Physiological Basis of the Selective Advantage of a *Spirillum* sp. in a Carbon-limited Environment

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A *Spirillum* sp. and a *Pseudomonas* sp. possessing crossing substrate saturation curves for L-lactate were isolated from fresh water by chemostat enrichment. Their K_s and $\mu_{\max}$ values for L-lactate were: *Spirillum* sp., 23 μM and 0.35 h^{-1} , respectively; *Pseudomonas* sp., 91 μM and 0.64 h^{-1} , respectively. Under L-lactate limitation, *Pseudomonas* sp. outgrew *Spirillum* sp. at dilution rates (D) above 0.29 h^{-1} , but the converse occurred at lower D values. The advantage of *Spirillum* sp. increased with decreasing D until, at $D = 0.05 \text{ h}^{-1}$ (i.e. L-lactate concentration of approximately 1 μM), *Pseudomonas* sp. was eliminated from the culture essentially as a non-growing population. In *Spirillum* sp. the K_m for L-lactate transport (5.8 μM) was threefold lower than in *Pseudomonas* sp. (20 μM); *Spirillum* sp. also possessed a higher $V_{\max}$ for the transport of this substrate. The surface to volume ratio was higher in *Spirillum* sp. and increased more markedly than in *Pseudomonas* sp. in response to decreasing D . Thus, a more efficient scavenging capacity contributes to the advantage of *Spirillum* sp. at low concentrations of the carbon source. Although most of the enzymes of L-lactate catabolism were more active in *Pseudomonas* sp., NADH oxidase activity was about twice as high in *Spirillum* sp.; and, unlike *Pseudomonas* sp., the cytochrome c content of this bacterium increased markedly with decreasing D . A more active and/or more efficient respiratory chain may therefore also play a role in the advantage of *Spirillum* sp. The other factors which appear to be involved include a lower energy of maintenance of *Spirillum* sp. [0.016 g L-lactate (g cell dry wt) $^{-1} \text{ h}^{-1}$ compared with 0.066 in *Pseudomonas* sp.] and a lower minimal growth rate.

INTRODUCTION

With the chemostat it is possible to investigate microbial selection and competition at different concentrations of a growth-limiting nutrient (Jannasch, 1967; Veldkamp & Jannasch, 1972; Veldkamp, 1976; Harder, Kuenen & Matin, 1977). Jannasch (1967) found that with a low, and growth-limiting, concentration of lactate added to a sample of sea water, a bacterial species was selected which was different from that selected at higher concentrations of the same substrate. Kuenen *et al.* (1977) reported that enrichment at different concentrations of phosphate as growth-limiting nutrient in the medium also led to the selection of different bacterial species. The bacteria selected at different concentrations of a growth-limiting substrate possessed substrate saturation curves which crossed over (see Fig. 1): those selected at the low concentration had a low saturation constant (K_s) and low maximum specific growth rate ($\mu_{\max}$); those selected at the higher concentration had a higher K_s and a higher $\mu_{\max}$. Subsequent studies have shown that bacteria with a low K_s also invariably have a relatively low $\mu_{\max}$. Since aquatic environments generally

contain low concentrations of nutrients (Duursma, 1961), it was inferred that the low K_s -low $\mu_{\max}$ type of bacteria play a dominant role in microbial transformations in these environments. Such bacteria are not isolated by traditional batch culture enrichments which, due to their high nutrient concentration, select exclusively for organisms with a high $\mu_{\max}$ (Veldkamp & Jannasch, 1972; Harder *et al.*, 1977). Little information exists on the physiology and ecology of this potentially important group of bacteria since nearly all the bacterial species which have been studied were isolated from batch culture enrichments.

This study was undertaken to investigate the physiological and biochemical basis of the capacity for relatively faster growth at low concentrations of carbon compounds. The low K_s -low $\mu_{\max}$ and high K_s -high $\mu_{\max}$ types of bacteria (a *Spirillum* sp. and a *Pseudomonas* sp., respectively) were isolated from a freshwater pond and a broad comparison was made of their characteristics during growth on different concentrations of L-lactate in chemostat culture. A preliminary report of these findings has been presented (Matin & Veldkamp, 1974) and different aspects of the physiology of these bacteria have been examined (Matin & Konings, 1973; Matin, Grootjans & Hogenhuis, 1976; Matin & Gottschal, 1976).

