The Effect of Growth Conditions on the Respiratory System of a Succinoglucan-producing Strain of *Agrobacterium radiobacter*

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The respiratory system of *Agrobacterium radiobacter* NCIB 11883, a producer of succinoglucan exopolysaccharide under ammonia-limited conditions, was studied following growth in continuous culture ($D = 0.045 \text{ h}^{-1}$) under glucose, oxygen and ammonia limitation. The respiratory chain contained *b*- and *c*-type cytochromes, plus two terminal oxidases (*aa*₃ and *co*) under all growth conditions, and exhibited a low transhydrogenase activity. An inactive apoenzyme form of the quinoprotein glucose dehydrogenase was also present which could be activated by adding pyrroloquinoline quinone (PQQ) to cell suspensions. The activity of the terminal region of the respiratory chain increased approximately fourfold following growth under oxygen limitation, and this was accompanied by a significant increase in the concentration of cytochrome oxidase *co*. Whole cells exhibited $\rightarrow \text{H}^+/\text{O}$ quotients of 5.5–6.3 for the oxidation of endogenous substrate depending on the nature of the growth-limiting nutrient. It is concluded that the respiratory chain energy conservation system of this organism is not significantly modified during ammonia-limited growth to offset the increased energy demands for exopolysaccharide synthesis.

INTRODUCTION

Succinoglucan exopolysaccharides are produced by several members of the genus *Agrobacterium* (Hisamatsu *et al.*, 1977, 1978) and on hydrolysis yield glucose, galactose, pyruvate, succinate and, in some cases, acetate. The polymer produced by *A. radiobacter* NCIB 11883 contains all these constituents in the molar ratio 7:1:1:1:0.1 and is synthesized at the highest rate during growth in continuous culture under ammonium limitation at a dilution rate of approximately 0.045 h^{-1} (Linton *et al.*, 1987*b*). Carbon-balance and yield data indicate that under these growth conditions polysaccharide production imposes a severe energy burden on the organism, the amount of ATP required for polymer synthesis being almost equal to that needed for production of cell material (Linton *et al.*, 1987*b*).

The objective of the present work was to investigate the composition of the respiratory chain of *A. radiobacter* following growth in continuous culture under different nutrient limitations, and to establish whether the energy conservation properties of this organism are modified during ammonia-limited growth in an attempt to offset the additional energy demand for polymer synthesis.

METHODS

Organism and growth conditions. *Agrobacterium radiobacter* NCIB 11883 was obtained from Dr J. D. Linton, Shell Research, Sittingbourne, Kent, UK. The organism was grown at 30 °C under glucose limitation in

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Abbreviations: PQQ, pyrroloquinoline quinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TMBZ, 3,3',5,5'-tetramethylbenzidine; GDH, glucose dehydrogenase.

continuous culture ($D = 0.045 \text{ h}^{-1}$) using a chemically defined medium (Linton *et al.*, 1987b) supplemented with glucose (4 g l^{-1}), ammonium sulphate (3 g l^{-1}) and 0.142 g nitrilotriacetic acid l^{-1} (steady state biomass density = $2.1 \text{ g dry wt l}^{-1}$). The organism was grown in an LH series 500 chemostat of 2.3 l working volume. In order to achieve ammonia-limited growth the input concentration of ammonium sulphate was reduced to 0.5 g l^{-1} (biomass density = $1.2 \text{ g dry wt l}^{-1}$). During oxygen-limited growth the biomass density was maintained at $0.5\text{--}0.8 \text{ g l}^{-1}$ by reducing both the air supply to the culture and the extent of agitation. Succinoglucon was produced only during growth under ammonia limitation when the polymer concentration in the medium was $0.98 \pm 0.03 \text{ g l}^{-1}$ (mean $\pm$ SEM of five determinations).

Preparation of cell suspensions and subcellular fractions. All operations were done at 4°C using pre-cooled buffers and glassware. HEPES/KOH buffer (20 mM , $\text{pH } 7.0$) was used throughout this work unless otherwise stated.

