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Alternative Function of the Electron Transport System in *Azotobacter vinelandii*: Removal of Excess Reductant by the Cytochrome *d* Pathway

J.-K. LIU,[1] F.-T. LEE,[1] C.-S. LIN,[2], X.-T. YAO,[3] J. W. DAVENPORT,[3] AND T.-Y. WONG[3]*

Institute of Life Sciences,[1] and Department of Marine Resources,[2] National Sun Yet-Sen University, Taiwan, Republic of China, and Molecular Sciences and Microbiology, University of Memphis, Memphis, Tennessee 38152[3]

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The N_2-fixing bacterium *Azotobacter vinelandii* was grown in an O_2-regulated chemostat with glucose or galactose as substrate. Increasing the O_2 partial pressure resulted in identical synthesis of the noncoupled cytochrome *d* terminal oxidase, which is consistent with the hypothesis that *A. vinelandii* uses high rates of respiration to protect the nitrogenase from oxygen. However, cell growth on glucose showed a lower yield of biomass, higher glycolytic rate, higher respiratory rate, and lower cytochrome *o* content than cell growth on galactose. Elemental analysis indicated no appreciable change in the C-to-N ratio of cell cultures, suggesting that the major composition of the cell was not influenced by the carbon source. A poor coordination of glucose and nitrogen metabolisms in *A. vinelandii* was suggested. The rapid hydrolysis of glucose resulted in carbonaceous accumulation in cells. Thus, *Azotobacter* species must induce a futile electron transport to protect cells from the high rates of glucose uptake and glycolysis.

The electron transport system (ETS) in bacteria exhibits considerable variation in complexity and organization. Branching at both the initial (dehydrogenase) and the terminal (oxidase) ends of the ETS is common. In addition, the number of energy-conserving sites of the ETS varies substantially. These variations are influenced by O_2 concentration, pH, temperature, the presence of certain substrates, and the age of the culture (12). This complexity allows aerobic metabolism to be modified to give selective growth advantages under a variety of environmental conditions; however, the nature of these advantages is obscure.

Azotobacter vinelandii is a free-living N_2-fixing bacterium commonly found in the soil. *A. vinelandii* uses aerobic respiration to produce the energy needed to support nitrogen fixation and growth. However, the two proteins constituting nitrogenase are rapidly inhibited by molecular O_2. Therefore, it has been proposed (30, 31) that the ETS of *A. vinelandii* serves not only to produce energy but also to scavenge cytoplasmic O_2 so as to allow proper functioning of the nitrogenase (respiratory protection). A proposed (11) ETS of *A. vinelandii* is shown in Fig. 1.

The extensive branching at the level of the primary dehydrogenases allows oxidation of various substrates of widely differing redox potentials. The NADH dehydrogenase (site I) has been shown to exhibit proton translocation. At a high degree of aeration, *A. vinelandii* exhibits a very high respiratory rate. The high O_2 uptake is associated with the de novo synthesis of both cytochrome *d* oxidase and NADH and NADPH dehydrogenases (2). At high concentrations of NADH, energy conservation at site I is lost (1, 2), further accelerating electron transport.

Electrons from the primary dehydrogenases are channeled to a central quinone-cytochrome *b* region that exhibits proton translocation properties (site II). At least two different branches of cytochromes can receive electrons from cytochrome *b*. The cytochrome $b \rightarrow d$ branch predominates when cells are growing under N_2-fixing conditions at a high degree of aeration. This pathway is uncoupled from energy conservation and thus allows rapid passage of electrons. It is this rapid oxidation which is thought to deplete cytoplasmic O_2 and thereby afford respiratory protection. When the O_2 tension is low, more electrons are transferred from cytochrome *b* through cytochrome c_{4+5} to the cytochrome $o + a_1$ terminal oxidase, which is coupled to ATP synthesis at site III.