METHODS

Growth conditions and enrichment procedure. The basal mineral salts medium contained (g l^{-1}): K_2HPO_4 , $3\text{H}_2\text{O}$, 1.13; KH_2PO_4 (anhydrous), 0.88; NH_4Cl , 1.00; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50; NaCl , 1.00; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.005; and 1.20 ml of the trace elements solution of Vishniac & Santer (1957). This medium was supplemented separately with lactate, succinate or L-alanine to 0.5 g l^{-1} to obtain culture media limiting in these carbon sources. The media were prepared and sterilized as previously described (Matin *et al.*, 1976). Enrichment and competition experiments were carried out at 28°C in a chemostat with a working volume of 200 ml. The pH was automatically maintained at 7.0 ± 0.1 by adding sterile 0.2 M-HCl solution. The oxygen concentration in the culture vessel was periodically monitored by an oxygen electrode and was never below 80% air saturation. The flow rate of fresh medium into the culture vessel was controlled with a peristaltic pump (LKB, Varioperpex, 12000). The inflowing air was passed through a water column (about 25 cm long) to minimize evaporation of the culture fluid. Other features of the chemostat were as described by Veldkamp (1976).

For enrichment, L-lactate-limited medium was supplied to two identical chemostats from a 15 l reservoir. Each of the vessels was inoculated with 10 ml of a sample from a freshwater pond which had been passed through a $1.2 \mu\text{m}$ membrane filter to remove debris and larger organisms such as protozoa. After 24 h of batch culture, the fresh medium flow was initiated to give dilution rates (D) of 0.05 h^{-1} in one vessel and 0.30 h^{-1} in the other. Different dominant bacterial populations were found in the two vessels after approximately five volume changes and these were isolated by repeated streaking on solid L-lactate medium (composition as described above, but with 20 g agar l^{-1} added). Stock cultures of the two bacteria were maintained on the same medium. These bacteria have been deposited with Deutsche Sammlung von Mikroorganismen, München, West Germany. The accession numbers are: *Spirillum* sp., DSM 1109; *Pseudomonas* sp., DSM 1110.

Procedure for competition experiments. The organisms were pre-grown in either batch or continuous culture at the dilution rate to be used in the competition experiment. They were harvested aseptically, mixed in suitable proportion and inoculated in a chemostat run at the specified D value. The inoculum size was adjusted to give an initial concentration of about $10^9 \text{ cells ml}^{-1}$. This concentration remained approximately constant during the experiment, only the relative proportions of the two bacteria changing. Saturating initial cell densities were employed to ensure rapid establishment of L-lactate limitation. At $D = 0.05 \text{ h}^{-1}$, viable cell counts were begun after the chemostat had been run for 4 h; at other D values, measurements were begun immediately after the two populations were mixed in the chemostat. Viable counts on samples removed during competition experiments were determined by spreading appropriate dilutions on L-lactate agar plates. Colonies of the two bacteria could be easily distinguished by their size and shape.

The washout rate of a non-growing population from the chemostat vessel was calculated from the equation (Jannasch, 1969):

$$\ln X_2 = \ln X_1 (t_2 - t_1) D \quad (1)$$

where X_2 is the number of the non-growing population at a given time (t_2) to be determined, and X_1 is the number of this population at any previous time (t_1).

The difference in the growth rates of the two populations in the competition experiments was calculated from the equation:

$$(\mu_1 - \mu_2) = \frac{\ln R_2 - \ln R_1}{t} \quad (2)$$

where μ_1 and μ_2 are the specific growth rates of the two populations, R_2 is the ratio of the two populations at a given time (t_2), R_1 is this ratio at any previous time (t_1), and $t = t_2 - t_1$.

Culture viability determination. The percentage viability of the two bacteria in chemostat cultures at various D values was determined by the slide-culture technique (Postgate, 1969). For this, 0.2 ml of 0.01 % (w/v) L-lactate medium [supplemented with 2 % (w/v) prefiltered agar] was allowed to set in stainless steel rings (inner diameter, 17 mm; height, 1 mm) placed on glass slides. A low L-lactate concentration was used to approximate as closely as possible to the conditions of the chemostat environment and to minimize the possibility of substrate-accelerated death (Calcott & Postgate, 1972). A suitable dilution of the culture was spread on the surface of the agar, so that each microscopic field at 400 $\times$ magnification contained about 50 organisms. The slides were incubated in a moist atmosphere at 28 °C and examined under a phase-contrast microscope every 3 h for up to 28 h. Each single cell was counted as one dead unit, each micro-colony as one viable unit. Each time 400 to 500 units were counted and the percentage viability was calculated from the numbers of viable and dead cells. The maximum value was usually attained by 21 h of incubation.

