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The Low Biomass Yields of the Acetic Acid Bacterium *Acetobacter pasteurianus* Are Due to a Low Stoichiometry of Respiration-Coupled Proton Translocation

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Growth energetics of the acetic acid bacterium *Acetobacter pasteurianus* were studied with aerobic, ethanol-limited chemostat cultures. In these cultures, production of acetate was negligible. Carbon limitation and energy limitation were also evident from the observation that biomass concentrations in the cultures were proportional to the concentration of ethanol in the reservoir media. Nevertheless, low concentrations of a few organic metabolites (glycolate, citrate, and mannitol) were detected in culture supernatants. From a series of chemostat cultures grown at different dilution rates, the maintenance energy requirements for ethanol and oxygen were estimated at 4.1 mmol of ethanol · g of biomass^{-1} · h^{-1} and 11.7 mmol of O_2 · g of biomass^{-1} · h^{-1}, respectively. When biomass yields were corrected for these maintenance requirements, the Y_{max} values on ethanol and oxygen were 13.1 g of biomass · mol of ethanol^{-1} and 5.6 g of biomass · mol of O_2^{-1}, respectively. These biomass yields are very low in comparison with those of other microorganisms grown under comparable conditions. To investigate whether the low growth efficiency of *A. pasteurianus* might be due to a low gain of metabolic energy from respiratory dissimilation, $\rightarrow H^+/O$ stoichiometries were estimated during acetate oxidation by cell suspensions. These experiments indicated an $\rightarrow H^+/O$ stoichiometry for acetate oxidation of 1.9 ± 0.1 mol of H^+/mol of O. Theoretical calculations of growth energetics showed that this low $\rightarrow H^+/O$ ratio adequately explained the low biomass yield of *A. pasteurianus* in ethanol-limited cultures.

Acetic acid bacteria are applied on a large scale for the industrial production of organic acids. An example is the classical vinegar process, in which acetic acid is produced via the incomplete oxidation of ethanol by *Acetobacter* species (13). The membrane-associated, pyrroloquinoline quinone (PQQ)-dependent dehydrogenases involved in ethanol oxidation have an extremely broad substrate specificity (2, 11) and, with some substrates, exhibit a high degree of enantioselectivity (14). This makes *Acetobacter* species interesting candidates for a potential role as biocatalysts in the industrial production of fine chemicals.

Production of large amounts of catalytically active biomass is a problem in laboratory studies as well as in industrial applications of *Acetobacter* species. In contrast to the promiscuity of their alcohol dehydrogenases, the typical acetic acid producers can use very few compounds as carbon sources for growth. Most of these strains only grow on ethanol, acetate, and, in some cases, lactate. Characteristically, their capacity for oxidation of ethanol and lactate to acetic acid far exceeds the capacity for complete oxidation of acetate to carbon dioxide (2, 9, 10). Growth on ethanol in batch cultures is therefore invariably accompanied by massive accumulation of acetic acid. As in other microorganisms (1, 37), the weak acid acetate causes dissipation of the transmembrane pH gradient when the pH of the extracellular medium is low. The resulting acidification of the cytoplasm has to be compensated for by proton consumption and/or translocation by the respiratory chain, thus explaining the rapid loss of viability in batch cultures of *Acetobacter* cells upon interruption of aeration (13).

Acetate accumulation by the acetic acid bacterium *Acetobacter pasteurianus* can be completely prevented by ethanol-limited chemostat cultivation. Although this mode of cultivation results in the production of biomass with a high and stable alcohol-oxidizing capacity, biomass yields on ethanol are still lower than those reported for other heterotrophic microorganisms (28). Therefore, acetate accumulation is apparently not the sole cause of the low biomass yield of *A. pasteurianus* in batch cultures.

In theory, the low biomass yield of acetic acid bacteria on ethanol might be due to one or more of several factors: (i) a high energy requirement for the assimilation of ethanol into biomass, (ii) a high energy requirement for maintenance purposes, or a (iii) a low yield of metabolically useful energy from dissimilatory metabolism (27). The bioenergetics of the initial steps of ethanol oxidation by the membrane-bound dehydrogenases have been studied extensively with resting cell suspensions and in vitro systems (for a review, see reference 20). However, in spite of the industrial relevance of ethanol metabolism by acetic acid bacteria, no data about the growth energetics of ethanol-grown cultures under defined conditions are available.

The aim of this study was to investigate the cause of the low biomass yield of acetic acid bacteria. To this end, biomass yields and maintenance energy requirements have been studied with ethanol-limited chemostat cultures of *A. pasteurianus*. Furthermore, the efficiency of energy coupling during respira-

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tion has been studied by determining the stoichiometry of respiration-driven proton translocation.