Samples withdrawn from glucose-limited cultures were centrifuged at 15000 g for 15 min . The cell pellet was washed once with a volume of buffer equal to the original culture volume and resuspended to give a cell density of approximately $10 \text{ mg dry wt ml}^{-1}$. Samples from ammonia-limited cultures containing succinoglucon were diluted with buffer ($5 \text{ vols buffer:1 vol. culture}$) before the initial centrifugation to facilitate separation of the cells from the highly viscous polymer. Samples from oxygen-limited cultures contained clumps of cellulose (J. D. Linton, unpublished observation). These were removed before centrifugation by passing the cells through a short column of non-absorbent cotton wool. The same procedures were used to prepare cell suspensions for measurement of $-\text{H}^+/\text{O}$ quotients, except that cells were washed and resuspended in 0.5 mM -HEPES ($\text{pH } 7.0$) containing 140 mM -KCl.

Cell-free extracts were prepared by sonicating washed-cell suspensions (cooled using an ice/water mixture) using repeated bursts of ultrasound ($6 \times 15 \text{ s}$ bursts, 1 min interval between bursts). Whole cells and debris were pelleted by centrifuging at 15000 g for 15 min , and the cell-free extract was removed and stored on ice. Cell-free extracts were separated into soluble and membrane fractions using high-speed centrifugation (150000 g for 1 h). Following removal of the supernatant, the membrane pellet was carefully resuspended using a glass homogenizer, then recentrifuged and finally resuspended in fresh buffer to yield the washed-membrane fraction.

Measurement of oxygen uptake. Rates of oxygen consumption (q_{O_2}) were measured at 30°C using a Rank oxygen electrode. The reaction chamber contained 3.8 ml buffer, to which were added 0.1 ml cell suspension ($0.5\text{--}1.0 \text{ mg dry wt cells}$) and finally 0.1 ml of substrate ($40 \mu\text{mol}$ carbohydrate substrates or $20 \mu\text{mol}$ ascorbate plus $4 \mu\text{mol}$ TMPD). For some experiments cells were incubated with $12 \mu\text{M}$ -PQQ plus 10 mM - MgCl_2 for 5 min before addition of substrate to allow for complete activation of quinoprotein glucose dehydrogenase (GDH). q_{O_2} values were corrected for endogenous rates of respiration measured before addition of substrate and, in the case of ascorbate-TMPD, for autooxidation. The oxygen concentration of air-saturated buffer at 30°C was taken as $230 \mu\text{M}$.

In situ measurements of q_{O_2} . In situ q_{O_2} values quoted in the text were taken from Linton *et al.* (1987b).

Measurement of $-\text{H}^+/\text{O}$ quotients. The organism was incubated at 30°C in a closed vessel of 5 ml working volume containing 4.7 ml cell suspension ($25 \text{ mg dry wt cells}$) prepared in 0.5 mM -HEPES ($\text{pH } 7.0$)/ 140 mM -KCl. Potassium thiocyanate was added to give a final concentration of 180 mM (0.3 ml of a 3 M -stock solution). The anaerobic suspension was mixed using a magnetic stirrer and the pH was monitored continuously using a glass combination micro-electrode (Russell pH Ltd) connected to a chart recorder. After 30 min incubation, $21 \mu\text{l}$ of a freshly prepared solution of carbonic anhydrase (12 mg ml^{-1}) was added to the suspension, the pH of which was then adjusted to 6.5 by the addition of 1 M -HCl or 1 M -KOH as appropriate and allowed to stabilize. The cell suspension was pulsed with oxygen by injecting known volumes ($10\text{--}35 \mu\text{l}$) of 140 mM -KCl saturated with air at 30°C , and the resulting pH changes were recorded. The oxygen concentration of the air-saturated KCl solution at 30°C was taken as $225 \mu\text{M}$. The pH recorder was calibrated by injecting small volumes ($1\text{--}6 \mu\text{l}$) of an anaerobic solution of 10 mM -KOH.