Energy of maintenance determination. Maintenance energy on L-lactate was determined by plotting $1/D$ against $1/Y$, where Y is the g dry weight cell material formed (g L-lactate consumed) $^{-1}$, as described by Pirt (1975). To determine Y at various D values, the organisms were grown in separate chemostats of 700 ml working volume, using 0.2 % (w/v) L-lactate medium. After a steady state was established at a given D , a 200 ml sample was removed, cooled and immediately centrifuged (8300 g; 20 min). The pellet was resuspended in 10 ml H₂O and quantitatively transferred to vials which had been pre-dried and weighed; the cells were not washed because of the predisposition of *Pseudomonas* sp. to lysis. The vials were dried to constant weight in an oven at 110 °C.

Saturation constant and maximum specific growth rate determinations. The saturation constant, K_s , was calculated from the equation:

$$K_s = \frac{\bar{s}(\mu_{\max} - D)}{D} \quad (3)$$

where $\bar{s}$ is the steady-state L-lactate concentration in the culture vessel at a given dilution rate (D), and $\mu_{\max}$ is the maximum specific growth rate of the bacterium.

To determine $\bar{s}$, each organism was grown separately at $D = 0.15 \text{ h}^{-1}$ in a chemostat with 600 ml working volume (Bioflo, New Brunswick Scientific Co), using L-lactate-limited medium (L-lactate concentration, 0.025 %, w/v). After five volume changes (approximately seven generations), four samples (each approximately 4 ml) were taken from each culture using a manifold, and filtered under vacuum. Millipore membrane filters (diam. 47 mm; pore size, 0.45 μm) were used and filtration was accomplished within 30 s to 1 min. Calculations based on the known respiratory capacity of the cells (see Fig. 5) and their K_s for L-lactate respiration (Matin & Konings, 1973) indicated that less than 1 % of the L-lactate in the culture filtrates could have been respired during sample collection. Portions (3 ml) of the filtrates were dried in a stream of air, resuspended in 1 ml distilled, deionized water and used in the L-lactate determination. Assay mixtures for L-lactate determination (Hohorst, 1963) contained (total volume, 1 ml): 0.5 ml of 0.4 M-hydrazine sulphate buffer, pH 9.5; 0.05 ml of 0.05 M-NAD; up to 0.43 ml of sample; and 0.02 ml of rabbit muscle L-lactate dehydrogenase (1500 I.U. ml $^{-1}$; Calbiochem). Lactate concentration in the sample was calculated from ΔA_{340} recorded spectrophotometrically; $\epsilon_{\text{NADH}} = 6.22 \times 10^6 \text{ l mol}^{-1} \text{ cm}^{-1}$.

To determine $\mu_{\max}$, the bacteria were grown in batch cultures in 100 ml medium containing 0.2 % (w/v) L-lactate in 250 ml Erlenmeyer flasks shaken at 28 °C, and ΔA_{660} was measured. The purity of the cultures was checked by streaking on 0.05 % (w/v) L-lactate and nutrient agar plates.

The Michaelis-Menten constant for L-lactate transport. Cells were grown in batch cultures in 0.2 % (w/v) L-lactate medium and harvested from late-exponential phase. They were washed three times in 0.1 M-potassium phosphate buffer, pH 6.6, and resuspended in the same buffer to a density of approximately 3.5 mg cell protein ml $^{-1}$. The uptake experiments were carried out as described by Matin & Konings (1973). The reaction mixtures contained (total volume, 100 μl): 50 mM-potassium phosphate buffer, pH 6.6; 10 mM-MgSO₄; 0.02 to 0.07 mg cell protein; and 1 to 20 μM -L-[U-¹⁴C]lactate (40 mCi mmol $^{-1}$; The Radiochemical Centre, Amersham). L-Lactate uptake was initially determined at 1, 3 and 20 μM , taking samples every 15 s. As the uptake was linear up to 45 s with 1 μM -L-lactate and up to 1 min or longer at other concentrations, the K_m values were determined by following uptake for 30 s at each concentration of L-lactate; see Matin & Konings (1973) for further details.

Surface to volume ratio. From steady-state cultures of *Pseudomonas* sp. and *Spirillum* sp. growing in separate chemostat vessels at various dilution rates under L-lactate- or succinate-limitation, 20 ml samples

were taken, centrifuged, washed in 0.1 M-ammonium acetate buffer, stained with 1 % (w/v) uranyl acetate and photographed, using a Philips EM300 electron microscope.

Each cell was considered to be made up of a cylinder and a sphere. The surface area and volume of the cylindrical part were calculated as $2\pi Rh$ and $\pi R^2 h$, respectively, where h is the height of the cylinder and R is its radius; the volume and surface area of the spherical part were calculated as $4\pi R^3/3$ and $4\pi R^2$, respectively, where R is the radius of the sphere. R was measured at the centre of a cell and was assumed to be identical for its cylindrical and spherical parts.

