Growth Efficiency of *Xanthomonas campestris* in Continuous Culture

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*Xanthomonas campestris* NCIB 11854 was grown glucose-limited in continuous culture at 28 °C, pH 6.8, in a defined minimal salts medium. Whole cells contained cytochromes b, c, aa₃, o-type cytochromes and possibly a₁, and yielded -H⁺/O quotients of up to approximately 6 for the oxidation of endogenous substrates. These data, by analogy with results obtained previously with other species of bacteria, suggest the presence of up to three sites of respiratory chain energy conservation. However, molar growth yields on glucose \[Y_{\text{max}}^{\text{glucose}} = 53.6 \text{ g dry wt bacteria (mol glucose)}^{-1}\] and oxygen \[Y_{\text{max}}^{\text{O}} = 26.4 \text{ g dry wt bacteria (mol O}_2\)^{-1}\] were extremely low and indicated an ATP/O quotient of approximately 1 which was only marginally increased when corrected for polymer production. A relatively rapid decay of the pH gradient generated by *in vitro* respiration was observed, probably indicating either an enhanced permeability of the cytoplasmic membrane to H⁺ or a rapid rate of ATP turnover, either of which could in part account for the observed low growth efficiency of the organisms.

INTRODUCTION

*Xanthomonas campestris* is the exopolysaccharide produced by the Gram-negative, obligately aerobic bacterium *Xanthomonas campestris*. The polysaccharide structure is based on a cellulose backbone having alternate glucosyl residues substituted by a trisaccharide chain of D-mannose, D-glucuronic acid and a terminal D-mannose. It is variably substituted by O-acetyl and pyruvic acid ketal groups (Kennedy & Bradshaw, 1984).

Theoretical studies of the energy requirements for xanthan synthesis (Jarman & Pace, 1984) have indicated that the maximum theoretical yield of xanthan from glucose is not strongly dependent on the ATP/O quotient for cell growth, whereas the theoretical xanthan yield on oxygen is very dependent on the ATP/O quotient exhibited by the organism. Energy for xanthan synthesis is derived from the oxidation of the carbon substrate, either completely to CO₂ or partially to various oxidized products which may be integral components of the xanthan molecule. The proportion of energy derived from the latter is strongly dependent on the xanthan composition and on the ATP/O quotient (Jarman & Pace, 1984). The ATP/O quotient has not so far been measured for *X. campestris*. As oxygen transfer into the viscous fermentation broth can limit xanthan productivity during the production process, a knowledge of the efficiency of energy conservation is important in determining how much energy, and hence oxygen, is required for culture growth and for xanthan production.

In this paper we report investigations of respiratory chain energy conservation in whole cells of *X. campestris* grown glucose-limited in continuous culture, conditions which minimize the production of polymer and hence facilitate the determination of the energetics of cell growth without the complication of polymer formation.

Abbreviations: TMPD, \(N,N,N',N'-\text{tetramethyl-p-phenylenediamine}\); DCCD, dicyclohexylcarbodiimide; \(\Delta \psi\), transmembrane electrical potential difference.
METHODS

Organism and growth medium. Xanthomonas campestris NCIB 11854 was grown in a minimal salts medium designed for glucose limitation, which contained (g l−1): glucose, 6.0; (NH₄)₂SO₄, 3.52; KH₂PO₄, 0.68; MgSO₄·7H₂O, 0.40; FeSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 6.25×10⁻⁴; CoCl₂·6H₂O, 2.97×10⁻⁴; NaMoO₄·2H₂O, 3.12×10⁻⁴; KI, 2.07×10⁻⁴; H₂BO₃, 7.75×10⁻⁵.

The medium was sterilized by autoclaving at 121 °C. Glucose was sterilized separately and added aseptically before use. Solutions of FeSO₄·7H₂O were filter sterilized (Millipore 0.45 μm filter) and added to the medium aseptically.