Although the ETS in *A. vinelandii* has been characterized and its response to aeration has been described, relatively little is known about the carbon metabolism that supplies the electrons for the ETS and nitrogen fixation. *A. vinelandii* uses different pathways to metabolize monosaccharides (24, 25). *A. vinelandii* metabolizes glucose oxidatively by the Entner-Doudoroff pathway (16, 17, 21). A similar pathway, the DeLey-Doudoroff pathway (7), is induced during growth on galactose (29). The end products of both pathways are the same, i.e., glyceraldehyde-3-P and pyruvate. Glyceraldehyde-3-P can be converted to pyruvate by enzymes of the remaining Embden-Meyerhof-Parnas pathway. The pyruvate is then converted to acetyl coenzyme A, which is oxidized to CO_2 by the tricarboxylic acid (TCA) cycle (19).

The transport systems for glucose (4, 9, 15) and galactose (27) in *A. vinelandii* are inducible by their respective sugars. The rate of glucose transport is much higher than that of galactose (27), and the Entner-Doudoroff pathway is more active than the DeLey-Doudoroff pathway (28). These data imply that *A. vinelandii* can metabolize glucose faster than galactose. Paradoxically, *A. vinelandii* produces more biomass

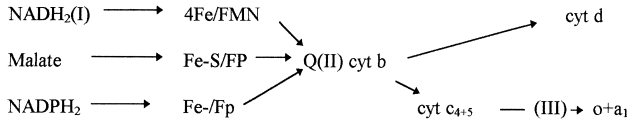

FIG. 1. Proposed ETS of *A. vinelandii*. cyt, cytochrome.

* Corresponding author. Phone: (901) 678-4462. Fax: (901) 678-4457. Electronic mail address: WONGT@CC.MEMPHIS.EDU.

TABLE 1. Cell yields and concentrations of cytochrome oxidases *d* and *o* of *A. vinelandii* grown under various levels of dissolved O_2 with either glucose or galactose as substrate[a]

Dissolved O_2 (%)	Cell yield (mg of dry wt/g of substrate)		Cytochrome *d* (nmol/mg of protein)		Cytochrome *o* (nmol/mg of protein)		Total cytochrome *d* + cytochrome *o* (nmol/mg of protein)	
	Glu	Gal	Glu	Gal	Glu	Gal	Glu	Gal
100	31.2 (± 1.9)	55.0 (± 1.9)	1.038 (± 0.02)	1.144 (± 0.01)	0.062 (± 0.02)	0.129 (± 0.015)	1.10	1.273
79	50.1 (± 1.7)	78.0 (± 2.0)	0.532 (± 0.01)	0.585 (± 0.021)	0.070 (± 0.01)	0.132 (± 0.018)	0.602	0.717
60	58.3 (± 1.5)	84.7 (± 1.7)	0.380 (± 0.012)	0.452 (± 0.011)	0.063 (± 0.01)	0.126 (± 0.015)	0.443	0.578
50	68.3 (± 4.0)	100.2 (± 3.4)	0.240 (± 0.016)	0.320 (± 0.01)	0.056 (± 0.01)	0.122 (± 0.01)	0.296	0.442
26	106.6 (± 1.4)	134.3 (± 0.7)	0.141 (± 0.015)	0.230 (± 0.021)	0.051 (± 0.021)	0.120 (± 0.014)	0.192	0.350
10	147.8 (± 0.1)	147.2 (± 0.1)	0.079 (± 0.02)	0.159 (± 0.0)	0.044 (± 0.006)	0.115 (± 0.02)	0.123	0.274

[a] Cells were assayed at mid-log phase. Values are the means (± standard deviations) from at least two independent preparations. Glu, glucose; Gal, galactose.

from galactose than from glucose (24). Additionally, when cells are placed in a mixture of glucose and galactose, *A. vinelandii* prefers galactose as its main source of catabolite and exhibits diauxic growth (28). This pattern is an exception to the general tendency of microbes to use the most rapidly consumed substrate (usually glucose) first before using other sugars.

