Cultivation of *Aquincola tertiaricarbonis* L108 on the fuel oxygenate intermediate tert-butyl alcohol induces aerobic anoxygenic photosynthesis at extremely low feeding rates

Thore Rohwerder, Roland H. Müller, M. Teresa Weichler, Judith Schuster, Thomas Hübschmann, Susann Müller and Hauke Harms

Helmholtz Centre for Environmental Research – UFZ, Department of Environmental Microbiology, Permoserstr. 15, D-04318 Leipzig, Germany

Aerobic anoxygenic photosynthesis (AAP) is found in an increasing number of proteobacterial strains thriving in ecosystems ranging from extremely oligotrophic to eutrophic. Here, we have investigated whether the fuel oxygenate-degrading betaproteobacterium *Aquincola tertiaricarbonis* L108 can use AAP to compensate kinetic limitations at low heterotrophic substrate fluxes. In a fermenter experiment with complete biomass retention and also during chemostat cultivation, strain L108 was challenged with extremely low substrate feeding rates of tert-butyl alcohol (TBA), an intermediate of methyl tert-butyl ether (MTBE). Interestingly, formation of photosynthetic pigments, identified as bacteriochlorophyll *a* and spirilloxanthin, was only induced in growing cells at TBA feeding rates less than or equal to maintenance requirements observed under energy excess conditions. Growth continued at rates between 0.001 and 0.002 h\(^{-1}\) even when the TBA feed was decreased to values close to 30% of this maintenance rate. Partial sequencing of genomic DNA of strain L108 revealed a bacteriochlorophyll synthesis gene cluster (*bchFNBHL*) and photosynthesis regulator genes (*ppsR* and *ppaA*) typically found in AAP and other photosynthetic proteobacteria. The usage of light as auxiliary energy source enabling evolution of efficient degradation pathways for kinetically limited heterotrophic substrates and for lowering the threshold substrate concentration *S*\(_{\text{min}}\) at which growth becomes zero is discussed.

INTRODUCTION

Microbes growing on methyl tert-butyl ether (MTBE) and related fuel oxygenates as sole carbon and energy source are rare in nature. Despite the enormous environmental impact of these fuel additives as groundwater pollutants (Kolb & Püttmann, 2006; Moran *et al*., 2005; Schmidt *et al*., 2002; van Wezel *et al*., 2009), only a few bacterial strains capable of growth with MTBE and related ether compounds have been found (Hyman, 2013). Among them, the betaproteobacterium *Aquincola tertiaricarbonis* L108 utilizes fuel oxygenates most effectively (Müller *et al*., 2008), likely due to the employment of a suit of enzymes well-adapted to the bulky tert-butyl moiety of MTBE (Rohwerder *et al*., 2006; Schäfer *et al*., 2012; Schuster *et al*., 2013; Yaneva *et al*., 2012). Nearly identical sequences of the pathway-encoding genes in the MTBE-degrading strains L108, *Rhodococcus ruber* IFP 2001 and *Methylibium petroleiphilum* PM1 as well as in other bacteria not related to fuel oxygenate degradation (Schuster *et al*., 2012, 2013; Yaneva *et al*., 2012) indicate that horizontal gene transfer has played an important role in the evolution of such an efficient metabolism. However, the evolutionary drivers towards degradation rates supporting growth on MTBE as sole source of energy and carbon remain enigmatic. In a theoretical study, the use of MTBE as a heterotrophic substrate was evaluated from both stoichiometric and kinetic viewpoints (Müller *et al*., 2007). It became evident that energy delivered by reported rates of MTBE degradation was generally close to (below or above) typical maintenance demands of bacteria, thus either restricting them to co-metabolic MTBE degradation or allowing autarkic growth only at relatively low rates. Undoubtedly, overcoming these kinetic limitations is very crucial for the evolution of an efficient MTBE pathway.

To learn more about the productive utilization of fuel oxygenates and its restriction by bacterial maintenance...
requirements, we cultivated *A. tertiaricarbonis* L108 in a constantly substrate-fed bioreactor with complete biomass retention (also referred to as a recycling fermenter). This kind of cultivation had already been applied to other pollutant-degrading bacterial strains, such as the 2,4-dichlorophenoxycetate-degrading *Cupriavidus necator* JMP 134 and the 3-chlorobenzoate-degrading *Pseudomonas* sp. strain B13, to study growth and maintenance under limiting conditions (Müller & Babel, 1996; Tros et al., 1996). Biomass accumulation continues as long as the individuals receive more substrate than required for mere biomass maintenance (Chesbro et al., 1979; van Verseveld et al., 1984). Biomass retention also gives them time to adapt to subcritical substrate fluxes by, e.g. entering more economic modes of growth and maintenance or redistributing energy fluxes between these processes. From the rates of biomass formation and corresponding substrate consumption, changes in the distribution of energy between biomass build-up and maintenance become evident. Generally, when applying biomass recycling fermentation to an exponentially growing culture, growth proceeds through two distinct phases of linear growth characteristic by progressively reduced specific growth rates. Finally, when the specific feeding rate is equal to maintenance requirements, a zero growth phase can be reached (Muller & Babel, 1996; Tros et al., 1996).

