CO$_2$ production, O$_2$ consumption and isocitrate dehydrogenase in the marine bacterium _Vibrio natriegens_

Elisa Berdalet$^1$, *, Ted Packard$^2$, Bernadette Lagacé$^2$, Sylvie Roy$^2$, Lily St-Amand$^2$, Jean-Pierre Gagné$^3$

$^1$Institut de Ciències del Mar (CSIC), P. Joan de Borbó, Comte de Barcelona s/n, E-08039 Barcelona, Spain
$^2$Institut Maurice Lamontagne, Pêches et Océans, CP 1000, Mont-Joli, Québec, Canada G5H 3Z4
$^3$Université de Québec à Rimouski, 310, allée des Ursulines, Rimouski, Québec, Canada G5L 3A1

ABSTRACT: The respiratory metabolism of the marine bacterium _Vibrio natriegens_ growing in batch cultures with acetate and pyruvate as carbon sources was studied. In particular, the relationship of the activity of the enzyme isocitrate dehydrogenase (IDH) to physiological CO$_2$ production and O$_2$ consumption was examined. Gas measurements were performed by a new type of respirometer that combined O$_2$ detection by a Pb-O fuel cell and CO$_2$ detection by infrared absorption. Two different respiratory patterns were observed. On pyruvate, CO$_2$ production and O$_2$ consumption rates paralleled each other during the exponential and the stationary phases. On acetate, they did not. Growth based on acetate was characterized by a higher O$_2$ consumption, lower CO$_2$ production, lower respiratory quotient and lower IDH activity than on pyruvate. In both culture media, the _in vitro_ IDH activity remained elevated after the _in vivo_ CO$_2$ production had decreased when the carbon source was exhausted. The range of the respiratory quotients obtained in the acetate cultures suggests that the acetate is partitioned between the Krebs cycle and the glyoxylate bypass in the proportions of 1:4 to 1:2. In the pyruvate cultures, the range of the respiratory quotients indicates that, in the course of the different growth phases, the partitioning of the carbon source between the Krebs cycle and the anaplerotic pathways is variable.

KEY WORDS: CO$_2$ production rates. O$_2$ consumption rates. Isocitrate dehydrogenase activity. _Vibrio natriegens_. Micro-Oxymax

INTRODUCTION

Heterotrophic bacteria may use many different organic compounds as energy and carbon sources. The molecules that are used are channelled through central metabolic pathways (Holms 1986) to produce different carbon skeletons necessary for the synthesis of new cellular material. Under aerobic conditions, the carbon source is also completely oxidized to carbon dioxide. During the oxidation, reduced nucleotides are generated for use in the biosynthetic pathways and in energy production (i.e. ATP).

Perhaps the most important central metabolic pathway is the Krebs or tricarboxylic acid (TCA) cycle (Fig. 1). Through it the acetyl-CoA, formed from the degradation of carbohydrates, lipids and protein, is completely oxidized to CO$_2$. The reducing power [NAD(P)H, FADH$_2$] generated during this oxidation drives the electron transfer system (ETS) to phosphorylate ADP to ATP. Under aerobic conditions, the final electron acceptor is O$_2$. In addition, the Krebs cycle furnishes intermediates (alpha-ketoglutarate, oxaloacetate, succinate, etc.) which are precursors for biosynthesis of amino acids and polysaccharides.

The Krebs cycle is nearly universal in microbes. It allows growth on a variety of carboxylic acids with 4, 5 or 6 carbon atoms as carbon sources and electron donors. However, growth based on 2- or 3-carbon molecules can not be achieved by means of the Krebs cycle alone. For example, growth on acetate (a 2-carbon molecule) poses a particular problem. Acetate
During growth on acetate, IDH is partially inactivated (El-Mansi et al. 1985) in order to allow the operation of the glyoxylate cycle. Inactivation of IDH is accomplished by phosphorylation catalyzed by the bifunctional IDH kinase/phosphatase (LaPorte & Koshland 1982). While acetate serves to repress IDH activity, pyruvate (a 3-carbon molecule) serves to activate it (Bennett & Holms 1975).

