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Availability of O_2 as a Substrate in the Cytoplasm of Bacteria under Aerobic and Microaerobic Conditions

TANJA ARRAS, JAN SCHIRAWSKI, AND GOTTFRIED UNDEN*

Institut für Mikrobiologie und Weinforschung, Universität Mainz, 55099 Mainz, Germany

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The growth rates of *Pseudomonas putida* KT2442 and mt-2 on benzoate, 4-hydroxybenzoate, or 4-methylbenzoate showed an exponential decrease with decreasing oxygen tensions (partial O_2 tension [pO_2] values). The oxygen tensions resulting in half-maximal growth rates were in the range of 7 to 8 mbar of O_2 (corresponding to 7 to 8 μM O_2) (1 bar = 10^5 Pa) for aromatic compounds, compared to 1 to 2 mbar for nonaromatic compounds like glucose or succinate. The decrease in the growth rates coincided with excretion of catechol or protocatechuate, suggesting that the activity of the corresponding oxygenases became limiting. The experiments directly establish that under aerobic and microaerobic conditions (about 10 mbar of O_2), the diffusion of O_2 into the cytoplasm occurs at high rates sufficient for catabolic processes. This is in agreement with calculated O_2 diffusion rates. Below 10 mbar of O_2, oxygen became limiting for the oxygenases, probably due to their high K_m values, but the diffusion of O_2 into the cytoplasm presumably should be sufficiently rapid to maintain ambient oxygen concentrations at oxygen tensions as low as 1 mbar of O_2. The consequences of this finding for the availability of O_2 as a substrate or as a regulatory signal in the cytoplasm of bacterial cells are discussed.

During aerobic growth, bacteria consume O_2 at high rates. The consumption of O_2 by oxidases takes place on the cytoplasmic side of the membrane. Since the diffusion of O_2 across the membrane is rapid, the supply of the oxidases with O_2 is guaranteed even at the very low O_2 tensions which are sufficient for aerobic growth (<1 mbar of O_2) (2, 4, 15, 16). Previously, the rate of O_2 diffusion into the cytoplasm of *Escherichia coli* was calculated from the cell dimensions and the diffusion coefficients and compared to the rates of O_2 consumption (2, 21, 22). It was estimated that at O_2 tensions as low as 0.2 mbar of O_2 (corresponding to 0.2 μM O_2), the supply of O_2 by diffusion exceeds the consumption by respiration. In agreement with this calculation, in *E. coli* the fermentation pathways were synthesized and used only at partial O_2 tension (pO_2) values well below 1 mbar of O_2 (3). Thus, O_2 is able to reach the active sites of the oxidases at rates sufficient to support aerobic respiration even at very low O_2 tensions.

The O_2 supply of the cytoplasmic space is not known and might be different from that of the membrane where the oxidases are located. From the diffusional parameters and the cell dimensions, it was calculated that the concentrations of O_2 should be the same within and outside the bacteria at O_2 tensions as low as 1 mbar of O_2 (21, 22). Therefore, we aimed for an experimental proof of the availability of O_2 in the bacterial cytoplasm under aerobic and microaerobic conditions.