In the present investigation, we cultivated *A. tertiaricarbonis* L108 in a recycling fermenter system on the MTEB intermediate tert-butyl alcohol (TBA), as this substrate has the practical advantage of a much lower volatility than MTBE. Surprisingly, when the specific TBA feed fell below maintenance requirements as calculated from chemostatic growth of strain L108 on this substrate, zero growth was not established but still an increase of biomass was observed. This was accompanied by the synthesis of bacteriochlorophyll *a* and carotenoids, a feature formerly unknown in strains of *A. tertiaricarbonis* (Lechner et al., 2007). The formation of these pigments was also induced by applying biomass recycling fermentation to an exponentially growing culture, and corresponding substrate consumption, thus changes in the distribution of energy between biomass build-up and maintenance become evident. Generally, when applying biomass recycling fermentation to an exponentially growing culture, growth proceeds through two distinct phases of linear growth characteristic by progressively reduced specific growth rates. Finally, when the specific feeding rate is equal to maintenance requirements, a zero growth phase can be reached (Muller & Babel, 1996; Tros et al., 1996).

**METHODS**

**Strain and cultivation.** For cultivation with biomass retention, *A. tertiaricarbonis* L108 (Lechner et al., 2007) was incubated in a laboratory fermenter (T4700CT; INFORS HT) at 20 °C and pH 6.8 ± 0.1. The fermenter was equipped with a hollow fibre cross-flow module (SPS 9005-4, 0.2 µm, 0.7 m²; Fresenius) operated in a bypass through which fermenter suspension was pumped at 60 l h⁻¹ (gear pump model MC 7970; Gather Industrie GmbH). After achieving chemostatic steady state at a dilution rate of 0.015 h⁻¹, cultivation was switched to the biomass retention mode. Then, only effluent cleared of cells in the cross-flow module left the fermenter via a pump connected to a level control electrode, thus holding the working volume of 2 l constant. A mineral salt medium (MSM) composed of (in mg l⁻¹): NH₄Cl, 760; KH₂PO₄, 340; K₂HPO₄, 485; CaCl₂ × 6 H₂O, 27; MgSO₄ × 7 H₂O, 71.2; CuSO₄ × 5 H₂O, 0.785; MnSO₄ × 4 H₂O, 0.81; ZnSO₄ × 7 H₂O, 0.44; Na₂MoO₄ × 2 H₂O, 0.25 and FeSO₄ × 7 H₂O, 47.6 was used for cultivation containing TBA at 1 g l⁻¹ (13.5 mM). This medium was supplied at 0.03 l h⁻¹. A vitamin solution was fed at 0.3 ml h⁻¹ from a separate reservoir stored in a refrigerator to give bulk concentrations of (in µg l⁻¹) biotin, 20; folic acid, 20; pyridoxine–HCl, 100; thiamine–HCl, 50; riboflavin, 50; nicotinic acid, 50; d-l-Ca-pantothenate, 50; p-amino- benzoic acid, 50; lipoic acid, 50 and cobalamin, 50. For additional chemostat cultivations, a Biostat D (B. Braun Biotech) fermenter (first experiment) and the INFORS T4700CT (second experiment) were used with working volumes of 1.2 and 2 l, respectively. Cultivation conditions and feeding medium were as described for the biomass retention experiment. Dilution rates and TBA concentrations in the inflowing medium are indicated below. Incubation in the dark was achieved by completely covering the fermenter with aluminium foil. Cultivation of batch cultures in MSM containing TBA and vitamins was performed in glass serum bottles with horizontal shaking as previously described (Rohwerder et al., 2006). Shifts to low nutrient conditions were achieved by washing exponential phase cells in MSM and incubating further in MSM plus vitamins at an optical density of 700 nm (OD₇₀₀) of 0.5 and at TBA concentrations of 0, 10, 25, 50, 100 and 250 mg l⁻¹ either in the dark or under laboratory illumination (TLD 58W/840, Philips).

**Flow cytometry.** Flow cytometry was used for accurately counting cells and for determining cell growth by cell size and DNA content measurements using a MoFlo cytometer (DakoCytomation). To samples of 450 µl bacterial suspension, 50 µl of a 30 % NaN₃ solution was immediately added and then stored at 4 °C until analysed. Just prior to being stained, the cells were washed in a phosphate buffer (400 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.2) and diluted to OD₇₀₀ 0.05 in the same buffer. Diluted cell suspensions (2 ml) were treated with 1 ml solution A (2.1 g citric acid/0.5 g Tween 20 in 100 ml distilled water) for 10 min. Then, the cells were washed and resuspended in 2 ml solution B (0.68 µM DAPI, 400 mM Na₂HPO₄, pH 7.0) for at least 20 min in the dark at room temperature. Excitation by 500 mW at 488 nm was used to analyse the forward scatter whereas side scatter was used as the trigger signal at the first observation point. DAPI was excited by 150 mW of ML-UV (333–365 nm) at the second observation point. The orthogonal signal was first reflected by a beam-splitter and then recorded after reflection by a 555 nm long-pass dichroic mirror, passage by a 505 nm short-pass dichroic mirror and a BP 488/10. DAPI fluorescence was passed through a 450/65 band pass filter. Photomultiplier tubes were obtained from Hamamatsu Photonics (models R 928 and R 3896). Amplification was carried out at linear or logarithmic scales, depending on the application. Fluorescent beads (Poly bead Microspheres; diameter, 0.483 mm; flow check BB/Green compensation kit, Blue Alignment Grade, ref. 23520, Polyscience) were used to align the MoFlo (coefficient of variation about 2 %). Also, an internal DAPI-stained bacterial cell standard was introduced for tuning the device up to a coefficient of variation value not higher than 6 %.