Analyses. Cell extracts were prepared and enzyme activities were measured as described by Martin *et al.* (1976). The reaction mixture for estimating total cytochrome *c* content contained (total volume, 1 ml): 50 μ mol potassium phosphate buffer, pH 7.0; 2.5 to 3 mg crude cell extract protein; and a few crystals of sodium dithionite (sample cuvette) or 0.05 μ mol $K_3Fe(CN)_6$ (reference cuvette). Absorption spectra were obtained using a Cary 14 recording spectrophotometer and $\Delta A_{555} - \Delta A_{540}$ was used to calculate the cytochrome *c* content (for reduced cytochrome *c*, $\epsilon = 19.1 \times 10^6 \text{ l mol}^{-1} \text{ cm}^{-1}$).

The respiratory capacity of the organisms grown at different D values was determined by quickly transferring organisms from a steady-state culture to a biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The oxidation rate of L-lactate was determined using a reaction mixture which contained (total volume, 3 ml): 50 mM-potassium phosphate buffer, pH 7.0; 0.1 to 0.2 mg cell protein; and 8 mM-sodium L-lactate. Oxygen uptake was measured at 29 °C for a period of 2 to 5 min; see Martin & Konings (1973) for further details.

Nucleic acids were determined by the method of Munro & Fleck (1966), and protein by that of Lowry *et al.* (1951).

RESULTS

Isolation and K_s values

A small, non-motile *Spirillum* sp. became the dominant population at $D = 0.05 \text{ h}^{-1}$, and a motile *Pseudomonas* sp. at $D = 0.30 \text{ h}^{-1}$. This dominance became apparent within five volume changes and persisted for as long as the experiment was continued (10 volume changes). The $\mu_{\max}$ values on L-lactate were: for *Spirillum* sp., 0.35 h^{-1} ; and for *Pseudomonas* sp., 0.64 h^{-1} . At $D = 0.15 \text{ h}^{-1}$, the residual L-lactate concentrations (average of four determinations $\pm$ S.E.M.; see Methods) were: for *Spirillum* sp., $0.017 \pm 0.003 \mu\text{mol ml}^{-1}$; and for *Pseudomonas* sp., $0.028 \pm 0.004 \mu\text{mol ml}^{-1}$. Thus the K_s values were calculated to be: for *Spirillum* sp., 23 μM ; and for *Pseudomonas* sp., 91 μM . From these data the substrate saturation curves of the two bacteria were calculated (Fig. 1), employing equation (3). The K_s of *Spirillum* sp. for L-lactate was approximately fourfold lower than that of *Pseudomonas* sp. and the crossing point of the curve was at about $D = 0.25 \text{ h}^{-1}$.

Kinetics of competition

Competition between the two bacteria was studied with a mixed culture growing in a chemostat operating at various D values, with L-lactate as the limiting nutrient (Fig. 2). At $D \leq 0.24 \text{ h}^{-1}$, *Spirillum* sp. outcompeted *Pseudomonas* sp. and its advantage became more pronounced with decreasing D ; indeed at $D = 0.05 \text{ h}^{-1}$, the ratio *Pseudomonas* sp./*Spirillum* sp. changed at a rate that indicated that under these conditions *Pseudomonas* sp. was not appreciably multiplying. At $D \geq 0.29 \text{ h}^{-1}$ *Pseudomonas* sp. outgrew *Spirillum* sp. (the kinetics of competition are shown in Fig. 2 only for $D = 0.32 \text{ h}^{-1}$). Similar results were obtained regardless of whether the inocula for the competition experiments were grown in batch or continuous culture. The possible influence of population density on these results was checked by increasing the L-lactate concentration in the inflow medium to 0.2 % and studying competition at a low (0.05 h^{-1}) and a high (0.28 h^{-1}) D value. The outcome, which was judged qualitatively by microscopic examination, agreed with previous findings. Thus, an approximately fourfold change in population density did not affect the outcome of competition.

The average difference in the specific growth rates of the two organisms during competition was calculated from the data of Fig. 2 using equation (2). At $D = 0.05$ and 0.24 h^{-1} , at which *Spirillum* sp. won the competition, this difference was about 0.06 and 0.11 h^{-1} ,

Fig. 1

Fig. 2

Fig. 1. Substrate saturation curves for L-lactate of *Spirillum* sp. and *Pseudomonas* sp. calculated from K_s and $\mu_{\max}$ data; $\mu_{\max}$ was determined as described in the text and K_s was calculated from the residual L-lactate concentration in the culture medium at $D = 0.15 \text{ h}^{-1}$. The growth constants for the two bacteria are: *Spirillum* sp.: $\mu_{\max}$, 0.35 h^{-1} , K_s , $23 \mu\text{M}$; *Pseudomonas* sp.: $\mu_{\max}$, 0.64 h^{-1} , K_s , $91 \mu\text{M}$.

