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Respiration quotient variability: bacterial evidence

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ABSTRACT: Respiratory metabolism was compared between 2 different physiological states of acetate- and pyruvate-grown cultures of *Pseudomonas nautica* and *Vibrio natriegens*. Here, we analyze 35 h and 520 h experiments in which time-courses of protein, pyruvate, acetate, respiratory CO_2 production (R_{CO_2}), respiratory O_2 consumption (R_{O_2}), isocitrate dehydrogenase (IDH) activity, and potential respiration (Φ) were measured. Respiratory quotients (RQs) were calculated as the ratio of the respiration rates (R_{CO_2}/R_{O_2}). Such RQs are widely used in ocean ecosystem models, in calculations of carbon flux, and in evaluations of the ocean's metabolic balance. In all the cultures, the RQ tended to increase. In the case of *P. nautica* on acetate, the RQ rose nearly an order of magnitude from values below 1 during carbon-substrate sufficiency to values close to 10 during carbon-substrate deficiency. In all the cultures, the respiration rates during the growth period paralleled the biomass increase, but after the substrates were exhausted, the respiration rates fell. In contrast, through this same transition period, the IDH activity and the Φ remained relatively high for the first 10 h of carbon-substrate deprivation, and then, these enzyme activities fell slowly, along with the biomass, as the carbon-substrate deprivation continued. The nutritional state of the bacteria affected the RQ, rendering the RQ variable for physiological and ecological purposes. These results argue that ecosystem models, oceanographic calculations of carbon flux, and evaluations of the ocean's metabolic balance that are influenced by bacterial metabolism need to be reconsidered in light of RQ variability.

KEY WORDS: O_2 consumption · CO_2 production · Isocitrate dehydrogenase · IDH · Electron transport system · ETS · Potential respiration · Growth

INTRODUCTION

The respiration quotient (RQ), the ratio of the CO_2 produced to the O_2 consumed in respiration, is an old concept, dating back to the 1860s. Armsby & Moulton (1925) and Lusk (1928) discuss its use in animal husbandry and human physiology, presenting research of Pettenkofer & Voit (1866), Warburg (1926), and others who were measuring RQ much earlier. RQ is a dimensionless ratio, calculated mole per mole, and is an index of the type of organic matter being oxidized in respiration. From the shift in RQ from 0.7 to 1.0, an investigator can differentiate lipid-based biological oxidation (metabolism) from carbohydrate-based metabolism. The majority of studies of respiration in aquatic ecosystems are based on RQ ranging from 0.7 to 1.2 (Berggren et al. 2012), but in many ecological calculations of microbial respiration, an RQ of 1 is assumed (González et al. 2003, Bühring et al. 2006). This practice occurs even though it is known that the RQ varies from 0.65 to 1.4 in individual ecological microbial communities (del Giorgio et al. 2006). Amado et al. (2013) argue that such an assumption is the only feasible option given the dearth of physio-

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logical studies that document the RQ variability in bacterial respiration and they conclude that more investigations of RQ variability are needed. A recent study of respiration in yeast found an RQ range of 0.4 to 1.4 (Slavov et al. 2014). When an organism is oxidizing carbohydrate, its CO_2 production rate and its O_2 consumption rate are equal, i.e. the RQ = 1. When an organism is oxidizing protein, the RQ is around 0.8, and when oxidizing lipids, it is close to 0.7 (Cantarow & Schepartz 1967, Guyton 1971, Hoar 1975, Gnaiger 1983, Stanier & Forsling 1990). When burning organic compounds richer in O_2 than carbohydrates, such as carboxylic acids, and when converting carbohydrate to fat, RQ values can be >1. When metabolism consumes oxygen-rich oxalic acid ($C_2H_2O_4$), RQ can rise to 4 (Dilly 2001). At the other end of the scale, gluconeogenesis (glucose synthesis) occurs when RQ falls below 0.7 (Cantarow & Schepartz 1967). In this way, RQ variations are generally explained by the fact that the amounts of CO_2 and O_2 produced are dependent on the oxidation state of the substrate and the pathways by which the substrate is metabolized (Burton 1982, Kader 1987). Berggren et al. (2012) argue from their field studies of Quebec lakes that variability in RQ indicates shifts in bacterial physiology and carbon consumption that cannot be deduced from other measurements. However, in oceanography, only a handful of RQ measurements have been made (Oviatt et al. 1986, Robinson et al. 2002). Nevertheless, when the effort is made to determine the RQ for each situation, as did Obernosterer et al. (2008) in their Southern Ocean iron-fertilization experiment, an R_{CO_2} production or a carbon oxidation calculation is more accurate.

Whether in the laboratory or in the field, an RQ is essential in calculating either organic carbon consumption or CO_2 production from R_{O_2} consumption measurements (Boucher et al. 1994, Bergström 2011, Giering et al. 2014). In this way, RQ becomes an influential factor in ocean carbon-cycle studies, for calculating carbon flux from plankton metabolism (Packard & Christensen 2004, Steinberg et al. 2008, Packard & Gómez 2013, Osma et al. 2014), and in investigating whether the ocean is autotrophic or heterotrophic (Ducklow & Doney 2013). Here, we measure the RQ in cultures of the marine bacteria *Pseudomonas nautica* and *Vibrio natriegens* as they pass from carbon substrate sufficiency to carbon substrate limitation.

P. nautica is an oil-degrading bacterium from the Gulf of Fos, France (Bonin et al. 1987a,b), and was used to advance studies of oil-spill bio-remediation (Swannell et al. 1996). *V. natriegens* is a well-studied non-pathogenic marine bacterium discovered by Payne et al. (1961) in a Georgia salt-marsh (Lee & Levy 1987, Lee 1995). Carbon-substrate limitation conditions are common in the ocean as marine microbial communities pass from bloom to post-bloom conditions (Liu et al. 2013), and are likely dominant in oligotrophic ecosystems where bacterial respiration accounts for up to 59% of plankton respiration (Robinson & Williams 2005). Here, we show that in our cultures, the RQ can range higher than the upper values reported above. To investigate this wide range, we examine time-courses of the physiological respiration rates (R_{O_2} and R_{CO_2}) and the enzymatic activities from the respiratory electron transport system (ETS) and isocitrate dehydrogenase (IDH). ETS and IDH activities are the biochemical enzyme activities that largely control the R_{O_2} consumption and R_{CO_2} production (Packard et al. 1996a, Nelson & Cox 2005, Gnaiger 2009). Via aerobic respiration, marine organisms obtain the energy to live from a wide range of compounds that are reduced in different, but well coordinated biochemical pathways. Two of these key pathways are the Krebs or tricarboxylic acid (TCA) cycle and ETS. IDH is proposed to be a future analytical method for CO_2 calculations in the ocean (Packard et al. 1996a, Roy & Packard 2001), but it needs further investigation, improvement (Robinson & Williams 2005), and calibration. In our study, we work with 2 substrates: acetate and pyruvate. Acetate, a 2 carbon molecule, is transformed into acetyl-CoA at the entry of the TCA and, via IDH and alpha-keto glutarate dehydrogenase, loses both carbons as CO_2 and produces 8 reducing equivalents (e^-) that in turn, lead to the consumption of 2 molecules of O_2 ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$) at cytochrome oxidase in the ETS. Accordingly, the potential RQ for acetate would be 1.0. When pyruvate (a 3-carbon molecule) is cycled through the TCA, it produces 10 reducing equivalents and 3 molecules of CO_2 and it consumes 2.5 O_2 molecules. This would result in an RQ of 1.2. Thus, the use of these different carbon substrates becomes a tool to generate different values of RQ. In *P. nautica*, we found that acetate-grown cultures showed an RQ increase to 10 during carbon-limitation, whereas RQ in pyruvate-grown cultures remained near 1.0 (Roy et al. 1999). However, because of the continued lack of published physiological information about RQ, we wanted to investigate this topic with another species and over longer periods of carbon-limitation. We expected that different levels of carbon limitation and different durations of starvation would generate a wider range of RQs.