Continuous culture. Bacteria were grown in a chemostat under glucose-limited conditions using a Chemap GF7 litre fermenter. The culture (4.0 l) was maintained at 28 °C and pH 6.8±0.02 by the addition of 1 M NaOH/1 M-KOH. The gas supply was air (1.0 l min⁻¹) and the culture was kept aerobic by stirring at 1500 r.p.m.

Gas analysis. Inlet and outlet gas streams were analysed using an on-line mass spectrometer (VG gas analysis, model MM 8-80).

Bacterial dry weight. Culture samples (30 ml) were washed twice with distilled water and dried to constant weight (Linton et al., 1975).

Polymer dry weight. A 30 ml sample of culture supernatant was added to 120 ml 2-propanol. The resultant precipitate was removed by winding round a glass rod, placed in a preweighed foil dish and dried to constant weight at 100 °C.

Total bacterial carbon. Analysis of total carbon, supernatant carbon and cell carbon was done using a Tocamaster total carbon analyser (Beckman).

Glucose. The concentration of glucose in the growth medium and the culture supernatant were measured using the hexokinase/glucose-6-phosphate dehydrogenase assay system (Sigma technical bulletin no. 15-UV).

Bacterial nitrogen. The percentage of elemental nitrogen in bacterial cells was measured on a Dohrmann nitrogen analyser (Techmann). Bacterial nitrogen was estimated from the difference between total culture nitrogen and total supernatant nitrogen.

Dissolved oxygen. Dissolved oxygen tension was measured with a galvanic oxygen electrode (Uniprobe). Calibration of the electrode was at atmospheric pressure with air and nitrogen.

Bacterial yields on glucose and oxygen. Bacterial yields on glucose (Yglucose, YO₂) were calculated respectively from the in situ rates of glucose and oxygen consumption (qglucose, qO₂) of steady-state chemostat cultures (Linton et al., 1975). Maximum growth yields (Ymax, Ymax) and maintenance coefficients (mO₂, mglucose) were obtained from plots of qglucose and qO₂ against dilution rate where Ymax = 1/slope and maintenance = intercept (Pirt, 1975; Fieschko & Humphrey, 1984).

Cytochrome content. This was determined as described by Porter et al. (1983). Concentrations of total b-type cytochromes were determined from the trough at 570 nm to a peak at 560 nm (mm⁻¹ cm⁻¹, 17-5), c-type from the trough at 540 nm to a peak at 550 nm (mm⁻¹ cm⁻¹, 7-3) and aa₃ from the trough at 620 nm to a peak at 603 nm (mm⁻¹ cm⁻¹, 11-7) in dithionite-reduced minus H₂O₂-oxidized difference spectra. The concentration of o-type cytochrome oxidase was determined from the peak at 418 nm to a trough at 430 nm (mm⁻¹ cm⁻¹, 17-0) in a dithionite-reduced + CO minus dithionite-reduced spectrum.

→H⁺/O quotients. →H⁺/O quotients [g-equiv. H⁺ translocated (g-atom O consumed)⁻¹] were measured as previously described (Mitchell & Moyle, 1967, as modified by Jones et al., 1975). Cells were harvested from the fermenter, washed twice and resuspended in 140 mM KCl/1 mM-Tris/HCl to give a final bacterial suspension of 6 g dry weight l⁻¹. This suspension was equilibrated anaerobically for 15 min at 28 °C, pH 6.5–7.0 in the presence of either potassium thiocyanate (optimal concentration 50 mM) or valinomycin [optimal concentration 1 μg (mg bacterial dry wt)⁻¹] before pulsing with air-saturated KCl. The system was calibrated with anaerobic 10 mM-HCl and 10 mM-NaOH. The concentration of exogenous substrates was 10 mM.

Preparation of respiratory membranes. Cells were harvested from a glucose-limited chemostat operating at a dilution rate of 0.10 h⁻¹, 28 °C and pH 6.8. Cells were then centrifuged at 4 °C, homogenized in glass-distilled water, recentrifuged and finally resuspended in 25 mm-sodium phosphate buffer, pH 7.0 (20 mg dry wt ml⁻¹). Cells were disrupted by sonication at 5–10 °C using an MSE 100 W ultrasonic disintegrator at an amplitude of 8 μm peak to peak. Cells were sonicated for a total time of 5 min, suspensions being recooled in ice every 1 min.