Nitrogen fixation is an important process in the nitrogen cycle. However, the role of free-living nitrogen fixing bacteria in soil improvement is not clear. This is largely due to the lack of understanding of these soil microbes. In this paper, we demonstrate that over a 10-fold range of oxygen concentrations, glucose selectively decreases the amount of cytochrome *o*. It also induces a high capacity for glucose metabolism. We suggest that a futile function of the ETS induced by glucose may be the cause of the low cell yield in glucose-grown cultures.

MATERIALS AND METHODS

All chemicals used are reagent grade and were purchased from Sigma (St. Louis, Mo.).

Organism and chemostat culture. *A. vinelandii* OP (ATCC 13705) was grown in 1.3 liters of modified Burk's buffer (26) supplemented with 0.5% of either glucose or galactose at 30°C in a 2-liter fermentor (KMJ-23; Mituwa Co., Osaka, Japan) with continuous agitation at 400 rpm. Precultures were grown in 1% test sugar twice before inoculation. The volume of the inoculum was 13 ml. Dissolved O_2 in the medium was maintained at a fixed saturation (±2%) by an oxygen electrode connected to an electronic controller valve that regulated the type and amount of input aeration gas (O_2 or N_2). Cultures were grown at dissolved O_2 levels of 7.55, 6, 5, 3.77, 2, and 0.75 ppm. These levels were equal to 100, 79.5, 66, 50, 27, and 10% of air saturation, respectively. Growth was monitored photometrically at 600 nm and by dry weight measurements. When needed, sodium nitrate was added to Burk's buffer at 0.05 or 0.1%. Unless otherwise specified, cultures were harvested and analyzed at mid-log phase.

Elements and biomass analysis. Samples in triplicate from the 100 and the 10% dissolved O_2 cultures were washed twice with deionized water, freeze-dried, weighed, and analyzed for percentages of carbon, nitrogen, hydrogen, and oxygen with a Heraeus CHN-O Rapid Element Analyzer (Heraeus Co., Harau, Germany) at 390°C according to the manufacturer's recommended procedure.

Preparation of cell extracts and cell membranes. Cell extracts and cell membranes were isolated by differential centrifugation according to the method described by Jurtshuk et al. (14).

Quantification of CO_2 production. Mid-log-phase cells from 100% air-saturated cultures were used to analyze the relative rates of CO_2 production with 1% (wt/vol) of their corresponding sugars as substrate. The carbon dioxide produced by the cells was trapped for 1 h and was quantified by the method described previously (29). The efficiency of CO_2 trapping was more than 87%.

O_2 uptake rate. O_2 uptake by mid-log-phase cells was analyzed by an oxygen monitor (YSI model 53) at 30°C. The incubation chamber contained 5 ml of air-saturated Burk's buffer and 5 mM test sugar. After 5 min of equilibration, 0.05 ml of mid-log-phase culture was injected into the chamber, and the rate of O_2 uptake was recorded. The rates of malate, succinate, hydrogen, and NADH oxidation of the cell membranes were analyzed similarly with the appropriate concentrations of the substrate in 50 mM phosphate buffer (pH 7.6).

Cytochrome analysis. The cytochrome content was estimated by using standard wavelength pairs and extinction coefficients (20). For cytochrome *d*, the assay curvette contained a grain of dithionite while the reference curvette contained oxygen (ferricyanide). For cytochrome *o*, the assay curvette contained carbon monoxide and a grain of dithionite while the reference curvette contained dithionite.

Pyruvate quantification. The conditions for pyruvate production from galactose or from glucose by the cell extracts and for the extraction of pyruvate have been described previously (27). The extracted pyruvate was quantified by high-performance liquid chromatography (Dionex 2000I; Dionex Corp.) with a cationic analytical column (IonPac AS4A). Samples were eluted with 1.8 mM Na_2CO_3–1.7 mM $NaHCO_3$ at a rate of 2 ml/min. Dilutions of a standard pyruvate solution were used to construct a standard curve.

Other enzyme assays. The activities of several other enzymes in cell extracts from 100% air-saturated samples were measured. Pyruvate dehydrogenase was assayed by the method described by Senior and Dawes (19). Glyceraldehyde-3-P dehydrogenase was assayed by the procedure described by Allison (3). Isocitrate dehydrogenase was assayed by the method described by Barrera and Jurtshuk (5). The amounts of protein were determined by the Bio-Rad method with bovine serum albumin as the standard.