MATERIALS AND METHODS

Experimental design

To investigate the RQ in different bacterial growth stages, time-course experiments were run on batch cultures at 22°C, maintained on pyruvate or acetate as described by Berdalet et al. (1995) and Packard et al. (1996b). Short-term experiments were run for a maximum of 35 h, and a (different) set of long-term experiments were run for 2 to 3 wk. The bacteria cultures were grown in 25 cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of media kept slowly rotating on an 'orbital shaker table'. At intervals over the different time courses, 2 flasks were chosen randomly, 25 ml of culture were transferred to the airtight respirometer flasks, and the respiration was measured. Then, samples of the remaining culture in the chosen flasks were taken in duplicate for growth (optical density at 550 nm [OD550], turbidity measured as absorbance at 550 nm), protein, pyruvate, acetate, R_{CO_2}, R_{O_2}, IDH activity, and potential respiration (Φ) as the cultures grew exponentially, reached steady state, and passed into senescence.

Bacterial cultures

Vibrio natriegens (ATCC 33788) and *Pseudomonas nautica* (Strain 617 from Dr. P. Bonin, Université de la Méditerranée, Marseille, France) were used for these cultures. Before any experiment, the bacteria had been adapted for a minimum of 15 generations to the experimental media (pyruvate or acetate). To inoculate the cultures, a sample from a mother culture in exponential or early stationary phase was used. Cultures were continuously agitated on an orbital shaker at 100 rpm at 22°C, and growth was followed spectrophotometrically at 550 nm (OD550). The OD550 had an initial value after inoculation of 0.1 absorbance units.

Culture media

The general culture procedures have been described by Berdalet et al. (1995) and Roy et al. (1999). More specifically, *P. nautica* was cultured according to the method of Packard et al. (1996a), and the medium for *V. natriegens* was developed from the media of Niven et al. (1977), Baumann & Baumann (1981), King & Berman (1984), and Nissen et al. (1987). The optimal conditions for growth of *V. natriegens* were experimentally established. They were grown on 400 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 25 mM NH_4Cl, 0.33 mM phosphate buffer, 0.01 mM $FeSO_4 \cdot 7H_2O$. Initial concentration of the culture-medium carbon-source was 30 mM sodium acetate or 20 mM pyruvate. (Note that these concentrations would provide the same amount of organic carbon at the start of an experiment.) The mean coefficient of variation was 2.1% (n = 20) for acetate and pyruvate. Reagents for the culture media were obtained from Sigma-Aldrich. All components were dissolved in 0.22 µm filtered deionized water (except $FeSO_4 \cdot 7H_2O$ and phosphate buffer), and pH was adjusted to 7.5 with 1 N NaOH. To remove particles, the medium was filtered through a GF/F glass fiber filter. Later, the medium was autoclaved for 45 min at 121°C. Then, to avoid precipitation during the autoclaving, the phosphate buffer (0.67 M, pH 7.5) and the iron sulphate solution ($FeSO_4 \cdot 7H_2O$, 0.1 mM) were prepared separately. The PO_4 buffer was sterilized by autoclaving, and the $FeSO_4$ solution was filtered through 0.22 µm acrodiscs. Finally, both solutions were kept frozen and were thawed and added to the culture medium just before use.

Protein measurements

For every flask, protein samples were taken in duplicate. Five to 10 ml of culture, depending on the level of biomass, were centrifuged at $10000 \times g$ at 4°C for 15 min and then frozen in liquid N_2 (Ahmed et al. 1976). Later, the bacterial pellets were defrosted, mixed well with 2 to 4 ml 1 N NaOH (at 22°C), and analyzed for protein in aliquots of 0.5 ml by the Lowry method (Lowry et al. 1951) in according with Berdalet et al. (1995). If the absorbance at 750 nm of the samples exceeded 0.4, the homogenates were diluted and analyzed again. For standardization, duplicate measurements of bovine serum albumin (BSA) from Sigma Chemical Company were used. Their range around the average of these duplicates increased from 2% during exponential growth to 14% during stationary phase (after 15 h). The mean of these ranges averaged 4.1%.

Biochemical parameters

For acetate and/or pyruvate and enzyme activity (IDH and ETS), samples were also taken in duplicate from every experimental flask and prepared as for the protein samples (Berdalet et al. 1995). The super-

natant fluid was collected in an acid-rinsed Corex tube and then stored in liquid N_2 for acetate or pyruvate analysis after centrifugation. Samples were thawed and adjusted to pH 2 by adding concentrated phosphoric acid and analyzed by high performance liquid chromatography.

Pellets for IDH extraction were resuspended in 2 ml of buffer at 0 to 4°C, and activity was determined spectrophotometrically at 340 nm following the NADPH production (Reeves et al. 1971, 1972, Holms & Bennett 1971, Berdalet et al. 1995) after $NADP^+$ addition. IDH catalyzes the reaction producing CO_2 during isocitrate oxidation.

$$\text{Isocitrate} + NADP^+ \leftrightarrow \alpha\text{-ketoglutarate} + NADPH + CO_2$$

Results are given as µm CO_2 min^{-1} per liter of culture. The IDH activity was calculated from the regression line of OD340 versus time. NADPH was used as the standard converting OD340 to CO_2 (µmol) because, from the equation above, NADPH production is stoichiometrically equal to CO_2 production (1:1). Each data point represented 4 analyses. The mean coefficient of variation was 5.0% (n = 20).

Samples for ETS were resuspended at 0 to 4°C in 2 ml of homogenizing buffer and measured kinetically for ETS activity with a modification of the Packard & Williams (1981) method as described by Packard & Christensen (2004). To calculate Φ, ETS in µmol e^- min^{-1} l^{-1} was divided by 4 ($4e^- + 4H^+ + O_2 \rightarrow 2H_2O$) to give µmol O_2 min^{-1} l^{-1} of culture. Again, each data point represents 4 analyses. In the case of ETS, the mean coefficient of variation was 7.2% (n = 20).

