich). *N. hamburgensis* was inoculated into these sealed bottles to  $\text{OD}_{600}$  0.4–0.6. Each culture bottle contained a 1.5 ml plastic tube containing a piece of Whatman filter paper soaked in 100  $\mu\text{l}$  18 M KOH to trap evolved  $\text{CO}_2$ . 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 Ecolume scintillation fluid (ICN), and  $^{14}\text{CO}_2$  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  $\mu\text{l}$  12.1 M hydrochloric acid to measure the residual  $^{14}\text{CO}_2$  dissolved in the incubation medium. This quantity was added to the radiolabel measured in the first KOH trap to determine the total  $^{14}\text{CO}_2$  released from lactate. Cellular incorporation of radioactive  $^{14}\text{C}$  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  $\mu\text{l}$  aliquot of the cell suspension was added to a vial containing 3.5 ml scintillation fluid, and cellular  $^{14}\text{C}$  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  $\text{NO}_2^-$ , to assess its mixotrophic and organotrophic potential. When *N. hamburgensis* was grown in medium supplemented with D-lactate and  $\text{NO}_2^-$ , the growth rate and final cell yield increased 50 and 60 %, respectively, compared with cultures grown lithoautotrophically on medium containing  $\text{NO}_2^-$  and  $\text{CO}_2$  (Fig. 1a). Concomitantly, an increase in the total protein content was also observed in *N. hamburgensis* cells grown



**Fig. 1.** Growth response of *N. hamburgensis* to lactate in the presence and absence of  $\text{NO}_2^-$ . Turbidometric growth,  $\text{NO}_2^-$  consumption and the protein content of whole cells were measured, in response to D- or L-lactate, in panels (a), (b) and (c), respectively. Cultures were amended with either 1 mM D-lactate (○), 1 mM L-lactate (△), 45 mM  $\text{NO}_2^-$  (□), 45 mM  $\text{NO}_2^-$  and 1 mM D-lactate (●), or 45 mM  $\text{NO}_2^-$  and 1 mM L-lactate (▲). Measurements depicted in (c) are from a representative experiment harvested at 100 h; filled and chequered bars represent  $\text{OD}_{600}$  and protein content, respectively.

mixotrophically with D-lactate and  $\text{NO}_2^-$  (Fig. 1c). Because D-lactate did not affect the gross amount of  $\text{NO}_2^-$  consumed (Fig. 1b), the increased growth rate and cell yield indicated that less  $\text{NO}_2^-$  was consumed per  $\text{OD}_{600}$  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  $\text{NO}_2^-$ . Similarly, when the amount of protein recovered from these cultures was normalized to  $\text{OD}_{600}$ , organotrophically grown cells contained 30 % less protein per  $\text{OD}_{600}$  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  $\text{NO}_2^-$  and  $\text{CO}_2$ , and growth was not detected when L-lactate was added in the absence of  $\text{NO}_2^-$ . 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  $\text{NO}_2^-$  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  $1.17 \mu\text{mol D-lactate (mg protein)}^{-1} \text{h}^{-1}$ . This short-term rate of lactate consumption by lithoautotrophically grown cells was not significantly affected by the addition of  $\text{NO}_2^-$ . Mixotrophically grown cells consumed lactate at a faster rate [ $1.67 \mu\text{mol D-lactate (mg protein)}^{-1} \text{h}^{-1}$ ] than lithoautotrophically grown cells, suggesting that *N. hamburgensis* had adapted to growth on lactate. In the presence of  $\text{NO}_2^-$ , 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,  $\text{NO}_2^-$ -dependent respiration was reduced after organotrophic growth on lactate, whereas mixotrophically grown cells maintained high rates of both  $\text{NO}_2^-$ - and lactate-dependent  $\text{O}_2$  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

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

Growth condition*	Assay condition†	D-Lactate consumed‡ [ $\mu\text{mol lactate (mg protein)}^{-1} \text{ h}^{-1}$ ]
$\text{NO}_2^-$	D-Lactate	$1.17 \pm 0.06$
$\text{NO}_2^- + \text{D-lactate}$	D-Lactate	$1.67 \pm 0.07$
$\text{NO}_2^-$	$\text{NO}_2^- + \text{D-lactate}$	$1.14 \pm 0.07$
$\text{NO}_2^- + \text{D-lactate}$	$\text{NO}_2^- + \text{D-lactate}$	$1.43 \pm 0.13$

\**N. hamburgensis* cells were harvested from 4-day cultures amended with 45 mM  $\text{NO}_2^-$ , or 45 mM  $\text{NO}_2^-$  and 0.5 mM D-lactate.