Fig. 2. Ratio of viable counts of *Pseudomonas* sp. and *Spirillum* sp., during competition in a mixed culture with L-lactate as limiting nutrient, as a function of growth rate (the abscissa shows the number of complete changes of medium that have passed through the chemostat). $D = 0.05 \text{ h}^{-1}$ (●), 0.24 h^{-1} (▲) and 0.32 h^{-1} (■). The dashed line indicates the change in this ratio if the organism losing the competition were being eliminated as a non-growing population (calculated from equation 1). The ordinate specifies the ratio of *Pseudomonas* sp. to *Spirillum* sp. (●, ▲), or *Spirillum* sp. to *Pseudomonas* sp. (■).

respectively. This observed difference, especially at $D = 0.24 \text{ h}^{-1}$, is much greater than predicted by the Monod relationships (Fig. 1); according to these relationships the maximum difference in the specific growth rates of the two organisms, at D values where *Spirillum* sp. wins the competition, is about 0.04 h^{-1} . The advantage of *Spirillum* sp. during competition cannot, therefore, be explained solely on the basis of these Monod relationships. To test whether *Spirillum* sp. excreted an inhibitor of the growth of *Pseudomonas* sp., which may contribute to this advantage, a steady-state culture of *Spirillum* sp. at $D = 0.05 \text{ h}^{-1}$ was filtered, L-lactate was added to the filtrate to 0.2% (w/v), and the resulting medium was inoculated with *Pseudomonas* sp. The organism grew at its usual rate indicating that no inhibitor had accumulated in the culture of *Spirillum* sp.

Transport parameters and surface to volume ratio

Spirillum sp. could owe its advantage over *Pseudomonas* sp. at low L-lactate concentrations to a high-affinity transport system for L-lactate. The kinetic constants for L-lactate transport were therefore determined for the two bacteria: *Spirillum* sp. had a threefold lower K_m value for L-lactate than *Pseudomonas* sp. ($5.8 \mu\text{M}$ versus $20 \mu\text{M}$); $V_{\max}$ for L-lactate transport was also significantly higher in *Spirillum* sp. [14.7 versus $10.0 \text{ nmol L-lactate (mg protein)}^{-1} (30 \text{ s})^{-1}$]. Essentially identical values were obtained in two separate determinations. In both bacteria, K_m for L-lactate was approximately fourfold lower than K_s for growth on L-lactate.

Another reason for the ability of *Spirillum* sp. to grow more rapidly at low concentrations of L-lactate could be a higher surface/volume ratio in this organism compared with

Table 1. *Effect of dilution rate on cell dimensions of Spirillum sp. and Pseudomonas sp. grown under L-lactate limitation*

Organisms grown in chemostat cultures at various dilution rates, with 0.2% (w/v) L-lactate in the inflow medium, were used to prepare electron micrographs as described in Methods. For each dilution rate, 40 to 50 cells (actual numbers given in parentheses) were measured and the different dimensions were calculated as described in Methods. Data given are mean values $\pm$ S.E.M. *Sp.*, *Spirillum* sp.; *Ps.*, *Pseudomonas* sp.

Dilution rate (h ⁻¹)	Length (μ m)		Breadth (μ m)		Surface area (μ m ²)		Volume (μ m ³)		Surface/volume (μ m ⁻¹)	
	<i>Sp.</i>	<i>Ps.</i>	<i>Sp.</i>	<i>Ps.</i>	<i>Sp.</i>	<i>Ps.</i>	<i>Sp.</i>	<i>Ps.</i>	<i>Sp.</i>	<i>Ps.</i>
0.01 (50)	2.16 $\pm$ 0.05	1.92 $\pm$ 0.04	0.47 $\pm$ 0.004	0.70 $\pm$ 0.005	3.18	4.22	0.34	0.65	9.30	6.49
0.06 (40)	2.10 $\pm$ 0.07	2.05 $\pm$ 0.04	0.55 $\pm$ 0.005	0.73 $\pm$ 0.002	3.58	4.68	0.44	0.75	8.05	6.24
0.16 (50)	2.21 $\pm$ 0.06	2.26 $\pm$ 0.07	0.56 $\pm$ 0.006	0.80 $\pm$ 0.004	3.89	5.70	0.50	1.01	7.82	5.66
0.27 (50)	2.40 $\pm$ 0.06	2.39 $\pm$ 0.05	0.62 $\pm$ 0.007	0.83 $\pm$ 0.006	4.69	6.20	0.67	1.13	7.04	5.49
0.33 (43)	2.19 $\pm$ 0.05	2.52 $\pm$ 0.06	0.67 $\pm$ 0.006	0.84 $\pm$ 0.008	4.57	6.68	0.69	1.25	6.67	5.34
0.35 (50) (batch)	2.17 $\pm$ 0.05	—	0.72 $\pm$ 0.007	—	4.89	—	0.78	—	6.27	—
0.46 (49)	—	2.75 $\pm$ 0.07	—	0.84 $\pm$ 0.006	—	7.26	—	1.38	—	5.26
0.64 (50) (batch)	—	3.55 $\pm$ 0.09	—	0.96 $\pm$ 0.008	—	10.66	—	2.32	—	4.59

