A Comparison of Respiratory Processes in *Spirillum volutans*, *Spirillum itersonii* and *Spirillum serpens*

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SUMMARY

Respiratory processes in *Spirillum serpens* and *S. itersonii* were compared with those in the microaerophile *S. volutans*. Rates of oxygen consumption with various substrates and the catalase activity were lower in *S. volutans*. The activities of five citric acid cycle enzymes and reduced nicotinamide adenine dinucleotide oxidase in cell-free extracts were also 10- to 200-fold lower. The concentration of cytochrome *c* was greater in the oxygen-insensitive spirilla. This may explain the poor growth of *S. volutans*: it is unlikely that the complete inhibition of growth in aerated cultures is due to a haemoprotein deficiency.

INTRODUCTION

Spirillum volutans, first mentioned in 1830, was described by Ehrenberg (1838). It is a large, polarly flagellated spirillum containing characteristic storage granules. The width of the organism, about 2 μm ., is constant, but its length varies from 10 μm . in young, rapidly dividing cultures to more than 100 μm . during the late stationary phase (Williams & Rittenberg, 1957).

Wells & Krieg (1965) cultured *Spirillum volutans* in nutrient broth without supplementation by equilibrating the medium with environments of reduced oxygen concentration. Growth occurred in a 9% but not in a 12% oxygen atmosphere with an inoculum of about 1.4×10^8 spirilla/ml. In its sensitivity to oxygen, *S. volutans* differed from the smaller spirilla which grew readily from small inocula in media equilibrated with air. In this work the respiratory processes in *S. volutans* were compared to *S. itersonii* and *S. serpens*, to determine whether significant differences exist which might explain its oxygen sensitivity.

METHODS

Organisms and media. The Pringsheim strain of *Spirillum volutans* studied by Rittenberg & Rittenberg (1962) was used. *Spirillum itersonii* and *S. serpens*, from the departmental culture collection, were maintained on Krieg's (McElroy, Wells & Krieg, 1967) medium (1 g. sodium succinate, 10 g. Bactopeptone (Difco), 1 g. $(\text{NH}_4)_2\text{SO}_4$, 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2 mg. MnSO_4 , 1000 ml. tap water, pH adjusted to 6.8 with KOH) supplemented with 1% (w/v) Nutrient Broth (Difco). *Spirillum volutans* was cultured in soft agar deeps of the same enriched medium except that the final concentration of agar was 2 g./l.

Spirillum volutans grew poorly when subcultured from soft agar into liquid media. Additional stock cultures were maintained in unsupplemented Krieg's medium which was also

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used for growth experiments and for obtaining bacteria for respiration studies and enzyme analyses. Inocula of 1.0 ml. were added to 100 ml. of medium contained in a 250 ml. Erlenmeyer flask. After 48 h. at 30°, these and similar *S. serpens* and *S. itersonii* cultures were used to seed 1.2 l. cultures contained in 2 l. Fernbach flasks. These were incubated at 30° for 2 days without agitation. All media to be inoculated with *S. volutans* were boiled to expel dissolved oxygen, and cooled immediately before use. Cultures were incubated in an air atmosphere. Growth was followed turbidimetrically with a Klett–Summerson colorimeter using a number 42 filter (520 nm.). Bacteria were harvested by centrifugation at 10,000 g for 10 min., resuspended in 50 mM-potassium phosphate buffer (pH 7.4) resedimented by centrifugation and again suspended in phosphate buffer to give a final concentration of 20 to 40 mg. cell protein/ml.

Preparation of bacterial extracts. Bacteria were fractionated by the procedure of Gray, Wimpenny, Hughes & Mossman (1966). Bacteria were ruptured by passing washed suspensions through an automated French pressure cell (Aminco, Silver Spring, Maryland, U.S.A.) cooled to 4°. Microscopic observations showed that breakage was complete after one passage. The treated suspension was centrifuged at 24,000 g for 20 min. and the supernatant fluid recentrifuged at 144,000 g for 60 min. The resultant high-speed supernatant is referred to as the HSS fraction. The residue from the initial centrifugation sedimented in two layers – an upper gelatinous layer above a paler, tightly packed pellet. The upper layer was removed with a spatula, resuspended in 50 mM-potassium phosphate buffer (pH 7.4) and homogenized in a glass homogenizer (Kontes Glass Co., Vineland, New Jersey, U.S.A.). The suspension was again centrifuged at 24,000 g and the upper layer of sediment removed and homogenized as before. The final suspension of cell-wall membrane (CWM) contained 5 to 15 mg. protein/ml. All manipulations were performed at 4°.