Cytochrome difference spectra. These were recorded using a Perkin Elmer λ -5 split beam spectrophotometer. The organism contained relatively low concentrations of cytochromes and it proved difficult to obtain good quality spectra using whole-cell suspensions. Measurements were therefore done on crude extracts obtained by briefly centrifuging disrupted cells (13000 g for 2 min) using an Eppendorf microfuge. The cytochrome profile of the samples was not affected by this treatment and less than 10% of the total cytochrome was lost in the pellet. Reduced-minus-oxidized spectra were recorded following oxidation of the contents of a reference cuvette using $10 \mu\text{l}$ of a saturated solution of ferricyanide and reduction of the contents of a sample cuvette using a few grains of dithionite. Concentrations of cytochromes were estimated using the following molar absorption coefficients ($\text{mm}^{-1} \text{ cm}^{-1}$): cytochrome *c* 17.3 ($552\text{--}538 \text{ nm}$); cytochrome *b* 17.5 ($560\text{--}575 \text{ nm}$); cytochrome *aa*₃ 11.7 ($604\text{--}625 \text{ nm}$) (see Jones & Poole, 1985). In order to record CO-binding spectra (reduced + CO-minus-reduced), a 2 ml sample was reduced using a few grains of dithionite, divided into two 1 ml portions and used to record the reduced-minus-reduced spectrum. The contents of the sample cuvette were then bubbled with carbon monoxide for 1 min after which time a baseline-corrected CO-binding spectrum was recorded.

SDS-PAGE. Electrophoresis of proteins was done using 12% (w/v) acrylamide gels according to the method of Laemmli (1970). Samples of cells (about $500 \mu\text{g dry wt}$) or membranes (about $100 \mu\text{g protein}$) were boiled for 4 min

in dissolving buffer (Laemmli, 1970) without mercaptoethanol, and were then stained for haem-associated peroxidase activity using the method of Thomas *et al.* (1976).

Assay of cell-free GDH activity. This was done polarographically in the presence of 12 μM -PQQ as described by van Schie *et al.* (1985) except that phenazine ethosulphate (1 mM) was used to mediate electron flow between GDH and oxygen.

Assay of membrane-bound transhydrogenase activity. This was done using washed-membranes as described by Jones *et al.* (1975).

Measurement of protein. This was done using the modified Lowry assay of Peterson (1977) using bovine serum albumin as a standard.

Measurement of bacterial dry weight. Suspensions of *A. radiobacter* from different nutrient limitations were diluted using distilled water and the optical density was measured at 600 nm. The dry weights of cell suspensions were determined by interpolation using the appropriate optical density/dry weight standard curves.

Chemicals. The trisodium salt of PQQ was a gift from Professor J. A. Duine, University of Delft, The Netherlands. All other chemicals were from Fisons and were of the highest grade available.

Presentation of data. Where appropriate, values have been quoted as the mean $\pm$ SEM with the number of determinations in parentheses.

RESULTS

Effects of nutrient limitation on rates of substrate-dependent oxygen consumption by washed cell suspensions

Rates of oxygen consumption (q_{O_2}) with various substrates were measured using washed cells prepared from cultures grown under different nutrient limitations (Table 1). Significant rates of respiration were observed in the absence of added substrate irrespective of the conditions under which the organism had been grown; these endogenous rates are probably due to mobilization of an intracellular polyglucose storage polymer that *A. radiobacter* produces constitutively and which accounts for about 3% of the bacterial dry weight during growth under glucose-limited growth and up to 30% of the dry weight during ammonia-limited growth (J. D. Linton, unpublished observations; Linton *et al.*, 1987b).