MATERIALS AND METHODS

Microorganisms and maintenance. *A. pasteurianus* LMG 1635 was obtained from the culture collection of the Laboratory of Microbiology, Gent, Belgium. After 48 h of cultivation in 500-ml shake flasks containing 100 ml of YPCE medium (per liter of demineralized water: Difco peptone, 10 g; Difco yeast extract, 10 g; $CaCO_3$, 20 g; ethanol, 20 ml), dimethyl sulfoxide was added to a final concentration of 10% (wt/vol). Aliquots of 1 ml were frozen and stored at −70°C. After inoculation of a frozen stock culture, starter cultures (100 ml of YPCE in a 500-ml shake flask) were incubated for 48 h on a rotatory shaker (200 rpm, 30°C). *Paracoccus pantotropha* LMD 82.5 (25) was obtained from The Netherlands Culture Collection, Delft. Starter cultures were prepared as described above.

Chemostat cultivation. Aerobic chemostat cultivation was carried out at 30°C in 2-liter Applikon laboratory fermenters at a stirrer speed of 1,000 rpm and at different dilution rates. The cultures were flushed with air (1 liter · min^{-1}). The dissolved-oxygen concentration in the cultures was measured with a polarographic oxygen electrode (Ingold) and was always above 25% of air saturation. The culture pH was controlled at either 6.0 (*A. pasteurianus*) or 7.0 (*P. pantotropha*) by automatic addition of 2 M KOH. The condenser was connected to a cryostat and cooled at 2°C. The working volume of the culture was kept at 1.0 liter by a peristaltic pump coupled to an Applikon level controller. The mineral medium contained the following (per liter of demineralized water): $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; $MnCl_2 \cdot 2H_2O$, 0.84 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg; $CaCl_2 \cdot 2H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3.0 mg; H_3BO_3, 1.0 mg; Kl, 0.1 mg; and British Drug House (BDH) silicone antifoam, 50 μl. The mineral medium was autoclaved at 120°C. Pure ethanol was added aseptically to the sterile medium without prior sterilization. The standard ethanol concentration in the feed was 10 g · $liter^{-1}$. This was the highest reservoir concentration at which the dissolved oxygen concentration could be kept above 25% of air saturation at all dilution rates (0.02 to 0.08 h^{-1}) without application of overpressure or sparging with pure oxygen.

Definition of steady state and control of culture purity. Cultures were deemed to be in the steady state when biomass concentration varied by less than 2% and the specific rate of oxygen consumption of the cultures varied by less than 5% over a period of 3 days. After a change in growth conditions, 10 to 15 volume changes were typically required to reach this situation. The purity of chemostat cultures was routinely examined by phase-contrast microscopy at a magnification of ×1,000. Furthermore, the absence of catalase activity, a striking characteristic of this aerobic organism (38), was checked daily by addition of 10 μl of hydrogen peroxide (Perhydrol; Merck) to a 1-ml culture sample. Contaminations were detected by visible gas formation.

Determination of culture dry weight. Dry weights of 10- to 20-ml culture samples were determined with a microwave oven and 0.45-μm-pore-diameter membrane filters (24). The dry weights of parallel samples varied by less than 1%.

Analysis of substrate and metabolites. Concentrations of ethanol in reservoir media were determined by high-performance liquid chromatography (HPLC) on an organic acid column HPX87-H from Bio-Rad at 60°C. Detection was by means of an Erma 7515 A refractive index detector coupled to a Hewlett-Packard integrator. Peak areas were linearly proportional to concentrations. A Waters 441 UV detector at 214 nm coupled to a Waters data module and an Erma ERC 7510 refractive index detector coupled to a Hewlett-Packard 3390A RI detector were used to detect metabolites. For the detection of possible low concentrations of ethanol in culture supernatants, a sensitive colorimetric assay based on *Hansenula polymorpha* alcohol oxidase (kindly provided by Bird Engineering, Schiedam, The Netherlands) was used (35).

Gas analysis. Prior to analysis of oxygen and carbon dioxide concentrations, the off gas of chemostat cultures was dried with a Permapure dryer (Inacom Instruments). Oxygen was measured with a Servomex 1100A paramagnetic oxygen analyzer, and carbon dioxide was measured with a Rosemount 870 infrared analyzer. Specific rates of oxygen consumption and carbon dioxide production were calculated as described previously (33).

Ethanol-dependent oxygen uptake. Ethanol-dependent oxygen uptake by culture samples was assayed with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). After appropriate dilution in air-saturated buffer (100 mM potassium phosphate buffer, 10 mM $MgSO_4$ [pH 6.0]), cells were incubated for 4 min at 30°C in a 4-ml thermostatted reaction chamber. Endogenous oxygen uptake rates by cells from carbon-limited chemostat cultures were negligible. Specific oxygen uptake rates were calculated based on an oxygen concentration in air-saturated buffer of 236 μM at 30°C. Ethanol was added to a concentration of 10 mM with a Hamilton syringe.