**Spectra of cytochromes and photosynthetic pigments.** Cytochromes were measured by differential spectroscopy of dithionite-reduced cells minus oxidized cells (Chance, 1952). The density of cell suspensions was adjusted to an OD₇₀₀ of 20. Dithionite was added at a concentration of 4 mg ml⁻¹ followed by 2 min incubation. Oxidation was obtained by sparging with oxygen for 1 min. Measurements were conducted right after these treatments with a Cary 400 Scan spectrometer at wavelengths between 400 and 650 nm. Bacteriochlorophyll and carotenoids were extracted with 1 ml of either tetrahydrofuran, methanol or methanol/acetone (30:70, v/v) from wet pellets obtained after brief centrifugation of 2 to 10 ml of cell suspension in a microcentrifuge (Eppendorf, type 5415 D). After addition of the solvent, samples were vortexed for 1 min at room temperature and thereafter extracted for 10 min at 23 °C and 1400 r.p.m. in a thermomixer. Particle-free extract was obtained by
centrifugation for 7 min at 13 200 r.p.m. The supernatant was used for chromophore characterization recording absorption from 300 to 900 nm.

Sequencing of L108 DNA. Genomic DNA of A. tertiaricarbonis L108 was extracted using the MasterPure DNA Purification kit (Epicentre) and sequenced by Illumina’s HiSeq 2000 technology (GATC Biotech). The obtained DNA sequences were analysed for open reading frames using Rast (Rapid Annotation using Subsystem Technology, http://rast.nmpdr.org/). An 11 801 bp sequence including the bchFNBHLC, ppAA, ppS1R and bchG genes was obtained. Sequence similarities were evaluated using the BLAST alignment tool (Altschul et al., 1997).

Other analyses. Biomass was routinely followed by measuring OD700 or by determining the protein concentration in aliquots of suspension (washed cells) according to Lowry et al. (1951). For calculating biomass values, a correlation with photometric OD700 values was established by drying samples to weight constancy at 105 °C resulting in a multiplicative factor of 0.38 to give the dry mass in g l⁻¹. TBA was determined by headspace gas chromatography as described elsewhere (Rohwerder et al., 2006; Schafer et al., 2011). A detection limit for TBA of 6.8 µM (0.5 mg l⁻¹) was achieved. Purity of cultures was tested by inspection of colonies formed after plating diluted suspension on rich agar (containing 10 g l⁻¹ peptone, 10 g l⁻¹ yeast extract and 1.8 g l⁻¹ fructose). In addition, purity was ascertained by 16S rRNA gene sequencing throughout the cultivation experiments as previously described (Jechalke et al., 2011) using the primer pair unibac27f and univ519r.

Calculation of growth parameters. Increase of biomass Δx (in mg l⁻¹ h⁻¹) during recycling fermentation was calculated from linear regression analysis of biomass values of the respective growth phases. Using the constant TBA feeding rate \( q_S = 0.2025 \text{ mmol l}^{-1} \text{ h}^{-1} \), TBA concentrations in the culture remained below detection limit at all times, resulting in a specific yield coefficient \( Y \) of

\[
Y = \frac{\Delta x}{q_S} \text{ (mg dry biomass mmol}^{-1} \text{TBA)} \tag{1}
\]

Specific growth rates \( \mu \) and specific TBA feeds \( F \) were derived according to

\[
\mu = \frac{\Delta x}{X} \text{ (h}^{-1} \text{)} \tag{2}
\]

and

\[
F = \frac{q_S}{X} \text{ (mmol TBA g}^{-1} \text{ biomass h}^{-1} \text{)} \tag{3}
\]

where \( X \) is the biomass at a specific time obtained from the linear regression analysis. Maintenance coefficients \( m_z \) were calculated according to the Pirt equation (Pirt, 1982) as

\[
m_z = \left(1 - \frac{1}{Y} \right) \mu \text{ (mmol TBA g}^{-1} \text{ biomass h}^{-1)} \tag{4}
\]

where \( Y_{\text{max}} \) is equal to the theoretical yield coefficient \( Y_{\text{theor}} \) on TBA which takes only into account the energy expenditure for biosynthesis, calculated as described elsewhere (Muller et al., 2007; Rohwerder et al., 2011) to be 62.53 mg biomass mmol⁻¹ TBA. For the determination of the limiting substrate concentration \( S_{\text{min}} \) at which \( \mu \) becomes zero (Muller et al., 2007), an extended Monod model was applied with

\[
\mu = \frac{m_S Y_{\text{max}}}{K_S + S} - m_z Y_{\text{max}} \text{ (h}^{-1} \text{)} \tag{5}
\]

in which \( S \) is the substrate concentration, \( m_S Y_{\text{max}} \) the decay rate accounting for biomass maintenance and \( K_S \) the substrate concentration required for half maximum growth. Due to maintenance costs, the true maximum growth rate \( \mu_{\text{max}} \) cannot be reached and only \( \mu_{\text{max}} \) can be experimentally observed according to

\[
\mu_{\text{max}} = \mu_{\text{max}} + m_S Y_{\text{max}} \text{ (h}^{-1} \text{)} \tag{6}
\]