q_{O_2} values with glucose as substrate were largely independent of the growth-limiting nutrient [75–96 ng-atom O min^{-1} (mg dry wt) $^{-1}$]. These values were significantly higher than the *in situ* value measured during glucose-limited growth at a dilution rate of 0.045 h^{-1} [42 ng-atom O min^{-1} (mg dry wt) $^{-1}$] but were similar to the *in situ* value measured during ammonia-limited growth [85 ng-atom O min^{-1} (mg dry wt) $^{-1}$]. The q_{O_2} values of washed cells prepared from all three types of nutrient limitation increased significantly when incubated with PQQ before addition of glucose, suggesting that *A. radiobacter* possesses the inactive (apoenzyme) form of quinoprotein GDH which is activated on adding the PQQ cofactor as has also been found for other Gram-negative organisms (Hommes *et al.*, 1984; van Schie *et al.*, 1984). Consistent with this proposal is the observation that q_{O_2} values measured with xylose, an alternative substrate for quinoprotein GDH (Hauge, 1960), also increased following addition of PQQ, and GDH activity [equivalent to 211 nmol glucose oxidized min^{-1} (mg protein) $^{-1}$] could be measured in cell-free extracts of glucose-limited cells using the polarographic method of van Schie *et al.* (1985) provided that PQQ was added to the assay system. Furthermore, Linton *et al.* (1987a)

Table 1. q_{O_2} values for the oxidation of different respiratory substrates by washed cell suspensions of *A. radiobacter* following growth in continuous culture under different nutrient limitations

Growth-limiting nutrient	Substrate added	q_{O_2} [ng-atom O min^{-1} (mg dry wt) $^{-1}$]			
		None (endogenous respiration)	Glucose		Ascorbate-TMPD
			– PQQ	+ PQQ	
Glucose		15 $\pm$ 2 (9)	96 $\pm$ 4 (8)	210 $\pm$ 12 (3)	483 $\pm$ 26 (10)
Oxygen		19 $\pm$ 1 (12)	94 $\pm$ 5 (5)	278 $\pm$ 24 (3)	1780 $\pm$ 61 (5)
Ammonia		14 $\pm$ 1 (12)	75 $\pm$ 4 (5)	154 $\pm$ 7 (5)	396 $\pm$ 30 (4)

Fig. 1. Cytochrome spectra of *A. radiobacter* grown in continuous culture ($D = 0.045 \text{ h}^{-1}$) under glucose limitation. Washed cell suspensions ($10 \text{ mg dry wt ml}^{-1}$) were prepared for spectral analysis as described in Methods. (a) Dithionite-reduced-minus-ferricyanide-oxidized difference spectrum; (b) dithionite-reduced + CO-minus-dithionite-reduced difference spectrum.

Table 2. Cytochrome content of *A. radiobacter* following growth in continuous culture under different nutrient limitations

Growth-limiting nutrient	Total cytochrome <i>c</i>	Total cytochrome <i>b</i> [pmol (mg dry wt) $^{-1}$]	Cytochrome <i>aa</i> ₃
Glucose	178 ± 8 (5)	99 ± 12 (5)	29 ± 3 (5)
Oxygen	272 ± 18 (3)	200 ± 14 (3)	34 ± 9 (3)
Ammonia	144 ± 8 (3)	75 ± 7 (3)	26 ± 7 (3)

reported that growing cultures and washed-cell suspensions of this strain of *A. radiobacter* produce gluconate (the product of GDH-catalysed glucose oxidation) at high rates [$55\text{--}100 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$] following addition of PQQ.

A. radiobacter readily oxidized ascorbate-TMPD, the q_{O_2} being approximately fourfold higher when the organism was grown under oxygen limitation compared with either glucose or ammonia limitation (Table 1), thus indicating that the activity of the terminal segment of the respiratory chain is regulated by the availability of oxygen.