In contrast to growth on acetate, growth on pyruvate is facilitated by other anaplerotic reactions that replenish the carboxylic acid intermediates of the Krebs cycle. These reactions are not the same as those of the glyoxylate cycle. For example, oxaloacetate is synthesized from pyruvate by the addition of a carbon atom from CO₂. This reaction is catalyzed by the pyruvate carboxylase or the phosphoenolpyruvate carboxylase (Fig. 1) (Gottschack 1986).

The behavior of IDH activity in the bacterium Escherichia coli growing on glucose, acetate, glycerol and pyruvate has been described in detail (Holms & Bennett 1971, Bennett & Holms 1975, Walsh & Koshland 1984, El-Mansi et al. 1986, Holms 1986). It has been described in thermophilic and mesophilic bacteria by Edlin & Sundaran (1989) and Novotny & Perry (1991); in Acinetobacter calcoaceticus by Hoyt & Reeves (1992); in root-nodule bacteria by Mandal & Chakraborty (1992); in yeast by Satrustegui et al. (1983); in Chlamydomonas by LalLiberté & Noüe (1993) and Martínez-Rivas & Vega (1993); and in several fish tissues by Childress & Somero (1979). However, no data on IDH activity in marine bacteria have been previously reported.

In our laboratory, we are interested in the estimation of CO₂ production rates by bacterioplankton, in particular through the use of enzymatic indices. Based on the important role of IDH in the production of CO₂ we initiated a series of experiments in order to test if the model described in the literature for Escherichia coli operated also in the marine bacterium Vibrio natriegens. In particular, we investigated the relationship between IDH activity and the respiratory processes in batch cultures of V. natriegens growing on acetate and pyruvate. According to the IDH-iCL model described for E. coli, IDH activities in the acetate-based cultures should be lower than in the pyruvate cultures. In addition, we hypothesized that lower IDH activity would be accompanied by lower CO₂ production. This is the first report of simultaneous measurements of IDH activity, CO₂ production and O₂ consumption rates in marine bacteria. It demonstrates that when growing on acetate, in contrast to pyruvate, growth rate, IDH specific activity and CO₂ production rates of V. natriegens were lower, but O₂ consumption rates were much higher. In a subsequent paper we will describe the estimation of the CO₂ production rates by the measurement of IDH activity (Packard et al. unpubl.).
MATERIALS AND METHODS

Bacterial cultures. Vibrio natriegens (ATCC 33788) were adapted to the experimental media (pyruvate or acetate) for at least 15 generations prior to the experiment. A culture in late exponential or early stationary phase was used as inoculum for the experiment. Cultures were continually agitated on an orbital shaker at 100 rpm at 22°C. Growth was followed by absorbance at 550 nm (OD550).

Culture media. Reagents were obtained from Sigma. The medium was developed from the media of Niven et al. (1977), Baumann & Baumann (1981), King & Berman (1984) and Nissen et al. (1987) after experiments in our laboratory established the optimal growth conditions for Vibrio natriegens. The culture medium contained: 400 mM NaCl, 10 mM MgSO4.7H2O, 10 mM CaCl2.2H2O, 10 mM KCl, 25 mM NH4Cl, 0.33 mM phosphate buffer, 0.01 mM FeSO4.7H2O, and 30 mM sodium acetate or 20 mM pyruvate. All components (except FeSO4.7H2O and the phosphate buffer) were dissolved in 0.22 μm filtered deionized water. The pH was adjusted to 7.5 with 1 N NaOH. The solution was filtered through a GF/F glass fiber filter to remove particles, and autoclaved for 45 min at 121°C. To avoid precipitate formation during autoclaving, the phosphate buffer (0.67 M, pH 7.5) and the iron sulphate solution (FeSO4.7H2O, 0.1 mM) were prepared separately. The phosphate buffer was sterilized by autoclaving and the iron sulphate solution by filtration through 0.22 μm acrodiscs. Both solutions were kept frozen and added to the culture medium on the day of use.