Combining equations 5 and 6 gives the limiting substrate concentration \( S_{\text{min}} \) at \( \mu = 0 \) as

\[
S_{\text{min}} = \frac{K_S m_S Y_{\text{max}}}{\mu_{\text{max}}} \text{ (mM)} \tag{7}
\]

RESULTS

Cultivation with biomass retention

The cultivation of strain L108 with TBA was started in chemostat mode at a dilution rate \( D = 0.015 \text{ h}^{-1} \) and a constant substrate feeding rate \( q_S = 0.2025 \text{ mmol l}^{-1} \text{ h}^{-1} \) (with \( S_0 = 13.5 \text{ mM of TBA in the feeding medium} \)). Steady state was reached at a biomass concentration of 0.405 g l⁻¹ completely consuming the supplied substrate (TBA concentration in the culture was below detection limit). Then, the cultivation was continued in the biomass recycling mode while keeping \( q_S \) unchanged. This resulted in progressive biomass accumulation (Fig. 1a). Four linear growth phases I to IV were distinguished and interpreted in terms of shifted kinetic and stoichiometric parameters in response to decreasing specific substrate and/or energy provision. The increase of biomass \( \Delta x \) and the corresponding yield coefficient \( Y \) for the different growth phases were calculated according to equation 1 (Table 1). In phase I, \( \Delta x \) was about 3 mg l⁻¹ h⁻¹ which is already half of the productivity of the chemostat culture (6.08 mg l⁻¹ h⁻¹ resulted in \( \Delta x = 0 \) at \( D = 0.015 \text{ h}^{-1} \)). Hence, \( Y \) decreased from a chemostatic value of 30 to about 15 mg biomass mmol⁻¹ TBA (Table 1). As expected, the rate of biomass accumulation significantly decreased further in phase II, reaching a value close to 1 mg l⁻¹ h⁻¹. Then, the expected transition to zero growth was not observed but, surprisingly, phases III and IV with much better growth than in phase II followed, showing \( \Delta x \) values of about 4 and 2.5 mg l⁻¹ h⁻¹, respectively. Even after 37 days in the recycling mode, biomass accumulation continued, exceeding values of 2.6 g l⁻¹. However, these higher biomass concentrations could not be determined any further with sufficient precision, as the bacterial cells did not completely stay in suspension but tended to clog the filter system significantly (data not shown).

The stark contrast between growth phases II and III was also observed by flow cytometry analysis (Fig. 1b) when comparing cell size and DNA content to characterize changes in growth velocity (Muller et al., 2010). Samples taken during phase II of recycling fermentation showed the typical pattern of a slowly growing culture. The majority of cells possessed the same number of chromosome copies, while only a few bacteria were characterized by increased DNA content and forward scatter-related cell size increase (Fig. 1b, B2 and B3). This pattern is quite similar to the slow growth at the transition to the stationary phase.
Fig. 1. Cultivation of *A. tertiaricarbonis* L108 on TBA with biomass retention. The culture was pre-incubated on TBA in chemostat mode until steady state was reached ($D=0.015$ h$^{-1}$). (a) Biomass accumulation during recycling mode (TBA feed $q_S=0.2025$ mmol h$^{-1}$; filled circles). For visualization of the progressively decreasing substrate flux, specific TBA feeding rates (as percentage of chemostatic maintenance requirements $m_S=0.261$ mmol g$^{-1}$ h$^{-1}$; open circles) are also shown. Growth phases I to IV were defined by linear regression analysis. Incubation times B1 to B9, at which samples were submitted to flow cytometric analysis, are indicated by crosses. (b) Flow cytometric histograms of forward light scatter (related to cell size information) and DAPI-mediated fluorescence (related to DNA content) are shown for samples B1 to B9.

Table 1. Kinetic and stoichiometric parameters during cultivation of *A. tertiaricarbonis* L108 on TBA with biomass recycling