$\rightarrow H^+$/O measurements. The experimental procedures for measuring respiration-driven proton translocation were essentially as described by Boogerd et al. (5). Cells were harvested from steady-state chemostat cultures by centrifugation (10 min at 10,000 × *g*) and resuspended in proton-translocation buffer (HEPES, 0.5 mmol · $liter^{-1}$; KCl, 100 mmol · $liter^{-1}$; KSCN, 100 mmol · liter; pH 6.0) to give a biomass concentration of 30 g (dry weight) · $liter^{-1}$. A total of 3.0 ml of this concentrated cell suspension was transferred to an anaerobic, thermostatted (30°C) reaction chamber and kept under a flow of water-saturated argon. The reaction vessel was equipped with a Ross combination pH electrode (Orion) connected to a Kipp BD40 recorder (Salm and Kipp; Breukelen, The Netherlands). After addition of acetate (10 mmol · $liter^{-1}$) and valinomycin (10 mg · $liter^{-1}$), the cell suspension was preincubated for 1 h. After preincubation, a defined amount of oxygen was added to the suspension by injection of a known volume (100 mmol · $liter^{-1}$) of air-saturated KCl, equilibrated at 30°C, with a gas-tight Hamilton syringe. $\rightarrow H^+$/O ratios were calculated after linear extrapolation of the decay following the initial rapid acidification after an oxygen pulse to the time point of oxygen addition. After each oxygen pulse, the pH electrode was calibrated by addition of known volumes of a freshly prepared anaerobic solution of oxalic acid (1 mmol · $liter^{-1}$). Neither doubling of the concentrations of valinomycin and/or thiocyanate nor addition of the membrane potential uncoupler triphenylmethyl-phosphonium chloride significantly affected the measured $\rightarrow H^+$/O values. This indicates that dissipation of the transmembrane electrical potential ($\Delta\psi$) was not limiting proton translocation (5).

Organic carbon analysis. The organic carbon content of culture supernatants was determined with a Dohrmann DC190 total organic carbon analyzer (Rosemount Analytical). Dried potassium phthalate (BDH Ltd.) was used as a standard.

Identification of metabolites by NMR spectroscopy. After lyophilization, a sample of culture supernatant was redissolved in D_2O. ^1H and ^1H-^{13}C correlation spectra were measured at 300 K on a Bruker AMX600 nuclear magnetic resonance (NMR) spectrometer equipped with an inverse triple resonance probe and a pulse field gradient system.

Preparation of cell extracts. Cells (ca. 50 mg [dry weight]) were harvested from steady-state cultures by centrifugation at 10,000 × *g* (10 min at 4°C), washed once with 100 mM phosphate buffer (pH 7.5, 4°C), and resuspended in 100 mM potassium-phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM dithiothreitol. The extracts were prepared directly after washing by sonication of the cells with glass beads (0.7-mm diameter) at 0°C for 3 min (six bursts of 30 s with intermediate cooling). Whole cells and debris were removed by centrifugation at 20,000 × *g* (20 min at 4°C). The clear supernatant typically contained 2 to 3 mg of protein · ml^{-1}.

Determination of enzyme activities. Activities of malate synthase (EC 4.1.3.2), isocitrate lyase (EC 4.1.3.1) and acetyl coenzyme A synthetase (EC 6.2.1.1) in cell extracts were assayed as described previously (8, 32). Activity of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was assayed as described by Beudeker et al. (4). For the latter enzyme activity, a cell extract of an ammonium-limited, aerobic chemostat culture of *Nitrosomonas europaea* (7) was used as a positive control.

Protein determination. The protein content of culture samples was estimated by a modified biuret method (37). The protein contents of supernatants and cell extracts were determined by the Lowry method. For both determinations, bovine serum albumin (fatty acid free; Sigma) was used as a standard.

RESULTS

Ethanol-limited growth of *A. pasteurianus*. An important prerequisite for studies of growth energetics in chemostat cultures is that the nature of the growth-limiting compound is firmly established. In chemostat cultures of *A. pasteurianus* grown as described in Materials and Methods, ethanol concentrations were below the detection limit of the alcohol oxidase assay (ca. 0.1 mM). Although indicative for limitation by the carbon source, a low residual concentration of the carbon source is not proof that this nutrient is the sole growth-limiting factor.

To investigate whether ethanol was indeed the growth-limiting nutrient under the growth conditions used in this study, *A. pasteurianus* was grown in aerobic chemostat cultures at two concentrations of ethanol in the reservoir medium. If ethanol is the growth-limiting nutrient, the biomass concentration in the culture should be linearly proportional to the ethanol concentration in the reservoir medium, in which other nutrients are present at fixed concentrations (i.e., the biomass yield on ethanol should be independent of its concentration in the reservoir medium).