For the degradation of aromatic compounds like benzoate, oxygenases are required for oxidative cleavage of the aromatic ring (7, 10). Due to the cytoplasmic location of the oxygenases and the need for molecular oxygen as a cosubstrate, the turnover of aromatic compounds depends on the availability of O_2 in the cytoplasm. The rate of metabolism of aromatic compounds therefore provides information on the minimal rate of O_2 diffusion into the cytoplasm. To this end, the relation of metabolism of various aromatic compounds to the pO_2 of the medium was studied. *Pseudomonas putida* KT2442 degrades benzoate by benzoate-1,2-dioxygenase and catechol-1,2-dioxygenase (*ortho* pathway), whereas 4-hydroxybenzoate is degraded via 4-hydroxybenzoate monooxygenase and protocatechuate-3,4-dioxygenase (*ortho* cleavage). 4-Methylbenzoate is metabolized by *P. putida* mt-2 by toluate-1,2-dioxygenase and catechol-2,3-dioxygenase (*meta* cleavage) (5, 8). The K_m values for O_2 of the oxygenases (≥7 μM) (1, 6, 12, 13) are much higher than those of the oxidases (<0.1 μM) (4, 15, 16). Therefore, limitation of growth or catabolism by O_2 must be due to the oxygenases, and information on O_2 diffusion into the cytoplasm and the O_2 concentration in the cytoplasm can be drawn from the growth-limiting pO_2 values. Here we report on experimental proof of the availability of O_2 in the cytoplasm. This finding also provides a basis for our understanding of the O_2 sensing by cytoplasmic O_2 sensor proteins like FNR (fumarate nitrate reductase regulator) from *E. coli* (9, 19, 22, 23) and the homologous proteins from *Pseudomonas* (17, 25) which are supposed to react directly with O_2 in the cytoplasm (2, 22, 23).

MATERIALS AND METHODS

Bacteria and media. *P. putida* KT2442 and *P. putida* mt-2(pWWO) were provided by I. Wagner-Döbler (Braunschweig, Germany) and M. Schlömann (Stuttgart-Hohenheim, Germany) (5, 24). *P. putida* KT2442 was grown in a modified M9 mineral medium (pH adjusted to 7.1) supplemented with a mineral salts solution and with glucose, succinate, benzoate, or 4-hydroxybenzoate (10 mM each) as sources of carbon and energy. The mineral salts solution was a combination of the following: solution 1, containing 25.39 g of $MgCl_2$, 2.0 g of $CaCO_3$, 4.5 g of $FeSO_4 \cdot 7H_2O$, 0.85 g of $MnSO_4 \cdot H_2O$, 1.44 g of $ZnSO_4 \cdot 7H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.16 g of $CaSO_4 \cdot 0.5H_2O$, and 0.02 g of H_3BO_3 dissolved in 51.3 ml of concentrated HCl and with water added to 100 ml; solution 2, containing 360 mM $FeSO_4 \cdot 7H_2O$; and solution 3, containing 1 M $MgSO_4$. Solutions 1 and 2 were filter sterilized, and solution 3 was autoclaved. Then 50 ml of solution 1, 2.5 ml of solution 2, 25 ml of solution 3, and 22.5 ml of autoclaved H_2O were combined. The medium was supplemented with 0.25 ml of the resulting mineral salts solution per 100 ml. *P. putida* mt-2(pWWO) was grown in a phosphate-buffered medium (14.0 g of $Na_2HPO_4 \cdot 12H_2O$, 2.0 g of KH_2PO_4 per liter) supplemented with a salts solution (20 ml/liter of medium) containing 2.5 g of $Ca(NO_3) \cdot 4H_2O$ (autoclaved separately) per liter, 0.5 g of Fe(III)NH_4-citrate per liter, 10 g of $MgSO_4 \cdot 7H_2O$ per liter, 50 g of $(NH_4)_2SO_4$

* Corresponding author. Mailing address: Institut für Mikrobiologie und Weinforschung, Universität Mainz, Becherweg 15, 55099 Mainz, Germany. Phone: 49-6131-393550. Fax: 49-6131-392695. E-mail: unden@mzdmza.zdv.uni-mainz.de.

per liter, and 50 ml of the Pfennig SL6 metal salts solution (14) per liter. The C source for *P. putida* mt-2 was 4-methylbenzoate (10 mM). *E. coli* MC4100 (18) was grown in M9 medium (11) supplemented with an amino acid mixture (20) and glucose (10 mM) or succinate (10 mM).

Growth. *P. putida* was grown at 30°C. Growth under anaerobic conditions was performed in sealed bottles under an atmosphere of nitrogen (2, 3). For aerobic conditions, the bacteria were grown in Erlenmeyer flasks filled to within 10% of the maximal volume under vigorous shaking (3). The medium was inoculated from cultures grown overnight under aerobic conditions in the mineral medium (same C source as that in the main culture) to an A_{578} not higher than 0.06.