Table 2. *Comparison of enzyme activities of Spirillum sp. and Pseudomonas sp.*

Organisms were grown at dilution rates of 0.04 and 0.28 h⁻¹ under L-lactate limitation and enzyme activities were measured as described by Martin *et al.* (1976). Specific activities are expressed as μ mol substrate converted min⁻¹ (mg protein)⁻¹.

Enzyme	Specific activity at $D = 0.04 \text{ h}^{-1}$		Specific activity at $D = 0.28 \text{ h}^{-1}$	
	<i>Spirillum</i> sp.	<i>Pseudomonas</i> sp.	<i>Spirillum</i> sp.	<i>Pseudomonas</i> sp.
NAD-independent L-lactate dehydrogenase	0.70	0.97	0.34	0.54
NAD-dependent L-lactate dehydrogenase (EC 1.1.1.27)	1.10	2.40	0.55	0.95
Aconitase (EC 4.2.1.3)	0.015	0.150	0.01	0.04
Isocitrate dehydrogenase (EC 1.1.1.42)	1.05	1.20	0.96	0.96
2-Oxoglutarate dehydrogenase (EC 1.2.4.2)	0.01	0.02	0.005	0.018
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	0.034	0.076	0.02	0.03
NADH oxidase	0.040	0.018	0.07	0.04

Pseudomonas sp., enabling it to concentrate a limiting nutrient more effectively. We therefore determined the cell dimensions of the two bacteria after growth at different D values. Under L-lactate limitation (Table 1), the cell length of *Spirillum* sp. did not change significantly in response to changes in D , but in *Pseudomonas* sp. it decreased nearly linearly with decreasing D . Both the bacteria became thinner with decreasing D values (Fig. 3): in *Spirillum* sp., the reduction in cell width was almost linear with decreasing D ; in *Pseudomonas* sp., it was non-linear and less pronounced. At all D values examined, cells of *Pseudomonas* sp. were wider than those of *Spirillum* sp.; cells of *Spirillum* sp. also showed a more pronounced curvature at low D values (Fig. 3). It is generally believed that cell width is a constant characteristic of a bacterium; these data show that this is not the case.

Total surface area as well as total volume decreased with decreasing D in both the bacteria, but the surface to volume ratio increased (Table 1). At all D values, the surface to volume ratio was greater in *Spirillum* sp. than in *Pseudomonas* sp., and the difference became more pronounced at lower D values because *Spirillum* sp. exhibited a greater increase in surface/volume ratio with decreasing D than did *Pseudomonas* sp.

Fig. 3. Effect of dilution rate on cell size and shape of *Spirillum* sp. and *Pseudomonas* sp. grown in L-lactate-limited medium. Bar markers represent 1 μm .

Enzymes of intermediary metabolism

Another reason for the advantage of *Spirillum* sp. could be that it has higher levels of enzymes of lactate catabolism. We therefore compared the specific activity of several enzymes of L-lactate metabolism in *Spirillum* sp. and *Pseudomonas* sp. Data are presented (Table 2) for two D values, 0.04 h^{-1} , at which in mixed cultures *Spirillum* sp. outcompetes *Pseudomonas* sp., and 0.28 h^{-1} , at which the converse occurs. At both D values *Pseudomonas* sp. possessed higher levels of most of the enzymes examined. However, NADH oxidase activity was approximately twofold higher in *Spirillum* sp. at both the D values. The two bacteria exhibited an interesting variation in their cytochrome c content at different D values (Fig. 4). At $D = 0.30 \text{ h}^{-1}$, they contained similar levels of this enzyme; as D was decreased, the level of cytochrome c decreased slightly in *Pseudomonas* sp. but increased markedly in *Spirillum* sp. so that at low D values (e.g. 0.04 h^{-1}) *Spirillum* sp. possessed three times as much cytochrome c as *Pseudomonas* sp.

As has been discussed in detail elsewhere (Matin *et al.*, 1976), both the organisms had higher activities of the enzymes examined, with the exception of NADH oxidase, after growth at the lower D value (Table 2).