Respiration of bacterial suspensions. In most experiments oxygen consumption of bacterial suspensions was followed polarographically in an apparatus similar to that described by Lloyd & Brookman (1967). Samples of the suspension (1 to 10 mg. protein), substrates and inhibitors were mixed with air-saturated 50 mM-potassium phosphate buffer (pH 7.4) to give a final volume of 2.0 ml. contained in the magnetically stirred reaction chamber. The sensitivity of respiration to cyanide and carbon monoxide was determined manometrically (Umbreit, Burris & Stauffer, 1964). Flasks were incubated at 30° after flushing for 10 min. with air or with an equal mixture of air and carbon monoxide.

Enzyme assays. Cytochrome oxidase and catalase were measured in bacterial suspensions. Cytochrome oxidase was assayed by measuring polarographically the rate of oxygen consumption in the presence of 1.5 mM-ascorbate and 0.25 mM-*NNN'*-tetramethyl *p*-phenylenediamine (Slater, 1950). Assays were performed at room temperature, 22 to 24°. Catalase activity was determined polarographically by following O₂ release from hydrogen peroxide by bacterial suspensions: activities were too low to measure spectrophotometrically. Washed bacterial suspension (0.2 to 5 mg. protein), 1.8 ml. oxygen-free 50 mM-potassium phosphate buffer (pH 7.4) and 0.1 ml. 250-fold diluted commercial hydrogen peroxide (reagent grade, 30% (w/v) solution) were added to the reaction vessel, and the rate of increase of dissolved oxygen concentration (initially about 20% saturated) was determined. The observed rate was corrected by subtracting the rate of oxygen release from H₂O₂ in the absence of bacteria and by adding the endogenous rate of oxygen consumption by bacteria in the absence of H₂O₂. Since there are uncertainties in both corrections, the activities of catalase cited (Table 5) are approximations. Corrected activities were proportional to the volume of bacterial suspension assayed.

Aconitase, fumarase, malate dehydrogenase and isocitrate dehydrogenase activities of the

high-speed supernatant and NADH oxidase and succinate dehydrogenase in the cell-wall membrane fractions were determined spectrophotometrically. Absorbancy changes at 30° were recorded with a Unicam S.P. 500 spectrophotometer coupled to a Gilford 2000 multi-sample attachment (Gilford Instrument Labs. Inc., Ohio, U.S.A.).

Aconitase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) was measured by observing the increase in extinction at 233 nm. in the presence of citrate (Anfinsen, 1955). Fumarase (L-malate hydrolase, EC 4.2.1.2) was assayed by observing the increase in extinction at 240 nm. in the presence of malate (Massey, 1955).

Malate dehydrogenase (L-malate: NAD⁺ oxidoreductase, EC 1.1.1.37) was determined by measuring the oxidation of NADH at 340 nm. in the presence of oxaloacetate (Ochoa, 1955*a*) and isocitrate dehydrogenase (DL-isocitrate: NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.42) by following the reduction of NADP⁺ at 340 nm. in the presence of DL-isocitrate (Ochoa, 1955*b*).

NADH oxidase was measured by observing the decrease in NADH extinction at 340 nm. (Slater, 1950) and succinic dehydrogenase (succinate: phenazine methosulphate oxidoreductase, EC 1.5.99.1) by following the decrease in extinction at 600 nm. of 2:6-dichlorophenolindophenol (Veeger, Der Vartanian & Zeylemaker, 1969). The validity of each assay for each species was checked by establishing that reaction rates were independent of the substrate concentration(s), but were proportional to the volume of bacterial extract added.

