SHORT COMMUNICATIONS

Abnormally Low Activities of Fumarate Hydratase and Malate Dehydrogenase in Oxygen-sensitive Cultures of *Spirillum volutans*

By J. A. COLE

Department of Biochemistry, The University of Birmingham, Birmingham B15 2TT

(Received 5 April 1973; revised 10 May 1973)

INTRODUCTION

A recent comparison of respiratory processes in three spirilla provided a reasonable explanation for the slow growth and poor yield of *Spirillum volutans*, but failed to reveal the biochemical basis of its oxygen sensitivity (Cole & Rittenberg, 1971). Although several enzyme activities were lower in extracts of *S. volutans* than in *S. serpens* or *S. itersonii*, none was totally absent. It was possible, therefore, that *S. volutans* failed to grow in aerated cultures because excess oxygen directly or indirectly inhibited a vital metabolic process rather than prevented the synthesis of key enzymes. This suggestion has now been investigated by comparing the growth yield, respiratory activity and rates of DNA and protein synthesis in aerated and in microaerobic cultures of *S. volutans*.

METHODS

The Pringsheim strain of *Spirillum volutans* was maintained and cultured as described previously, except that 2 l conical flasks replaced 2 l Fernbach flasks (Cole & Rittenberg, 1971). Aerated cultures (500 ml Krieg's medium in 2 l conical flasks) were incubated at 30 °C in a New Brunswick model G-25 gyrotory shaker (New Brunswick Scientific Co., New Jersey, U.S.A.) operating at 200 cycles/min. After 8 h or 12 h, as noted in the text, bacteria were centrifuged for 10 min at 10000 g, resuspended in 50 mM-K⁺ phosphate buffer (pH 7.4), centrifuged as before and resuspended in the phosphate buffer (40 mg cell protein/ml). Part of the suspension was sonicated for 3 × 20 s at 4 °C with a Mullard ultrasonic power unit operating at 18000 to 20000 cycles/s (Measuring & Scientific Equipment Ltd., Crawley, Sussex; 1.0 cm diam. probe). Cell membrane and soluble extracts were prepared, and tri-carboxylic acid (TCA) cycle enzymes were assayed as described previously (Cole & Rittenberg, 1971). Activities were independent of substrate concentrations, but proportional to the volume of cell-free extract in the assay.

The effect of oxygen on the incorporation of radioactive thymidine or arginine into perchloric acid (PCA)-precipitable cell material was measured with 50 ml cultures in 250 ml conical flasks supplemented with 2 µCi [³H]thymidine (2.0 µCi/mM) or 2 µCi [¹⁴C]arginine (220 mCi/mM). Duplicate flasks were inoculated with 1 ml from a 12-h microaerobic culture which had previously been subcultured daily for 5 days. They were incubated at 30 °C for 12 h with or without aeration. Samples (5.0 ml) were transferred at intervals to 2 ml 5% (v/v) PCA and either 5 mM-unlabelled arginine or thymidine at 4 °C. After 5 min on ice, 1 ml 2.4 N-KOH was added, and precipitates were collected on Whatman GF/C 25 mm filter discs, washed twice with 10 ml 1 mM-unlabelled arginine or thymidine and counted. Radioactivity (c.p.m.) was proportional to the sample volume throughout each experiment.

Table 1. Comparison of the growth yield, respiratory rate, enzyme activities and rates of DNA and protein synthesis in aerated and microaerobic cultures of *Spirillum volutans*

Enzyme activities are the average of three to seven independent preparations, unless otherwise indicated. Microaerobic cultures were harvested after 18 h at 30 °C.

Assay	Cultures aerated for		Micro-aerobic cultures
	8 h	12 h	
Growth yield (mg dry wt/l)	ND	320-410	90-140
Respiratory rate (nmole O ₂ reduced/2.0 ml/min)	26	6	13
[³ H]thymidine in perchloric acid precipitate (c.p.m. in precipitate/ml culture)	ND	435	204
[¹⁴ C]arginine in perchloric acid precipitate (c.p.m. in precipitate/ml culture)	ND	216	87
NADH oxidase (μmole NADH oxidized/mg protein/h)	2.4; 4.0	3.2 ± 0.8	0.7
Succinate dehydrogenase (EC. 1.5.99.1) (μmole dye reduced/mg protein/h)	2.5; 3.0	2.7 ± 1.2	3.8
Fumarate hydratase (EC. 4.2.1.2) (μmole fumarate formed/mg protein/h)	4.0; 13.1	0.01	1.2
Malate dehydrogenase (EC. 1.1.1.37) (μmole NADH oxidized/mg protein/h)	10.1; 7.7	0.01	1.5
Aconitate hydratase (EC. 4.2.1.3) (μmole cis-aconitate formed/mg protein/h)	17.1; 40.1	2.9-17.8	3.8
Isocitrate dehydrogenase (EC. 1.1.1.42) (μmole NADP ⁺ reduced/mg protein/h)	ND; 29.2	8.1-35.8	5.3

ND, Not determined.

Samples of similar cultures without added arginine or thymidine were transferred to the reaction vessel of the oxygen electrode to determine respiratory rates (Cole & Rittenberg, 1971). Microaerobic cultures were swirled to increase the concentration of dissolved oxygen before samples were taken.