Respiration measurements

R_{CO_2} and R_{O_2} were simultaneously measured by a Micro-Oxymax system (Columbus Instruments International). The Micro-Oxymax is a computer-controlled closed-circuit respirometer. The system monitors gas concentrations in the headspace above the culture (www.colinst.com) via 2 well-sealed tubes. The culture itself was sampled by syringe through a septum in each experimental flask. Calculations of incremental and accumulated values for consumption of O_2 and production of CO_2 were possible because of periodic sensing of the gas concentration. Oxygen measurements were carried out with an oxygen detector based on the principle of a PbO_2 fuel cell. CO_2 measurements were made with an infrared CO_2 detector (sensitive to the 2000 µm absorption peak of CO_2). Both were part of the Micro-Oxymax system. The detection limit was 10 ppm of O_2 and CO_2. This is equivalent to 0.44 µmol l^{-1}. The maximum sensitivity for the 2 rates was 8.9 nmol h^{-1}. The instrument had a multiple sample chamber (for up to 20 channels), a reference chamber, and a computerized data acquisition and analysis system. The Micro-Oxymax maintained aerobic conditions because it periodically refreshed (replenished) the air in the headspace if the O_2 level fell below 19.3% to avoid oxygen limitation. Cultures were maintained at 22°C and continuously shaken (to optimize gas exchange). Respiration is given as µmol O_2 min^{-1} l^{-1} and µmol CO_2 min^{-1} l^{-1}. Note that here, R_{CO_2} refers to CO_2 generated by intact bacteria suspended in their growth medium, and R_{O_2} refers to O_2 consumed under the same conditions. A normal measurement took ~30 min. Measurements over 30 min intervals were referenced against a baseline and a control and were made in duplicate. The calibration of the oxygen detector was done with high-precision gas standards. The respirometry for all the original measurements was described by Berdalet et al. (1995) and Packard et al. (1996b). The mean coefficient of variation was 5.1% (n = 20) for R_{O_2} and 5.2% (n = 20) for R_{CO_2}.

Statistical analysis

Data were analyzed using the program R (R Development Core Team 2010). Relationships between R_{CO_2}/R_{O_2} and IDH/Φ over different time scales (short- and long-term experiments) were obtained from the regression equations, using confidence limits of 95% and Pearson correlation coefficients. ANCOVA was applied to determine statistical differences between slopes and ordinates in the regression lines. Normality of residuals was confirmed using the Shapiro-Wilks test.

RESULTS

Data summary

Two types of experiments were conducted. Short-term experiments that ran up to 35 h and long-term (2 to 3 wk) experiments that ran for 330 and 520 h. In all these experiments, we used the 2 bacterial species *Pseudomonas nautica* and *Vibrio natriegens* growing on either acetate or pyruvate. In Fig. 1, measurements of substrate (carbon source), protein, enzyme activity (IDH and Φ), R_{CO_2}, and R_{O_2} are shown. Fig. 2 presents comparable long-term experiments in which we made the same measurements. Data for both fig-

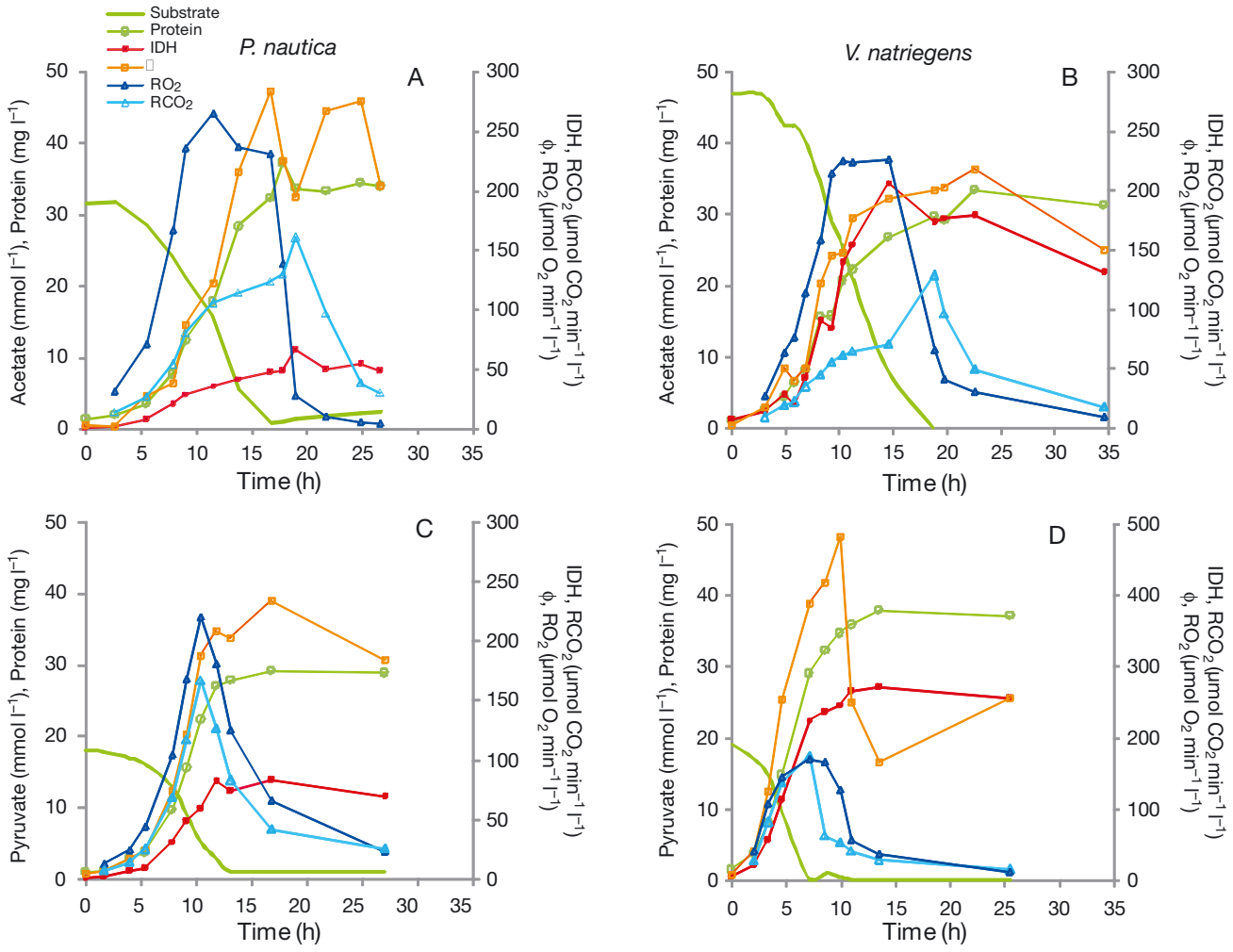

Fig. 1. Short-term experiments. Time courses of respiration, enzyme activity, biomass, and carbon source: (A) *Pseudomonas nautica* in acetate, (B) *Vibrio natriegens* in acetate, (C) *P. nautica* in pyruvate, (D) *V. natriegens* in pyruvate. Note that, depending on the experiment, the cultures are substrate-sufficient in the first 5 to 15 h and substrate-limited (starved) afterwards. For display clarity, the protein data have been divided by 10, and R_{CO_2} and R_{O_2} have been multiplied by 5. IDH: isocitrate dehydrogenase; ϕ: potential respiration; R_{O_2}: O_2 consumed; R_{CO_2}: CO_2 generated

ures are represented on the same scale so that all the time-courses could be shown in the same graph. However, this required the use of multipliers, as explained in the legends for Figs. 1 & 2.