Cytochromes. Cytochromes in both CWM and HSS fractions were identified and measured with a Cary 15 recording spectrophotometer, and with a Cary 14 spectrophotometer adapted for low-temperature spectroscopy. Difference spectra, dithionite reduced against ferricyanide oxidized, were obtained and cytochrome concentrations calculated by the method of Estabrook & Holowsinsky (1961) using the extinction coefficients for the analogous pigments in rat-liver mitochondria. For low-temperature spectroscopy (Estabrook, 1961) each cell extract, 0.8 ml., was mixed with 1.2 ml. 50% (w/v) sucrose in 50 mM-potassium phosphate buffer (pH 7.4) and divided into two parts. These were oxidized and reduced at room temperature and injected with a syringe into perspex cuvettes immersed in liquid nitrogen.

The pyridine haemochromogens were determined by twice extracting bacterial suspensions with two volumes 0.4% (w/v) hydrochloric acid in acetone (Jacobs & Wolin, 1963). The cell debris remaining after extraction was retained, and used to assay haem *c*. The acetone extracts were collected by centrifugation and decantation, combined, and evaporated to dryness in dimmed light at 40° on a rotary evaporator. The residue was dissolved in petroleum ether (ligroine, b.p. 30 to 60°) and shaken with 50 ml. 0.1 M-sodium bicarbonate. If an emulsion formed, this was broken by adding a few crystals of sodium chloride. The petroleum layer, which contained haem *b*, was collected and evaporated to dryness.

To the acetone extract or cell debris were added 0.5 ml. N-NaOH, 1.0 ml. reagent grade pyridine and 4.5 ml. water (Jacobs & Wolin, 1963). Reduced against oxidized difference spectra were recorded within 1 min. of adding alkali and pyridine. The reducing agent was taken from a fresh batch of reagent grade sodium dithionite, and the samples were shielded from light whenever possible. The cellular concentrations of protoporphyrin IX and haem *c* were determined from the intensities of the α bands of their corresponding pyridine haemochromogens.

Cytochrome *o* (Castor & Chance, 1959) was determined by recording carbon monoxide difference spectra of unfractionated cell-free extracts supplemented with sodium succinate (10 mM-final concentration). An extract was flushed for 2 min. with air-free carbon monoxide and its spectrum, relative to the untreated extract, was measured. The test sample was

again flushed with carbon monoxide, and the cycle repeated until no further increase in extinction was observed at 419 nm.

Benzoquinones were isolated from dense suspensions (40 to 100 mg. protein/ml.) by treating the bacteria with 4 volumes of methanol which had been cooled in acetone and dry ice, and then extracting lipids with 5 volumes of either chloroform or petroleum ether (ligroine, b.p. range 30 to 60°) (Redfearn, 1967). The solvent layer was collected by centrifugation before repeating the extraction. The combined extracts were evaporated to dryness at 40° on a rotary evaporator, and the residue was dissolved in 5.0 ml. ethanol. Difference spectra, silver oxide oxidized against sodium borohydride reduced, were recorded and the concentration of quinone determined from the peak height of 275 nm. The spectra were identical to those of commercially available benzoquinone-6 and benzoquinone-10. As the individual quinones were not identified, concentrations are expressed as absorbancy units/g. bacterial protein.

Protein was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951). Bacterial numbers were determined microscopically by counting the number of organisms from a suitable dilution on a haemocytometer grid.

RESULTS

Growth characteristics. *Spirillum volutans* grew in air from small inocula only if the Krieg's medium was boiled for several minutes and rapidly cooled immediately before introducing the inoculum (Table 1). Even with pretreatment, 1 % (v/v) inocula were required to initiate growth when bacteria were taken from a 4-day or older culture, and no growth was observed when freshly inoculated cultures were shaken in air. Large inocula taken from young, motile cultures initially grew well even in media that had not been boiled, but growth often ceased after several generations. *Spirillum itersonii* and *S. serpens* gave dense cultures irrespective of the inoculum size or the pretreatment of the medium.

