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Cytochrome and Ubiquinone Patterns During Growth of *Azotobacter vinelandii*

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After synthesis during the early log phase, the concentrations of ubiquinone and cytochromes did not vary during the growth cycle of *Azotobacter vinelandii*, when grown with either high or low aeration on nitrogen-free or urea-containing media. The level of aeration had no effect on the concentrations of the electron carriers synthesized, but affected the growth rate. On urea-containing medium, the concentration of cytochrome a_2 was low, but it was synthesized at a linear rate when the bacteria were transferred to nitrogen-free medium. *A. vinelandii* was shown to utilize sufficient medium urea to account for all of the cell nitrogen. Growth on urea-containing medium with an oxygen atmosphere resulted in low growth yields, and cytochromes $c_4 + c_5$ were not synthesized; the concentrations of ubiquinone and cytochromes b_1, a_1, and a_2 were only 12 to 18% of the values for growth on nitrogen-free medium with high aeration.

The rate of synthesis of the respiratory enzyme systems of microorganisms appears to be dependent on the culture conditions, and the formation of individual electron carriers is dependent on the conditions of aerobiosis (15), electron acceptor (3, 16), carbon source (17), or assimilatory nitrogen source (8).

Furthermore, the levels of the cytochrome oxidases may be dependent on both oxygen tension and growth phase of the organism (11, 12, 16, 17).

In a strain of *Azotobacter vinelandii*, a strictly aerobic bacterium capable of fixing atmospheric nitrogen, the addition of sources of combined nitrogen to the medium affects the degree of synthesis of the electron transport system. In particular, urea as a source of combined nitrogen affects both the structure and function of the respiratory enzyme system and the level of formation of cytochrome a_2 is depressed (8).

The present paper presents the results of a study on the effects of variations in growth conditions, both of nitrogen source and degree of aeration, on the synthesis of the respiratory enzyme system of *A. vinelandii* throughout the growth cycle. The degree of aeration for growth on both nitrogen-free and urea-containing media had no effect on the concentrations of the cytochromes or ubiquinone formed but affected the growth rate. Furthermore, there was little variation in the electron carrier concentrations during the growth cycle. Cytochrome a_2 was present at about a fourfold higher concentration with growth on nitrogen-free medium as compared to growth on urea-containing medium. Under an atmosphere of oxygen in the presence of urea, the concentrations of the electron carriers were much lower than with growth under either nitrogen-fixing conditions or on urea-containing medium with an atmosphere of air. Furthermore, with the growth on urea-containing medium in an oxygen atmosphere, cytochromes $c_4 + c_5$ were not formed.

MATERIALS AND METHODS

Chemicals. Urease, type III, was obtained from Sigma Chemical Co. Ltd., London. All other chemicals were obtained from British Drug Houses, Ltd., Poole, England, and were of the finest available grade. Glass-distilled water was used throughout this work.

Growth and fractionation of A. vinelandii. *A. vinelandii* [National Collection of Industrial Bacteria (NCIB) 8660] was grown on the nitrogen-free medium described by Jones and Redfearn (7); for growth with urea present (1 g per liter), the $NaMoO_4$ was omitted.

For the experiments in which the changes in electron carrier concentrations were measured during the growth cycle, the bacteria were grown in 700 ml of medium in 2-liter flasks with shaking on a gyratory incubator shaker. For high aeration, the shaker was set at the maximum shake rate and for low aeration at the minimum rate. Several 2-liter flasks were grown, and we harvested either one or two flasks at each point throughout the growth cycle. For all other experiments, growth was with the high shake rate. In

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the growth-transfer experiment, a suitable number of the flasks was harvested on a Sharples continuous flow centrifuge, washed with 25 mM KH_2PO_4-Na_2HPO_4 buffer (*p*H 7.4), and added to the nitrogen-free medium.

Growth of the bacteria under an atmosphere of 100% oxygen was achieved by use of 5-liter carboys with an oxygen flow rate of approximately 2 liters per min fed via a sparger into the urea-containing medium, which was stirred with a magnetic stirrer.

A mutant of *A. vinelandii* strain O (ATCC 13266) that was incapable of fixing atmospheric nitrogen (13) was grown to the mid-log phase on urea-containing medium. Harvesting, rupture, and fractionation of this mutant and that of the NCIB 8660 strain was by the method described by Jones and Redfearn (7). The small particulate fraction refers to the cytochrome-rich fraction containing high oxidase activities and sedimenting between 35,000 × *g* and 105,000 × *g* (7).