Experimental design. Cultures were grown in 25 cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of media. Initial absorbances at 550 nm after inoculation were 0.1. At intervals over the time course, 2 flasks were chosen randomly, and 25 ml of culture were transferred to the Oxymax flasks for the respiration measurements. After the respiration measurements, the corresponding Erlenmeyer flasks were sampled for OD550 (optical density at 550 nm), protein, IDH activity and carbon source (see below).

Respiration measurements. The Micro-Oxymax respirometer (Columbus Instruments International Corporation, Columbus, OH, USA) measured changes in concentration of CO2 and O2 in the head space of the experimental flasks. The apparatus consisted of an infrared detector (sensitive to the 200 μm absorption peak of CO2), an oxygen detector (based on the principle of the PbO2 fuel cell), a multiple sample chamber (for up to 20 channels), a reference chamber and a computerized data analysis system. A measurement normally required 30 min. Aerobic conditions were assured because the Micro-Oxymax changed the air in the head space if the O2 level fell below 18%.

In this paper in vivo respiratory rates are the CO2 production or O2 consumption rates as measured by the Micro-Oxymax. They are reported as nmol CO2 (or O2) min⁻¹ (mg protein)⁻¹.

Biochemical parameters. For every experimental flask, samples were taken in duplicate for the carbon source (acetate or pyruvate), protein concentrations and IDH activity. Then 5 to 10 ml of culture (depending on the biomass) were centrifuged at 10000 × g for 15 min at 4°C. The supernatant fluid was collected in an acid-rinsed Corex tube, and stored in liquid nitrogen for acetate or pyruvate analysis. The pellets were resuspended in 2 ml of the IDH extraction buffer at 0 to 4°C for IDH analysis, and in 2 to 4 ml 1 N NaOH (at 22°C) for protein analysis. The samples were mixed well, immediately transferred to cryovials, and frozen in liquid nitrogen.

Protein analysis was performed using the method of Lowry et al. (1951). The homogenates were defrosted and well mixed. An aliquot of 0.5 ml was analysed. The homogenates were diluted if the absorbance at 750 nm exceeded 0.4 and analysed again. Bovine Serum Albumin (BSA) from Sigma Chemical Company was used as a standard.

The IDH extraction buffer consisted of 25 mM MOPS (pH 7.5) containing 167 μg ml⁻¹ lysozyme. A detailed series of experiments carried out with Vibrio natriegens had concluded that this was the best extraction solution. The homogenates were thawed and put on ice. The assay was carried out in spectrophotometer quartz cuvettes (1 cm light path, 1 ml capacity), at 22°C. Changes in the absorbance (OD340) were continuously followed over time. The reaction mixture contained 0.2 ml of the extract, 0.4 ml of 10 mM D,L-isocitrate solution (Na salt, Sigma 176-1), 0.2 ml of 10 mM MnCl2.4H2O (Sigma 150-2A) and 0.2 ml of 4.16 mM NADP⁺-2H2O (Na salt, Sigma 240-301). In a blank cuvette, isocitrate was omitted. The reaction was initiated by the addition of NADP⁺-2H2O. The slope of the regression line of OD340 versus time gave the IDH activity. Since NADPH production is stoichiometrically equal to CO2 production in μmoles, NADPH was used as a standard to convert OD340 to μmol CO2. IDH activity constitutes the potential rate of CO2 production and as such it is reported as nmol CO2 min⁻¹ (mg protein)⁻¹.

Acetate and pyruvate were separated and quantified in their acid form by high performance liquid chromatography (HPLC). The analytical system consisted of 2 pumps (Perkin-Elmer, Norwalk, CT, USA; Series 3B), a 20 μl sample loop injector (Rheodyne, model 7125), a standard 4.6 mm i.D. reverse-phase C18 column (Supelcosil LC 18, dₚ = 3 μm), a precolumn (Supelcosil LC 18) and a UV-VIS variable wavelength detector (Perkin-Elmer LC-85 and the autocontrol module). The
Carboxylic acids were quantified by monitoring the absorbance at 210 nm. Acetic acid sodium salt (Aldrich) and pyruvic acid sodium salt (Sigma), more than 99% pure, were used as reference compounds for the external standardization. Mobile phase was prepared using HPLC grade phosphoric acid and water (Milli-Q system, Millipore, Bedford, MA, USA). All chromatographic measurements were carried out at 0.7 ml min⁻¹ using 0.05 M phosphoric acid as the mobile phase. Samples were thawed and adjusted to pH 2 by adding 3 μl concentrated phosphoric acid prior to the analysis.