Quantitative estimation of the viability of a *Spirillum volutans* culture is difficult because colonies do not form on solid media. Qualitative tests for viability were made by transferring 0.5 ml aseptically to fresh medium which had been boiled and cooled rapidly to expel dissolved oxygen. Viable cultures gave rise to short, motile spirilla after 12 h at 30 °C. Non-viable cultures were not motile and showed no growth even after 2 days of incubation in fresh medium. Growth was measured by counting the number of bacteria on a haemocytometer grid, and by measuring the culture turbidity in a Klett-Summerson colorimeter. Motility was examined by phase contrast microscopy.

RESULTS

The effect of vigorous aeration on the growth of *Spirillum volutans* depended on at least two factors, the physiological state of the inoculum and its size. In general, the number of bacteria required to initiate growth in media equilibrated with air increased with the inoculum age. Growth from 2% (v/v) inocula occurred when bacteria were taken from early exponential phase cultures which had been incubated microaerobically for 12 h after five daily subcultures into fresh medium. The spirilla divided once during the first 5 h of aeration, three times in 12 h, and subsequently grew with a mean generation time of 2.8 h. The dissolved oxygen concentration was approximately 0.25 mM during the first 5 h of growth, but fell to 0.05 mM after 8 h. Comparable microaerobic cultures grew slightly faster in the first 12 h, but the generation time increased as the rate of oxygen dissolution became limiting. Bacteria from 48 to 72 h microaerobic cultures only initiated growth in medium which had been boiled to expel dissolved oxygen. In aerated media no more than one division occurred

before motility and viability were lost. In contrast, the growth-rate was stimulated by aeration if these cultures were first incubated microaerobically for 12 h: this was the preferred method of culturing bacteria for enzyme and respiratory studies.

Subsequent experiments investigated the effect of aerating exponential phase cultures on respiratory and TCA cycle enzyme activities, cell yield, DNA synthesis and protein synthesis. Bacteria from microaerobic cultures were assayed for comparison (Table 1). The yield of bacteria after 12 h of aeration was 3 times that of microaerobic cultures, but respiratory activity was optimal after 8 h of aeration. The rates of oxygen consumption after 8 h and 12 h were 300 and 60% of the microaerobic rates, respectively. If 8 mM-K⁺-succinate was added after 8 h of aeration, further increases in the bacterial density and respiratory rate were observed. The spent medium supported further growth only when additional succinate was added, suggesting that all of the initial succinate is metabolized after 8 to 12 h of aeration. Aeration stimulated the rate of arginine and thymidine incorporation into PCA-precipitable cell material, the radioactivity of aerated bacteria after 12 h being 2.3–2.5-fold that of microaerobic cultures. Neither [³H]uracil nor [³H]uridine was assimilated by *Spirillum volutans*, so attempts to measure rates of RNA synthesis were unsuccessful.

Aconitate hydratase and isocitrate dehydrogenase activities varied considerably in extracts of bacteria harvested after 12 h of aeration: the range of observed activities has therefore been shown in Table 1. The average activities of both enzymes and NADH oxidase were approximately four-fold higher than in microaerobically grown bacteria, but fumarate hydratase and malate dehydrogenase were not detected. However, all enzymes except succinate dehydrogenase were more active in bacteria harvested after 8 h of aeration.

DISCUSSION

The effect of aerating *Spirillum volutans* cultures was critically dependent on the physiological state of the bacteria. Although small inocula from stationary or late exponential phase cultures were killed by aeration, the stimulation of metabolic activity of larger inocula from early exponential phase cultures was unexpected (Wells & Krieg, 1965; Cole & Rittenberg, 1971) (Table 1). One reasonable explanation for the oxygen sensitivity of *Spirillum volutans* is as follows. In microaerobic cultures, oxygen is completely reduced to water by succinate or endogenous substrates such as poly- β -hydroxybutyrate (Martinez, 1963). The rate of oxygen reduction decreases when succinate is exhausted, so oxygen or toxic oxidation products (for example, peroxides) accumulate within the bacteria. If oxygen directly or indirectly inhibits the synthesis or activity of key catabolic enzymes, adenosine triphosphate (ATP) synthesis would be insufficient to supply maintenance energy for viability (Bauchop & Elsdén, 1960). According to this hypothesis, loss of both motility (which is ATP dependent) and viability would be prevented by transferring bacteria to fresh, oxygen-depleted medium, but accelerated by increased exposure to oxygen, particularly in the absence of a readily oxidizable substrate such as succinate. Both predictions have been verified. The latter occurred in aerated cultures between the eighth and twelfth hours of incubation when fumarate hydratase and malate dehydrogenase activities were also completely lost (Table 1). These TCA cycle enzymes have an essential role in the energy metabolism of aerobic organisms. It is possible, therefore, that fumarate hydratase and malate dehydrogenase are the key enzymes whose synthesis or activity is inhibited by oxygen.

I am grateful to Dr S. C. Rittenberg, in whose laboratory this investigation was initiated, and Dr N. R. Krieg for helpful comments and criticism.

REFERENCES

- BAUCHOP, T. & ELSDEN, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *Journal of General Microbiology* **23**, 457-467.
- COLE, J. A. & RITTENBERG, S. C. (1971). A comparison of respiratory processes in *Spirillum volutans*, *Spirillum itersonii* and *Spirillum serpens*. *Journal of General Microbiology* **69**, 375-383.
- MARTINEZ, R. J. (1963). On the nature of the granules of the genus *Spirillum*. *Archiv für Mikrobiologie* **44**, 334-343.
- WELLS, J. S. & KRIEG, N. R. (1965). Cultivation of *Spirillum volutans* in a bacteria-free environment. *Journal of Bacteriology* **90**, 817-818.