Presentation of results. Where appropriate, results are presented as the mean ± SEM with the number of determinations in parentheses. Lines are fitted to data points by linear regression analysis using Grafit/1000 software (Graphic User Systems) on an HP3357 (Hewlett Packard).

RESULTS

Determination of glucose limitation. In order to determine whether growth was glucose limited, X. campestris was grown in chemostat culture (D = 0.05 h⁻¹) at inlet glucose concentrations
Growth efficiency of Xanthomonas campestris

Fig. 1. Effect of dilution rate on substrate uptake in a glucose-limited chemostat culture of X. campestris. ○, $q_{O_2}$; ●, $q_{glucose}$.

Fig. 2. Effect of dilution rate on the rate of product formation by a glucose-limited chemostat culture of X. campestris. △, $q_{CO_2}$; ▲, $q_{polymer}$.

Table 1. Respiration-driven proton translocation by whole cells of X. campestris grown under glucose-limited conditions

X. campestris was grown in continuous culture at a dilution rate of 0.09 h$^{-1}$, then prepared and assayed for $-\Delta^+H/\Delta^+O$ quotients as described in Methods.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta^+\Psi$-collapsing system</th>
<th>$-\Delta^+H/\Delta^+O$ quotient</th>
<th>$t_{1/2}$ for decay of $\Delta^+H$ gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>SCN$^-$</td>
<td>$5.8 \pm 0.5$ (3)</td>
<td>$4.2 \pm 0.3$ (12)</td>
</tr>
<tr>
<td>Glucose</td>
<td>SCN$^-$</td>
<td>$5.1 \pm 0.2$ (3)</td>
<td>$4.35 \pm 0.3$ (14)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>Valinomycin/K$^+$</td>
<td>$4.6 \pm 0.4$ (5)</td>
<td>$13.9 \pm 1.2$ (11)</td>
</tr>
<tr>
<td>Glucose</td>
<td>Valinomycin/K$^+$</td>
<td>$4.7 \pm 0.2$ (5)</td>
<td>$10.8 \pm 2.3$ (9)</td>
</tr>
<tr>
<td>Succinate</td>
<td>Valinomycin/K$^+$</td>
<td>$3.4 \pm 0.3$ (4)</td>
<td>$11.4 \pm 2.5$ (16)</td>
</tr>
</tbody>
</table>

between 3 and 9 g$^{-1}$. The cell dry weight increased linearly with increasing glucose concentrations, thus confirming glucose-limited growth. A small amount of polymer was produced under these conditions, the amount of which also increased linearly as a function of glucose concentration over the range that was tested. The specific rate of polymer production ($q_{polymer}$; g polymer produced (g bacterial dry wt$^{-1}$ h$^{-1}$) did not change significantly. No other products (i.e. protein or organic acids) could be detected apart from polymer, which accounted for approximately 95% of the supernatant carbon.

Determination of biomass yields on glucose and oxygen. Maximum molar growth yields for glucose-limited X. campestris (corrected for maintenance substrate consumption) were calculated from the in situ rates of substrate consumption (Fig. 1). These were: $Y_{max}^{\text{glucose}}$, 53.6 g dry wt bacteria (mol glucose)$^{-1}$; and $Y_{max}^{\text{O}_2}$, 26.4 g dry wt bacteria (mol O$_2$)$^{-1}$. The maintenance energy requirements were 0.04 mmol glucose (g dry wt bacteria)$^{-1}$ h$^{-1}$ and 0.41 mmol O$_2$ (g dry wt bacteria)$^{-1}$ h$^{-1}$. The specific rates of CO$_2$ production and polymer formation are shown in Fig. 2. The cell composition throughout these studies was essentially unchanged over the range of dilution rates tested, viz. percentage cell nitrogen 12.3 ± 0.4 ($n = 6$) and percentage cell carbon 49.3 ± 0.3 ($n = 6$). Mean carbon recoveries for all steady-states were 93.6% ± 3.6 ($n = 13$).