Enzyme activity: IDH and Φ

Short-term experiments. Enzymatic activity is present even during starvation (Fig. 1). In general, the IDH activity and Φ rise during the hours of substrate sufficiency. After substrate limitation sets in, both parameters in the *P. nautica* cultures stay relatively constant with Φ higher than IDH. This can be seen when either enzyme activity is normalized by biomass because both the enzyme activities and the biomass trend in parallel with culture age (Fig. 1). During the same conditions of substrate limitation, IDH in the *V. natriegens* cultures retains similarly high activity levels.

Long-term experiments. Enzymatic processes in the long-term experiments (Fig. 2) after 24 h varied little. In all experiments, enzymatic activity was observable even during carbon substrate limitation. Almost all IDH and Φ values were similar; IDH ranged from 0.5 to 66 µmol CO_2 min^{-1} l^{-1} of culture, while Φ ranged from 1.3 to 284 µmol O_2 min^{-1} l^{-1} of culture. In *P. nautica* on acetate (Fig. 2A), IDH rose with carbon substrate limitation and then decreased with Φ until ~350 h. In *V. natriegens* growing on

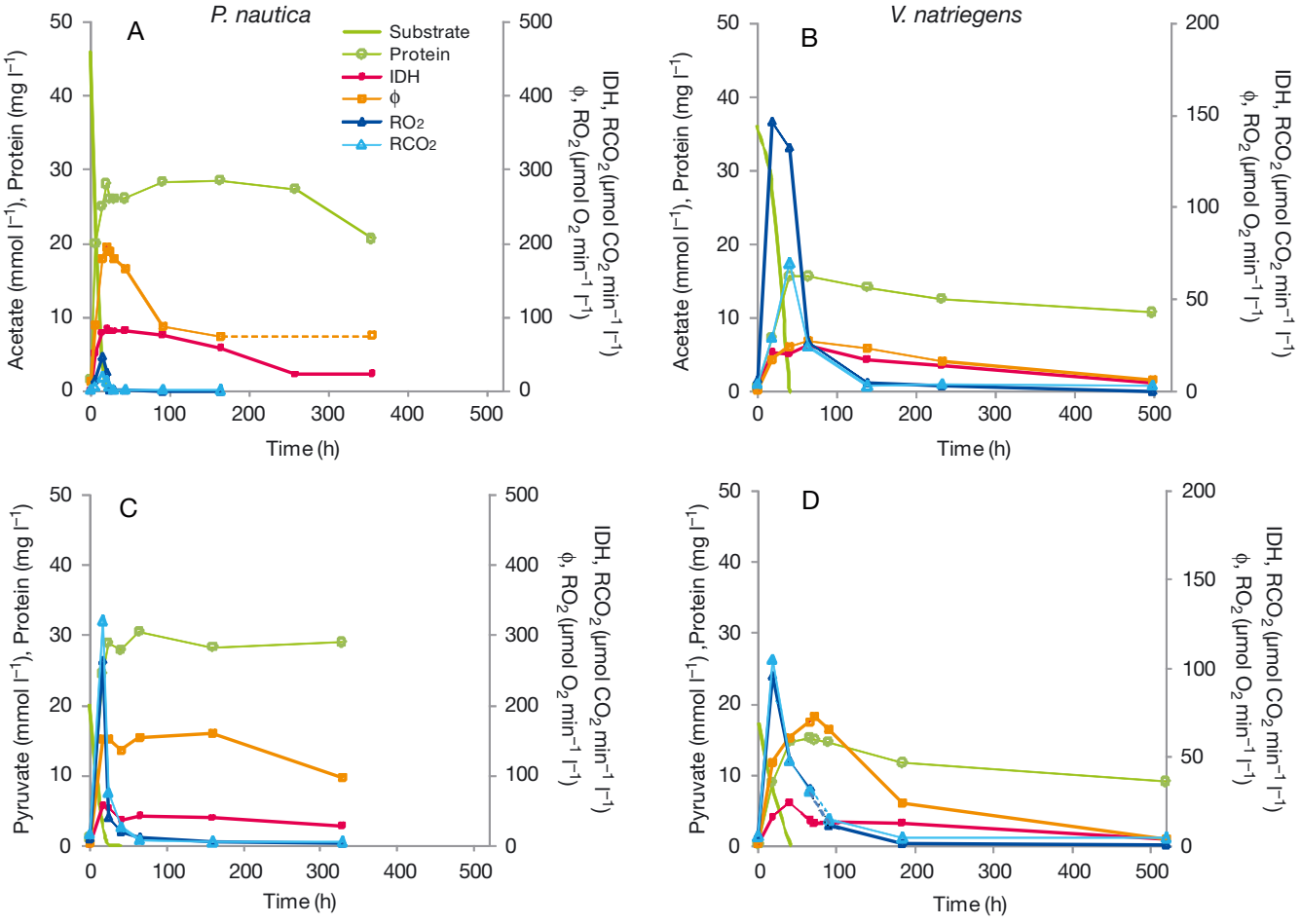

Fig. 2. Long-term experiments. Time courses of respiration, enzyme activity, biomass, and carbon source: (A) *Pseudomonas nautica* in acetate, (B) *Vibrio natriegens* in acetate, (C) *P. nautica* in pyruvate, (D) *V. natriegens* in pyruvate. Note that, except for the first 30 to 35 h, the cultures are continually starved for carbon; they are substrate-limited. During this period, the biomass and the enzyme activities decline, while the respiration (R_{O_2} and R_{CO_2}) is barely measureable. For display clarity, protein data have been divided by 10, have R_{O_2} and R_{CO_2} been multiplied by 10. Dotted lines symbolize absent data. IDH: isocitrate dehydrogenase; ϕ: potential respiration; R_{O_2}: O_2 consumed; R_{CO_2}: CO_2 generated

acetate, both IDH and Φ dropped after carbon substrate limitation to levels lower than 4.7 µmol CO_2 min^{-1} l^{-1} and 6.2 µmol O_2 min^{-1} l^{-1} (Fig. 2B), respectively. In experiments with *P. nautica* growing on pyruvate, Φ and IDH remained almost constant for 350 h after carbon substrate limitation (Fig. 2C). In experiments with *V. natriegens* growing on pyruvate, Φ maintained activity around 72 µmol O_2 min^{-1} l^{-1} of culture for the first 100 h and then decreased to barely detectable levels of activity after 500 h. IDH activities decreased more rapidly in the first 100 h to 13 µmol CO_2 min^{-1} l^{-1} of culture and then more slowly in the next 400 h to 4.5 µmol CO_2 min^{-1} l^{-1} of culture (Fig. 2D). Substrate deficiency characterized most of the culture's life. Note that both IDH and Φ tended to decrease with time as the bacterial cultures starved.