Cytochrome analysis

Analysis of the cytochrome complement of *A. radiobacter* using difference spectrophotometry revealed that the organism contained *b*- and *c*-type cytochromes plus cytochrome oxidase *aa*₃ during growth under glucose limitation as indicated by peaks in the reduced-minus-oxidized difference spectrum at 552 nm and 604 nm representing the α -bands of cytochromes *c* and *aa*₃ respectively, and by an inflexion at 560 nm due to the α -band of cytochrome *b* (Fig. 1*a*). The absolute concentration of each type of cytochrome was similar in cells grown at high ambient concentrations of oxygen (i.e. under glucose or ammonia limitation), but when the organism was grown under oxygen limitation the concentrations of the *b*- and *c*-type cytochromes approximately doubled whilst the concentration of cytochrome *aa*₃ remained constant (Table 2).

The nature of the potential terminal oxidases present under different nutrient limitations was investigated further by recording reduced + CO-minus-reduced difference spectra (Fig. 1*b*). All of the latter exhibited a peak at 428 nm and a trough at 443 nm indicating that cytochrome *a*₃ was present under all growth conditions at a concentration of $9\text{--}15 \text{ pmol (mg dry wt)}^{-1}$. Most CO-binding spectra also contained peaks at 413 nm and troughs at 522 nm and 552 nm which

Fig. 2. Dixon plots showing the effect of cyanide on the oxidation of ascorbate-TMPD by whole cells of *A. radiobacter* grown in continuous culture ($D = 0.045 \text{ h}^{-1}$) under glucose limitation. The concentrations of TMPD used were $100 \mu\text{M}$ ($\circ$), $200 \mu\text{M}$ ($\bullet$), $500 \mu\text{M}$ ($\square$) and $1000 \mu\text{M}$ ($\blacksquare$). Plots in (a) and (b) were obtained using different batches of cells.

were due to cytochrome *c* binding carbon monoxide; this phenomenon, which has been observed with many bacterial *c*-type cytochromes, is probably due to displacement of one of the ligands coordinating the haem and is not in itself indicative of an oxidase function. Spectra of cells grown under oxygen limitation sometimes contained an additional peak at 418 nm and a shoulder at 558 nm , which suggest the presence of an *o*-type oxidase, but these peaks were often obscured by the peaks at 413 nm and 552 nm due to cytochrome *c*. In view of our failure to detect an *o*-type oxidase consistently by means of spectral analysis it was decided to investigate the cytochrome complement by first separating the polypeptides using SDS-PAGE and then staining for haem-associated peroxidase activity using TMBZ (Thomas *et al.*, 1976). Washed cells and membranes prepared from cultures grown under all nutrient limitations contained two polypeptides (M_r 29000 and 24000) which stained for haem and were present at much higher concentrations following growth under oxygen limitation (not shown). The M_r values of these polypeptides are similar to those of cytochrome oxidase *co* components isolated from other Gram-negative organisms (see Discussion).

Analysis of the terminal segment of the respiratory chain

The composition of the terminal segment of the respiratory chain was investigated further by examining the effect of cyanide on the oxidation of ascorbate-TMPD since it has previously been observed that TMPD oxidation is inhibited by cyanide in an uncompetitive fashion when respiratory chains are terminated by cytochrome oxidase *aa₃* whereas non-competitive inhibition is observed with *o*-type oxidases (Jones, 1973; Carver & Jones, 1983). Dixon plots constructed using cells grown under glucose limitation showed two distinct types of inhibition: non-competitive inhibition ($K_i = 4.1 \pm 0.6 \mu\text{M}$; $n = 5$) was observed at low concentrations of cyanide ($0\text{--}10 \mu\text{M}$) whilst uncompetitive inhibition ($K_i \leq 80 \mu\text{M}$) was noted when the cyanide concentration exceeded $10 \mu\text{M}$ (Fig. 2*a, b*). Similar Dixon plots were obtained with cells grown under ammonia or oxygen limitation, although in the latter case the uninhibited rate of TMPD oxidation was considerably higher (see Table 1).