General assays. Oxygen tension was assayed throughout the growth cycle (with a Clark electrode) by removing a sample and comparing its oxygen tension with that of an uninoculated sample that had been subjected to identical conditions.

The ubiquinone (Q-8) content of the small particulate fraction was assayed by the chemical extraction technique developed by Pumphrey and Redfearn (14), but omitting the partition with 95% methanol.

The urea content of the medium was estimated with urease. A 1-ml amount of the urea solution was added to 1 ml of 0.2 M phosphate buffer, *p*H 7.4, and 5 ml of urease (0.2 mg per ml) and was incubated at 30 C for 30 min. A 1-ml amount of 1 M KCl was then added and the solution was shaken for 30 sec. Then 1 ml of this solution was added to 0.5 ml of a solution containing 11.5 ml of sodium acetate buffer (0.5 M, *p*H 5.5), 1.0 ml of KCl (0.06 M), and 37.5 ml of 2-methoxy-ethanol. An 0.5 ml amount of a solution composed of 0.5 ml of ninhydrin reagent in 4.8 ml of 2-methoxy-ethanol was then added, and the solution was boiled for 15 min and cooled; 4 ml of 50% ethyl alcohol was added. The absorbance was measured at 570 nm. The concentration was determined from a standard curve plotted with known concentrations of urea (0 to 100 μg).

Nitrogen was determined by the micro-Kjeldahl method (10) with the indicator of Ma and Zuazaga (9). A distilled water blank was performed with each estimation.

The total concentrations of the individual cytochromes and total flavoprotein were assayed from the $Na_2S_2O_4$ reduced minus oxidized difference spectra (7).

Protein was assayed by the modified biuret method of Gornall, Bardawill, and David (5). Growth was measured by the optical density at 680 nm, or by the absorbance in an Eel colorimeter (no filter). Unit optical density at 680 nm corresponded to 0.14 mg (dry weight) of bacteria.

RESULTS

With the low shake rate on nitrogen-free medium, the oxygen tension of the medium decreased to less than 15% of the original and maximal growth was attained after 180 to 200 hr (Fig. 1). The concentrations of the cytochromes and ubiquinone increased to a maximum during the early log phase and remained approximately constant during the mid-log to stationary phase; the average values of the concentrations at the mid-log phase were identical to those reported previously (7). A similar pattern was found for the synthesis of the electron carriers with growth on nitrogen-free medium at a high shake rate, where the oxygen tension of the medium remained at the initial concentration, but the growth rate was faster and maximal growth was attained after 50 to 70 hr.

Similar results were obtained for growth with high aeration on urea-containing medium, with constant concentrations of the cytochromes and ubiquinone from the mid-log to stationary phase. In this case, however, the concentration of cytochrome a_2 was always less than 0.15 μmole per g of protein in the small particulate fraction,

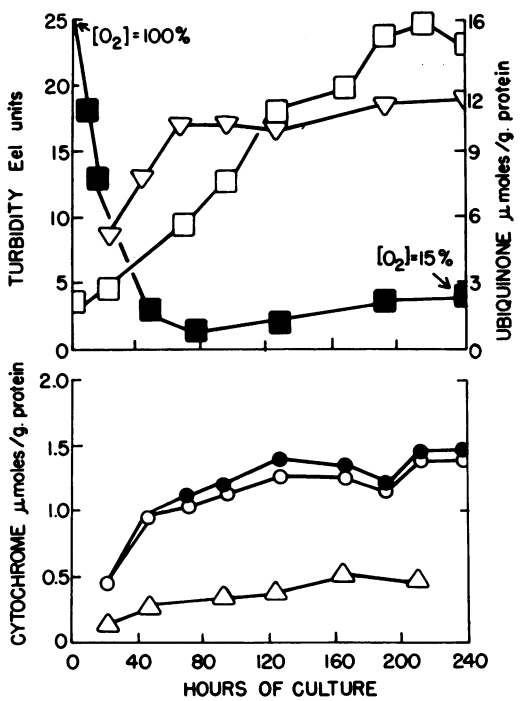

FIG. 1. *Variation of the ubiquinone and cytochrome contents of A. vinelandii during growth on nitrogen-free medium with low aeration. The concentrations of ubiquinone* (▽), *cytochromes* $c_4 + c_5$ (○), *cytochrome* b_1 (○), *and cytochrome* a_2 (△) *were measured throughout the growth cycle* (□). *The oxygen tension* (■) *decreased to less than 15% of the initial value at the start of the growth phase. The methods used are described in the text.*

rather than the 0.5 to 0.7 μmole per g of protein found in the nitrogen-free particles. (The terms nitrogen-free particles and urea particles refer to the small particulate fractions derived from cells grown in nitrogen-free and urea-containing medium, respectively.)