Physiological measurements: R_{CO_2} and R_{O_2}

Short-term experiments. In all short-term experiments, R_{O_2} was higher than R_{CO_2} before starvation was reached (Fig. 1). Experimental results with *P. nautica* and *V. natriegens* on acetate were similar, but the 2 curves were out of phase. The peaks were displaced in time. In these experiments, R_{O_2} rose rapidly, but as starvation began, around 15 h, it fell. During this R_{O_2} fall, R_{CO_2} peaked and then rapidly declined (Fig. 1A,B). In the 2 pyruvate-based experiments, pyruvate decreased from a initial level of 20 mmol l^{-1} to nearly zero after ~10 h (Fig. 1C,D). In the *P. nautica* culture, both R_{CO_2} and R_{O_2} rose and fell in parallel (Fig. 1C). The decrease in respiration occurred before carbon substrate depletion. In the comparable *V. natriegens* pyruvate culture (Fig. 1D),

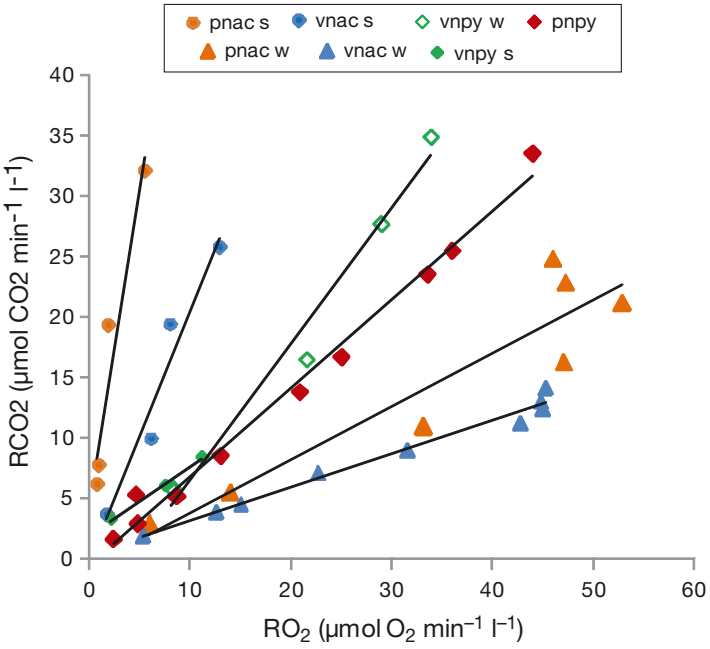

Fig. 3. Short-term experiments. R_{CO_2} production versus R_{O_2} consumption; w is for well-fed (substrate-sufficient), and s is for starvation conditions (substrate-starved). pnac: *Pseudomonas nautica* in acetate, pnpy: *P. nautica* in pyruvate, vnac: *Vibrio natriegens* in acetate, vnpy: *V. natriegens* in pyruvate. For pnpy, the well-fed and starved conditions had the same slope

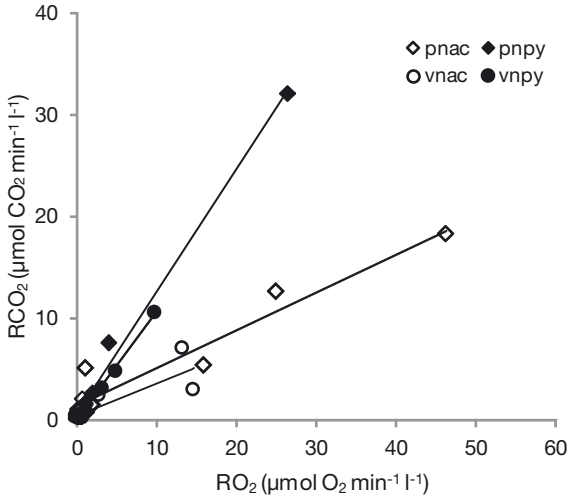

Fig. 4. Long-term experiments. R_{CO_2} production versus R_{O_2} consumption with regression equations that include all data points. pnac: $R_{CO_2} = 0.3681\ R_{O_2} + 1.5454$; $r^2 = 0.93$; n = 9; $p < 0.05$; pnpy: $R_{CO_2} = 1.204\ R_{O_2} + 0.3718$; $r^2 = 0.99$; n = 7; $p < 0.05$; vnac: $R_{CO_2} = 0.3103\ R_{O_2} + 0.5962$; $r^2 = 0.65$; n = 6; p = 0.05; vnpy: $R_{CO_2} = 1.0624\ R_{O_2} + 0.0701$; $r^2 = 0.99$; n = 7; $p < 0.05$. Note that the carbon sources dictate the parallelism of the curves, not the bacterial species. In addition, note that the respiration quotient (slope) is higher for the 3-C substrate (pyruvate) than for the 2-C substrate (acetate). This difference cannot be seen in the short-term experiments (Fig. 3)

the respiration signals were out of phase; however, in both of the acetate-based cultures, they were even more out of phase.

In comparing the R_{CO_2} to R_{O_2} in the short-term experiments (Figs. 1 & 3), the relationship between R_{CO_2} and R_{O_2} differs with each experiment. High variability is clear (Fig. 3). However, note that in the *P. nautica* in pyruvate (pnpy) culture (Figs. 1C & 3), the data from both the carbon-sufficient and the carbon-limited phases follows the same correlation ($R_{CO_2} = 0.7295\ R_{O_2} - 0.5969$, n = 10, $r^2 = 0.9882$, $p < 0.05$). For all the experiments, if the substrate-starved and the substrate-sufficient data are considered separately, 2 different relationships appear (Fig. 4). From the equations in Table 1, it is clear that the starved acetate-based cultures have different RQs (slopes) than the starved pyruvate-based cultures.

Long-term experiments. R_{CO_2} and R_{O_2} decreased to low values within the first 150 h of the experiment (Fig. 2).