RESULTS

The cell yield of the N_2-fixing bacterium *A. vinelandii* is influenced both by the O_2 tension and by the kind of substrate being used. To separate these parameters, we conducted our experiments in an O_2-regulated chemostat. The effects of O_2 tension on dry cell yield, cytochrome *d*, and cytochrome *o* of *A.*

TABLE 2. Compositions of nitrogen, carbon, hydrogen, and oxygen of *A. vinelandii* grown under 100 and 10% dissolved O_2 with either glucose or galactose as substrate

Dissolved O_2 (%)	Substrate	% Elements (molar ratio)[a]			
		N	C	H	O
100	Glucose	11.4 ± 0.01 (0.23)	42.54 ± 0.01 (1)	7.2 ± 0.03 (2.03)	38.8 ± 0.03 (1.2)
	Galactose	11.09 ± 0.01 (0.23)	41.38 ± 0.01 (1)	6.8 ± 0.03 (1.98)	40.7 ± 0.03 (1.2)
10	Glucose	12.24 ± 0.01 (0.24)	43.45 ± 0.01 (1)	7.23 ± 0.03 (1.99)	34.8 ± 0.03 (1.09)
	Galactose	12.65 ± 0.01 (0.24)	45.19 ± 0.01 (1)	7.4 ± 0.03 (1.96)	37.1 ± 0.03 (1.11)

[a] Data are the means ± standard deviations from three different preparations. Molar ratios were calculated by dividing each element by its corresponding atomic weight and were normalized, with carbon as 1. The remaining ashes from each sample were less than 0.05%.

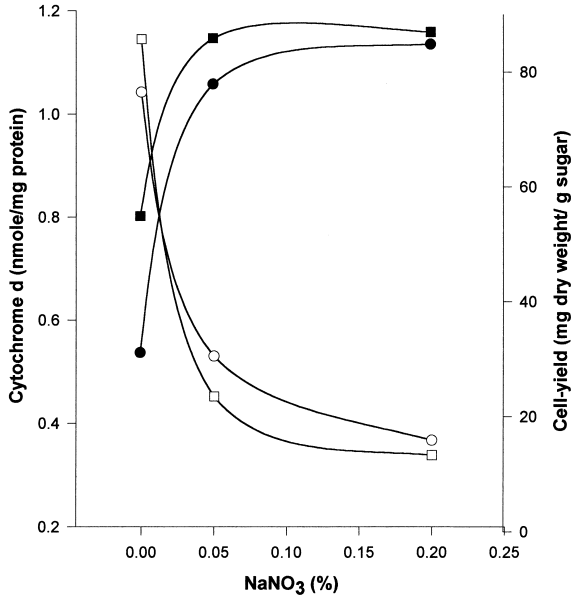

FIG. 2. Effects of $NaNO_3$ on cell yield (right axis) of galactose-grown (■) and glucose-grown (●) cultures and on the concentrations of cytochrome *d* (left axis) of galactose-grown (□) and glucose-grown (○) mid-log cells of *A. vinelandii*. Cultures were grown under 100% air-saturated conditions.

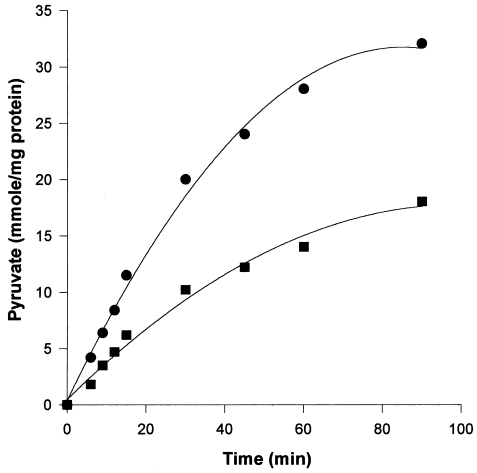

FIG. 3. Formation of pyruvate by the cell extracts of *A. vinelandii*. Pyruvate produced from glucose (●) was generated from the cell extracts of glucose-grown cells. Pyruvate produced from galactose (■) was produced from the cell extracts of galactose-grown cells.