Growth in an oxystat. Growth at defined O_2 tensions (pO_2) was performed in an oxystat (chemostat with constant pO_2) (Biostat MD; Braun, Melsungen, Germany) in batch culture (1.5 liters) with constant stirring (400 rpm) (2, 3). The pO_2 value of the medium was measured continuously with an O_2 electrode. The pO_2 was maintained at a constant level by supplying air (valve I) and N_2 (valve II) to the vessel. When the pO_2 fell below 98% of the set value, valve I opened and sterile air was supplied till the set value was reached. The flow of air was increased manually from about 0.16 to 1.6 liters min^{-1} during growth to compensate for the increasing O_2 consumption. The flow of N_2 (0.1 liters min^{-1}) was decreased or switched off as required. *E. coli* was grown in the oxystat in the supplemented M9 medium as described previously (2, 3). Growth rates were calculated from $\mu = \ln (A_{578,t_2}/A_{578,t_1}) \cdot (t_2 - t_1)^{-1}$, where t_2 and t_1 are the times of measurement and A_{578,t_1} and A_{578,t_2} are the absorbance values at 578 nm measured at t_1 and t_2, respectively.

Analytical procedures. Substrates (glucose, succinate, benzoate, 4-hydroxybenzoate, and 4-methylbenzoate) and products (catechol, protocatechuate) were determined from the supernatants of the cultures after removal of the bacteria by centrifugation. The substances were analyzed by high-performance liquid chromatography (HPLC) on an Aminex HPX87H column (300 by 7.8 mm; Bio-Rad) with 6.5 mM H_2SO_4 as the eluent (flow rate, 0.55 ml min^{-1}) as described previously (20). The following substrates and products were determined and quantified with standard solutions by a refractive index and by a UV light detector (215 nm): glucose, glycerol, acetate, ethanol, formate, pyruvate, fumarate, succinate, and lactate. Benzoate (retention time [R_t] = 68 min), 4-hydroxybenzoate (R_t = 51 min), catechol (R_t = 32.0 min), and protocatechuate (R_t = 33.3 min) were identified by the R_t values of authentic substances, and the ratio of the refractive index/UV absorption at 215 nm was used to confirm the identities.

RESULTS AND DISCUSSION

Growth of *P. putida* on aromatic compounds at limiting pO_2. *P. putida* was grown on nonaromatic and aromatic substrates like glucose, succinate, and benzoate in an oxystat at defined pO_2 values. In the oxystat, the set pO_2 values could be maintained constant for the duration of the growth experiment. With glucose or succinate as the substrate, the growth behavior changed only when the pO_2 fell below 10 mbar of O_2 (corresponding to about 10 μM O_2). At lower pO_2 values, the growth rate and final cell density decreased, and under anaerobic conditions, no growth was observed. With benzoate or 4-hydroxybenzoate as the substrate, under aerobic conditions (212 mbar of O_2; air saturation), growth of *P. putida* (Fig. 1A) was similar to that on glucose or succinate. However, with decreasing pO_2 values, growth rate and yield decreased significantly.

In Fig. 1B, the rate constants for growth on aromatic and nonaromatic substrates are plotted versus the pO_2 values. With glucose and succinate, growth of *P. putida* commenced at very low pO_2 values and showed a saturation curve with increasing pO_2. With the aromatic substrates benzoate and 4-hydroxybenzoate, growth started only at pO_2 values above 4.2 mbar. With 4-methylbenzoate, the O_2 requirement was even higher (data not shown). The maximal growth rates for succinate and benzoate corresponded to doubling times of 46 and 51 min, respectively. When *E. coli* was grown on succinate or glucose, the growth rates increased immediately from 0 mbar, similar to the growth rates of *P. putida* on the same substrates (data not shown). For growth on glucose, however, the growth rates did not drop to zero at 0 mbar of O_2 due to the presence of fermentative growth. Thus, the growth rate at 0 mbar of O_2 ($\mu = 0.011\ min^{-1}$) was about half that of *E. coli* grown under aerobic conditions on glucose ($\mu = 0.020\ min^{-1}$).