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>$Y$ mg mmol$^{-1}$</th>
<th>Incubation time</th>
<th>$\mu$ $10^{-3}$ h$^{-1}$</th>
<th>$F$ mmol g$^{-1}$ h$^{-1}$</th>
<th>Apparent $m_S$ mmol g$^{-1}$ h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemostat</td>
<td>30.0 ± 1.0</td>
<td></td>
<td>15</td>
<td>0.50 ± 0.02</td>
<td>0.261 ± 0.016</td>
</tr>
<tr>
<td>Phase I</td>
<td>14.7 ± 3.2</td>
<td>B1</td>
<td>7.9 ± 1.8</td>
<td>0.54 ± 0.05</td>
<td>0.411 ± 0.149</td>
</tr>
<tr>
<td>Phase II</td>
<td>5.1 ± 1.0</td>
<td>B2</td>
<td>1.5 ± 0.3</td>
<td>0.28 ± 0.02</td>
<td>0.259 ± 0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B3</td>
<td>1.4 ± 0.3</td>
<td>0.27 ± 0.02</td>
<td>0.251 ± 0.070</td>
</tr>
<tr>
<td>Phase III</td>
<td>18.6 ± 1.8</td>
<td>B4</td>
<td>4.0 ± 0.9</td>
<td>0.21 ± 0.04</td>
<td>0.151 ± 0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B5</td>
<td>3.3 ± 0.7</td>
<td>0.18 ± 0.03</td>
<td>0.126 ± 0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B6</td>
<td>2.3 ± 0.4</td>
<td>0.12 ± 0.02</td>
<td>0.085 ± 0.019</td>
</tr>
<tr>
<td>Phase IV</td>
<td>12.4 ± 0.7</td>
<td>B7</td>
<td>1.4 ± 0.1</td>
<td>0.11 ± 0.01</td>
<td>0.089 ± 0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B8</td>
<td>1.2 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>0.078 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B9</td>
<td>1.0 ± 0.1</td>
<td>0.08 ± 0.01</td>
<td>0.065 ± 0.007</td>
</tr>
</tbody>
</table>
observed in a batch culture of strain L108 growing on lactate (Figs S1 and S2, available in Microbiology Online). Samples from phase III, however, stand clearly out by having a significant fraction of cells with increased cell size and DNA content (Fig. 1b, B4 to B6), more resembling the transition of exponential to linear growth in the reference culture (Fig. S2). In line with this, specific growth rate $\mu$ (equation 2) decreased dramatically in phase II to values of less than 10% of the initial growth rate of 0.015 h$^{-1}$ (chemostat steady state before recycling mode), whereas a three times higher value of 0.004 h$^{-1}$ was obtained at the beginning of phase III (Table 1, sample B4).

The observed accumulation of biomass at the constant TBA feed $q_S$ resulted in an ongoing decrease of substrate flux per cell. Throughout the experiment, a nearly sevenfold reduction of the specific feed $F$ (equation 3) was achieved (Table 1). Consequently, only during growth phase I, $F$ exceeded the maintenance coefficient $m_S\sim0.261$ mmol TBA g$^{-1}$ biomass h$^{-1}$ calculated from the growth data obtained for the chemostatic steady state (at $\mu=0.015$ h$^{-1}$; equation 4). For visualizing this dramatic reduction, the specific TBA feed as percentage of the chemostatic $m_S$ is given in Fig. 1(a) for all data points. At the beginning of the recycling mode, the feed represented about 200% of the maintenance requirements, while decreasing rapidly to values close to 100% in phase II. Then, it even dropped to rates of less than 50% of chemostatic $m_S$ in phases III and IV. Considering only the substrate TBA as energy source, this reduction of the specific feed would result in a significant decrease of the actual maintenance requirements of the culture growing in the recycling mode, with final $m_S$ values representing only 25% of the chemostatic maintenance (Table 1, sample B9). As the minimum substrate concentration $S_{\text{min}}$ is proportional to $m_S$ (equation 7), this fourfold maintenance reduction also means that the threshold TBA concentration at which growth becomes zero equally diminished from chemostatic growth with excess substrate flux to the end of phase IV (Table 1, sample B9).

Detection of cytochromes and photosynthesis pigments

Remarkably, during phases II, III and IV of recycling mode cultivation, the colour of the culture changed gradually from the pale creamy appearance known for A. tertiaricarbonis L108 to a deep cherry-like tint. As there was no indication of contamination by other organisms throughout the whole experiment, the origin of the observed pigmentation was investigated. Under conditions of chemostatic growth characterized by excess substrate, TBA-grown cells displayed an absorption spectrum with maxima typical for cytochrome $c$ at around 550–552 nm and for cytochrome $a$ at around 600 nm, whereas no signals typical of $b$-type cytochromes (560–570 nm) were found (Fig. 2a). Shoulders/peaks at 514–522 nm were interpreted as $\beta$-bands of $a$- and $c$-type cytochromes (Chance, 1952). In contrast, cells collected at the end of the recycling experiment showed a pronounced maximum at around 516 nm and only little adsorption between 550 and 570 nm. Using methanol as a solvent, a green extract was obtained which displayed a spectrum typical for bacteriochlorophyll $a$ with an absorption maximum at 767 nm (Fig. 2b). Methanol/acetone (30:70, v/v) and tetrahydrofuran extracted bacteriochlorophyll with absorption maxima at 769 and 771 nm, respectively. In addition, a further pigment was detected with a spectrum typical for carotenoids of the spirilloxanthin type (Agalidis et al., 1999; Fuchs et al., 2007) with absorption maxima of 464, 495 and 529 nm in methanol/acetone and 473, 502 and 537 nm in tetrahydrofuran.