†The initial concentrations of D-lactate and  $\text{NO}_2^-$  in the assay were 1 and 40 mM, respectively.

‡D-Lactate was measured periodically over a 3 h incubation. Values represent the mean  $\pm$  SD of triplicate samples for each assay condition.

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).

### CO<sub>2</sub> 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  $\text{NO}_2^-$  and in the absence of CO<sub>2</sub>. Growth and  $\text{NO}_2^-$  consumption were monitored in a closed system (preventing the influx of atmospheric CO<sub>2</sub>) during treatment with sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) 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  $\text{NO}_2^-$  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  $\text{NO}_2^-$  consumption was similar to that of the negative control cultures without an added carbon source. When a limiting amount of Na<sub>2</sub>CO<sub>3</sub> (0.19 mM) was added, in the presence or absence of D-lactate, similar amounts of growth and  $\text{NO}_2^-$  consumption were measured (Table 3). When Na<sub>2</sub>CO<sub>3</sub>

was provided in excess (0.75 mM), the OD<sub>600</sub> increased twofold over cultures containing 0.19 mM Na<sub>2</sub>CO<sub>3</sub>, and nearly all of the  $\text{NO}_2^-$  was consumed. In contrast to the lack of stimulation observed when D-lactate was added to CO<sub>2</sub>-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<sub>2</sub>CO<sub>3</sub> alone. Growth and  $\text{NO}_2^-$  consumption were not affected when a higher concentration (1.5 mM) of Na<sub>2</sub>CO<sub>3</sub> was added, or if parallel culture bottles were exposed to atmospheric CO<sub>2</sub> (data not shown), indicating that 0.75 mM Na<sub>2</sub>CO<sub>3</sub> was saturating lithoautotrophic growth.

### Effect of $\text{NO}_2^-$ on lactate and CO<sub>2</sub> metabolism

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

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

Growth condition	O <sub>2</sub> uptake* (nmol min <sup>-1</sup> mg <sup>-1</sup> )	
	$\text{NO}_2^-$	Lactate
$\text{NO}_2^-$	$630.9 \pm 17.3$	$5.9 \pm 1.0$
$\text{NO}_2^- + \text{D-lactate}$	$685.9 \pm 31.6$	$16.3 \pm 0.8$
D-Lactate	$367.7 \pm 10.3$	$16.2 \pm 1.4$

\*Endogenous rates of O<sub>2</sub> uptake were subtracted from all reported values. Respiration rates were measured after a single passage for each respective growth condition. Values indicate the mean  $\pm$  SD of triplicate samples for each growth condition.

**Table 3.** Growth and  $\text{NO}_2^-$  consumption by *N. hamburgensis* in response to the presence of carbonate and D-lactate

$\text{CO}_2^{3-}$ concn (mM)	D-Lactate concn (mM)	$\text{OD}_{600}^*$	$\text{NO}_2^-$ consumed (mM)*
0	0	$0.016 \pm 0.002$	$14.3 \pm 0.3$
0.19	0	$0.023 \pm 0.001$	$23.4 \pm 0.3$
0.75	0	$0.047 \pm 0.008$	$31.4 \pm 2.4$
0	0.5	$0.016 \pm 0.001$	$15.3 \pm 0.3$
0.19	0.5	$0.027 \pm 0.004$	$25.2 \pm 0.3$
0.75	0.5	$0.068 \pm 0.005^\dagger$	$33.0 \pm 0.3$

