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SHORT COMMUNICATION

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Oxygen uptake kinetics of *Pseudomonas chlororaphis* grown in glucose- or glutamate-limited continuous cultures

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Abstract Oxygen uptake and glucose and glutamate oxidation kinetics of the heterotrophic bacterium *Pseudomonas chlororaphis* grown in glucose- or glutamate-limited cultures under oxygen-saturating or oxygen-limiting conditions were determined. K_m values for oxygen were 1.4–5.6 μM. Only in the case of glucose were significantly lower K_m values and enhanced specific oxygen affinity (V_{max}/K_m) per cell found under oxygen-limiting conditions. Both K_m and specific affinity values for glucose and glutamate oxidation were apparently affected by oxygen concentration, although a statistically significant enhancement of the oxidation kinetics was found only for glutamate. The kinetic data found for *P. chlororaphis* support the conclusion that the outcome of competition for oxygen with *Nitrosomonas europaea* in the rhizosphere of oxygen-releasing macrophytes will primarily be determined by oxidation kinetics of the electron donor instead of the oxygen uptake kinetics of the respective organisms.

Key words Oxygen kinetics · K_m · *Pseudomonas chlororaphis* · *Nitrosomonas europaea* · Chemostat · Competition

Introduction

On the basis of literature values reported for oxygen uptake kinetics, heterotrophic bacteria should outcompete autotrophic ammonia oxidisers in oxygen-limited environments such as the rhizosphere of oxygen-releasing plants. The ammonia oxidisers are proposed to be outcompeted because of their inferior oxygen consumption kinetics (Sharma and Ahlert 1977; Prosser 1989; Van Niel 1991). The fact that ammonia-oxidising bacteria can be active in the rhizosphere of oxygen-releasing plants (Bodelier et al. 1996; Engelaar et al. 1995) implies that an excessive amount of oxygen is available, that carbon is limiting the heterotrophic bacteria, or that ammonia oxidisers compete successfully for oxygen by using more favourable oxygen uptake kinetic parameters than have been reported to date. Published half-saturation constants for oxygen uptake by heterotrophic bacteria are in the range of 0.018–6.5 μM O_2 depending on the type of cytochrome oxidase present (Poole 1983), while values for ammonia oxidisers are reported in the range of 7–40 μM (Sharma and Ahlert 1977; Stenstrom and Poduska 1980; Prosser 1989). To date, there is only one study presenting a lower K_m value (1.3 μM), determined for the ammonia-oxidising species *Nitrosomonas europaea* grown in oxygen-limited continuous cultures (Laanbroek and Gerards 1993).

Prediction of the competitive outcome on the basis of kinetic values presented in the literature may be suspect because of the variety of methods used to measure uptake kinetic parameters and differences in growth conditions prior to kinetic measurements. The latter can result in the presence of different cytochromes with different kinetics properties (Poole 1983). Moreover, it is more appropriate to compare specific affinities, defined as the ratio V_{max}/K_m, in order to study competitiveness at low substrate concentrations (Healy 1980).

The goal of this study was to determine oxygen uptake kinetics of the heterotrophic bacterium *Pseudomonas chlororaphis*, which is common in the rhizosphere of the oxygen-releasing macrophyte *Glyceria maxima* (Nijburg et al. 1997), and to compare these kinetic values with those of the ammonia-oxidising bacterium *N. europaea* on the basis of specific affinity (e.g. V_{max}/K_m). *N. europaea* also appeared to be abundant in the root zone of *G. maxima*, as demonstrated by a positive reaction to specific antibodies

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for *N. europaea* in the most diluted positive tube of an MPN enumeration (Bodelier, unpublished results). The heterotrophic bacterium was grown under energy-limited conditions, as has been done for *N. europaea* (Laanbroek and Gerards 1993), with glucose or glutamate as substrate, both of which are representative root exudates (Curl and Truelove 1986).

Materials and methods

Organism and culture conditions

P. chlororaphis ATCC 43928 (Christensen and Tiedje 1988) was grown in continuous culture in a 1,250-ml Biostat M fermenter (Braun, Melsungen, Germany) at a dilution rate of 0.015 h^{-1} (pH 7.5, 25° C, 300 rpm). Duplicate chemostats were operated to reach steady states under excessive (80% air saturation; ≈ 214 μM O_2; maintained by sparging with air) and limited oxygen supply (oxygen diffusion from the headspace) with glucose or glutamate as the electron donors and the sole carbon source. When the oxygen supply was reduced, 0% air saturation was maintained by the oxygen-consuming activity of the cells. The medium contained per litre: 330 mg $(NH_4)_2SO_4$, 100 mg KH_2PO_4, 40 mg $MgSO_4 \cdot 7H_2O$, 20 mg $CaCl_2$, 500 mg NaCl, 396 mg glucose · H_2O or 353 mg glutamic acid and 1 ml trace element solution (Verhagen and Laanbroek 1991). The pH of the culture was maintained at pH 7.5 by automatic titration with 0.1 M NaOH. Steady state was assumed when optical density (660 nm), cell numbers and glucose/glutamate concentrations remained constant for five volume changes.