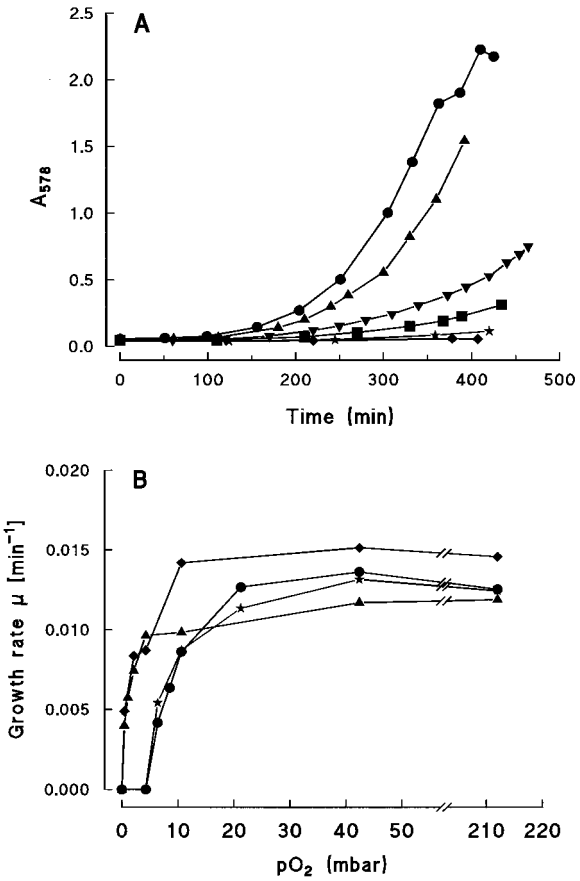

FIG. 1. Growth (A) and growth rates (B) of *P. putida* KT2442 grown in an oxystat on aromatic and nonaromatic substrates as a function of pO_2. (A) Growth with benzoate in the oxystat at different pO_2 values. Growth was performed in the mineral medium with 10 mM benzoate at 212 (●), 21 (▲), 8 (▼), 6 (■), 4 (★), and 0 (◆) mbar of O_2. (B) The rate constants for growth (μ) were determined from the growth curves shown in panel A. Substrates (10 mM each) for growth: benzoate (●), 4-hydroxybenzoate (★), glucose (◆), and succinate (▲).

$pO_{0.5}$ values for growth on aromatic substrates are higher than those for growth on nonaromatic substrates. For *P. putida*, from the relation of the growth rates to the pO_2 values, the $pO_{0.5}$ values for the substrates can be determined. The $pO_{0.5}$ value corresponds to the pO_2 value yielding half-maximal growth rates (2, 3). The measured $pO_{0.5}$ values can be classified into two groups. For growth of *P. putida* and *E. coli* on glucose and succinate, low values ($pO_{0.5} \leq 2$ mbar of O_2) were found. For growth on aromatic compounds, the $pO_{0.5}$ values were distinctly higher and corresponded to about 8 mbar for growth on benzoate and 4-hydroxybenzoate and to 19 mbar for growth on 4-methylbenzoate.