$-\Delta^+H/\Delta^+O$ quotients. $-\Delta^+H/\Delta^+O$ quotients for whole cells of X. campestris grown under glucose limitation at a dilution rate of 0.09 h$^{-1}$ were determined in the presence of either SCN$^-$ or valinomycin + K$^+$ to collapse $\Delta^+\Psi$ (Table 1). The oxidation of endogenous substrates [presumed to be mainly NAD(P)H] or added glucose yielded $-\Delta^+H/\Delta^+O$ quotients up to 5.8 depending on the exact conditions of the assay, whereas significantly lower values were obtained for the oxidation of exogenous succinate (FAD-linked). However, even after optimizing the concentration of
Table 2. Cytochrome contents of whole cells of X. campestris grown under glucose-limited conditions at various dilution rates

Whole cells were prepared and assayed for cytochromes as described in Methods. A cytochrome ‘a₁’ peak was present at all dilution rates indicated.

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Cytochrome content [nmol (g bacterial cells)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>0.014</td>
<td>89</td>
</tr>
<tr>
<td>0.022</td>
<td>66</td>
</tr>
<tr>
<td>0.067</td>
<td>49</td>
</tr>
<tr>
<td>0.10</td>
<td>73</td>
</tr>
<tr>
<td>0.12</td>
<td>60</td>
</tr>
</tbody>
</table>

ND, Not detected.

valinomycin this counter-ion system did not appear to be as effective in collapsing Δψ as did SCN⁻. In all cases, however, a very rapid decay of the pH gradient was observed, with $t_{1/2}$ values of <13.9 s compared with approximately 45 s or longer for most species of mesophilic bacteria (C. W. Jones, unpublished data).

**Transhydrogenase activity of respiratory membranes.** Investigation of the electron transfer activities of respiratory membranes prepared from X. campestris revealed that this organism was capable of oxidizing NADH at a substantially faster rate than NADPH [16.5 vs. 1.6 nmol O min⁻¹ (mg protein)⁻¹]. The addition of NAD⁺ (2 mM) had no effect on the rate of NADPH oxidation, thus indicating the absence of an active, membrane-bound transhydrogenase.

**Cytochrome content of X. campestris.** Room temperature dithionite-reduced minus H₂O₂-oxidized and dithionite-reduced + CO minus dithionite-reduced difference spectra of whole cells indicated the presence of cytochromes b, c, aa₃ and o-type cytochrome oxidase. Cytochrome aa₃ was detected only at the higher dilution rates; b and c type cytochromes and o-type cytochromes were present at all dilution rates (Table 2). A small absorbance at approximately 590 nm in reduced minus oxidized spectra suggested that ‘cytochrome a₁’, may have been present, particularly at the higher dilution rates.

**Effect of temperature on growth efficiency.** Most of the results that have previously been reported with various strains of X. campestris have been obtained at growth temperatures between 28 and 30 °C (Moraine & Rogovin, 1966, 1971; Silman & Rogovin, 1972; Davidson, 1978). However, earlier work in this laboratory using X. campestris NCIB 11854 demonstrated that the maximum temperature for growth is 32 °C (A. J. Rye, unpublished results), so that at 28 °C the organism is very near to its maximum growth temperature. As the optimum temperature for growth rate is often higher than that for growth yield the effect of decreasing the growth temperature from 28 to 20 °C during glucose-limitation was investigated. No significant differences in the molar growth yields were observed.