Measurements of oxygen uptake and substrate oxidation kinetics

Oxygen uptake and substrate oxidation kinetics were determined as initial rate measurements in a closed chamber with a polarographic oxygen electrode with washed cell suspensions from steady-state cultures using a biological oxygen monitor (Oxygen meter 781, Strathkelvin Instruments Glasgow, Scotland) according to Laanbroek and Gerards (1993). Oxygen uptake kinetics were determined as the tangent of oxygen concentration versus time plots at different oxygen concentrations of 1–230 μM at 28° C in the presence of excess glucose or glutamate. Substrate oxidation kinetics were determined by measuring initial oxygen consumption rates at concentrations of glucose or glutamate of 10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0.005 mM and saturating oxygen concentrations (90–100% air saturation). Substrate uptake/oxidation curves obeyed Michaelis-Menten kinetics as is shown in Fig. 1. Maximal uptake/oxidation rates (V_{max}) and saturation constants (K_m) were estimated with the computer program ENZPACK version 2.0 (P. A. Williams, Bangor, Wales, UK) using the „direct linear" method of Eisenthal and Cornish-Bowden (1974).

Enumeration of *P. chlororaphis* cells

Cell counts were performed microscopically using a Bürker-Türk counting chamber immediately upon sampling of the chemostat. Cell counts were also performed on the washed cell suspensions that were used for biological oxygen monitor measurements.

Analytical methods

Glucose concentration was measured with a glucose test combination (Boehringer, Mannheim, Germany) based on the GOD-POD (glucose oxidase-peroxidase) method described by Werner et al. (1970). Glutamate concentration was measured by means of a test combination (Boehringer, Mannheim, Germany) based upon an enzymatic assay using glutamate dehydrogenase and diaphorase as described by Beutler (1985). Total, particulate and dissolved organic carbon were determined according to Verhagen and Laanbroek (1991) using a total organic carbon analyzer (model 700, O. I. Corporation, College Station, Tex., USA).

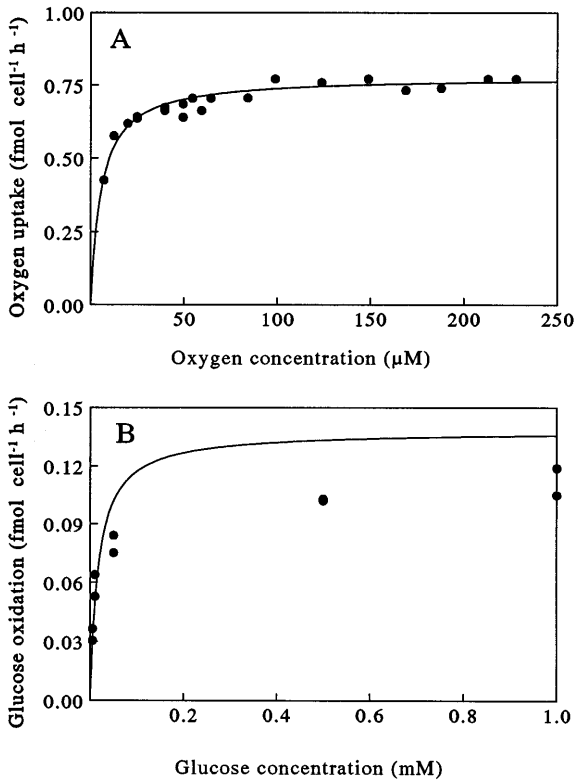

Fig. 1 Typical oxygen uptake (**A**) and glucose (**B**) oxidation plots of washed steady-state cell suspensions (8.4×10^8 ml^{-1}) of *Pseudomonas chlororaphis* as determined using a biological oxygen monitor. The curves were drawn using the respective K_m and V_{max} values as estimated with the direct linear method. Data fitted the Michaelis-Menten equation; R^2 = 0.92 and 0.86 for oxygen uptake and glucose oxidation, respectively