Energy of maintenance

The energy of maintenance (Pirt, 1975) was compared in the two organisms. The yield corrected for maintenance requirement on L-lactate was comparable in the two bacteria [0.38 and $0.41 \text{ g cell dry wt (g L-lactate consumed)}^{-1}$ in *Spirillum* sp. and *Pseudomonas* sp., respectively], but the energy of maintenance was fourfold lower in *Spirillum* sp. [$0.016 \text{ g L-lactate (g cell dry wt)}^{-1} \text{ h}^{-1}$ compared with 0.066 in *Pseudomonas* sp.].

Fig. 4

Fig. 5

Fig. 4. Effect of dilution rate on the levels of cytochrome *c* in *Spirillum* sp. (●) and *Pseudomonas* sp. (○).

Fig. 5. Percentage viability (▲, ●) and respiratory capacity for L-lactate (△, ○) in cultures of *Spirillum* sp. (▲, △) and *Pseudomonas* sp. (●, ○) as a function of dilution rate.

Role of 'minimal growth rate'

Recent evidence suggests that the growth rate of micro-organisms can be decreased only to a finite value, up to approximately 6% of their maximum rate of growth (Pirt, 1972). If so, the 'minimum' rate of growth ($\mu_{\min}$) of *Pseudomonas* sp. would be approximately twofold higher than that of *Spirillum* sp. and this difference could contribute to the advantage of *Spirillum* sp. at low *D* values. To check this point, we determined the viability of the cultures of the two bacteria at different *D* values. This was between 90 and 98% at *D* values between 0.47 h⁻¹ and 0.05 h⁻¹ (Fig. 5). Below *D* = 0.05 h⁻¹, there was an abrupt decline in the percentage viability of the culture of *Pseudomonas* sp., but not of *Spirillum* sp. As a further check on the physiological state of the cultures of the two bacteria after growth at different *D* values, we determined their maximal respiratory capacity for L-lactate. A sharp discontinuity was observed in the respiratory capacity of *Pseudomonas* sp. below *D* = 0.08 h⁻¹ but not in that of *Spirillum* sp. (Fig. 5).

As has been pointed out by Pirt (1972), the data of Tempest, Herbert & Phipps (1967) also showed sharp discontinuities in the RNA content of *Klebsiella (Aerobacter) aerogenes*, when it was grown at below 6% of its $\mu_{\max}$. No such discontinuity could be discerned in the RNA or DNA content of *Pseudomonas* sp. at growth rates down to 4% of its $\mu_{\max}$. In both the bacteria, in the entire range of *D* values examined (0.02 to 0.45 h⁻¹), the RNA content increased linearly with increasing *D*, but the DNA content declined, somewhat slowly up to about *D* = 0.2 h⁻¹ and then more rapidly.

DISCUSSION

A comparative study has been made of several characteristics of *Spirillum* sp. and *Pseudomonas* sp. to aid understanding of the physiological basis of the advantage of the former bacterium at low L-lactate concentrations in the environment. *Spirillum* sp. had a greater capacity to concentrate the limiting L-lactate from the environment. This was indicated by its higher affinity and more active transport system for L-lactate and by its higher surface to volume ratio. The increase in surface to volume ratio in the two bacteria

in response to growth at decreasing concentrations of L-lactate was significantly greater in *Spirillum* sp. than in *Pseudomonas* sp. This greater uptake capacity for L-lactate must contribute to the advantage of *Spirillum* sp. at low L-lactate concentrations.

An assessment of the value of $\mu_{\min}$ in the two bacteria was made by investigating the influence of D on their viability, total respiratory capacity and nucleic acid content. The percentage viability of the culture of *Pseudomonas* sp. dropped markedly below $D = 0.05 \text{ h}^{-1}$ and there was a marked discontinuity in the respiratory capacity of the culture below $D = 0.08 \text{ h}^{-1}$, suggesting that the $\mu_{\min}$ of *Pseudomonas* sp. lay in this region. The value obtained from the respiration data is perhaps the more reliable: the recorded percentage viability may be an overestimation of the actual number of growing bacteria in the chemostat since it was determined on a medium which contained a much higher concentration of L-lactate than the chemostat culture at low D values (1 mM as opposed to 1 to 10 μM). Neither loss of viability nor discontinuity in the respiratory capacity of the *Spirillum* sp. culture was observed in the range of D values examined, indicating that the $\mu_{\min}$ of this bacterium must be below the lowest D value examined. Thus, at D values approximately equal to $\mu_{\min}$ of *Pseudomonas* sp., *Spirillum* sp. can still grow normally. This must therefore contribute to its advantage at these D values. Similarly, a lower energy of maintenance on L-lactate, by enabling *Spirillum* sp. to devote a greater portion of the total available energy to growth processes, must also contribute to its advantage at low L-lactate concentrations. No discontinuity in the nucleic acid content of *Pseudomonas* sp. was observed when D was lowered below its apparent $\mu_{\min}$ value which is in contrast to the finding of Tempest *et al.* (1967; see Pirt, 1972).