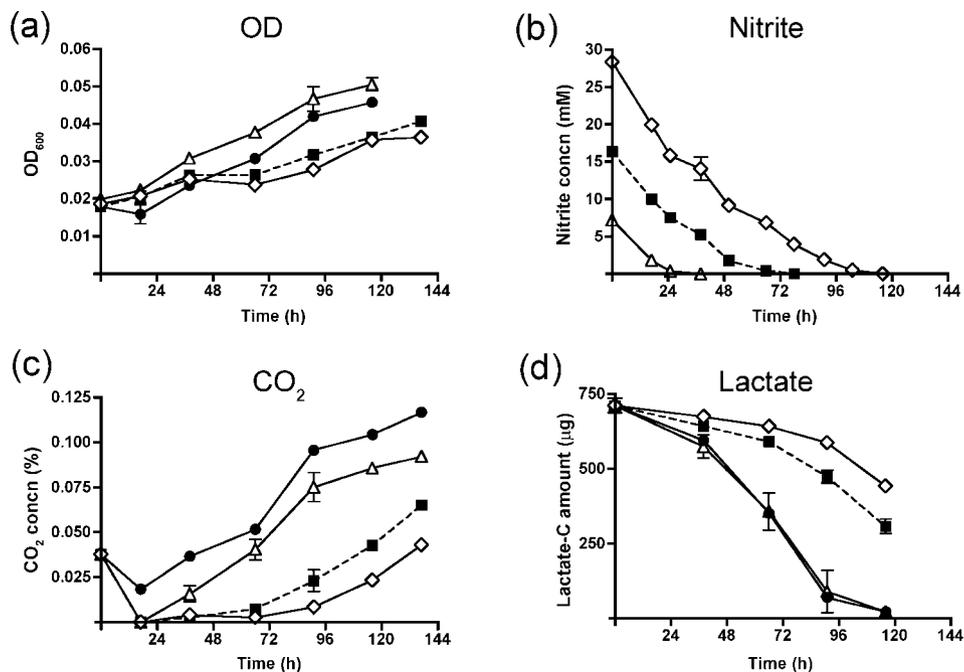
\* $\text{OD}_{600}$ , and  $\text{NO}_2^-$  consumed, after 72 h of growth. The initial  $\text{OD}_{600}$  and concentration of  $\text{NO}_2^-$  were 0.015 and 35 mM, respectively.

†Statistically significantly difference ( $P \leq 0.005$ ) from cultures containing carbonate without lactate.

from 39 to 130  $\mu\text{g}$  lactate-C ( $\text{OD}_{600}$  unit) $^{-1}$  h $^{-1}$ . Lactate consumption increased to a maximum rate of 284  $\mu\text{g}$  lactate-C ( $\text{OD}_{600}$  unit) $^{-1}$  h $^{-1}$  by 66 h in cultures not amended with  $\text{NO}_2^-$ . Yet, in cultures amended with 15 mM  $\text{NO}_2^-$ , a low rate of lactate consumption [69  $\mu\text{g}$  lactate-C ( $\text{OD}_{600}$  unit) $^{-1}$  h $^{-1}$ ] was sustained until after the  $\text{NO}_2^-$  was depleted, reaching a maximum rate [182  $\mu\text{g}$  lactate-C ( $\text{OD}_{600}$  unit) $^{-1}$  h $^{-1}$ ] by 118 h. Taken together, these results suggest that  $\text{NO}_2^-$  is suppressing lactate consumption and that  $\text{CO}_2$  fixation continues to occur in the presence of  $\text{NO}_2^-$  and lactate. Nevertheless, because lactate was consumed constitutively at a low rate, once the  $\text{NO}_2^-$

was depleted (or the  $\text{NO}_2^-$ -induced  $\text{CO}_2$  limitation was relieved) growth was able to occur with all treatments.

To further assess the effect of  $\text{NO}_2^-$  on lactate and  $\text{CO}_2$  assimilation, the consumption and distribution of [1- $^{14}\text{C}$ ]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  $\text{NO}_2^-$ , most  $^{14}\text{C}$  from lactate was retrieved as  $\text{CO}_2$ , and only 31–42% of the  $^{14}\text{C}$  was incorporated into cells. In contrast, in the presence of



**Fig. 2.** Effect of  $\text{NO}_2^-$  on growth, and on  $\text{CO}_2$  and lactate consumption. *N. hamburgensis* cultures were amended with 0 (●), 7.5 (△), 15 (■) and 30 mM  $\text{NO}_2^-$  (◇). Growth (a), concentration of  $\text{NO}_2^-$  (b),  $\text{CO}_2$  (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  $\text{CO}_2$  (~0.038%). Data points indicate the mean of triplicate cultures of each treatment.

**Table 4.** Fate of [1-<sup>14</sup>C]lactate

Values shown are in  $\mu\text{mol lactate (mg protein)}^{-1}$ . Each value represents the mean  $\pm$  SD of three biological replicates.

Growth condition	Assay condition	Fate				
		Cells	(%)	CO <sub>2</sub>	(%)	Total consumed*
NO <sub>2</sub> <sup>-</sup>	Lactate	0.34 $\pm$ 0.01	31	0.74 $\pm$ 0.08	69	1.07 $\pm$ 0.08
	Lactate + NO <sub>2</sub> <sup>-</sup>	0.73 $\pm$ 0.01	61	0.47 $\pm$ 0.08	39	1.21 $\pm$ 0.07
NO <sub>2</sub> <sup>-</sup> + D-lactate	Lactate	0.49 $\pm$ 0.02	42	0.68 $\pm$ 0.05	58	1.18 $\pm$ 0.07
	Lactate + NO <sub>2</sub> <sup>-</sup>	0.66 $\pm$ 0.07	68	0.31 $\pm$ 0.05	32	0.97 $\pm$ 0.04
D-Lactate	Lactate	0.67 $\pm$ 0.05	37	1.12 $\pm$ 0.06	63	1.80 $\pm$ 0.11
	Lactate + NO <sub>2</sub> <sup>-</sup>	0.85 $\pm$ 0.05	63	0.50 $\pm$ 0.08	37	1.35 $\pm$ 0.13