RESULTS

Growth

Fig. 2 shows the development of the culture biomass (in terms of protein) and the consumption of the carbon sources. In both experiments, Vibrio natriegens cells entered the exponential phase during the first 30 to 60 min of the experiment. On pyruvate, cells grew faster (k₀ = 0.74 h⁻¹) and reached the stationary phase earlier (4.6 h) than those growing on acetate (k₀ = 0.38 h⁻¹, duration of the exponential phase = 9.3 h). In both media, the final crop in terms of protein was almost the same (350 ± 20 and 375 ± 14 mg protein l⁻¹ on acetate and pyruvate, respectively). The end of the exponential growth phase occurred slightly prior to the carbon source exhaustion. This suggests that something other than the carbon source was the growth-limiting factor.

Protein concentration was related to OD₅₅₀ in the acetate cultures by the equation: mg protein l⁻¹ = -19.2 + 214.7(OD₅₅₀), r² = 0.997. In the pyruvate culture, it was not significantly different: mg protein l⁻¹ = -19.9 + 221.8(OD₅₅₀), r² = 0.9871.

Respiratory metabolism

The time courses of IDH activity and the in vivo CO₂ production rates are plotted in Fig. 3. In both acetate and pyruvate media, IDH activity increased during the exponential phase, decreased slightly during the stationary phase and remained relatively constant until

![Fig. 2. Evolution of the biomass in terms of protein concentration and the consumption of the carbon source in the acetate (A) and the pyruvate (B) cultures. Vertical bars indicate range of values (n = 2).](image)

![Fig. 3. Evolution of the IDH activity and CO₂ production rates during the experiments on (A) acetate and (B) pyruvate. Vertical bars indicate range of values (n = 2). *End of the exponential growth phase. Arrows indicate exhaustion of the carbon source. Note the different scales of the y-axes.](image)
the end of the experiment. *In vivo* CO<sub>2</sub> production rates increased at the beginning of the experiment and started to decrease towards the end of the exponential phase before the carbon source was completely exhausted in the culture medium. It can be clearly observed that the enzymatic activity remained high compared to the *in vivo* CO<sub>2</sub> production rates after carbon was exhausted.

When comparing Fig. 3A & B, it can be noted that higher IDH activity was measured in the pyruvate cultures than in the acetate cultures. Also, *in vivo* CO<sub>2</sub> production rates were higher in the pyruvate cultures than in the acetate ones especially during the exponential growth phase (Fig. 3, data points before the asterisk).

The differences in the respiration associated with the different culture media can be observed in Fig. 4. O<sub>2</sub> consumption rates are represented by negative values. In the pyruvate cultures, CO<sub>2</sub> production and O<sub>2</sub> consumption rates appeared almost as mirror images of each other (Fig. 4A), although the absolute rates of O<sub>2</sub> consumption were higher than those of CO<sub>2</sub> production. In contrast, in the acetate cultures, O<sub>2</sub> consumption rates followed very different dynamics than CO<sub>2</sub> production rates (Fig. 4A). The absolute rates of O<sub>2</sub> consumption were much greater than the CO<sub>2</sub> production rates. Comparing both culture media, the acetate cultures exhibited higher O<sub>2</sub> consumption and lower CO<sub>2</sub> production rates.

Fig. 4B shows the cumulative respiration per unit biomass. The total CO<sub>2</sub> produced was slightly higher in the pyruvate cultures than in the acetate ones during the first 18 h. The total O<sub>2</sub> consumed was markedly higher in the acetate cultures.