The final bacterial density in Krieg's medium differed markedly between species, but

Table 1. *The effect of aeration, and the treatment of the growth medium before inoculation, on the growth of Spirillum volutans*

Pretreatment of medium	Inoculum size* (organisms/ml.)	Culturing conditions	Cell density (organisms/ml.) after	
			48 h.	96 h.
Boil; cool	5×10^5	Stationary	7×10^6	8×10^6
	4×10^3	Stationary	8×10^4	9×10^5
	4×10^3	Shake	2×10^3	1×10^3
Untreated	5×10^5	Stationary	5×10^8	5×10^6
	4×10^3	Stationary	8×10^3	8×10^3 †
	4×10^3	Shake	2×10^3	1×10^3
Boil; cool	5×10^5	Stationary, pH frequently readjusted to 6.8–7.0	1×10^7	—
	5×10^5	Stationary, pH readjusted and 6 μ mole/ml. succinate added after 24 h.	2×10^7	—

* The inoculum was taken from a culture 48 h. old grown without shaking in unsupplemented Krieg's medium.

† These cultures gave variable results: growth usually stopped after one or two cell divisions, but occasionally more extensive growth was observed.

varied little for duplicate cultures of the same species (Table 2). The pH of the medium increased during growth, which ceased when the pH rose to 8.4, or somewhat lower in the case of *Spirillum volutans*. Attempts to increase the growth of *S. volutans* by buffering the medium were unsuccessful: 20 mM-tris (hydroxymethyl) aminomethane (tris) (pH 7.0) 5 mM-potassium phosphate buffer (pH 6.8) completely inhibited its growth, although no similar effects were noted for *S. serpens* or *S. itersonii*. At concentrations above 10 mM, sodium ions were also inhibitory for *S. volutans*. The final cell yield of *S. volutans* was greater when the pH was repeatedly adjusted to 6.8 with sterile sulphuric acid, and a further increase resulted on addition of more succinate (Table 1).

Oxygen consumption of bacterial suspensions. Washed suspensions of *Spirillum volutans* consumed oxygen in the absence of added substrate but at a rate less than that of the other two spirilla (Table 3). Of the substrates tested, only succinate caused a marked stimulation of *S. volutans* respiration. The respiration of succinate and peptone by *S. serpens* and *S. itersonii* was 2- to 35-fold greater on an equivalent protein basis.

Table 2. *Growth yields and final pH in cultures of different spirilla in Krieg's medium*

Organism	Increase in density after 48 h. (mg. dry wt/l.)	Final pH
<i>Spirillum volutans</i>	90 to 140	7.4 to 8.1
<i>S. itersonii</i>	450 to 480	8.1 to 8.4
<i>S. serpens</i>	250 to 290	8.1 to 8.4

Table 3. *Rates of oxygen consumption by washed suspensions of three spirilla with various substrates*

Substrate	Oxygen consumption (nmole/mg. protein/min.)		
	<i>S. volutans</i>	<i>S. serpens</i>	<i>S. itersonii</i>
None	2.2	32	38
5 mM-Succinate	30	127	60
5 mM-Glycerol	2.2	42	52
5 mM-Malate	5.8	43	50
1 % (v/v) Peptone	3.7	128	58
5 mM-Acetate	3.7	65	32
5 mM-Glutamate	2.2	48	45

Table 4. *Activities of some tricarboxylic acid cycle enzymes, NADH oxidase and catalase in three spirilla*

Enzyme	Cell fraction	Enzyme activity (μ mole/g. protein/h.)*		
		<i>S. volutans</i>	<i>S. serpens</i>	<i>S. itersonii</i>
Fumarase	HSS	1.2	25.1	21.8
Malate dehydrogenase	HSS	1.5	332	151
Aconitase	HSS	3.8	31.2	1.9
Isocitrate dehydrogenase	HSS	5.3	16.9	18.9
Succinic dehydrogenase	CWM	3.8	7.2	7.1
NADH oxidase	CWM	0.7	9.3	24.7
Catalase	Whole cells	2	16	75

* Average of duplicate assays on 3 to 5 independent preparations.