On the transfer from urea-containing to nitrogen-free medium at the end of the log phase when there was linear growth, there was a linear rate of formation of cytochrome a_2 to the level normally found in the nitrogen-free particles, and there was little or no lag in growth (Fig. 2).

Using a ^{15}N-enriched atmosphere, Wilson, Hull, and Burris (18) showed that all or most of the cell nitrogen of *A. vinelandii* strain O was derived from the medium nitrogen when urea was the source of combined nitrogen. The strain of *A. vinelandii* used in these studies similarly utilized sufficient medium nitrogen to account for all the cell nitrogen formed (Fig. 3). Moreover, in a typical experiment, there was a total loss of medium nitrogen of 320 μg per ml, which compares with a cell nitrogen formation of 290 μg per ml; however, the overall loss of nitrogen from the medium urea was greater (400 μg per ml). Thus, the bacterium must utilize the medium urea and then donate some nitrogen-containing component to the medium. Growth on urea-containing medium was accompanied by the increasing appearance of a medium component (after centrifuging to remove the bacterial cells) absorbing at 317 and 268 nm; this component did not form with growth on nitrogen-free medium. This was not the previously reported pigment formed by growth

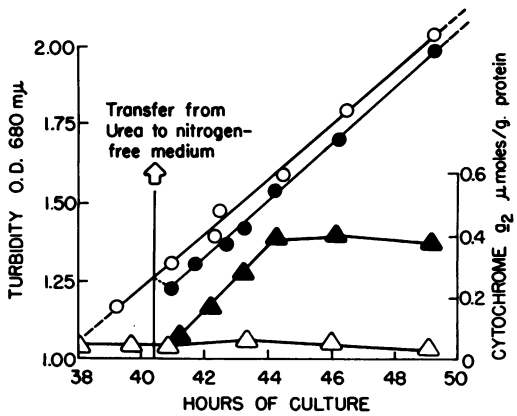

FIG. 2. *Synthesis of cytochrome a_2 on the transfer of A. vinelandii from urea-containing to nitrogen-free medium. The concentration of cytochrome a_2 on urea medium (△) remained a low constant value during growth on this medium (○), but there was a linear synthesis of this cytochrome on the transfer to nitrogen-free medium (▲), with no lag in growth on the nitrogen-free medium (●).*

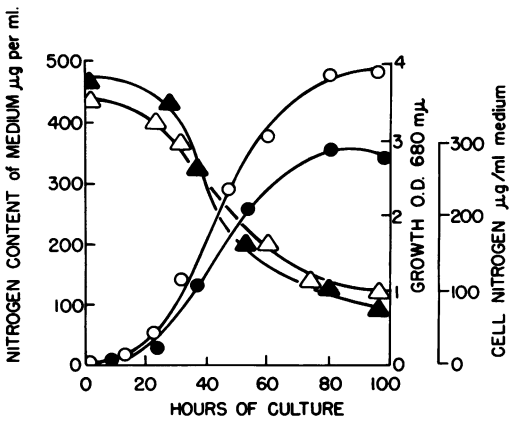

FIG. 3. *Decrease in the nitrogen-content of the medium during growth of A. vinelandii on urea-containing medium. There was a loss of medium urea nitrogen (▲) and total medium nitrogen (△) with the increase in bacterial whole-cell nitrogen (●) during the growth cycle (○).*

on iron-deficient medium (4), and preliminary attempts to isolate the pigment were unsuccessful.

That more than sufficient medium nitrogen was incorporated into cellular nitrogen to account for all of the cellular nitrogen formed with growth on urea-containing medium did not necessarily preclude the possibility that the bacterium was still utilizing some atmospheric nitrogen. A mutant of *A. vinelandii* (AV-3) that was unable to fix atmospheric nitrogen (13) was therefore grown on urea-containing medium. An electron transport system was formed that was identical in composition to that of *A. vinelandii* (strain NCIB 8660) grown on urea-containing medium (Table 1; also see Table 1, reference 8).