These observations suggest that a considerable proportion of the electron flow from TMPD to oxygen can occur by way of an oxidase (probably cytochrome *co*) that is highly sensitive to cyanide; when the concentration of cyanide exceeds $10 \mu\text{M}$, this oxidase is rendered inactive and electron flow continues by way of cytochrome *aa₃*. The effect of cyanide on glucose-dependent respiration was also examined using cells grown under glucose and oxygen limitation and with both types of cells there was little diminution in the measured rate of oxygen consumption until the cyanide concentration exceeded $75 \mu\text{M}$ ($I_{50} = 100 \mu\text{M}$ for glucose-limited cells and $95 \mu\text{M}$ for oxygen-limited cells). Electron flow from glucose to oxygen is evidently not impeded at

Table 3. $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates by washed cell suspensions of *A. radiobacter* following growth in continuous culture under different nutrient limitations

Growth-limiting nutrient	$\rightarrow H^+/O$ quotient [ng-ion H^+ (ng-atom O) $^{-1}$]	t_1 for collapse of H^+ gradient (s)
Glucose	6.3 ± 0.1 (18)	37.5 ± 5.0 (10)
Oxygen	5.5 ± 0.1 (6)	33.5 ± 1.5 (6)
Ammonia	5.9 ± 0.2 (5)	10.0 ± 1.0 (4)

concentrations of cyanide which render cytochrome *co* inactive, presumably because cytochrome *aa₃* is not saturated by the relatively low rate of electron transfer.

Measurement of $\rightarrow H^+/O$ quotients

This was done for suspensions of *A. radiobacter* prepared from cultures grown under glucose, oxygen and ammonia limitation (Table 3). These values were obtained using cells respiring endogenous substrate (probably a glucose homopolymer; see above) which has been assumed to donate electrons to the respiratory chain via NAD(P)H. Unfortunately, it did not prove possible to deplete cells of endogenous substrate and hence discrete proton-ejecting segments of the respiratory chain could not be identified by measuring $\rightarrow H^+/O$ quotients in the presence of added substrates (e.g. succinate or ascorbate-TMPD).

$\rightarrow H^+/O$ quotients were close to 6 ng-ion H^+ (ng-atom O) $^{-1}$ for cells grown under either glucose- or ammonia-limited conditions, but were significantly lower than 6 for cells grown under oxygen limitation (Table 3). Interestingly, the rate of decay of the proton gradient following an oxygen pulse was much more rapid with cells grown under ammonia-limited conditions. However, as a normal rate of decay was later observed with cells grown under K^+ limitation (A. Cornish, unpublished), which also produce polymer, it seems likely that the rapid decay rate reflects uptake of NH_4^+ rather than polymer synthesis.

Membrane-bound transhydrogenase activity

The activity in membranes prepared from glucose-limited cells was 19.7 nmol min $^{-1}$ (mg protein) $^{-1}$ compared to 93.5 nmol min $^{-1}$ (mg protein) $^{-1}$ for the NADH oxidase system. As this activity is only approximately 20% of the rate of NADH oxidation, site 0 is unlikely to have contributed significantly to the $\rightarrow H^+/O$ quotients.

DISCUSSION

The results described above indicate that the respiratory chain of *A. radiobacter* NCIB 11883 is fairly typical of Gram-negative aerobes in that it contains *b*- and *c*-type cytochromes together with two terminal oxidases (*aa₃* and *co*) which can be distinguished by their sensitivity to cyanide, but exhibits only very low transhydrogenase activity. The only apparent change in the respiratory chain as a result of growth under different nutrient limitations occurs in oxygen-limited cells where the activity of the terminal region of the respiratory chain increases approximately fourfold, presumably to compensate for the low ambient concentration of oxygen. This change is probably due to the synthesis of cytochrome oxidase *co* since it is accompanied by an increase in the concentrations of two membrane-bound polypeptides (M_r 29000 and 24000) that stain positively for haem. In this context it is worth noting that cytochrome oxidases *co* from *Azotobacter vinelandii* (Jurtshuk & Yang, 1980), *Pseudomonas aeruginosa* (Matsushita *et al.*, 1982), *Rhodopseudomonas palustris* (King & Drews, 1976) and *Methylophilus methylotrophus* (Carver & Jones, 1983; Froud & Anthony, 1984) contain a *b*-type cytochrome (M_r 29000) plus a *c*-type cytochrome (M_r 24000 $\pm$ 2000) and are inhibited non-competitively by low concentrations of cyanide.