The different patterns of the CO<sub>2</sub> produced versus O<sub>2</sub> consumed in *vivo* was reflected in the different respiratory quotients (RQ, molar ratio of the CO<sub>2</sub> produced to O<sub>2</sub> consumed; Fig. 4C). In the pyruvate cultures, RQ values were 0.7 to 1.3 while in the acetate ones RQ values were, in general, lower than 0.5.

**DISCUSSION**

The results show that *Vibrio natriegens* grew more slowly on acetate than on pyruvate, but the final crop (in terms of protein) was similar in both cultures (Fig. 2). On pyruvate, IDH activity (Fig. 3), CO<sub>2</sub> production rate (Figs. 3A, B, or 4A) and total CO<sub>2</sub> produced (Fig. 4B) were higher than on acetate. The growth on acetate was also characterized by a notably high O<sub>2</sub> consumption. The shapes of the CO<sub>2</sub> production and the O<sub>2</sub> consumption time courses were different on acetate than on pyruvate.

These differences suggest that *Vibrio natriegens* shifts its metabolism in order to grow on the 2-carbon (acetate) and 3-carbon (pyruvate) sources. Lower IDH activity in the acetate cultures (Fig. 3) is evidence that...
the glyoxylate bypass is operating. This short circuit of the Krebs cycle (Fig. 1) facilitates growth on acetate in *Escherichia coli* (Holms 1986). In that species, IDH is partially inactivated by the IDH kinase/phosphatase while growing on acetate and, when this substrate is exhausted, IDH activity is completely restored (Holms & Bennett 1971, El-Mansi et al. 1985, Holms 1986).

However, in all our experiments with *V. natriegens* IDH activity never increased after the exhaustion of acetate (Fig. 3A), even in experiments that lasted for 15 d (Berdalet unpubl.). This difference suggests physiological differences between *V. natriegens* and *E. coli*. Differences in the control of IDH and ICL as well as the presence of different forms of IDH have been reported in *Acinetobacter calcoaceticus* (Hoyt & Reeves 1992).

In other bacteria there is considerable variability in the structure and coenzyme specificity of IDH (Edlin & Sundaran 1989, Leyland & Kelly 1991).

Because IDH is important in CO₂ production (Fig. 1) we hypothesized that growth on acetate would result in lower CO₂ produced compared to the pyruvate based cultures. In fact, lower CO₂ production was observed in the acetate cultures while acetate was present in the medium (Fig. 4A, B, data points before carbon was exhausted; note that the acetate levels are illustrated in Fig. 2A). After acetate exhaustion, the CO₂ production was maintained for a few hours and at the end of the experiment the total CO₂ produced was slightly higher than in the pyruvate cultures (Fig. 4B). This argues for 2 types of metabolism in the acetate cultures. During the exponential phase, the metabolism was essentially based on acetate, while during steady state and senescence, metabolism was based on another carbon source (probably from excreted material).

Another characteristic of the growth on acetate was the markedly higher O₂ consumption (Fig. 4A, B). The completion of the glyoxylate bypass requires 2 mol acetate and results in the consumption of ½ mol O₂ without CO₂ production (Fig. 1). In contrast, when 1 mol acetate is driven through the Krebs cycle it generates 2 mol CO₂ and consumes 2 mol O₂ (Fig. 1). The high O₂ consumption suggests an important use of the glyoxylate bypass during growth on acetate, i.e. that a higher proportion of the acetate is driven through the glyoxylate bypass than through the Krebs cycle. Holms (1986) suggested a partition of 2:1 between the Krebs cycle and the glyoxylate bypass when *Escherichia coli* grows on acetate. With such a partition 4 mol of acetate would be driven through the Krebs cycle for every 2 mol driven through the glyoxylate bypass, thus resulting in an RQ of 

\[
\frac{8\text{CO}_2}{8\text{O}_2 + \frac{3}{2}\text{O}_2} = 0.84
\]

This value is much higher than that observed in the cultures of *Vibrio natriegens* growing on acetate (Fig. 4C). We estimate that the range of RQ obtained in the acetate cultures in our study can be explained assuming the acetate is partitioned in the proportions 1:4 to 1:2 between the Krebs and the glyoxylate bypass.