**Effect of yeast extract on growth efficiency.** In a defined minimal salts medium at 28 °C X. campestris has a maximum specific growth rate of approximately 0.15 h⁻¹, whereas in a complex medium containing yeast extract or other complex organic nitrogen sources maximum specific growth rates up to approximately 0.3 h⁻¹ have been reported (Moraine & Rogovin, 1966). The strain of X. campestris used in these current studies exhibited a similar increase in growth rate in the presence of yeast extract. To determine if it showed a change in growth efficiency, X. campestris was grown in continuous culture ($D = 0.10$ h⁻¹) in the minimal salts medium previously described for glucose limitation but with the addition of yeast extract (3 g l⁻¹). The observed molar growth yields were 60.3 g dry wt bacteria (mol glucose)⁻¹ and 21.1 g dry wt bacteria (mol O₂)⁻¹, i.e. not significantly different to those exhibited by cells grown on the minimal salts medium alone. Similarly no significant changes were observed in whole cell →H⁺/O quotients for the oxidation of endogenous substrates or in the $t_{1/2}$ for H⁺ re-entry. The observed $Y_{NH₄}$ increased by approximately 70% in the presence of 3 g yeast extract l⁻¹.
indicating that the assimilable nitrogen in yeast extract is used in preference to (NH₄)₂SO₄ at this dilution rate.

**DISCUSSION**

The maximum molar growth yields of *X. campestris* are low compared with those of other species of heterotrophic bacteria which have been grown under glucose-limited conditions with oxygen as the terminal electron acceptor. Mean $Y_{\text{max glucose}}$ values of 100 and 110 g dry wt bacteria (mol glucose)$^{-1}$ and $Y_{\text{O}_2}^\text{max}$ values of 62 and 91 g dry wt bacteria (mol O$_2$)$^{-1}$, have been reported for bacteria containing respectively two and three sites of respiratory chain energy conservation (Jones, 1977), compared with values of 53·6 and 26·4 respectively for *X. campestris*. Assuming a mean $Y_{\text{ATP}}^\text{max}$ value of 13·5 g dry wt bacteria (mol ATP)$^{-1}$ for growth on glucose, (Jones, 1977), and ignoring any ATP generated by substrate level phosphorylation, then ATP/O = $Y_{\text{O}_2}^\text{max}/2 \cdot Y_{\text{ATP}}^\text{max}$ which gives an ATP/O quotient of approximately 1. However, under these conditions *X. campestris* synthesized significant amounts of polymer which will lead to an underestimation of the molar growth yields, the magnitude of which will depend on the oxygen and glucose requirements for xanthan synthesis. Under conditions of glucose limitation where the only products are cells and CO$_2$ and the respiratory quotient is close to unity theoretical stoichiometric calculations indicate that for the cell composition obtained a $Y_{\text{O}_2}^\text{max}$ of 26·4 g dry wt bacteria (mol O$_2$)$^{-1}$ is incompatible with a $Y_{\text{max glucose}}$ of 53·6 g dry wt bacteria (mol glucose)$^{-1}$. Theoretical xanthan yields on oxygen and glucose at different ATP/O quotients have been previously calculated for xanthan containing 1 acetyl group and 0·5 pyruvic acid ketal groups per sub-unit (Jarman & Pace, 1984). The xanthan composition found in the present study was very similar (0·3 to 0·5 pyruvic acid ketal groups and 0·5 to 1·0 acetyl groups per sub-unit). The theoretical yields for xanthan synthesis at an ATP/O quotient of 1 are 0·82 g xanthan (g glucose)$^{-1}$ and 6·3 g xanthan (g O$_2$)$^{-1}$. Using these theoretical yields and the observed values obtained for $q_{\text{polymer}}$, the observed $q_{\text{glucose}}$ and $q_{\text{O}_2}$ values can then be corrected for xanthan synthesis and thus $Y_{\text{max glucose}}$ and $Y_{\text{O}_2}^\text{max}$ values corrected for xanthan synthesis can be derived. These were 74·1 g dry wt bacteria (mol glucose)$^{-1}$ and 29·3 g dry wt bacteria (mol O$_2$)$^{-1}$. Hence correcting the yields for polymer production results in only a slight increase in the ATP/O quotient derived from the $Y_{\text{O}_2}^\text{max}$, but a much more significant increase in the $Y_{\text{max glucose}}$. These corrected yields are now in agreement with the theoretical stoichiometric yields.