Table 1. Minimum, maximum, and mean respiratory quotient (RQ) from exponential phase (w: well fed) and stationary phase (s: starvation) for short-term and long-term experiments. (Note that in all experiments, the transition points between the well-fed and starved conditions were excluded). Regression equations correspond to $R_{CO_2} = mR_{O_2} + b$

Bacteria	Condition	RQ MIN.	RQ MAX.	RQ Mean	RQ SD	Regression[a] $y =$	r^2	n
Short-term experiments								
pnac	w	0.3	0.5	0.4	0.06	$0.4054x - 0.4045$	0.92	6
	s	5.7	9.59	7.2	1.66	$5.2962x + 3.4891$	0.92	4
vnac	w	0.3	0.3	0.3	0.02	$0.2769x + 0.3332$	0.98	9
	s	1.6	2.4	2.0	0.32	$2.0564x - 0.4225$	0.95	4
pnpy	w	0.6	0.8	0.6	0.07	$0.7598x - 1.2327$	0.99	5
	s	0.6	1.1	0.9	0.3			2
vnpy	w	0.7	1.0	0.9	0.16	$1.126x - 5.0411$	0.97	4
	s	0.7	1.3	1.0	0.34	$0.5737x + 1.7177$	0.99	3
Long-term experiments								
pnac	w	0.3	0.8	0.5	0.25	$0.383x + 0.1797$	0.99	3
	s	2.4	7.8	4.7	2.23	$4.2758x - 0.1633$	0.76	5
vnac	w	0.2	0.7	0.4	0.33			2
	s	0.6	1.2	0.9	0.29	$0.928x - 0.0242$	0.99	3
pnpy	w	1.2	1.9	1.6	0.34	$1.1533x + 1.5905$	0.99	3
	s	0.6	1.2	0.9	0.30	$0.3266x + 0.3467$	0.98	3
vnpy	w	0.8	1.1	1.0	0.18			2
	s	1.0	4.5	2.4	1.64	$0.86x + 0.3836$	0.99	4

[a]Regression lines: p-values < 0.05 in all cases

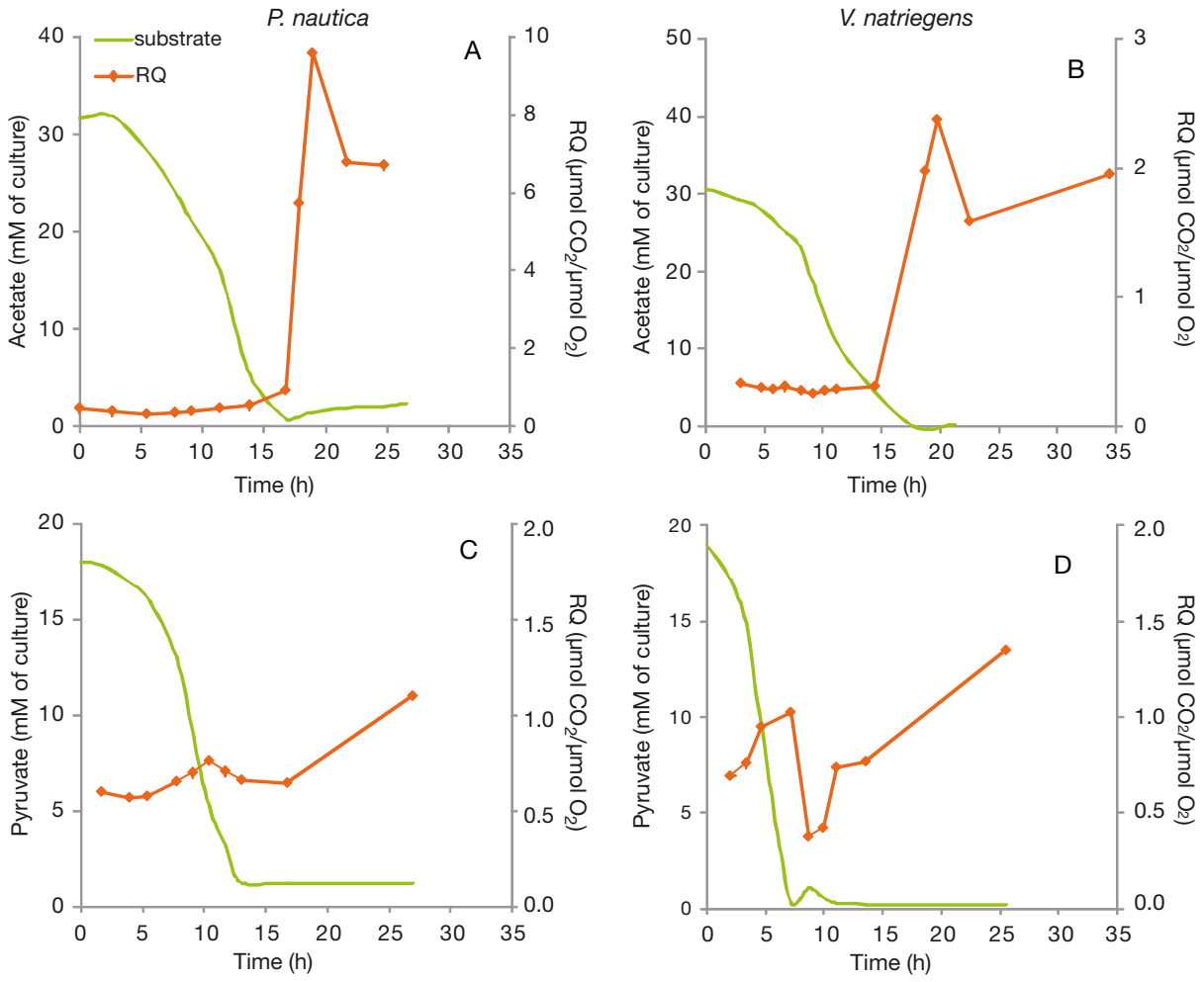

Fig. 5. Short-term experiments: RQ and substrate (carbon source) versus time. Note the carbon substrate sufficiency on the left and carbon substrate depletion (starvation) on the right of each panel. Top panels are acetate-based cultures; bottom panels are pyruvate-based cultures. Left panels are *Pseudomonas nautica*, right panels are *Vibrio natriegens*. In all experiments, RQ rises during starvation

After that time, R_{CO_2}, although low, steadily increased in relation to R_{O_2}. This trend contrasted greatly with both the biomass and the enzyme activity data, all of which maintained relatively high values throughout substrate limitation.

All the slopes between the 2 respiration measurements (R_{CO_2} and R_{O_2}) for the long-term experiments were compared (Fig. 4) and displayed a good correlation in all cases except in *V. natriegens* on acetate. The slope of these functions was the overall RQ. This is another way to estimate RQ, but requires sufficient samples collected during the same culture condition. ANCOVA analysis between cultures that were growing on the same carbon substrate (i.e. *P. nautica* or *V. natriegens* on acetate) shows that the slopes were not significantly different (Fig. 4; $p > 0.05$). In contrast, for the same bacterial species but for different substrates (i.e. *P. nautica* on acetate or pyruvate), the slopes appeared to be significantly different ($p < 0.05$). All the slopes (RQs) during starvation conditions were significantly different ($p < 0.05$).