It is possible that higher concentrations of key catabolic enzymes and/or a higher affinity of these enzymes for their substrate also contribute to the advantage of *Spirillum* sp. at low L-lactate concentrations. An example of increased activity of a catabolic enzyme leading to faster growth at low substrate concentrations is provided by the 'hyper' mutants of *Escherichia coli*, which outgrew the wild type at low lactose concentrations, evidently because they synthesized increased levels of β -galactosidase (Horiuchi, Tomizawa & Novick, 1962; Smith & Dean, 1972). The work of Rigby, Burleigh & Hartley (1974) illustrates the role of the affinity of a catabolic enzyme in the outcome of competition: a mutant of *Klebsiella aerogenes* possessing a lower K_m for the first enzyme in xylitol metabolism (ribitol dehydrogenase) outcompeted the wild type during xylitol-limited growth in a chemostat.

Spirillum sp. did not prove to be analogous to the 'hyper' *E. coli* mutants with respect to most of the lactate catabolic enzymes, since these activities were significantly lower in *Spirillum* sp. than in *Pseudomonas* sp., even after growth at L-lactate concentrations which, in mixed cultures, support faster growth of the former bacterium. The only exceptions were NADH oxidase and cytochrome *c* levels and, curiously, the cytochrome *c* content in *Spirillum* sp., but not in *Pseudomonas* sp., increased markedly in response to decreasing L-lactate concentration in the environment. We are now investigating whether the higher levels of these respiratory chain components in *Spirillum* sp. are indicative of a more efficient energy generation during respiration. Attempts were made to compare the K_m values of L-lactate dehydrogenase (NAD-independent) for L-lactate in the two bacteria. In *Spirillum* sp., this enzyme consisted of three species with different pH optima and there was a selective increase in two of these activities as the L-lactate concentration in the environment was decreased (A. Matin & J. Steenhuis, unpublished). It is conceivable that these enzyme species differ in their K_m values for L-lactate and attempts are currently in progress to purify them and determine their K_m values.

The Monod relationships, calculated from $\mu_{\max}$ data and the K_s values determined at $D = 0.15 \text{ h}^{-1}$, did not account for the degree of advantage of *Spirillum* sp. over *Pseudomonas* sp. at $D \leq 0.24 \text{ h}^{-1}$. According to these relationships, the difference between the the growth rates of the two bacteria at these D values should not be greater than 0.04 h^{-1} ;

in fact, however, *Spirillum* sp. outgrew *Pseudomonas* sp. at a rate of 0.06 h^{-1} at $D = 0.05\text{ h}^{-1}$, and 0.11 h^{-1} at $D = 0.24\text{ h}^{-1}$. The reason(s) for this discrepancy is not known. The difference in the μ_{min} of the two bacteria is probably a contributing factor. Another possibility is that the μ - s relationships differed at different D values. Several physiological traits in these bacteria were altered in response to culture D . Besides the changes reported here, there was, with decreasing D , a marked increase in the specific activity of several enzymes of intermediary metabolism as well as in the NAD content of the two bacteria (Matin *et al.*, 1976; Matin & Gottschal, 1976); such changes could indeed influence the μ - s relationships. The change in the K_s values of a *Spirosoma* sp. and a *Spirillum* sp. in response to temperature have been reported (Wirsén & Jannasch, 1970; Harder *et al.*, 1977). Furthermore the postulated mechanism of the selection of 'hyper' *E. coli* mutants (Horiuchi *et al.*, 1962) assumes that the increased β -galactosidase levels in these mutants influence their K_s value for lactose (see Dean, 1972). Whether the μ - s relationships were influenced in our bacteria by culture D was not determined. A third possibility for the discrepancy is that factors related to the interaction of the two populations in mixed cultures contributed to the advantage of *Spirillum* sp. Apart from excluding the production by *Spirillum* sp. of an inhibitor of the growth of *Pseudomonas* sp., the data provide no further information on this point.

Although much remains to be learned about the reasons for the selective advantage of *Spirillum* sp. in carbon-limited environments, the data clearly establish that in response to the different niches which these bacteria occupy in nature – *Spirillum* sp., those scarce in nutrients, and *Pseudomonas* sp., richer ones capable of supporting rapid growth – the two bacteria have evolved a variety of different physiological and morphological characteristics.

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