At a dilution rate of 0.05 h^{-1}, the biomass yield on ethanol was the same when cultures were grown with either 10 or 20 g of ethanol · $liter^{-1}$ (Table 1). In both cases, the concentrations of ethanol and acetate in culture supernatants were negligible, in accordance with an earlier report (28). However, when carbon balances were constructed over the cultures, including

TABLE 1. Growth of *A. pasteurianus* LMG 1635 in aerobic chemostat cultures on two different concentrations of ethanol in reservoir medium

Ethanol concn ($g \cdot liter^{-1}$) in reservoir	Dilution rate (h^{-1})	Biomass		Residual concn ($mmol \cdot liter^{-1}$)		qCO_2 ($mmol \cdot g^{-1} \cdot h^{-1}$)[a]	% Carbon recovery[b]	
		Concn ($g \cdot liter^{-1}$)	Yield ($g \cdot g^{-1}$)	Ethanol	Organic carbon		Biomass, CO_2	Biomass, TOC, CO_2
10.21	0.053	1.42	0.139	<0.2	21.3	12.88	91.9	96.7
19.81	0.055	2.70	0.136	<0.2	45.8	14.17	93.5	98.8

[a] qCO_2, specific rate of CO_2 production.
[b] Carbon recoveries were calculated on the basis of the assumption that 50% of the biomass dry weight consists of carbon. TOC, total organic carbon.

only ingoing substrate, biomass, and carbon dioxide, the carbon recovery was only about 92 to 94% (Table 1).

To check whether this incomplete carbon recovery was due to the production of soluble organic metabolites, total organic carbon contents of culture supernatants were determined. At both reservoir concentrations of ethanol tested, ca. 5% of the carbon present in the feed was recovered in the supernatants (Table 1). This was not due to extracellular protein, the concentration of which was below 0.05 $g \cdot liter^{-1}$ (data not shown). When the dissolved organic carbons were taken into account, carbon recoveries increased to 97 to 99%. The observation that the concentration of the unknown organic compounds was proportional to the ethanol concentration in the reservoir medium is consistent with the cultures being ethanol limited.

Metabolite production in ethanol-limited chemostat cultures. To identify the organic compounds present in the supernatants of ethanol-limited chemostat cultures, the supernatants were subjected to NMR analysis. By means of ^{1}H and ^{1}H-^{13}C correlated spectroscopy, three metabolites could be identified. The major metabolite detected in culture supernatants was glycolate, the concentrations of which (determined by HPLC) were 3.1 mM in a culture grown on 10 g of ethanol $\cdot$ $liter^{-1}$ and 5.3 mM in a culture grown on 20 g of ethanol $\cdot$ $liter^{-1}$. The two other compounds identified were citrate and mannitol, the concentrations of which were below 1 mM. Together, these three compounds accounted for approximately half of the organic carbon present in the culture supernatants. When the production of glycolate, citrate, and mannitol was taken into account, carbon balances gave a recovery of ca. 95% (Table 2).

In contrast to citrate, glycolate and mannitol are not normal intermediates of ethanol metabolism. Glycolate is a well-known by-product of autotrophic bacteria that fix carbon dioxide via ribulose bisphosphate carboxylase/oxygenase. No ribulose bisphosphate carboxylase/oxygenase activity was detected in cell extracts of ethanol-limited chemostat cultures (dilution rate [D] = 0.05 h^{-1}). These extracts did, however, contain high activities of isocitrate lyase and malate synthase (0.43 $\pm$ 0.02 U $\cdot$ mg of protein^{-1} and 0.30 $\pm$ 0.03 U $\cdot$ mg of protein^{-1}, respectively), indicating involvement of the glyoxylate cycle in the assimilation of C_2 compounds. It is conceivable that glycolate is formed by reduction of glyoxylate, an intermediate of this pathway. Such a reduction does, however, seem peculiar for an oxidative organism like *A. pasteurianus.*

Formation of mannitol from ethanol requires gluconeogenesis and reduction of a hexose. In algae and fungi, polyols act as compatible solutes (17). Since acetic acid bacteria are known to thrive at high ethanol and/or sugar concentrations (31), it is tempting to speculate that mannitol plays a similar role in these organisms. The low concentrations of mannitol found in culture supernatants might then reflect some leakage from the cells. However, experimental evidence to support this hypothesis is lacking.