Fig. 1. Difference spectra of high-speed supernatant (HSS) extracts of *Spirillum volutans*, *S. itersonii* and *S. serpens*. The bacterial extract (3.0 ml.) was transferred to two cuvettes. A few grains of sodium dithionite were added to the test sample, and one drop (approximately 0.05 ml.) of 10 mM-potassium ferricyanide was added to the control cuvette. Spectra were recorded at room temperature with the Cary 15 spectrophotometer. The length of the vertical bar represents an absorbancy increment of 0.01. The protein concentrations for the three HSS samples were 6.6 mg./ml. (*S. itersonii*), 5.4 mg./ml. (*S. serpens*) and 4.2 mg./ml. (*S. volutans*).

Fig. 2. Difference spectra of cell-wall-membrane fractions of *Spirillum volutans*, *S. itersonii* and *S. serpens*. The experimental details were the same as for Fig. 1. The protein concentrations were 13.0 mg./ml. (*S. itersonii*), 4.1 mg./ml. (*S. serpens*) and 10.0 mg./ml. (*S. volutans*).

Cyanide-inhibited *Spirillum volutans* respiration by 95 %, but carbon monoxide had little effect even after prolonged incubation of the organism in its presence. Tris, phosphate and sodium ions at concentrations that inhibit growth also caused a 30 to 40 % decrease in the respiration of *S. volutans*. There was a significant decrease in respiration rate at pH values above the growth limiting level of about 8.0. *Spirillum serpens* and *S. itersonii* were sensitive to cyanide (95 % at 5 mM-KCN) but not to any of the other compounds mentioned above.

Tricarboxylic acid cycle enzyme activities. HSS fractions of all three spirilla contained malate dehydrogenase, NADP⁺ dependent isocitrate dehydrogenase, aconitase and fumarase

activities, but succinic dehydrogenase was sedimented in the CWM fraction (Table 4). The activities of all tricarboxylic acid (TCA) cycle enzymes were lower on a protein basis in *Spirillum volutans* extracts, but only fumarase and malate dehydrogenase were especially deficient compared to the oxygen-insensitive spirilla. CWM fractions oxidized NADH, but once again this activity was low in *S. volutans*. *Spirillum volutans* can thus oxidize succinate to fumarate, but subsequent pyridine nucleotide dependent activities are weak.

Electron transfer-chain components. As succinate oxidation in the spirilla was cyanide-sensitive, and membrane preparations oxidized NADH and succinate, the presence of a cytochrome-linked terminal electron transfer pathway to oxygen is probable. This was confirmed spectrophotometrically.

The cytochrome spectra of HSS and CWM extracts are shown in Fig. 1 and 2. All three spirilla synthesized appreciable quantities of cytochrome *b* and *c*, but *a*-type cytochromes were not detected. This agrees with the insensitivity of oxygen consumption in the presence of succinate to carbon monoxide. Low-temperature spectra of whole-cell, soluble and membrane-bound cytochromes revealed that more than one *b*- and *c*-type cytochrome was formed by the three spirilla. Although small variations in the positions of absorption maxima were noted, the spectra were similar in each species. The individual components are now being characterized, and will be the subject of a subsequent report. The concentrations of cytochromes in each cell fraction are compared in Table 5.

Spirillum volutans appeared to contain less cytochrome *c* than the other two spirilla. This was confirmed by converting the cytochromes to their pyridine haemochromogen derivatives. Comparable quantities of the pyridine haemochromogen of protoporphyrin IX were obtained from all three organisms, but *S. volutans* contained less haem *c* (Table 5).

Cytochrome oxidase. The endogenous rates of oxygen consumption of all three organisms doubled when 1.5 mM-ascorbate was added as the oxidizable substrate. This rate was further stimulated in the presence of 0.25 mM-*NNN'*-tetramethyl *p*-phenylenediamine, which is thought to pass electrons directly to cytochrome oxidase. Although the latter activity was lowest in *Spirillum volutans* (72 nmole/mg. protein/min. compared with 240 nmole/mg./min. in *S. serpens* and 200 nmole/mg./min. in *S. itersonii*), it was still considerably greater than the peptone, succinate or malate supported respiration rates of this organism. It seems unlikely that cytochrome oxidase activity limits the rate at which oxygen can be reduced. This conclusion was supported by the observation that the respiration rate of a sonically

Table 5. Cytochrome *o*, haem and benzoquinone content of whole cells, and the concentration of cytochromes in cell-free extracts of spirilla