vinelandii growing on glucose or on galactose are summarized in Table 1. The concentrations of cytochrome *d* in both glucose- and galactose-grown cells increased in response to high O_2 tension. However, the amounts of cytochrome *o* in both cultures were much less responsive to O_2 tension. The biomasses of both cultures decreased as the O_2 tension increased; however, the glucose-grown cultures were more sensitive to O_2 tension than galactose-grown cultures.

This difference in growth efficiencies between these two sugars was not likely due to any major change in carbon metabolism that alters the composition of carbon in the cell (such as an increase in the formation of polyhydroxybutyrate or cell capsules). Elemental analysis of the cultures showed that the C-to-N-to-H-to-O ratios of glucose- and galactose-grown cultures were similar. The molar ratio of C to H to O in *A. vinelandii* was always approximately 1:2:1. However, the degree of aeration, but not the carbon substrate, could affect the carbon-to-nitrogen ratio. The ratio of C to N increased by approximately 4% in cultures growing at a low degree of aeration (Table 2). Analysis of the spent media of galactose- and glucose-grown cultures revealed less than 0.6% of the total carbon and only traces of fixed nitrogen remaining in the media in stationary-phase cultures (data not shown). This suggested that *A. vinelandii* utilized most of the carbon substrate but did not secrete a significant amount of organic molecules into the medium.

When *A. vinelandii* was grown in the presence of fixed nitrogen under 100% air-saturated conditions, the amount of cytochrome *d* decreased substantially. In addition, the effect of substrate on cell yield between glucose and galactose became only marginal (Fig. 2). This suggests that the biomass differences of glucose- and galactose-grown cultures are related to the processes controlling N_2 fixation. Because the biomass differences between glucose- and galactose-grown cultures were greatest under N_2-fixing conditions at high O_2 tension, subsequent experiments with 100% air-saturated cultures were performed.

In vitro analysis revealed that extracts from glucose-grown cells produced pyruvate from glucose at about twice the rate that extracts from galactose-grown cells produced pyruvate from galactose (Fig. 3). Examination of other cytoplasmic enzymes (Table 3) showed that the glyceraldehyde-3-P dehydrogenase of glucose-grown cells was very active, while that of the galactose-grown cells was not as active. However, the pyruvate dehydrogenase and the isocitrate dehydrogenase were essentially the same in both glucose- and galactose-grown cells.

The efficiency of the TCA cycle in a growing culture can be measured by the rate of respiration and the amount of CO_2 produced by the culture. The rate of O_2 uptake and the formation of CO_2 were greatly influenced by the age of the culture (Table 4). At mid-log phase, glucose-grown cells were more active than galactose-grown cells. This suggested that enzymes of the TCA cycle in glucose-grown cells must operate at a higher rate to hydrolyze the pyruvate formed.

The high rate of respiration in glucose-grown cells requires a rapid electron flux into the electron transport chain. *A. vinelandii* produces several membrane-bound oxidoreductases. Comparative studies of these enzymes may shed some light on the differences in glucose and galactose metabolism. The rates of $NADH^+$, malate, succinate, and hydrogen oxidation by the membrane fractions of *A. vinelandii* growing on either glucose or galactose are summarized in Table 5. Except for NADH oxidation, all enzyme activities tested were essentially the same in glucose- or galactose-grown cells. The NADH oxidation rate in glucose-grown cells was about twice that in their galactose-grown counterpart.