In addition to the three identified compounds, some small unidentified peaks were seen in HPLC chromatograms and NMR spectra. Attempts to identify these by various analytical techniques (liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry, and electrospray ionization analysis) proved unsuccessful, but suggested that they represented compounds with seven or more carbon atoms (data not shown). Since it was deemed unlikely that the low concentrations of these unknown metabolites would substantially affect growth energetics, no further attempts were made to fully close the carbon balances.

Maintenance energy requirement and "true" biomass yield on ethanol. The biomass yields on ethanol (Table 1) are much lower than those observed for other microorganisms. To further investigate the low growth efficiency of *A. pasteurianus* in ethanol-limited chemostat cultures, its maintenance energy requirement and "true" biomass yields on ethanol and oxygen

TABLE 2. Metabolite concentrations, specific conversion rates, and carbon recoveries of aerobic ethanol-limited chemostat cultures of *A. pasteurianus* LMG 1635 grown at various dilution rates

Dilution rate (h^{-1})	Concn ($g \cdot liter^{-1}$) of ethanol in reservoir	Biomass		Protein content (g of protein $\cdot g^{-1}$)	Concn (mM) of:					Specific rate ($mmol \cdot g^{-1} \cdot h^{-1}$) of:			% Carbon recovery[a]
		Concn ($g \cdot liter^{-1}$)	Yield ($g \cdot g^{-1}$)		Organic carbon	Glycolate	Citrate	Mannitol	Acetate	Ethanol	O_2	CO_2	
0.019	9.72	0.72	0.074	0.61	22.8	2.5	0.8	0.1	0.15	5.60	15.5	9.6	96
0.030	10.03	1.06	0.106	0.57	15.0	2.5	0.3	<0.1	0.17	6.26	17.0	10.4	95
0.042	9.95	1.24	0.125	0.61	19.9	3.5	0.7	<0.1	0.14	7.24	18.8	11.7	95
0.053	10.21	1.42	0.139	0.61	21.3	3.1	0.9	0.1	0.17	8.21	21.6	12.9	95
0.063	9.90	1.53	0.155	0.63	22.6	2.8	0.7	<0.1	0.16	8.82	22.9	13.5	94
0.071	10.01	1.60	0.160	0.64	19.0	2.3	0.9	<0.1	0.17	9.70	24.2	14.7	93
0.086	8.00	1.41	0.176	0.63	20.9	2.3	0.7	0.4	1.12	10.6	27.2	15.8	96

[a] The calculated carbon recovery includes identified compounds only; higher values were obtained when the total organic carbon concentrations in culture supernatants were used.

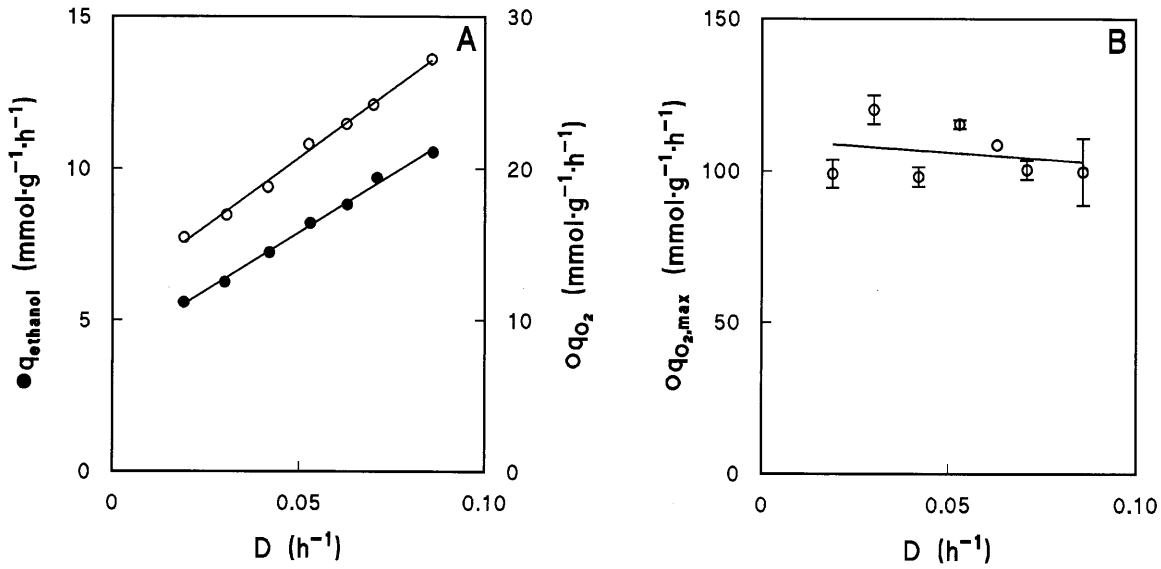

FIG. 1. (A) Specific rates of ethanol consumption ($q_{ethanol}$) and oxygen consumption (q_{O_2}) in aerobic, ethanol-limited chemostat cultures of *A. pasteurianus* LMG 1635 grown at various dilution rates. Cells were grown at 30°C and pH 6.0. Other culture parameters are present in Table 2. (B) Effect of dilution rate (*D*) on the maximum specific rates of ethanol-dependent oxygen uptake, as determined with a Clark-type oxygen electrode.