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

NO<sub>2</sub><sup>-</sup>, a greater percentage of lactate-C was assimilated (61–68%) and the percentage of <sup>14</sup>C retrieved as CO<sub>2</sub> decreased significantly. More [<sup>14</sup>C]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 NO<sub>2</sub><sup>-</sup>, more <sup>14</sup>C was incorporated into cell material, regardless of how cells were grown, yet the proportional distribution and amount of <sup>14</sup>C in cellular material versus CO<sub>2</sub> did not shift as drastically in cells that had been exposed to lactate previously. Furthermore, the total amount of [<sup>14</sup>C]lactate consumed by lithoautotrophically grown cells was unaffected by the presence of NO<sub>2</sub><sup>-</sup>, whereas lactate consumption decreased 18 and 25% in mixotrophically and organotrophically grown *N. hamburgensis*, respectively.

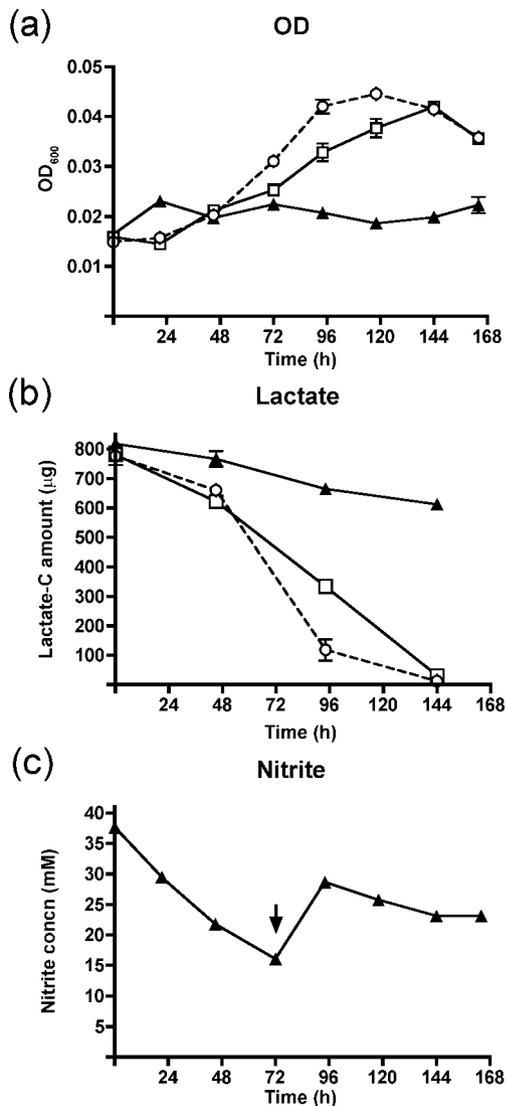
Because (1) the amount and rate of lactate consumption correlated with changes in both NO<sub>2</sub><sup>-</sup> and CO<sub>2</sub>, and (2) CO<sub>2</sub> fixation appeared to be induced by NO<sub>2</sub><sup>-</sup>, we wanted to determine whether the suppressive effect of NO<sub>2</sub><sup>-</sup> on lactate metabolism was caused indirectly by a CO<sub>2</sub> limitation. *N. hamburgensis* was grown organotrophically on lactate in sealed culture bottles with and without a CO<sub>2</sub> trap. In organotrophic cultures without a CO<sub>2</sub> trap, almost 90% of the lactate (0.5 mM) was consumed by 96 h (Fig. 3b). When CO<sub>2</sub> 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 NO<sub>2</sub><sup>-</sup> was added in lieu of a CO<sub>2</sub> 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 NO<sub>2</sub><sup>-</sup> 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 NO<sub>2</sub><sup>-</sup> stimulates acetate assimilation by both lithoautotrophically and organotrophically grown cells of *N. winogradskyi* (Smith & Hoare, 1968), NO<sub>2</sub><sup>-</sup> did not affect lactate consumption by lithoautotrophically grown cells of *N. hamburgensis*, and NO<sub>2</sub><sup>-</sup> 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 NO<sub>2</sub><sup>-</sup> oxidation in other *Nitrobacter* species (Steinmuller & Bock, 1976; Tandon & Mishra, 1968), our data suggest that lactate does not increase the