Excretion of intermediates under O_2 limitation. The growth medium was analyzed for products or intermediates excreted by the bacteria during growth in the oxystat at different pO_2 values (Fig. 2). The medium was analyzed by HPLC for the presence of organic acids, alcohols, sugars, and aromatic compounds, in particular for intermediates of the respective metabolic routes. During growth at high oxygen tensions, all types of substrates were completely oxidized by *P. putida* and no organic end products were detected in significant amounts (>0.05 mol/mol of substrate). From glucose and succinate, no end products were excreted even at decreased oxygen tensions,

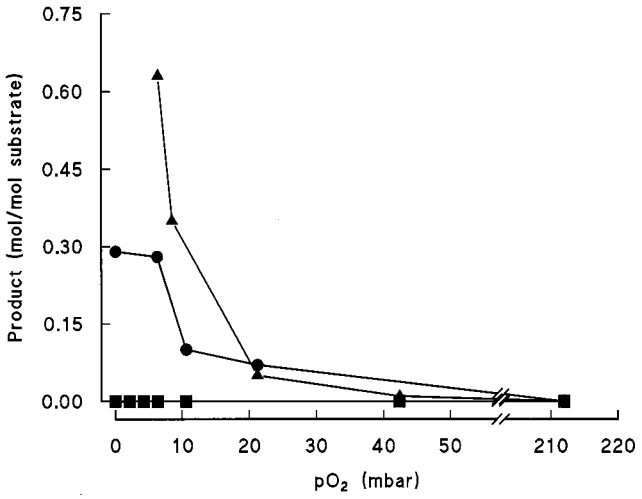

FIG. 2. Products in the culture medium of *P. putida* KT2442 excreted after growth at various pO_2 values in the oxystat: catechol (▲) excreted during growth on benzoate (10 mM), protocatechuate (●) excreted during growth on 4-hydroxybenzoate, and end products (■) from succinate and glucose. For end products tested, see Materials and Methods.

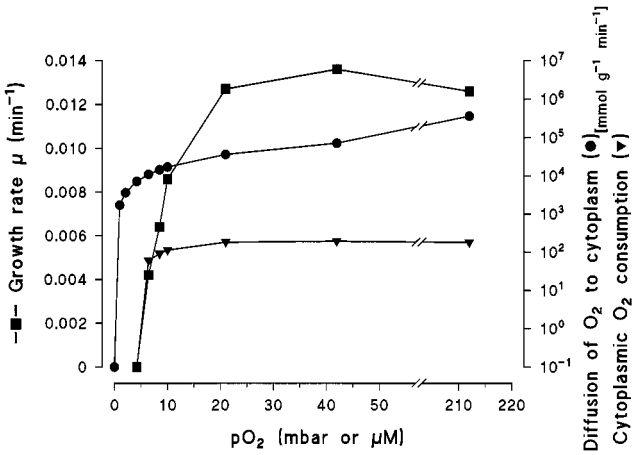

FIG. 3. Growth rate, O_2 consumption by dioxygenases, and maximal rate of O_2 diffusion into the bacteria (*P. putida* KT2442) as a function of the pO_2 in the medium during growth on benzoate. The growth rate was determined as described in the legend to Fig. 1. The O_2 consumption by the dioxygenases was calculated from μ and from the molar growth yield on benzoate ($Y_{benzoate}$) under the respective conditions as shown in Table 1. The rate of diffusion into the bacteria was calculated as a function of external oxygen concentration (with the internal concentration set at zero). For the calculation, the diffusion coefficients of O_2 in water, phospholipid, and cytoplasm and the cell dimensions were used (2, 22).

indicating complete oxidation. When *P. putida* KT2442, however, was grown on benzoate, a product was found in the medium at oxygen tensions below 20 mbar which was identified as catechol. Catechol is an intermediate of the *ortho* cleavage pathway of benzoate. Up to 0.65 mol of catechol per mol of benzoate was measured, indicating a severe limitation in the *ortho* cleavage pathway resulting in the excretion of the intermediate without complete oxidation. During growth on 4-hydroxybenzoate, protocatechuate was excreted in large amounts (0.28 mol/mol of 4-hydroxybenzoate) at oxygen tensions below 20 mbar. Obviously, limitation of protocatechuate-3,4-dioxygenase activity (6) by low O_2 tensions causes accumulation and excretion of the intermediate protocatechuate. Therefore, in both pathways, central steps, i.e., the dioxygenases reacting on catechol and protocatechuate, are limiting under microaerobic conditions.