were estimated. A series of aerobic, ethanol-limited chemostat cultures were grown at dilution rates ranging from 0.02 to 0.08 h^{-1}. (The maximum specific growth rate of *A. pasteurianus* under the experimental conditions is ca 0.10 h^{-1} [data not shown].) In all cultures, the carbon recovery (including identified compounds only) was around 95%. Neither the protein content of the biomass nor the concentration of identified metabolites exhibited a clear correlation with the dilution rate (Table 2).

The specific oxygen and ethanol consumption rates increased linearly with increasing dilution rate (Fig. 1A), consistent with a growth rate-independent maintenance energy requirement (23). Consequently, the maintenance energy requirement and the "true" biomass yield corrected for maintenance were estimated from equation 1:

$$q_s = \frac{D}{Y_{max}} + m_s \quad (1)$$

In this equation, q_s is the specific rate of consumption of the energy substrate (ethanol or oxygen), Y_{max} is the biomass yield corrected for maintenance energy requirement, D is the dilution rate, and m_s is the maintenance energy requirement.

The maintenance energy requirements for oxygen and ethanol estimated from Fig. 1A were 11.7 mmol of $O_2 \cdot g^{-1} \cdot h^{-1}$ and 4.1 mmol of ethanol $\cdot g^{-1} \cdot h^{-1}$, respectively. Because the substrate used for maintenance purposes is completely dissimilated, the ratio between the maintenance requirements for oxygen and ethanol should equal 3 ($C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3\ H_2O$). The observed ratio was 2.9, which is in good agreement with the expected value. From the slopes of the lines shown in Fig. 1A, the molar growth yields on ethanol and oxygen (corrected for maintenance) were estimated at 13.1 g of biomass $\cdot$ mol of ethanol^{-1} and 5.6 g $\cdot$ mol of oxygen^{-1}, respectively.

In all steady-state cultures, rates of ethanol-dependent oxygen uptake in the presence of excess substrate were even higher than the rates in situ (Fig. 1B). These oxygen uptake capacities, which probably mainly reflect incomplete oxidation of ethanol to acetic acid, were virtually independent of the dilution rate. The increase in the oxygen uptake rate upon the addition of excess substrate is a further indication of the cultures being ethanol limited.

Only a few literature data about biomass yields and maintenance energy requirements of heterotrophic, aerobic microorganisms grown on ethanol are available. A comparison with the Y_{max} and m_s of *Acinetobacter calcoaceticus* and the yeast *Candida utilis* (Table 3) indicates that *A. pasteurianus* exhibits a combination of a high ethanol requirement for maintenance and a low Y_{max}. At least in theory, this might reflect a low efficiency of dissimilatory ethanol metabolism (i.e., a low ATP yield per mole of dissimilated ethanol). This possibility was further investigated by estimating the stoichiometry of respiration-driven proton translocation in *A. pasteurianus.*

Stoichiometry of respiration-driven proton translocation. The stoichiometry of respiration-driven proton translocation (number of protons translocated by the respiratory chain during the reduction of one oxygen atom; $\rightarrow H^+/O$ ratio) is a measure of the efficiency of respiratory energy coupling (18, 21). The $\rightarrow H^+/O$ ratio of *A. pasteurianus* was estimated with cells grown in aerobic, ethanol-limited chemostat cultures (D = 0.05 h^{-1}).

In the experimental setup used for $\rightarrow H^+/O$ measurements, use of ethanol as a substrate would lead to its almost stoichiometric oxidation to acetic acid. This process represents only 4 of the 12 electrons available from the complete oxidation of

TABLE 3. Biomass yields and maintenance requirements of heterotrophic microorganisms in aerobic ethanol-limited chemostat cultures with ammonium salts as the nitrogen source

Organism	Y_{max} (g $\cdot$ mol of ethanol^{-1})	m_s (mmol of ethanol $\cdot$ $g^{-1} \cdot h^{-1}$)	Reference
A. calcoaceticus	38.9	2.5	12
C. utilis	38.2	0.4	16
A. pasteurianus	13.1	4.1	This study

ethanol to carbon dioxide; the remaining 8 electrons are derived from the further oxidation of the intermediate acetate. Moreover, the protons produced during incomplete oxidation of ethanol might obscure the measurements. Therefore, acetate rather than ethanol was used as the electron donor.