In common with many Gram-negative organisms (Hommes *et al.*, 1984; van Schie *et al.*, 1984), *A. radiobacter* synthesizes the inactive apoenzyme of quinoprotein GDH. Although the

function of this enzyme in such organisms is still open to conjecture (see Duine *et al.*, 1986), it is clear that the GDH holoenzyme donates electrons to the respiratory chain via the quinone pool (Beardmore-Gray & Anthony, 1986), and using membrane vesicles it has been shown that the reconstituted enzyme can generate a protonmotive force *in vitro* following addition of glucose (van Schie *et al.*, 1985). Linton *et al.* (1987a) have shown that addition of PQQ to ammonia-limited cultures of *A. radiobacter* causes gluconate to be formed at high rates but does not affect the rate of succinoglucon production or the composition of the polymer.

The $\rightarrow\text{H}^+/\text{O}$ quotient of almost exactly 6 which was observed for the oxidation of endogenous substrate by whole cells of *A. radiobacter* grown under ammonia limitation is fully compatible with a respiratory chain that contains cytochrome *c* but lacks significant transhydrogenase activity, and indicates the presence of three energy coupling sites (Jones, 1977). However, assuming that the nature of the endogenous substrate remains unchanged, the slightly (but significantly) higher and lower values which were obtained with cells grown under glucose- or oxygen-limited conditions respectively suggests that the terminal respiratory chain may be more complex. Indeed, it is tempting to speculate that energy conservation in this region may reflect a variable mixture of a proton-pumping cytochrome oxidase (e.g. *aa₃*) and a non-proton-pumping cytochrome oxidase which consumes 2H^+ on the periplasmic side of the membrane (e.g. *co*). Unfortunately, it has not proved possible to carry out a more detailed analysis of the proton-translocating properties of the terminal region of the respiratory chain (e.g. by measuring $\rightarrow\text{H}^+/\text{O}$ quotients with ascorbate-TMPD using initial rate procedures), since we have not been able to deplete the organism of endogenous substrate, and endogenous respiration could not be blocked using classical inhibitors of the cytochrome *bc* complex (e.g. antimycin A and hydroxyquinoline *N*-oxide) as these were found to be ineffective.

Since ATP/O quotients of 1.4–1.6 have been estimated from the growth yields of *A. radiobacter* under glucose-limited conditions (Linton *et al.*, 1987b), the $\rightarrow\text{H}^+/\text{O}$ quotients reported above are consistent with an overall $\rightarrow\text{H}^+/\text{ATP}$ quotient of approximately 4 g-ion H^+ (mol ATP)⁻¹ in this organism.

It is concluded that the energy conservation properties of *A. radiobacter* are not significantly modified during growth under ammonia-limited conditions in an attempt to offset the increased energy demands for exopolysaccharide synthesis.