The relatively high rate of O₂ consumption in the acetate-based culture could be related to its lower growth rate. On acetate, the growth rate was half that observed on pyruvate (0.38 h⁻¹ vs 0.74 h⁻¹). The slower growth on acetate was also reported by Holms (1986) for *Escherichia coli*. He proposed that growth was limited by the glyoxylate bypass and, in particular, by the enzyme ICL. In contrast, growth on pyruvate would be faster because pyruvate enters the Krebs cycle faster (El-Mansi & Holms 1989). It can be used in gluconeogenesis (Lehninger 1993) and is a direct precursor of some amino acids (El-Mansi et al. 1986).

Concerning the pyruvate cultures, a very obvious feature of the respiration was the parallelism between the CO₂ production and the O₂ consumption rates (Fig. 4) with an RQ range of 0.7 to 1.3. This range of RQ can be explained by different partitioning of the pyruvate between the Krebs cycle and the anaplerotic pathways [that replenish the oxaloacetate (OAA) used for biosynthesis] in the course of the different growth phases. When 1 mol of pyruvate is driven through the Krebs cycle, 3 mol CO₂ are liberated and \( \frac{3}{2} \) mol O₂ are consumed (Fig. 1), yielding an RQ of 1.2. The carboxylation of 1 mol of pyruvate to make OAA (Fig. 1, and Gottschalk 1986) would consume 1 mol of CO₂ without O₂ consumption. A partition of 1:1 between these 2 metabolic pathways would yield an RQ of (3CO₂ – 1CO₂)/(\( \frac{3}{2} \)O₂) = 0.8. Other anaplerotic reactions including the participation of the glyoxylate bypass may be involved. For example, a partition of 1:1:1 among the Krebs cycle, the carboxylation of pyruvate and the glyoxylate bypass would result in an RQ of (3CO₂ – 1CO₂ + 1CO₂)/(\( \frac{3}{2} \)O₂ + 2O₂) = 0.66, as found at the beginning of the experiment. In this last case, the participation of the glyoxylate bypass could be related to a lower IDH activity in the pyruvate cultures at the beginning of the experiment (Fig. 3B).

Our study constitutes the first IDH investigation in marine bacteria and is the first IDH investigation on any bacterium since 1971 to include measurements of the CO₂ and O₂ dynamics. In the previous study (Holms & Bennett 1971), the respiratory metabolism and IDH activity in *Escherichia coli* growing on glycerol (a 3-carbon molecule) and glucose were characterized. The CO₂ production paralleled the O₂ consumption rates during the exponential phase of the culture with an RQ of approximately 1.0 and 0.63 in the glucose and the glycerol cultures, respectively. When such cultures reached the stationary phase, the O₂ consumption rates dropped faster than the CO₂ production. During growth on glucose, *E. coli* excretes acetate, which is used when glucose is exhausted to
sustain respiration during an initial part of the stationary phase. In that part of the growth, the respiratory metabolism based on acetate is characterized by a slightly higher $O_2$ consumption than $CO_2$ production (Fig. 3 in Holms & Bennett 1971). In the present study we have shown in detail the different patterns of $O_2$ consumption and $CO_2$ production displayed by the marine bacterium *Vibrio natriegens* in the course of growth on acetate and pyruvate as $C$ sources. In summary, these experiments with *Vibrio natriegens* have clearly shown that this species grew twice as fast on pyruvate as it did on acetate, but the biomass yield was the same. Higher growth rates on pyruvate were associated with elevated levels of $CO_2$ production and IDH activity. Slower growth on acetate was associated with higher $O_2$ consumption rates. After nutrient exhaustion, respiratory $CO_2$ production dropped to a few percent of its maximum value while IDH activity remained relatively high (i.e. 60 to 90% of maximum activity). This situation suggests that IDH was not inactivated by the IDH kinase/phosphatase as described in *Escherichia coli*. The respiratory time-courses have demonstrated the capacity of the Micro-Oxymax to automate simultaneous measurement, calculation, and display of $O_2$ consumption, $CO_2$ production, and RQ in cultures of marine bacteria.

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**LITERATURE CITED**


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