The average $\rightarrow H^+/O$ ratio of *A. pasteurianus*, calculated from 16 independent experiments, was 1.9 ± 0.1. Control experiments performed under identical conditions (pH 6.0, 30°C) with *P. pantotropha* LMD 82.5 pregrown in aerobic carbon-limited chemostat cultures (30°C, D = 0.05 h^{-1} [pH 7.0]) yielded an $\rightarrow H^+/O$ ratio of 4.9 ± 0.3 (n = 7). This is close to $\rightarrow H^+/O$ ratios reported for starved cell suspensions of the related species *Paracoccus denitrificans* (18). This demonstrates that the low $\rightarrow H^+/O$ ratio found for *A. pasteurianus* was not an artifact of the experimental setup used for the proton translocation experiments. The biomass yield of *P. pantotropha* in the ethanol-limited chemostat culture was 25.2 g of biomass · mol of ethanol^{-1}, or about fourfold higher than the biomass yield of *A. pasteurianus* at the same dilution rate.

Can a low $\rightarrow H^+/O$ ratio explain the low biomass yields of *A. pasteurianus*? If the low biomass yield of *A. pasteurianus* is primarily caused by a low ATP yield from respiratory dissimilation of ethanol, its ATP requirement for biomass synthesis should be comparable to that of other microorganisms grown on ethanol (or acetate, which is assimilated via the same metabolic pathways). To estimate the biomass yield on ATP ($Y_{ATP/max}$; corrected for maintenance energy requirements) of ethanol-limited chemostat cultures, the following assumptions were made.

(i) The $\rightarrow H^+/O$ ratio of 2 measured with acetate as the electron acceptor was assumed to be representative for all electrons derived from the complete oxidation of ethanol to carbon dioxide. Electrons derived from the first steps in ethanol oxidation (i.e., the oxidation of ethanol to acetate) can enter the respiratory chain either at the level of NADH dehydrogenase (pyridine-nucleotide-dependent dehydrogenases) or via cytochrome *c* (PQQ-dependent dehydrogenases [20]). It is highly unlikely that these reactions will result in an $\rightarrow H^+/O$ ratio higher than that observed with acetate as the electron donor. Moreover, as discussed above, oxidation of acetate to carbon dioxide accounts for the majority of the electrons derived from the complete oxidation of ethanol to carbon dioxide.

(ii) The $\rightarrow H^+/ATP$ ratio of the proton-translocating ATP synthetase was assumed to be 4 (34). With the $\rightarrow H^+/O$ ratio of 2 determined in the present study, this results in a P/O ratio (ATP synthesized per electron pair donated to the respiratory chain) of 0.5.

(iii) Complete dissimilation of ethanol to carbon dioxide yields 6 mol of reduced cofactors [FADH, NAD(P)H and/or $PQQH_2$]. If, for all of these reduced cofactors, the P/O ratio = 0.5, this yields 3 ATPs · mol of ethanol^{-1}. Additionally, 1 mol of ATP is formed by substrate-level phosphorylation in the tricarboxylic acid cycle (succinate thiokinase reaction). Of the 4 ATPs formed per mol of ethanol, 2 have to be invested in the activation of ATP by acetyl coenzyme A synthetase as follows: acetate + ATP + coenzyme A → acetyl coenzyme A + AMP + PP_i. High levels of this enzyme were detected in cell extracts of *A. pasteurianus* (data not shown). Thus, at a P/O ratio of 0.5, the net ATP yield from the dissimilation of ethanol equals 2 mol of ATP/mol of ethanol.

(iv) To describe assimilation of ethanol, a biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ was used (1 C mol = 25 g of biomass [dry weight] including ash [26]).

(v) Production of carbon dioxide during assimilatory reactions was assumed to be 10.55 mmol · g of biomass^{-1} (15).

(vi) Organic metabolites present in culture supernatants were disregarded; ethanol not converted to biomass was assumed to be completely dissimilated to carbon dioxide and water.

On the basis of assumptions iv to vi, assimilation of *A. pasteurianus* biomass from ethanol can be described by equation 2:

$$0.632\ C_2H_6O + 0.200\ NH_3 + 0.846\ O_2 \rightarrow CH_{1.8}O_{0.5}N_{0.2}$$
$$(25\ [\text{g of biomass}]) + 0.264\ CO_2 + 1.296\ H_2O \quad (2)$$

In this assimilation equation, the oxygen requirement results from the net production of reducing equivalents in the metabolic network leading from ethanol to biomass. These reducing equivalents are formed because ethanol is more reduced than biomass, and, moreover, 100% carbon conversion in assimilation is not possible because of the obligatory formation of CO_2 (15).