Despite the apparent nonutilization of the atmospheric nitrogen with growth on urea-containing medium, the presence of atmospheric nitrogen is essential for the synthesis of the complete electron transport system. Growth on urea-containing medium under a stream of pure oxygen or a 4:1 mixture of helium and oxygen led to lower growth yields, and the electron transport system was of a different composition from that with growth on urea-containing medium in the air. The yields of ubiquinone and cytochromes b_1, a_1, and a_2 were only 12 to 18% of those of the nitrogen-free cells grown with vigorous aeration, and cytochromes $c_4 + c_5$ were not formed (Table 1). Figure 4 shows the cytochrome spectrum for growth under these conditions. The peaks at 560, 605, and 628 nm are the α-absorption peaks of cytochromes b_1, a_1, and a_2, respectively; there was a β-peak of cytochrome b_1 at 528 nm and a single Soret peak at 428 nm (not shown in Fig. 4). This

TABLE 1. *Concentrations of the electron transport enzymes of A. vinelandii and a mutant unable to fix atmospheric nitrogen, with growth on urea-containing medium*[a]

Strain	Atmosphere	Ubiquinone (Q-8)	Flavoprotein	Cytochrome			Ratio of Q-8 to cytochrome b_1
				$c_4 + c_5$	b_1	a_2	
NCIB 8660	O_2	1.38	0.5	ND[b]	0.22	0.09	6.3
AV-3[c]	Air	10.7	1.9	1.42	1.22	0.13	8.8

[a] Concentrations are given in micromoles per gram of small particulate fraction protein.
[b] Not spectrally detectable.
[c] Mutant of strain O, unable to fix atmospheric nitrogen.

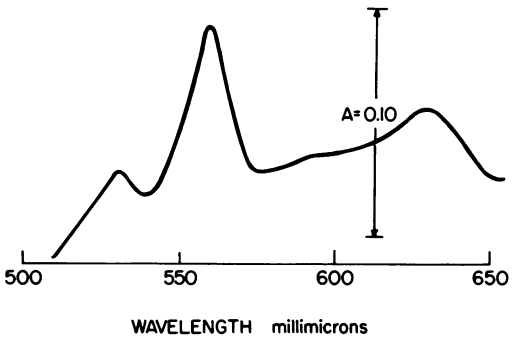

FIG. 4. *Cytochrome spectrum of A. vinelandii grown on urea-containing medium with a pure oxygen atmosphere. The bacteria were grown to the mid-log phase in 5-liter carboys with oxygen fed at 2 liters per min via a sparger; the bacteria were harvested and the small particulate fraction was isolated. The $Na_2S_2O_4$ reduced minus oxidized difference spectrum of 13.4 mg per ml of small particles is shown. Cuvettes having a 1 cm light path were used.*

spectrum is noticeably different from published cytochrome spectra of *A. vinelandii* grown in an atmosphere of air (7, 8), in which there is an α-peak at 551 nm due to cytochromes $c_4 + c_5$. Though ubiquinone was also found in lower concentrations than with growth on nitrogen-free medium (in air), it was present in a six- to nine-fold excess over cytochrome b_1 (Table 1), as has been found for other growth conditions (8).

DISCUSSION

A change from aerobic to anaerobic growth conditions may cause a repression in the synthesis of the cytochrome systems of facultative anaerobes (1, 15). A change from aerobic to anaerobic conditions, where nitrate is the terminal electron acceptor, may cause either the repression or the induction of cytochromes (2, 16). Similarly, a change in the level of aeration of facultative anaerobes affects the electron transport systems, as shown by the synthesis of cytochrome a_2 in *Aerobacter aerogenes* and *Escherichia coli*; in these organisms, synthesis of cytochrome a_2 is dependent on the aeration rate but synthesis of cytochrome b_1 is independent of the aeration rate (11, 12). In *Haemophilus parainfluenzae*, the relative ratios of the terminal oxidases formed are also dependent on the rate of aeration (16). In contrast to this, it has been shown in the above experiments that the concentrations of the electron carriers, including cytochrome a_2, formed in the strict aerobe *A. vinelandii* are similar with varying rates of aeration

After the synthesis of ubiquinone and cytochromes during the early log phase, their concentrations did not vary throughout the growth cycle of *A. vinelandii*. This, again, is in contrast with the situation in *H. parainfluenzae*, where the levels of the terminal oxidases and cytochrome c_1 varied throughout the growth cycle (16).

It has previously been shown (8) that the electron transport system formed by *A. vinelandii* on urea-containing medium is somewhat different from that formed on nitrogen-free medium, and, in particular, the level of cytochrome a_2 formation is very much lower. It has been shown here that the medium urea was utilized in sufficient quantities to account for all of the cell nitrogen formed. Thus, the medium urea was not acting simply as a repressor of this cytochrome. This was confirmed by the growth on urea-containing medium of a mutant (13) that was unable to fix atmospheric nitrogen and which formed a similar cytochrome a_2-deficient system.