Results and discussion

Cell numbers at 0% air saturation ($0.36–0.72 \times 10^9$ ml^{-1}) were approximately 50% of those at oxygen saturation ($0.96–1.3 \times 10^9$ ml^{-1}), indicating oxygen-limiting conditions. Residual glucose concentrations during steady state were always below the detection limit (15–20 μM) of the assay, while glutamate concentrations in the culture were 10 μM at saturating oxygen conditions and 15 μM at 0% of air saturation (data not shown). Besides the residual substrate carbon and biomass carbon, dissolved organic carbon in the cultures was relatively low (6–11% of the amount of carbon in the influent, data not shown) and independent of the oxygen concentration. The reduced cell yield and the absence of accumulation of substrate or intermediates at low oxygen concentration suggest uncoupling of energy generation and growth, as was demonstrated by Harrison and Loveless (1971) as the consequence of limited oxygen availability.

The K_m values for oxygen found for *P. chlororaphis* were in the range of those found for cytochrome *o* (1.8–

Table 1 Kinetics of oxygen uptake by *Pseudomonas chlororaphis* grown in continuous culture (D = 0.015 h^{-1}, 25° C, pH 7.5) at 80% and 0% air saturation and with glucose or glutamate as electron donor. Yields and product of specific affinity and yield are also presented. Values are the means (± 1 SE) of two replicate chemostats. Significant differences between means of the different parameters for oxygen are indicated by different letters (Tukey's test, $P < 0.05$, $n = 2$)

Substrate uptake studied	Oxygen (% air sat.)	K_m (μM)	V_{max} (fmol $cell^{-1}$ h^{-1})	Specific affinity (pl $cell^{-1}$ h^{-1})	Yield[a] (10^{13} cells mol^{-1})	Specific affinity × yield (1 h^{-1} mol^{-1})
Oxygen (*glucose* as e^- donor)	80	5.63 ± 0.11a	0.79 ± 0.01a	0.14 ± 0.00a	14.77 ± 1.71a	20,640
	0	1.38 ± 0.02b	1.05 ± 0.03a	0.76 ± 0.03b	6.20 ± 1.95a	46,750
Oxygen (*glutamate* as e^- donor)	80	4.80 ± 0.87a	0.64 ± 0.14a	0.13 ± 0.01a	12.25 ± 0.29a	16,370
	0	4.86 ± 0.11a	1.29 ± 0.34a	0.26 ± 0.06a	6.57 ± 2.07a	16,060

[a] Cells produced per mol oxygen were calculated assuming that the difference between total organic carbon present in the culture and the amount of carbon supplied was respired. The respired amount of carbon was stoichiometrically converted to the amount of oxygen consumed (× 6 and × 4.5 mol O_2 per mol glucose or glutamate, respectively)

Table 2 Kinetics of glucose and glutamate oxidation by *P. chlororaphis* grown in continuous culture (D = 0.015 h^{-1}, 25° C, pH 7.5) at 80% or 0% air saturation. Yields and product of specific affinity and yield are also presented. Values are the means (± 1 SE) of two replicate chemostats. Significant differences between means of the different parameters for glucose and glutamate, respectively, are indicated by different letters (Tukey's test, $P < 0.05$, $n = 2$)

Substrate uptake studied	Oxygen (% air sat.)	K_m (μM)	V_{max} (fmol $cell^{-1}$ h^{-1})	Specific affinity (pl $cell^{-1}$ h^{-1})	Yield[a] (10^{13} cells mol^{-1})	Specific affinity × yield (1 h^{-1} mol^{-1})
Glucose	80	17.6 ± 0[b]	0.14 ± 0.00c	7.91 ± 0.08ab[c]	56.55 ± 3.85a	4,476
	0	11.3 ± 0.4a	0.16 ± 0.00ab	14.00 ± 0.20a	23.85 ± 6.15b	3,351
Glutamate	80	30.5 ± 0.2b	0.13 ± 0.02c	4.27 ± 0.57b	36.60 ± 0.80ab	1,565
	0	15.1 ± 2.1a	0.23 ± 0.02a	15.90 ± 3.70a	19.85 ± 5.65b	2,947

[a] Calculated as the number of cells produced per mol glucose/glutamate consumed by the culture. The amount of substrate consumed is calculated as mol glucose/glutamate in the influent –mol glucose/glutamate in the culture at steady state

[b] Not included in statistical procedure because it is not possible to determine the homogeneity of variances with 0 SE

[c] Homogeneity of variances achieved after ln transformation

6.5 μM) and cytochrome aa_3 (4–7 μM) (Table 1), cytochrome *o* being the most common cytochrome in bacteria (Poole 1983). With glucose as the electron donor, specific oxygen affinity increased under oxygen-limiting conditions (Table 1), which was not the case with glutamate. The expression of higher affinity cytochromes such as cytochrome *d* under oxygen limitation has been reported earlier (Poole 1983). However, K_m values for this type of cytochrome are 0.018–0.35 μM and, thus, much lower than the values in this study.