Availability of O_2 as an intracellular substrate for aromatic substrate degradation. The data can be used to roughly estimate the rate of O_2 diffusion into the cells required for this process. The rate of O_2 consumption by the oxygenases in the cell interior (ν_{O_2in}) is twice the rate of benzoate metabolism ($\nu_{benzoate}$) (Table 1) corresponding to 0.22 mmol of benzoate · min^{-1} · g (dry weight)$^{-1}$ and 0.44 mmol of O_2 · min^{-1} · g (dry weight)$^{-1}$. The calculated rate of O_2 diffusion into the cells under aerobic conditions, 360 mmol of O_2 · min^{-1} · g (dry weight)$^{-1}$ (Table 1), exceeds the rate of intracellular O_2 consumption by the oxygenases by 3 orders of magnitude.

Plotting the rates of growth on benzoate as a function of the pO_2 shows that diffusion of O_2 is not limiting under aerobic or microaerobic conditions (Fig. 3): growth is limited apparently only at pO_2 values below 10 mbar of O_2. The growth limitation coincides with the excretion of the oxygenase substrates catechol and protocatechuate, demonstrating that oxygenation is the growth-limiting step. At 10 mbar of O_2, the calculated diffusion is still higher by 2 orders of magnitude than the O_2 consumption by the oxygenases (Fig. 3). Therefore, the decrease in the growth rate is presumably not caused by limiting O_2 diffusion but by the high K_m value (20 μM) of the oxygenase (Table 1). Thus, at pO_2 values as low as 10 mbar, there is substantial O_2 present in the cytoplasm. The high K_m values of the oxygenases prevented an analysis of the situation at lower pO_2 values. The calculation of the diffusion rates for O_2, however, also suggests that at distinctly lower oxygen tensions,

TABLE 1. Metabolic and energetic parameters for growth of *P. putida* on benzoate

Parameter[a] or data	Value	Reference or source
Experimental parameters		
Maximal growth rate on benzoate (μ_{max}) (min^{-1})	0.0136	Fig. 1B
Molar growth yield on benzoate ($Y_{benzoate}$) [g (dw) · mol^{-1}]	62	Fig. 1A
$pO_{0.5}$ for half-maximal growth rate (μM)	8.2	This work
K_m (catechol-1,2-dioxygenase) (μM O_2)	20	1, 12
Calculated data [mmol · g (dw)$^{-1}$]		
Benzoate consumption at μ_{max} ($\nu_{benzoate}$)	0.22	$\nu_{benzoate} = \mu/Y_{benzoate}$
Intracellular O_2 consumption (oxygenases, ν_{O_2in})	0.44	$\nu_{O_2in} = 2 \cdot \nu_{benzoate}$
Intracellular O_2 consumption (oxygenases plus oxidases, $\nu_{O_2in,\,t}$)	1.65	$\nu_{O_2in,\,t} = 7.5 \cdot \nu_{benzoate}$[b]
Maximal rate of O_2 diffusion into *P. putida* cells	360	2, 21

[a] g (dw), grams (dry weight).
[b] Based on the growth reaction (1 benzoate + $7.5O_2 \rightarrow 7CO_2 + 3H_2O$).

down to 1 mbar of O_2, the intracellular pO_2 equals the extracellular pO_2 (2, 21, 22). The O_2 present under aerobic and microaerobic conditions most likely is also used as the signal for O_2 sensor-regulator proteins like FNR from *E. coli* (9, 22) and homologous proteins from *Pseudomonas* strains (17, 25) which are thought to react directly with O_2 in the cytoplasm (2, 23). The regulatory $pO_{0.5}$ which causes a switch from active (anaerobic) to inactive (aerobic) FNR is in the range of 1 to 5 mbar of O_2 in the external medium for many target genes, which is in good agreement with the results found in the present work.

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