The linear rate of synthesis of cytochrome a_2 to the level normally found in the small particulate fraction of cells grown on nitrogen-free medium, on the transfer from urea-containing to nitrogen-free medium, suggests that the enzymes of the cytochrome a_2 biosynthetic pathway are present and need not be induced on the transfer of medium. Despite attempts to wash out the urea, the absence of any noticeable lag in growth on transfer possibly indicates the utilization of endogenous supplies of urea or its metabolites.

There was a loss of over 80% of the cyto-

chromes, ubiquinone, and flavoprotein with growth on urea-containing medium under an oxygen atmosphere when compared to growth in the air. The rate of growth was lower and the total growth dropped. Jacobs and Conti (6) found that the respiratory rate of extracts from anaerobically grown cells of *Staphylococcus epidermidis* was one-tenth of the respiratory rate of extracts from aerobically grown cells. Addition of hemin to the anaerobic growth medium resulted in extracts with an oxidase activity which was 60% of that of the extracts from aerobic cells and in the synthesis of the protoheme-containing cytochromes. They suggest that oxygen is required for the biosynthesis of the electron transport system. Similarly, atmospheric nitrogen may be required for the biosynthesis of the high concentrations of the respiratory enzymes found in *A. vinelandii*, despite its nonutilization in the presence of urea in the growth medium.

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LITERATURE CITED

1. Asnis, R. E., V. G. Veley, and M. C. Glick. 1956. Some enzymatic activities of a particulate fraction from sonic lysates of *Escherichia coli*. J. Bacteriol. 72:314–319.
2. Azoulay, E. 1964. Influence des conditions de culture sur la respiration de *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 92:458–464.
3. Azoulay, E., and P. Couchoud-Beaumont. 1965. Etude de la cytochrome-oxidase de *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 110:301–311.
4. Bulen, W. A., and J. R. Lecompte. 1962. Isolation and properties of a yellow-green fluorescent peptide from *Azotobacter* medium. Biochem. Biophys. Res. Commun. 9:523–528.
5. Gornall, A., C. S. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751–766.
6. Jacobs, N. J., and S. F. Conti. 1965. Effect of hemin on the formation of the cytochrome system of anaerobically grown *Staphylococcus epidermidis*. J. Bacteriol. 89:675–679.
7. Jones, C. W., and E. R. Redfearn. 1966. Electron transport in *Azotobacter vinelandii*. Biochim. Biophys. Acta 113:467–481.
8. Knowles, C. J., and E. R. Redfearn. 1968. The effect of combined-nitrogen sources on the synthesis and function of the electron transport system of *Azotobacter vinelandii*. Biochim. Biophys. Acta 162:348–355.
9. Ma, T. S., and G. Zuazaga. 1942. Micro-Kjeldahl determination of nitrogen: a new indicator and an improved rapid method. Ind. Eng. Chem. Anal. Ed. 14:280–282.
10. Markham, R. 1942. A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. 376:790.
11. Moss, F. 1952. The influence of oxygen tension on respiration and cytochrome a_2 formation in *Escherichia coli*. Australian J. Exptl. Biol. Med. Sci. 30:531–540.
12. Moss, F. 1956. Adaptation of the cytochromes of *Aerobacter aerogenes* in response to environmental oxygen tension. Australian J. Exptl. Biol. Med. Sci. 34:395–406.
13. Mumford, F. E., J. E. Carnahan, and J. E. Castle. 1959. Nitrogen fixation in a mutant of *Azotobacter vinelandii*. J. Bacteriol. 77:86–90.
14. Pumphrey, A. M., and E. R. Redfearn. 1960. A method for determining the concentration of ubiquinone in mitochondrial preparations. Biochem. J. 76:61–64.
15. Schaeffer, P. 1952. Disparition partielle des cytochromes par culture anaerobic chez certaines bacteries aerobies facultatives. Biochim. Biophys. Acta 9:261–270.
16. White, D. C. 1962. Cytochrome and catalase patterns during growth of *Haemophilus paralnfluenzae*. J. Bacteriol. 83:851–859.
17. White, D. C. 1967. Effect of glucose on the formation of the membrane-bound electron transport system in *Haemophilus parainfluenzae*. J. Bacteriol. 93:567–573.
18. Wilson, P. W., J. F. Hull, and R. H. Burris. 1943. Competition between free and combined nitrogen in nutrition of *Azotobacter*. Proc. Natl. Acad. Sci. U.S. 29:289–294.