We compared oxygen kinetics of *P. chlororaphis* with those of the autotrophic ammonia oxidiser *N. europaea* using exactly the same methods and growth conditions reported previously (Laanbroek and Gerards 1993; Laanbroek et al. 1994). Specific oxygen affinity for the ammonia oxidiser appeared to be higher - 1.37 and 1.09 nl $cell^{-1}$ h^{-1} for cultures grown at 80% and 0% air saturation, respectively (Laanbroek et al. 1994). This difference was due to a substantially higher V_{max} for *N. europaea* that at oxygen-limiting conditions was ten- and 29-fold the V_{max} for *P. chlororaphis* with glucose or glutamate as electron donor, respectively. However, besides the oxygen uptake kinetics, the yield has to be taken into account in predicting the outcome of the competition for oxygen between those organisms, as described by Button (1994) in the following equation:

$$A = \mu/(a°_A \times Y) \quad (1)$$

A = the substrate concentration required to reach growth rate (μ) given the specific substrate affinity ($a°_A$) and yield (Y). The product of specific affinity and yield determines the attainable growth rate at a certain substrate concentration. At oxygen limitation, this product for oxygen as a substrate is higher for *P. chlororaphis* for growth on glucose and glutamate (Table 1) as compared to the same value for *N. europaea*, which was 8,720 l h^{-1} mol^{-1} (Laanbroek et al. 1994). This difference is due to a lower yield value and not to inferior oxygen uptake kinetics of the ammonia oxidiser. This implies that the critical oxygen concentration for *N. europaea* at a dilution rate of 0.015 h^{-1} in chemostats is higher than that of *P. chlororaphis*, leading to washout of the nitrifier before oxygen-limiting conditions are even reached.

Assuming a situation where no washout takes place (e.g. in soil) and a cell-to-cell competition for oxygen, the oxygen concentration at which both organisms will acquire equal amounts of oxygen per cell can be calculated based on their K_m and V_{max} values. The substrate consumption rate, *V*, would be equal for both organisms at this oxygen concentration (S), as indicated by the Monod equation:

$$(V_{max1} \times S)/(K_{m1} + S) = (V_{max2} \times S)/(K_{m2} + S) \quad (2)$$

Solving for substrate concentration S produces Eq. 3:

$$S = (V_{max1} \times K_{max2} - V_{max2} \times K_{m1})/(V_{max2} - V_{max1}) \quad (3)$$

Substitution of the V_{max} and K_m values of *P. chlororaphis* and *N. europaea* at 0% air saturation (D = 0.015 h^{-1}) in this equation with the measured values produces S values of –0.50 and –4.48 μM O_2 for growth on glucose and glutamate, respectively. Thus, at all oxygen concentrations, the oxygen consumption rate per cell is higher for *N. europaea*, which must be regarded as the better competitor when no growth takes place.

Competition only for oxygen is probably rare under natural conditions since limiting amounts of the electron donors also influence the performance at low oxygen availability. From the glucose and glutamate oxidation kinetics of *P. chlororaphis* (Table 2) and the data of Laanbroek et al. (1994), it is evident that ammonia-oxidising bacteria have very poor electron donor kinetics. In fact, the products of specific affinity and yield reveal that according to Eq. 1, the ammonium concentration for maintaining a growth rate for 0.015 h^{-1} of *N. europaea* has to be 40- to 70-fold higher than the concentrations of glucose or glutamate for the growth of *P. chlororaphis*. This strongly suggests that a prerequisite for actual oxygen competition between ammonia oxidisers and heterotrophs is that ammonium be available in excess as compared to the availability of the electron donor concentration for the heterotroph.

Since ammonia-oxidising bacteria are not good in the acquisition of limiting amounts of ammonium and lose in competitions for this compound with plants and heterotrophic bacteria (Verhagen et al. 1994; Verhagen and Laanbroek 1991), we can conclude that in most natural ecosystems it is more likely that ammonia-oxidising bacteria will be limited by ammonium than by oxygen.

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