It should be noticed that this finding of photosynthesis pigments was unexpected and therefore, the transparent reactor had only been exposed to the normal day–night change of light in the laboratory rather than having been illuminated in a controlled way. In contrast to the pigment formation during recycling fermentation, chemostatic growth of strain L108 on TBA or acetate at a $D$ of 0.015 or 0.025 h$^{-1}$ (Müller et al., 2007) corresponding to specific feeds $\geq$ 200% of maintenance requirements, did not lead to induction of photosynthetic pigments at identical illumination. Likewise, batch cultures on TBA with substrate consumption rates exceeding 1000% of the observed chemostatic $m_S$ (Müller et al., 2008) did not show any remarkable pigmentation. In addition, rapid shifts transferring cells of strain L108 from batch cultures actively growing on TBA to low nutrient conditions (TBA concentrations in MSM between 0 and 3.4 mM) and incubating either at laboratory illumination or in the dark did not induce formation of photosynthetic pigments.

Chemostat experiments

Controlled induction of photosynthetic pigments was achieved in separate chemostat experiments in which strain L108 was cultivated on TBA at different levels of substrate limitation. As extraction with tetrahydrofuran was most efficient (Fig. 2b), pigment formation was checked by treating biomass samples with this solvent and measuring absorbance of extracts at 772 nm (bacteriochlorophyll $a$) and 501 nm (spirilloxanthin).

In a first approach, a biomass concentration of nearly 1.7 g l$^{-1}$ was reached under batch-like growth conditions with complete consumption of the initial TBA dosage. Then, the reactor was kept in darkness and the dilution rate was adjusted to $D=0.01$ h$^{-1}$ (with $S_0=40.5$ mmol l$^{-1}$ of TBA in the feeding medium). This resulted in $F=0.253$ mmol g$^{-1}$ h$^{-1}$, representing about 100% of the previously determined chemostatic $m_S$ (Table 1). After 10 days of incubation in the dark without any detectable formation of photosynthesis pigments (Fig. 3a), the reactor was exposed to the regular laboratory illumination for another week while keeping the TBA feeding rate unchanged. This led to the formation of bacteriochlorophyll and carotenoids. Throughout the cultivation, biomass
concentrations show high variations and bacteria were not completely suspended but partially attached to the fermenter wall. The biomass reduction at the end of the experiment, however, may also indicate that even under illumination growth rates of $\mu \geq 0.01 \text{ h}^{-1}$ could not be achieved.

In a second chemostat experiment, strain L108 was therefore incubated on TBA at a lower dilution rate. After reaching a biomass concentration of 1.6 g l$^{-1}$ under batch-like conditions and laboratory illumination, a dilution $D=0.002 \text{ h}^{-1}$ was applied with an initial...
$S_0=82$ mM of TBA and a reduction to $S_0=48$ mM after 10 days of incubation, corresponding to a TBA feed $F$ as low as 40 and 30%, respectively, of the chemostatic $m_S$. This extreme substrate limitation resulted in a washing out of the bacteria for 27 days (Fig. 3b). Then, biomass concentrations stabilized at about 1 g l$^{-1}$ indicating a growth rate close to 0.002 h$^{-1}$. According to equation 4, this would mean that the actual $m_S$ decreased to 25% of the chemostatic $m_S$ determined under excess energy conditions. A similar situation was observed at the end of phase IV of recycling fermentation (Table 1, sample B9).

In line with the assumption that induction of photosynthesis pigments was restricted to extremely low TBA feeding rates, carotenoids and bacteriochlorophyll were also synthesized in this experiment, although absorbance at 772 nm did not reach the level obtained in the first chemostat with the higher dilution rate. After 43 days of chemostat cultivation, the TBA flux was even lowered to $m_S$ at a chemostat with the higher dilution rate. After 43 days of chemostat cultivation, the TBA flux was even lowered to $m_S$, indicating that a further reduction of the heterotrophic substrate feed could not be compensated.

**Photosynthetic regulators PpsR and PpaA**

Partial sequencing of genomic DNA of strain L108 revealed a bacteriochlorophyll synthesis gene cluster ($bchFNHL$) and photosynthesis key regulator genes ($ppsR$ and $ppaA$) typically found in aerobic anoxygenic photosynthesis (AAP) as well as in proteobacteria with anaerobic anoxygenic photosynthesis activity (Fig. S3). The sequence of the predicted transcriptional regulator PpsR is similar to homologues of other proteobacteria, such as *Methylbacterium satilis universalis* FAM5 and the known AAP bacterium *Rhodospirillum centenum* SW with amino acid identities of 50 and 51%, respectively. Domain inspection revealed the typical C-terminal helix–turn–helix motif and a close cysteine residue (at position 441 in strain L108) highly conserved in PpsR (Masuda *et al.*, 2008). However, a corresponding cysteine residue at position 268 conserved in anaerobic photosynthetic purple non-sulfur bacteria, such as strains of *Rhodobacter sphaeroides* and *R. capsulatus*, is missing (Fig. 4 and Fig. S4), indicating that PpsR does not form intramolecular disulfide bonds under aerobic conditions in strain L108 (Masuda *et al.*, 2008).

The predicted PpaA protein from strain L108 is most similar to the sequence from *M. universalis* FAM5 showing 39% identical residues. Comparison with other PpaA from AAP bacteria and *Rhodobacter* strains revealed that the regulator from strain L108 also contains the haem-binding SCHIC (sensor containing haem instead of cobalamin) domain responding to oxygen (Fig. 5). This domain seems to be well conserved among PpaA and AppA regulators (Moskvin *et al.*, 2010). The latter is related to PpaA but responds to both oxygen and light. Thus far, it has only been found in *R. sphaeroides* as an additional photosynthetic regulator besides PpaA. In contrast to the high similarity of the SCHIC domains of these regulator proteins, the N-terminal sequence of PpaA not yet associated with any function shows high variability (Fig. S5).