The amount of ethanol used for dissimilation can be calculated from the difference between the total ethanol requirement for growth (Y_{max} = 13.1 g of biomass · mol of ethanol^{-1} or 1.908 mol of ethanol · C mol of biomass^{-1} [Fig. 1A]) and the ethanol used for assimilation. Thus, for the synthesis of 25 g of biomass, 1.908 − 0.632 = 1.276 mol of ethanol dissimilated according to equation 3:

$$1.276\ C_2H_6O + 3.828\ O_2 \rightarrow 2.552\ CO_2 + 3.828\ H_2O \quad (3)$$

Following assumption ii, the ATP yield from ethanol dissimilation by *A. pasteurianus* then equals 1.276 × 2 = 2.552 mol of ATP · C mol of biomass^{-1}. Some additional ATP is formed during the reoxidation of cofactors during assimilation. This amount can be estimated from the oxygen uptake during assimilation (0.846 mol of O_2 · C mol of biomass^{-1}) and the estimated P/O ratio of 0.5. This yields an ATP production of 0.846 mol · C mol of biomass^{-1}.

The total amount of ATP involved in the synthesis of 1 C mol of biomass amounts to 0.846 + 2.552 = 3.398 mol. The biomass yield on ATP corrected for maintenance ($Y_{ATP/max}$) then becomes 25:3.398 = 7.4 g of biomass · mol of ATP^{-1}. This is very similar to the estimated $Y_{ATP/max}$ of *Escherichia coli* during acetate-limited growth (7.1 g · mol^{-1} [30]) and that of the yeast *C. utilis* during ethanol-limited growth (7.6 g · mol^{-1} [36]). There are no indications that the metabolic pathways for assimilation of C_2 compounds are drastically different in the three organisms. It can therefore be concluded that the low biomass yield of *A. pasteurianus* is adequately explained by a low efficiency of respiratory energy transduction.

DISCUSSION

The acetic acid bacteria exhibit a low biomass yield on the carbon substrate and a high rate of metabolite formation under conditions of substrate excess, consistent with the conclusion of Linton and Rye (19) that energetic efficiency and rate of metabolite production in microorganisms are generally inversely related. Already in 1962, Stouthamer (29), based on measurements of ADP phosphorylation during substrate oxidation by crude cell extracts, proposed that the low biomass yields of the acetic acid bacterium *Gluconobacter liquefaciens* in batch cultures were due to a low efficiency of oxidative phosphorylation. The experimental data and calculations presented here demonstrate that, indeed, the low $\rightarrow H^+/O$ ratio of *A. pasteurianus* adequately explains its low growth efficiency under carbon-limited conditions. It is likely that a similar situation exists in other acetic acid bacteria. For example, the low biomass yields in glucose-limited chemostat cultures of the sugar-metaboliz-

ing species *Acetobacter diazotrophicus* (3) can, at least in theory, also be explained from a low P/O ratio (calculations not shown).

It has previously been demonstrated that the composition of bacterial proton-translocating respiratory chains has a substantial impact on biomass yields under carbon-limited growth conditions (6, 18). If the $\rightarrow H^+/O$ ratio of 2 estimated in this study accurately reflects the mechanistic stoichiometry of respiratory proton translocation, energy transduction by the respiratory chain of *A. pasteurianus* is solely based on a single redox loop (i.e., inward translocation of an electron pair coupled to consumption of two cytoplasmic protons by the terminal oxidase). Any involvement of proton-pumping dehydrogenases or oxidases should lead to higher $\rightarrow H^+/O$ ratios. Nevertheless, there is experimental evidence that proton-pumping oxidases are active in some of the acetic acid bacteria (20). Further work is required to investigate to what extent these systems are active during carbon-limited growth and whether their presence can be correlated to biomass yields under defined, carbon-limited growth conditions.

In many bacteria, growth efficiency is dependent on the nature of the growth-limiting nutrient, with less-efficient modes of dissimilation predominating under conditions in which the energy substrate is not growth limiting. In addition to changes in the pattern of metabolite production (22), such variations in energetic efficiency can be caused by redirection of the electron flow via alternative respiratory chain components (30). Our data indicate that although *A. pasteurianus* is able to prevent acetate production during ethanol-limited growth, it lacks the metabolic flexibility to switch to an energetically efficient mode of respiration during energy-limited growth.

From an ecophysiological point of view, the ethanol-limited chemostat cultures used in this study bear little resemblance to the natural environments from which acetic acid bacteria are most readily isolated. These environments are characterized by the presence of high concentrations of ethanol, lactate, and/or sugars (31). Under such conditions, energetic efficiency of substrate dissimilation is unlikely to exert a major selective pressure. Instead, competitive advantage may be derived from a high rate of substrate conversion. Both the low biomass yields of *A. pasteurianus* and the leakage of organic metabolites during ethanol-limited cultivation may be a reflection of optimization for speed rather than for efficiency.

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