Respiration quotient

Short-term experiments. RQ measurements in the short-term experiments are shown in Fig. 5. When the cultures were substrate-sufficient, RQs were low, but when carbon substrate limitation started in the acetate cultures of both *P. nautica* and *V. natriegens*, the RQ values rose dramatically (Table 1, Fig. 5A,B). In *P. nautica* on pyruvate, the RQ rose only at the end of the experiment (Fig. 5C). In contrast to the constant pre-starvation RQ values in the first 3 experiments, in the *V. natriegens* culture growing on pyruvate (Fig. 5D), the signal displayed noticeable noise

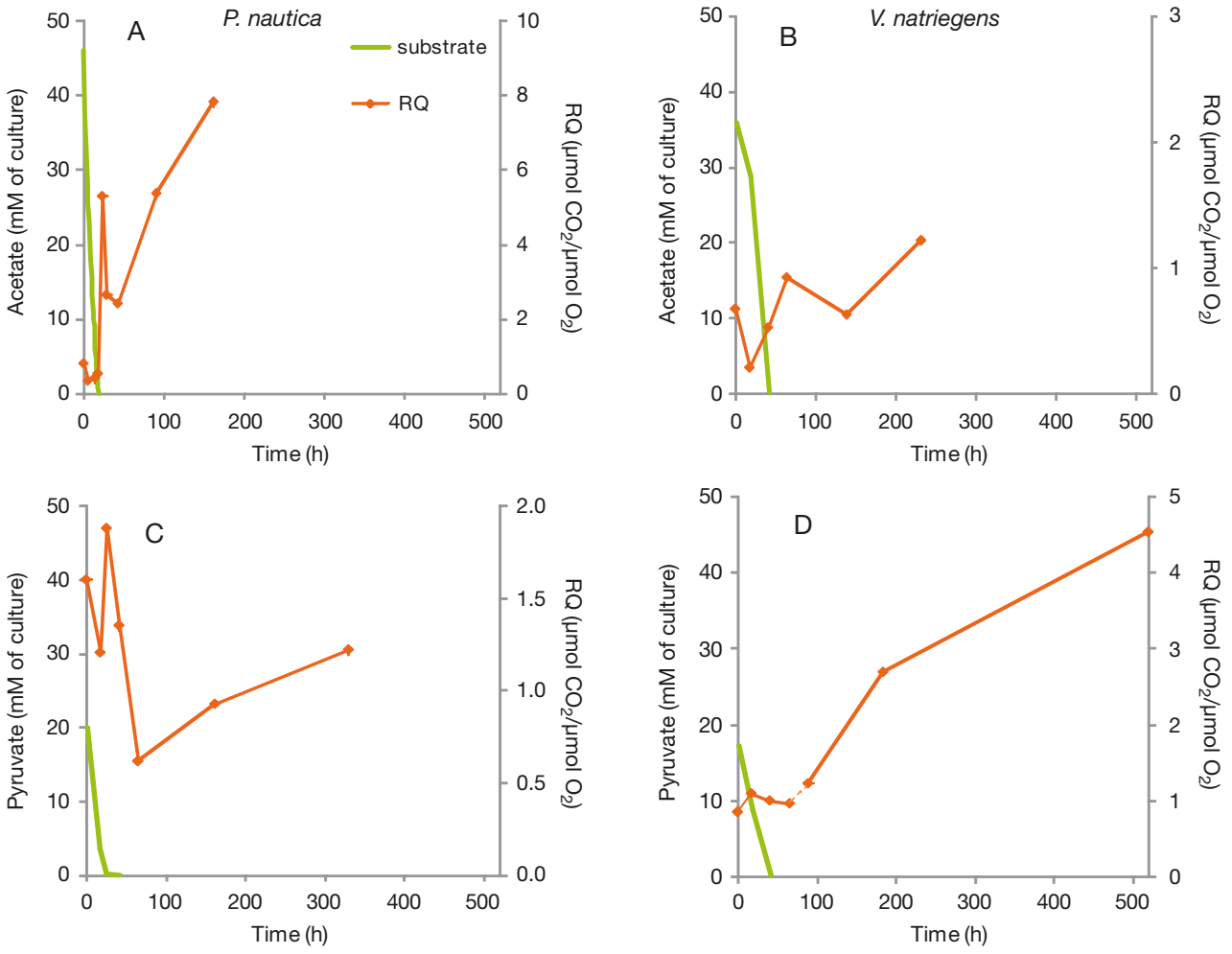

Fig. 6. Long-term experiments: RQ and substrate (carbon source) versus time. Top panels are acetate-based cultures; bottom panels are pyruvate-based cultures. Left panels are *Pseudomonas nautica*, right panels are *Vibrio natriegens*. In all panels, RQ increases during the late starvation period

between Hours 7 and 12 of the experiment. Afterwards, at 25 h, it rose to an RQ of 1.4.

In summary, these short-term experiments show a distinct shift in the RQ when either bacteria species was growing on acetate (Figs. 5A,B). In contrast, when growing on pyruvate, both species displayed an RQ shift, but it was less pronounced than in Figs. 5C,D.

Long-term experiments. The time-course signature of the long-term experiments (Fig. 6) is comparable to that of the short-term experiments (Fig. 5). Of the 4 experiments, *P. nautica* on acetate (Fig. 6A) behaved most similarly to the short-term experiments. In all long-term experiments, carbon substrate limitation began within the first 50 h. We see an RQ jump when carbon substrate limitation was reached, with a subsequent rise to the end of the experiment (Fig. 6A). When *P. nautica* was growing on pyruvate (Fig. 6C) and carbon substrate limitation set in after 65 h, the RQ rose. However, it rose at a lower rate than in the second experiment (*V. natriegens*) on pyruvate in which RQ rose to 4.5 at the experiment end (Fig. 6D). In *V. natriegens* growing on acetate (Fig. 6B), RQ rose to 1.2 after acetate exhaustion; however, this RQ was lower than the high value (8.0) found in the *P. nautica* culture growing on acetate (Fig. 6A, Table 1).