**DISCUSSION**

During cultivation of *A. tertiaricarbonis* L108 on the MTBE intermediate TBA at very low substrate feeding rates less than or equal to maintenance requirements observed under energy excess conditions, growth did not become zero but continued at low rates. This prolonged growth was accompanied by the formation of photosynthetic pigments, suggesting that strain L108 is able to compensate energy limitations by AAP.

The substrate-based maintenance coefficient $m_S=(0.261 \pm 0.016)$ mmol TBA g$^{-1}$ h$^{-1}$ determined for strain L108 growing under steady state chemostat conditions at a TBA feed $\geq 200\%$ of this $m_S$ corresponds well to $m_S=0.233$ mmol TBA g$^{-1}$ h$^{-1}$ calculated previously from the energy equivalent-based coefficient $m_S=4.2$ mmol ATP g$^{-1}$ h$^{-1}$ derived from chemostat experiments using acetate as growth substrate (Müller *et al.*, 2007). It is in the typical range of maintenance costs observed with many heterotrophic bacteria (Müller & Babel, 1996). Accordingly, as has been demonstrated for other bacteria and substrates when applying the recycling fermentation technique (Müller & Babel, 1996; Tros *et al.*, 1996), strain L108 showed two distinct phases I and II of linear growth while the TBA feed approached a value equal to the maintenance requirements. This typical behaviour has been explained as an event of energy redistribution, e.g. shifts in pathways and reduction of energy spilling reactions, induced by the progressively decreasing specific energy flux from the available heterotrophic substrate. However, the prolonged accumulation of biomass in phase III at even higher growth rates than observed in phase II and an apparent reduction of maintenance requirements to 25% of the initial (chemostatic) value in phase IV were unexpected and cannot be explained without postulating the recruitment of an additional energy source. In line with this, a second regulatory event seemed to initiate phase III by inducing a substrate-independent, phototrophic mode of energy generation. This was quite surprising, since a photosynthetic machinery had not been recognized before in this intensively studied strain (Lechner *et al.*, 2007). Obviously, synthesis of bacteriochlorophyll and carotenoids is only induced in strain L108 at substrate feeding rates less than or equal to maintenance rate, a condition rarely occurring in typical batch or chemostat cultivations. However, the induction of photosynthetic pigments was also established in the two chemostat experiments applying a TBA feed representing only 30 to 100% of the maintenance coefficient determined under energy excess conditions. This behaviour corresponds in general to an observation made with the closely related betaproteobacterium *Roseateles depolymerans* 61A. This strain showed induction...
of an AAP apparatus when the supply of the carbon source was suddenly reduced (Suyama et al., 2002). Starvation by a gradual decrease in the supply rate of carbon, in contrast, did not induce photosynthesis in this strain which clearly deviates from the results with A. tertiaricarbonis L108.

Whereas anoxygenic photosynthetic activity in bacteria was observed only under anaerobic conditions in former times, i.e. applied to purple photosynthetic bacteria (Imhoff & Trüper, 1989), the group of known AAP species is steadily increasing (Yurkov & Beatty, 1998; Csotonyi et al., 2010). However, this property is more distributed in alpha- and gammaproteobacterial strains (Suzuki & Béja, 2007; Yurkova et al., 2002; Yutin et al., 2007) than in the Betaproteobacteria, and A. tertiaricarbonis L108 is currently only one further example belonging to the latter phylogenetic group besides R. depolymerans 61A (Suyama et al., 1999) inducing an AAP apparatus. Regulation of this trait appears diverse. Besides available energy as discussed above, oxygen concentration and light intensity is implied (Berghoff et al., 2011; Tomasch et al., 2011). The inductive effect at deficient carbon conditions was also revealed in a study on facultative photosynthetic bacteria in the Atlantic and Pacific Oceans under oligotrophic conditions (Kolber

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Partial CLUSTAL W alignment of PpsR sequences from A. tertiaricarbonis L108, M. universalis FAM5 (METUNv1_03978), R. centenum SW (RC1_2118), Jannaschia sp. strain CCS1 (Jann_0164), R. capsulatus SB 1003 (RCAP_rcc00668) and R. sphaeroides 2.4.1 (RSP_0282). Black and grey shading indicate a pair of cysteine residues conserved in Rhodobacter strains forming a disulfide bond under aerobic conditions (Masuda et al., 2008). Asterisks (*) indicate identical residues in all sequences; double dots (.) mark conserved substitutions; single dots (.) assign semi-conserved substitutions with similar shapes.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{CLUSTAL W alignment of SCHIC domains of PpaA sequences from A. tertiaricarbonis L108, M. universalis FAM5 (METUNv1_03977), R. centenum SW (RC1_2118), Jannaschia sp. strain CCS1 (Jann_0163), R. capsulatus SB 1003 (RCAP_rcc00667) and R. sphaeroides 2.4.1 (RSP_0283). Black shading indicates identities within the group. The consensus is derived from a larger alignment of SCHIC domains according to Moskvin et al. (2010).}
\end{figure}
et al., 2001), but regulation in this case might be different from the above examples. On the other hand, AAP bacteria in the Mediterranean sea have been found to thrive better in more eutrophic environments suggesting that most of them are relatively poor competitors under nutrient limiting conditions (Hojerová et al., 2011).