TABLE 3. Specific activities of some cytoplasmic enzymes in glucose- or galactose-grown cells

Enzyme	Sp act (mmol/min/mg of protein)	
	Glucose-grown cells	Galactose-grown cells
Glyceraldehyde-3-P dehydrogenase	0.354 ± 0.073	0.051 ± 0.018
Pyruvate dehydrogenase	0.146 ± 0.077	0.143 ± 0.065
Isocitrate dehydrogenase	0.330 ± 0.112	0.334 ± 0.066

TABLE 4. Whole-cell respiration and CO_2 production rates of glucose- and galactose-grown cells[a]

Pregrown substrate	Respiration rate (μmol of O_2/min/mg of dry wt)		CO_2 production rate (% carbon trapped/h/mg of dry wt)	
	Mid-log phase	Stationary phase	Mid-log phase	Stationary phase
Glucose	31.5 ± 4.8	9.1 ± 3.2	37 ± 6	14 ± 2
Galactose	16.5 ± 2.1	7.8 ± 0.5	19 ± 4	13 ± 1

[a] Cells were assayed with 1% of their pregrown sugar in Burk's buffer. Values are the means ± standard deviations from two experiments.

DISCUSSION

Haaker and Veeger (10) point out that the respiratory protection of the *Azotobacter* nitrogen-fixing system is independent of the oxidation capacity of the cells. These authors show that the rate of respiration depends on the type of substrate oxidized. We found that the respiration rate of glucose-grown cells was higher than that of galactose-grown cells (Table 4); however, the rate of respiration was unrelated to the total amount of the terminal oxidase (Table 1). This suggests that *A. vinelandii* contains more cytochrome oxidase than that required for normal growth. Production of excess terminal oxidase seems common in bacteria. Other bacteria, such as *Klebsiella aerogenes* (13), *Beneckea natriegens* (23) and *Bacillus subtilis* (22), also produce more cytochrome oxidase than what is required for aerobic respiration.

The effect of oxygen on the N_2 fixation system of *A. vinelandii* has been studied extensively (1, 2, 6, 8, 30, 31). The organism can produce an FeS protein that binds to nitrogenase to form an O_2-stable but inactive complex. This conformational protection allows time for the cell to respond to the increased pO_2. During this transition period, a series of modifications in the respiratory system occurs. *A. vinelandii* adapts its metabolism to higher O_2 tensions by increasing electron flow from NAD(P)H to cytochrome *d*. As the activity of NADH dehydrogenase increases, it becomes uncoupled from ATP synthesis and thus achieves respiratory protection. We confirmed the previous observation that cell yield is inversely proportional to aeration (pO_2).

However, the theory of respiratory protection proposed by Drozd and Postgate (8) does not by itself explain the biomass differences between glucose- and galactose-grown cultures grown under identical O_2 tensions. The relationships between pO_2 and cell yields of glucose and galactose are shown in Fig. 4. This relationship can be represented by the formula $Y = (-k \cdot pO_2) + c$, where Y is cell yield, k is the decreasing slope of the curve due to O_2 tension, and c is the theoretical cell yield value when the pO_2 effect is 0. Except under very low aeration, at which the supply of O_2 may limit aerobic growth, the slopes

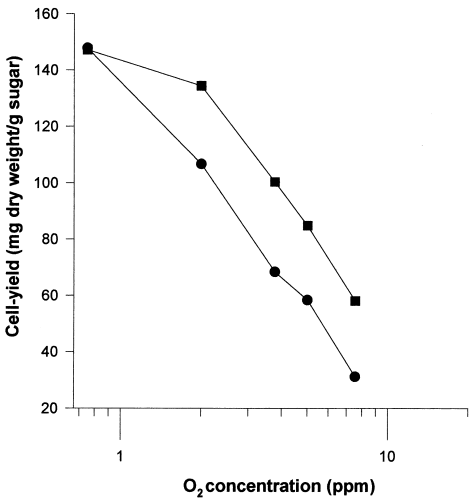

FIG. 4. Comparative studies of the effects of O_2 on cell yields of glucose-grown (●) and galactose-grown (■) cultures.

of the two curves are similar. This suggests that cells growing on glucose or galactose respond to O_2 tension in similar manners. In other words, the results from Fig. 4 suggest that the costs of respiratory protection in both glucose- and galactose-grown cells are similar. However, the cell yield of glucose-grown cells is consistently smaller than that of galactose-grown cells at the same pO_2. If growth is limited by nitrogen fixation and nitrogen fixation is limited by pO_2, the more rapidly respiring cells growing on glucose should grow better; they do not.