DISCUSSION

RQ and enzyme activity

RQ shifts can result from the variable activities of different biochemical pathways. In the Krebs cycle, R_{CO_2} is the collective activity of 3 enzymes: isocitrate dehydrogenase (IDH, EC 1.1.1.42), α-ketoglutarate

dehydrogenase (α-KGDH, EC 1.2.7.3), and pyruvate dehydrogenase (PDH, EC 1.2.2.2) (Walsh & Koshland 1984, Holms 1986a,b). R_{O_2}, although largely controlled by the ETS, is also influenced by all the oxidases, oxygenases, and hydroxylases in a cell. In Figs. 1 & 2, we see that in starvation, IDH and Φ activities are still present at relatively high levels, and an IDH dominance is not detectable. Consequently, the RQ elevation (Figs. 5 & 6) cannot be attributed to the relative activity trends of IDH and ETS. In nature, microorganisms are able to react quickly to environmental changes, adapting their metabolic profiles to organic inputs (Martinez et al. 1996, Mudryk & Donderski 1997, Sala & Güde 2004, Mudryk & Skórczewski 2006). Still, such a mechanism requires time-consuming enzyme induction or activation. Here, the maintenance of IDH and Φ levels after starvation suggests that *Vibrio natriegens* and *Pseudomonas nautica* are prepared to bypass this time-consuming step to be ready to react immediately via Michaelis-Menten kinetics to any fortuitous availability of substrates. Thus, the differential impact of declining substrates ($NADP^+$, isocitrate, NADH, and succinate) induced by substrate-limitation could provide the explanation for the declining physiological rates.

RQ variability and carbon limitation

In this study, we observed a rising RQ after nutrient depletion in cultures of 2 marine bacteria. The RQ ranged from a low of 0.3 during exponential growth to a high of 9.6 during senescence and nutrient depletion (Fig. 5A). In comparison, the RQs in fresh waters of Quebec ranged from 0.25 to 2.26 (Berggren et al. 2012). Those authors argue that the common assumptions of 0.8 to 1.0 RQ values in the literature are not justified. Furthermore, they argue that the use of a constant RQ depresses estimations of the metabolic balance between respiration and photosynthesis, i.e. the P/R ratio. They maintain that high RQs have a physiological explanation based on the different biochemical pathways of DOC degradation. However, it is not obvious how RQ could rise to 10 (Fig. 5A) by a biochemical mechanism. This would mean that O_2 consumption is severely repressed or that CO_2 production is highly stimulated. It is difficult to see how starvation could cause those shifts. Possibly, vegetative cells are being transformed into spores, but what reactions are involved are unknown. Here, the 2 physiological signals of the respiration do appear to shift in the right direction for the RQ to rise. As starvation sets in, R_{O_2} decreases before R_{CO_2}, causing the RQ rise (Fig. 1A,B). Why this uncoupling occurs is not obvious. Recent research with nematodes has shown that starvation decreases R_{O_2} without decreasing the activity of ETS (Chin et al. 2014). This impact was traced to increases in the concentration of alpha-keto glutarate (α-KG), the product of IDH activity. Since CO_2 is also a product of IDH activity, perhaps R_{CO_2} is also stimulated, resulting in a rise in the RQ. Here, we saw the decrease in R_{O_2} and stability in the ETS but not an increase in the IDH activity and R_{CO_2}. Nevertheless, could starvation have caused an increase in the α-KG by another mechanism in our cultures? Additional experimentation is required to address this question.

Another observed trend in our results was that the temporal signatures of the RQs differed between the acetate- and the pyruvate-grown cultures, independent of the species. This difference occurred in short-term experiments but not in the long-term ones (Figs. 5 & 6). A comparison of the slopes in the R_{CO_2}-R_{O_2} regressions (Fig. 4) showed that the biochemistry, not the bacterial species, determined the RQ. An ANCOVA test confirmed it by showing that there was no significant difference in the slopes of *P. nautica* and *V. natriegens* growing on the same substrate (Fig. 4). Because acetate and pyruvate are products of lipid and carbohydrate metabolism, respectively, the short-term experimental results (Table 1) confirmed that carbohydrate consumption leads to higher RQs (0.7 to 1) than does lipid consumption (0.4 to 0.3). This is consistent with the historical use of the RQ.

Future research

In the present study, we document the accompanying RQ changes that occurred in bacterial culture experiments designed to detect the shift in the relationship between respiratory enzyme activity and respiration as exponentially growing bacteria encounter carbon-substrate limitation. The RQ rose after the carbon limitation set in. We had seen this before with short-term experiments of *P. nautica*, but here, this rise is confirmed in long-term experiments with *P. nautica* and with both short-term and long-term experiments with *V. natriegens*. In future experiments, it would be interesting to focus on the shift in RQ during the opposite transition, from nutrient-depletion (senescence) to carbon-sufficiency, but adding more detailed measurements of the organic components of the culture media. Learning the

organic composition of the media during the 'nutrient-depletion' phase would be especially interesting. In addition, investigation should be extended to bacteria cultures growing on different substrates; to cultures of phytoplankton, macroalgae, zooplankton, and benthic invertebrates; as well as to natural assemblages of these organisms in field samples. Exploiting mesocosms, as is done in the KOSMOS programs (Riebesell et al. 2013), is an excellent way of achieving the later experiments.

Finally, RQ variability could be used to study biological energy production. At present, RQ is used to differentiate different types of biological oxidation. However, in the future, RQ could also be used as an index of the cost, in terms of organic carbon, of generating a unit of energy (energy production cost). Oxygen consumption is directly related, in aerobic metabolism, to ATP production, and the molar ratio of the ATP production to the reduction of an oxygen atom (ATP/$2e^-$ ratio) (Ferguson 2010) ranges from 2.5 (NADH oxidation) to 1.5 (succinate oxidation). Accordingly, high RQs would indicate that more organic matter is oxidized to produce a unit of ATP than in cases involving low RQs. An inverted RQ [1/RQ = (ΔO_2 consumed)/(ΔCO_2 produced)] is an index of energy production efficiency. Future research should explore using the RQ to assess both energy production costs and energy production efficiencies.

CONCLUSIONS

(1) RQ changes with nutritional impact on the physiological state. Bacteria under substrate-limitation have RQs higher than when they are growing under substrate-sufficient conditions.

(2) RQs rise by nearly a factor of 10 (Fig. 5A) during substrate-deficiency (senescence). This suggests that the nutritional conditions of bacteria should be considered, in the future, when selecting an RQ for calculation purposes.

(3) Should this level of RQ variability (Fig. 3) be found in phytoplankton, macroalgae, zooplankton, and benthic invertebrates, then ecosystem models, ocean carbon flux calculations, and predictions of the balance between ocean autotrophy and heterotrophy could be impacted.

(4) Respiration under starved and well-fed conditions of acetate- and pyruvate-based cultures of *P. nautica* and *V. natriegens* show that rates of O_2 consumption and CO_2 production during substrate-sufficiency parallels the biomass increase (Fig. 1), but that after nutrients are exhausted, both respiration rates fall rapidly. This drop in the respiration rates occurs much sooner and more rapidly than the slow decline in the enzyme activities.

(5) IDH activity and potential respiration (ETS activity expressed in terms of oxygen) can maintain high levels after nutrients are depleted (Figs. 1 & 2), but after 10 h of starvation, these values and the cell protein begin to slowly decline as substrate-deficiency persists. The decline in the enzyme activities is much slower after the point of nutrient exhaustion than is the rapid decline in the physiological respiration rates.

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