**Fig. 3.** Effect of CO<sub>2</sub> limitation on organotrophic growth of *N. hamburgensis* on D-lactate. Turbidimetric growth (a), and D-lactate (b) and NO<sub>2</sub><sup>-</sup> (c) consumption were monitored under different types of CO<sub>2</sub> limitation. Cultures (50 ml) in sealed bottles were inoculated with NO<sub>2</sub><sup>-</sup>-grown cells to an initial OD<sub>600</sub> of 0.015 into media containing 0.5 mM D-lactate (○), 0.5 mM D-lactate + KOH trap (□), or 35 mM NO<sub>2</sub><sup>-</sup> and 0.5 mM D-lactate (▲). An additional supplement of 20 mM NO<sub>2</sub><sup>-</sup> was added [indicated by an arrow in (c)] to the NO<sub>2</sub><sup>-</sup>-containing culture at 72 h to avoid NO<sub>2</sub><sup>-</sup> limitation.

rate of NO<sub>2</sub><sup>-</sup> oxidation in *N. hamburgensis*, but instead reduces the NO<sub>2</sub><sup>-</sup> 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 *N. hamburgensis* indicated that organotrophic growth rates on pyruvate, acetate or glycerol were faster than lithoautotrophic growth (Bock *et al.*, 1983). In our laboratory, the

generation time of 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, *N. hamburgensis* was grown in an undefined, complex medium containing yeast extract and peptone (Bock *et al.*, 1983; Harris *et al.*, 1988; Kirstein & Bock, 1993; Sundermeyer-Klinger *et al.*, 1984). We have observed that ammonium amendment of NO<sub>2</sub><sup>-</sup>-containing cultures enhances the growth rate and cell yield of lithoautotrophically grown *N. hamburgensis* (our unpublished results), implying that reduced forms of organic nitrogen could have stimulated the faster mixotrophic and organotrophic growth rates of *N. hamburgensis* reported in the earlier studies.

Although a lactate metabolic pathway in *N. hamburgensis* appears to be constitutively expressed, our results indicate that the physiology of *N. hamburgensis* changes in response to lactate, in both the presence and absence of NO<sub>2</sub><sup>-</sup>. The rates of D-lactate consumption and D-lactate-dependent O<sub>2</sub> 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 *N. hamburgensis* (Bock *et al.*, 1986; Kirstein *et al.*, 1986). These adaptive changes also complement our observation that lactate-dependent O<sub>2</sub> uptake rates were threefold higher in organotrophically and mixotrophically grown cells compared with cells harvested from lithoautotrophic cultures.

Despite these physiological adaptations, *N. hamburgensis* could only use D-lactate as the sole carbon source in the absence, but not the presence, of NO<sub>2</sub><sup>-</sup>, suggesting (but not proving conclusively) that lithoheterotrophic growth is not possible. The apparent inability of *N. hamburgensis* to grow on NO<sub>2</sub><sup>-</sup> and D-lactate (a C3 molecule) as the sole energy and carbon source, respectively, could be the result of an insufficient supply of the CO<sub>2</sub> 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 *N. winogradskyi* in the absence of CO<sub>2</sub> (Delwiche & Feinstein, 1965). Alternatively, the lack of growth and the reduced rate of lactate metabolism in the presence of NO<sub>2</sub><sup>-</sup> during CO<sub>2</sub> 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<sub>2</sub><sup>-</sup> to complex I (Sewell & Aleem, 1969), continuous consumption of reductant by CO<sub>2</sub> fixation (and the concomitant maintenance of an oxidized pool of electron carriers) may be required to sustain reverse electron flow from NO<sub>2</sub><sup>-</sup>. Use of CO<sub>2</sub> fixation as a 'reductant sink' is not unprecedented, as other alphaproteobacteria maintain redox balance by fixing CO<sub>2</sub> to regenerate oxidized electron

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 CO<sub>2</sub>, 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 CO<sub>2</sub> 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 NO<sub>2</sub><sup>-</sup>-dependent O<sub>2</sub> 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 NO<sub>2</sub><sup>-</sup>-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 NO<sub>2</sub><sup>-</sup> is present, the organism’s heterotrophic and mixotrophic potential is hampered by an inability to stop fixing CO<sub>2</sub> and efficiently switch to an organic carbon source. Further investigations of the obligate requirement of *Nitrobacter* for CO<sub>2</sub> during growth on NO<sub>2</sub><sup>-</sup> 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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