The data from Table 1 also reveal other interesting relationships among O_2 tension, the sugar substrates, and the two terminal oxidases in *A. vinelandii*. These relationships are best illustrated in the computer-generated three-dimensional mesh graph shown in Fig. 5. As shown, the induction profiles of cytochrome *d* in both glucose- and galactose-grown cultures by O_2 are very similar. Cytochrome *o*, on the other hand, was less sensitive to O_2 tension but was greatly affected by the type of substrate being used; galactose induced almost twice as much cytochrome *o* in *A. vinelandii* as did glucose. Electron transport through cytochrome *o* is coupled to ATP synthesis, while that through cytochrome *d* is not. The greater abundance of cytochrome *o* in galactose-grown cells increases the ATP yield per mole of carbohydrate oxidized and thus can offer an immediate explanation as to why galactose produced biomass more efficiently than glucose. The reason for a higher level of cytochrome *o* synthesis in the presence of galactose is still unknown.

TABLE 5. Rates of malate, succinate, hydrogen, and NADH oxidation by the membrane fractions of *A. vinelandii* grown on glucose or galactose as the sole carbon source

Membrane fraction	Rate of O_2 consumption (μmol of O_2/min/mg of protein)			
	NADH	Malate	Succinate	Hydrogen
Log phase				
Glucose-grown cells	0.773 ± 0.007	0.320 ± 0.096	0.059 ± 0.011	0.028 ± 0.006
Galactose-grown cells	0.385 ± 0.030	0.272 ± 0.064	0.051 ± 0.020	0.032 ± 0.010
Stationary phase				
Glucose-grown cells	0.184 ± 0.022	0.171 ± 0.022	0.059 ± 0.009	0.011 ± 0.004
Galactose-grown cells	0.153 ± 0.017	0.132 ± 0.018	0.050 ± 0.010	0.015 ± 0.008

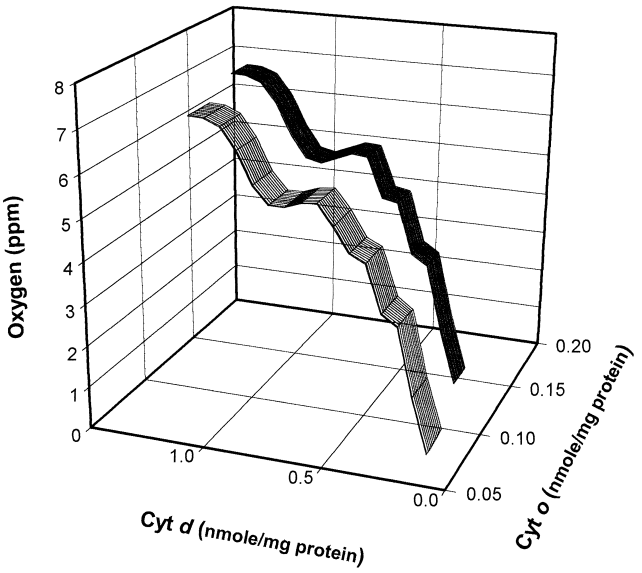

FIG. 5. Effects of O_2 on the production of cytochrome *d* (Cyt d) and cytochrome *o* (Cyt o) by glucose-grown (lightly shaded curve) and galactose-grown (darkly shaded curve) cultures. The three-dimensional mesh plot was generated from the data in Table 1 by SigmaPlot (Jandel Scientific, San Rafael, Calif.) software.

The rate of nitrogen fixation is thought to limit the growth of *A. vinelandii*. The C-to-N ratios of cultures growing on glucose and on galactose were nearly identical (Table 2) despite the differences in biomass. This result, together with our previous observation that the specific activities of the nitrogenase in glucose- and galactose-grown cultures were the same (28), suggests that the same pathway is used to assimilate nitrogen in the two cultures.