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REFERENCES

- BEARDMORE-GRAY, M. & ANTHONY, C. (1986). The oxidation of glucose by *Acinetobacter calcoaceticus*: interaction of the quinoprotein glucose dehydrogenase with the electron transport chain. *Journal of General Microbiology* **132**, 1257–1268.
- CARVER, M. A. & JONES, C. W. (1983). The terminal respiratory chain of the methylotrophic bacterium *Methylophilus methylotrophus*. *FEBS Letters* **155**, 187–191.
- DUINE, J. A., FRANK, J. & JONGEJAN, J. A. (1986). PQQ and quinoprotein enzymes in microbial oxidations. *FEMS Microbiology Reviews* **32**, 165–178.
- FROUD, S. J. & ANTHONY, C. (1984). The purification and characterization of the *o*-type cytochrome oxidase from *Methylophilus methylotrophus*, and its reconstitution into a 'methanol oxidase' electron transport chain. *Journal of General Microbiology* **130**, 3319–3325.
- HAUGE, J. G. (1960). Kinetics and specificity of glucose dehydrogenase from *Bacterium anitratum*. *Biochimica et biophysica acta* **45**, 263–269.
- HOMMES, R. W. J., POSMA, P. W., NEIJSSSEL, O. M., TEMPEST, D. W., DOKTOR, P. & DUINE, J. A. (1984). Evidence of a quinoprotein glucose dehydrogenase apo-enzyme in several strains of *Escherichia coli*. *FEMS Microbiology Letters* **24**, 329–333.
- HISAMATSU, M., OTT, I., AMEMURA, A. & HARADA, T. (1977). Change in ability of *Agrobacterium* to produce water-soluble and water-insoluble β -glucans. *Journal of General Microbiology* **103**, 375–379.
- HISAMATSU, M., SANO, K., AMEMURA, A. & HARADA, T. (1978). Acidic polysaccharides containing succinic acid in various strains of *Agrobacterium*. *Carbohydrate Research* **16**, 89–96.
- JONES, C. W. (1973). The inhibition of *Azotobacter vinelandii* terminal oxidases by cyanide. *FEBS Letters* **36**, 347–350.
- JONES, C. W. (1977). Aerobic respiratory systems in bacteria. *Symposia of the Society for General Microbiology* **27**, 23–59.
- JONES, C. W. & POOLE, R. K. (1985). The analysis of cytochromes. *Methods in Microbiology* **18**, 285–328.
- JONES, C. W., BRICE, J. M., DOWNS, A. J. & DROZD, J. W. (1975). Bacterial respiration-linked proton translocation and its relationship to respiratory chain composition. *European Journal of Biochemistry* **52**, 265–271.
- JURTSCHUK, P. & YANG, T. S. (1980). Oxygen-reactive

- haemoprotein components in bacterial respiratory systems. In *Diversity of Bacterial Respiratory Systems*, vol. 1, pp. 137–159. Edited by C. J. Knowles. Cleveland, Ohio: CRC Press.
- KING, M. T. & DREWS, G. (1976). Isolation and partial characterization of the cytochrome oxidase from *Rhodospseudomonas palustris*. *European Journal of Biochemistry* **68**, 5–12.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LINTON, J. D., WOODARD, S. & GOULDNEY, D. G. (1987*a*). The consequence of stimulating glucose dehydrogenase activity by the addition of PQQ on metabolite production by *Agrobacterium radiobacter* NCIB 11883. *European Journal of Applied Microbiology and Biotechnology* (in the Press).
- LINTON, J. D., EVANS, M. W., JONES, D. S. & GOULDNEY, D. G. (1987*b*). Exocellular succinoglucon production by *Agrobacterium radiobacter* NCIB 11883. *Journal of General Microbiology* **133**, 2961–2969.
- MATSUSHITA, K., SHINAGAWA, E., ADACHI, O. & AMEYAMA, M. (1982). *o*-Type cytochrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. *FEBS Letters* **139**, 255–258.
- PETERSON, G. L. (1977). A simplification of the protein assay of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* **83**, 346–356.
- VAN SCHIE, B. J., VAN DIJKEN, J. P. & KUENEN, J. G. (1984). Non-coordinated synthesis of glucose dehydrogenase and its prosthetic group PQQ in *Acinetobacter* and *Pseudomonas* spp. *FEBS Microbiology Letters* **24**, 133–138.
- VAN SCHIE, B. J., HELLINGWERF, K. J., VAN DIJKEN, J. P., ELFERINK, G. L., VAN DIJL, J. M., KUENEN, J. G. & KONINGS, W. N. (1985). Energy transduction by electron transfer via a pyrrolo-quinoline quinone-dependent glucose dehydrogenase in *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* (var. *lwoffii*). *Journal of Bacteriology* **163**, 493–499.
- THOMAS, P. E., RYAN, D. & LEVINE, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulphate polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.