Difference spectra of whole cells of *X. campestris* indicated the presence of cytochromes b, c, aa$_3$, an o-type cytochrome and possibly cytochrome a$_1$. However, in view of the present controversy over the identity of the ‘a$_1$’ absorbance at approximately 590 nm in reduced minus oxidized difference spectra (see Jones & Poole, 1985) the presence of a genuine cytochrome a$_1$ in this organism, rather than a high-spin cytochrome b$_{590}$ must currently be regarded as uncertain. It is not known if the o-type cytochrome is o or co.

--H$^+$/O quotients of up to approximately 6 were observed for the oxidation of endogenous substrates [usually NAD(P)H] and significantly lower values for the oxidation of added succinate. In the absence of an active, energy-linked transhydrogenase these data indicate the presence of three proton and/or charge translocating sites in the respiratory chain of *X. campestris*, a conclusion which is compatible with the observed redox carrier composition of the respiratory chain (see Jones, 1977).

The rate of decay of the pH gradient generated by respiration was at least three times faster ($t_{1/2} < 14$ s) than is observed with most other species of mesophilic bacteria under comparable conditions ($t_{1/2} > 40$ s) (C. W. Jones, unpublished data), although rapid decay rates have also been reported for *Rhizobium leguminosarum* (Ratliffe et al., 1983) and for the thermophiles *Thermus thermophilus* and *Bacillus acidocaldarius* (McKay et al., 1982; Farrand et al., 1983). It is tempting to suggest that the relatively rapid decay rate reflects energy dissipation and is thus at least partly responsible for the low growth yields exhibited by these organisms. It should be noted, however, that the rate constant of this process is at least two orders of magnitude slower than the rate constant for respiration (D. B. Kell, personal communication).

Several possible explanations for the relatively rapid rate of decay of the proton gradient can be suggested. Firstly, an inherent leakiness of the cytoplasmic membrane to H$^+$ such that the
overall $\frac{H^+}{ATP}$ quotient is significantly greater than the $\frac{H^+}{ATP}$ quotient exhibited by the ATP phosphohydrolase ($BF_0$, $BF_1$) per se, in this case up to 6 compared with $\leq 3$. This could be achieved either by passive $H^+$ leakage via a potentially controllable $H^+$ uniport system analogous to that present in brown fat mitochondria (Nicholls, 1979) or by electroneutral entry with an anion or in exchange for a cation. The second possible explanation is that a high rate of ATP utilization by intracellular metabolic reactions causes a high rate of proton influx via the ATP phosphohydrolase system in order to resynthesize the ATP and thus maintain the intracellular energy charge or phosphorylation potential. The third possibility is an effect of growth nutrients. The growth of $X. campestris$ in a complex medium containing yeast extract is significantly faster than in a defined minimal salts medium. This is unlikely to be due to an increase in growth efficiency as theoretical calculations have shown that the $Y_{X/ATP}$ values for growth on glucose and inorganic salts are not significantly different to those for growth on glucose and preformed monomers (Stouthamer, 1977). Indeed, when $X. campestris$ was grown in the presence of yeast extract, the molar growth yields were not significantly different from those obtained at a similar growth rate on the minimal salts medium. Furthermore, there was no significant difference in the rate of decay of the proton gradient between cells grown in the presence or absence of yeast extract.

The low in situ ATP/O quotient of approximately 1 supports earlier theoretical calculations by Jarman & Pace (1984). The maximum rates of production of xanthan gum are relatively high and have been reported to be in the range of 0.3 to 0.4 g xanthan (g cell dry wt)$^{-1}$ h$^{-1}$ (Evans et al., 1979; Jarman & Pace, 1984); this would appear to agree with data obtained from a wide range of micro-organisms indicating that there is an inverse correlation between the energetic efficiency of micro-organisms and their capacity for product excretion (J. D. Linton & A. J. Rye, unpublished data). However, at these relatively high rates of production combined with the low growth efficiency of $X. campestris$ there will be a high oxygen demand for bacterial growth and xanthan synthesis which must be taken into account when developing high productivity xanthan processes.

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REFERENCES