However, the rates of flow of carbon metabolites were significantly different during growth on glucose and on galactose. We previously suggested that the activity of the Entner-Doudoroff pathway in *A. vinelandii* is poorly regulated and responds mainly to the flux of glucose (28). In keeping with this, Table 4 shows that glucose is converted rapidly to CO_2.

We suggest the following hypothesis. The concentration of free glucose in the soil is usually low. *A. vinelandii* can metabolize the minute amount of glucose efficiently, but it lacks the ability to regulate the use of this sugar at the high concentrations used in laboratory media. Consequently, cells accumulate high concentrations of $NADH^+$ and ATP when *A. vinelandii* is grown on glucose medium. The reducing equivalents could be used for protein synthesis if enough ammonium were also available (Fig. 2). However, the ammonium output by the nitrogenase is fixed. Oxidative metabolism and thus growth itself would stop if the NAD^+ pool of the cell were completely reduced. To avoid this problem, *A. vinelandii* produces a highly active NADH dehydrogenase to oxidize NADH and regenerate NAD^+. The bacterium may also modify NADH dehydrogenase and utilize an effective electron transport system through cytochrome *d* to increase the overall rate of electron transport. This explains the high rate of respiration observed in glucose-grown cells. The modification at phosphorylation site I and the reduction in the energy-coupled cytochrome *o* pathway could also prevent accumulation of ATP that might tie up the phosphate pool of the cell. Thus, the loss of energy coupling at site I, the increase in the NADH dehydrogenase level, and changes in the concentrations of the two oxidases together may allow *A. vinelandii* to grow on glucose. A less likely alternative hypothesis is that glucose somehow directly represses the cytochrome *o* pathway in *A. vinelandii*. Cells may compensate for the loss of ATP by increasing the rates of glycolysis and respiration through the poorly coupled cytochrome *d* pathway.

Several questions remain unanswered. Pyruvate should be converted to acetyl coenzyme A by pyruvate dehydrogenase and should subsequently enter the TCA cycle. One would predict that glucose-grown cells would produce more pyruvate dehydrogenase to accommodate the higher concentration of pyruvate and that the TCA enzymes, such as isocitrate dehydrogenase, would be more active. Comparative studies of pyruvate dehydrogenase and isocitrate dehydrogenase showed that cells growing on glucose and galactose exhibited similar levels of these enzymes.

It is possible that the in vitro measurements of pyruvate dehydrogenase and isocitrate dehydrogenase represent the maximum enzyme potential achievable in the cells but not the actual activities involved in vivo. It is also possible that cells grown on glucose induce a new enzyme pathway to bypass pyruvate dehydrogenase and isocitrate dehydrogenase. We are now exploring the metabolism of pyruvate in this interesting organism.

The idea of futile reactions or energy-spilling reactions is well recognized for the anaerobes and the facultative fermentative cells. Russell and Cook (18) have recently reviewed this topic and concluded that when catabolism and anabolism are not fully coupled, energy-spilling reactions are essential for microbes to eliminate surplus energy and to avoid the accumulation of harmful metabolites inside the cell.

In addition to some specific ATP-spilling reactions (18), most fermentative microbes can remove their surplus metabolites and regenerate their reducing power by various modes of fermentation. For example, to regenerate NAD^+, cells reduce various organic molecules with NADH and then secrete the reduced metabolites as fermentative products. Some microbes can also generate H_2 directly from NADH. The particular mode of fermentation used varies with the growth environment and the physiological state of the cells and could undoubtedly increase the flexibility of microbes for surviving in various environments.

Most obligate aerobes cannot secrete metabolites outside themselves effectively. However, it is logical to believe that a certain mechanism must exit so that these aerobes can remove unwanted metabolites and regenerate their NAD^+ when needed. We suggest that obligate aerobes, such as *A. vinelandii*, use the enzymes of the TCA cycle to oxidize and thus remove unwanted carbons. Rapid oxidation of these carbonaceous compounds would generate high levels of reducing power. Cells may rely on the flexibility of the ETS to recycle their electron carriers.

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