From the partial sequence of the photosynthesis gene cluster obtained for strain L108, regulatory units explaining the exclusive induction at low carbon fluxes cannot clearly be identified. Generally, the arrangement of photosynthetic genes in AAP bacteria seems to be quite diverse, suggesting that these micro-organisms form a heterogeneous group among the Proteobacteria phylogenetically (Igarashi et al., 2001; Zheng et al., 2011). Consequently, regulations of AAP could be likewise diverse. However, at least PpsR and PpaA seem to be key regulators of photosynthesis in all aerobic and anaerobic anoxygenic proteobacteria (Elsen et al., 2005; Masuda et al., 2008; Zheng et al., 2011). In anaerobic photosynthetic bacteria, this regulation represses expression of photosynthesis genes under aerobic conditions, thus preventing formation of reactive oxygen species (Elsen et al., 2005). In AAP bacteria, the PpsR- and PpaA-mediated repression is reversed somehow. In the case of the AAP bacterium R. centenum, this reversion could be partially explained by the inability of its PpsR to form an intramolecular disulfide bond (Masuda et al., 2008), which is stimulating DNA binding to target promoters in R. capsulatus and other purple non-sulfur bacteria. The absence of a cysteine residue in the sequence from strain L108 at position 268 (Fig. 4) resulted in a similar inability of disulfide bond formation likely weakening the role of PpsR as aerobic repressor. However, mechanisms for either inducing or repressing photosynthesis in response to oxygen cannot explain the regulation observed with strain L108 when incubated under low carbon fluxes. Consequently, a not yet identified regulation strictly coupling induction of photosynthesis with the energetic state of the cell obviously plays a more important role than PpsR and PpaA in this AAP bacterium.

Although the induction leading to co-utilization of light energy only at extremely low heterotrophic substrate fluxes might be surprising at first sight, it could also be seen as a particular adaptation of strain L108 enabling evolution of degradation pathways for the exploitation of new carbon and energy sources. In case of kinetic limitations due to the lack of efficient enzymes, the rate of energy generation from an organic compound can be below the cells’ demand of maintenance energy (Müller et al., 2007). As a consequence, autarkic growth is not possible but only co-metabolic growth might occur. This is particularly relevant for xenobiotic compounds only recently introduced into the environment where efficient degradation pathways have not yet been evolved. In this situation, co-metabolism of one or even several other substrates, such as co-pollutants, could enable degradation of the new compound. In case of MTBE contamination, however, it has been demonstrated that in the presence of easily metabolizable gasoline compounds, degradation of the fuel oxygenate can even be inhibited (Deeb et al., 2001; Wang & Deshusses, 2007). Consequently, as long as the kinetically limited degradation of a new pollutant does not result in a clear growth advantage, a driver for the evolution of a more efficient pathway is missing. Generally, competition among the microbial community for the better accessible substrates is likely to be strong, making it quite difficult to favour the new compound as a carbon and energy source, i.e. to back the wrong horse. More seriously, in case co-metabolic substrates for energy generation are not available at sufficient concentrations, degradation might be impossible. On the other hand, using light as auxiliary energy could compensate the deficit and thus enable productive degradation despite kinetic limitations. Then, a more efficient pathway might evolve by adaptation processes and sequestering new metabolic sequences via horizontal gene transfer.

Another consequence of light co-utilization via AAP is a significant reduction of the minimum substrate concentration $S_{\text{min}}$. Among the productive degraders of fuel oxygenates and their intermediates, A. tertiaricarbonis L108 is relatively efficient (Müller et al., 2008), at least when the substrates are provided at concentrations between 1 to 10 mM which is the upper limit of oxygenate concentrations typically found in the environment. In many cases, however, water systems containing fuel oxygenates at $\leq 100 \mu\text{M}$ have to be treated, e.g. at the Leuna test site in Germany where ponds and wetland systems have been constructed for the removal of MTBE from contaminated groundwater (Jechkal et al., 2011; Seeger et al., 2011). Based on the growth performance of strain L108 during batch experiments without formation of photosynthetic pigments, we recently calculated an $S_{\text{min}}$ for MTBE of about 300 to 400 $\mu\text{M}$ (Rohwerder et al., 2011). Now, considering the significant reduction of the $S_{\text{min}}$ value for TBA observed during the recycling fermentation, employing AAP when degrading MTBE might also result in a fourfold or even higher reduction of the threshold concentration. Thus, degradation capacities more relevant for the treatment of low MTBE concentrations might be achieved. However, this requires exposure to light which excludes in situ bioremediation of groundwater. Consequently, besides other advantages, constructed wetlands may also be favoured due to enabling not only plant growth but also bacterial AAP for the bioremediation of waters contaminated with pollutants at concentrations suboptimal for productive degradation.

REFERENCES


Growth of Aquincola tertiaricarbonis at very low rates