Pigment assayed	Units	<i>S. volutans</i>	<i>S. serpens</i>	<i>S. itersonii</i>
Protohaeme (pyridine haemochromogen)	pmole/g. dry wt	21	22	26
Haeme <i>c</i> (pyridine haemochromogen)	pmole/g. dry wt	25	72	78
Cytochrome <i>o</i>	Peak height at 420 nm./g. dry wt	5	19	12
Cytochrome <i>c</i> in HSS	pmole/mg. protein	107	193	343
Cytochrome <i>c</i> in CWM	pmole/mg. protein	99	708	220
Cytochrome <i>b</i> in HSS*	pmole/mg. protein	82	158	236
Benzoquinone	Extract volume × A 275 nm./g. dry cell wt	4.4	5.4	2.7

* Cytochrome *b* in the CWM fractions was masked by the high concentration of cytochrome *c*. No *a*-type cytochromes were detected.

disrupted suspension of *S. volutans* was a little higher when both NADH and succinate were present than the sum of the rates with the individual substrates (Scholes & Smith, 1968).

Quinones and catalase. Oxidation-reduction reactions have been described in which flavo-proteins and quinones mediate the reduction of oxygen to hydrogen peroxide (Meister & Wellner, 1963). These reactions by-pass the energy-conserving reactions of the cytochrome chain (but see Cox, Snoswell & Gibson, 1968) and would be favoured in organisms which contain high concentrations of quinones. The resulting peroxide might be toxic if cells contained weak catalase activity. We therefore looked for quantitative differences in both the quinone concentrations and the catalase activity of the three spirilla. Similar quantities of benzoquinones were extractable from each organism (Table 5). No naphthoquinones were detected. In contrast, *Spirillum volutans* has a very low catalase activity (Table 4) which is, nevertheless, higher than its respiratory rate. Thus, accumulation of toxic peroxides (Stephenson, 1939; Dickens, 1946, 1962) is an unlikely mechanism for oxygen inhibition.

DISCUSSION

The data confirm the sensitivity of *Spirillum volutans* to oxygen first reported by Wells & Krieg (1965). Compared to two oxygen insensitive spirilla, *S. serpens* and *S. itersonii*, *S. volutans* grows slowly and less abundantly and has a lower respiratory activity. Qualitatively, the three organisms have similar electron transfer-chain components and possess all enzymes of the Krebs cycle for which assays were performed. On a comparable weight or protein basis, total cytochromes and particularly cytochrome *c* are significantly lower in *S. volutans*; its fumarase, malate dehydrogenase, and NADH oxidase activities are 10- to 200-fold less; and its catalase activity is very low. These findings suggest a possible explanation for its low growth rate and yield.

Succinate is the major energy source for *Spirillum volutans* growing in Krieg's medium. The enzyme data suggest that only the succinate dehydrogenase reaction could have major significance in funnelling electrons into its electron-transport chain. As a consequence, per unit of succinate metabolized, ATP yields and growth yields would be much lower in *S. volutans* than in the other two spirilla in which no enzymic impediment exist to the generation of NADH via the Krebs cycle and its subsequent oxidation. The slower growth and respiration rate of *S. volutans* may simply be a consequence of the low absolute level of succinate dehydrogenase. The low respiratory activity is not due to a cytochrome deficiency since the electron transfer to oxygen is not rate-limiting in the organism's respiration.

The low respiration rate of *Spirillum volutans* may account for its sensitivity to inhibition by oxygen, which is a widespread, if not universal, attribute of cells; the differences between different organisms being one of degree (Haugaard, 1968). It would appear *a priori* that the intracellular concentration (or the oxygen concentration at the cell boundary) would be the determinant. If oxygen entrance into a cell is an unregulated diffusion process, at a given external oxygen concentration *S. volutans*, which has a low respiration rate, should have a higher intracellular oxygen concentration than *S. itersonii* or *S. serpens*, which respire more rapidly. If some critical cellular process in all three organisms were equally sensitive to oxygen, inhibition of *S. volutans* would be expressed at a lower external partial pressure or concentration.

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