Physiology and morphology of *Legionella pneumophila* in continuous culture at low oxygen concentration

W. STUART MAUCLINE,* ROSA ARAUJO,† ROBIN WAIT, A. BARRY DOWSETT, P. JULIAN DENNIS‡ and C. WILLIAM KEEVIL

Pathology Division, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wilts. SP4 0JG, UK

(Received 22 May 1992; revised 17 July 1992; accepted 13 August 1992)

Two strains of *Legionella pneumophila* serogroup 1 monoclonal subgroup Pontiac were grown for the first time in continuous culture using a chemically defined medium. The influence of temperature on physiology and morphology was investigated by fixing the growth rate (equal to the dilution rate, D) at 0.08 h^{-1} and controlling the pH and dissolved oxygen concentration of the culture. Serine provided the principal source of carbon and energy but growth was limited by tyrosine. The bacterium behaved as a microaerophile in this medium, with maximal growth occurring at $0.31\text{ (mg O}_2\text{)}\text{ l}^{-1}$ (equivalent to a dissolved oxygen tension of 4% (v/v) air saturation at 30°C). The cultures consisted of flagellated, short rods at 24°C , but exhibited an increased level of pleomorphism and the loss of flagella as the temperature was increased to 37°C . The presence of intracellular granules was noted, and their abundance was temperature-dependent. Polyhydroxybutyrate was present in *L. pneumophila*, and the proportion of the cell dry weight that it accounted for varied with temperature, being maximal at 24°C . The ratio of saturated to unsaturated fatty acids in the cells decreased as the temperature was reduced towards 24°C , so as to maintain membrane fluidity at low growth temperature.

Introduction

The principal aetiological agent of Legionnaires' disease, *Legionella pneumophila*, has been isolated from a wide range of habitats, including natural surface waters (Fliermans *et al.*, 1981) and man-made systems (Dennis *et al.*, 1982; Bartlett *et al.*, 1983), which vary both physically and chemically. Thermotolerance is one of the most interesting characteristics of legionellae: Wadowsky *et al.* (1986) demonstrated that naturally occurring *L. pneumophila* could multiply between 25 and 37°C ; Dennis *et al.* (1984) showed that there was no significant loss of viability at 46°C ; and Fliermans *et al.* (1981) isolated *L. pneumophila* from natural surface waters between 5.7 and 63°C . Man-made water systems such as cooling towers, hot water systems and whirlpool spas can act as amplifiers of legionellae numbers and as

generators of respirable aerosols, and have thus been the main sources of both cases and outbreaks of legionellosis.

In such systems, the growth of bacteria is probably sub-optimal due to the low availability of nutrients. It is not possible to sustain growth in batch culture under such conditions; in continuous culture however, it is possible to maintain steady-state growth at almost any growth rate for as long as is required. In addition to providing a constant chemical environment, chemostat culture allows the growth rate to be fixed whilst environmental parameters are changed (Monod, 1950; Novick & Szilard, 1950; Herbert *et al.*, 1956). For these reasons the chemostat was chosen as the most appropriate tool for investigating the influence of environment on *L. pneumophila*.

Early studies suggested that legionellae were fastidious and required amino acids, especially L-cysteine (George *et al.*, 1980; Tesh & Miller, 1981; Ristroph *et al.*, 1981), and iron, possibly in the ferric form (Feeley *et al.*, 1978). In the environment legionellae may obtain essential nutrients via associations with amoebae (Rowbotham, 1980) or with other micro-organisms in biofilms (Keevil *et al.*, 1988). Legionellae are unusual among Gram-negative bacteria in that their principal sources of

* Author for correspondence. Tel. (0980) 610391 ext. 2390; fax (0980) 611096.

† Present address: Departamento de Microbiología, Universidad de Barcelona, Av. Diagonal 645, Barcelona 08071, Spain.

‡ Present address: Spencer Harris Laboratory, Thames Water Utilities, Manor Farm Road, Reading RG2 0JN, UK.

carbon and energy are amino acids; glucose and polysaccharides being used very poorly (Hoffman, 1984).

Previous studies have shown that *L. pneumophila* can be grown in continuous culture, but only using complex media (Berg *et al.*, 1985; Dennis, 1986). Such media do not reflect the conditions experienced by legionellae in the environment, either in water systems or *in vivo*. Moreover, complex media are inappropriate for physiological studies since it is not only difficult to determine the limiting nutrient but also almost impossible to define the available concentrations of nutrients such as amino acids and vitamins. Accordingly, we have used the defined medium of Pine *et al.* (1986) which has proved suitable for the growth of legionellae in batch culture. This present study describes the influence that temperatures which are normally encountered *in vitro* and *in vivo* have on the physiology and morphology of two strains of *L. pneumophila* serogroup 1 monoclonal subgroup Pontiac.

Methods

Strain and culture. The chemostat was inoculated with either the environmental strain 74/81 or the clinical isolate Corby. The strains used were stored as individual aliquots at -80°C , and were grown on Buffered Charcoal Yeast Extract (BCYE) agar (Edelstein, 1981) for 72 h at 37°C prior to inoculation of the chemostat. The one litre chemostat vessel contained 500 ml ACES-buffered chemically defined medium (ABCD; Pine *et al.*, 1986). The culture medium was initially purged with oxygen-free nitrogen (BOC) and maintained at an oxygen concentration of 0.31 mg l^{-1} [equivalent to 4% (v/v) of air saturation at 30°C] through feedback control of the agitation rate, by an Anglicon Microlab Fermentation Control System (Brighton Systems, Newhaven, UK) linked to a galvanic oxygen electrode (Uniprobe, Cardiff). Temperature and pH were controlled using appropriate Anglicon Microlab controllers linked to an Anglicon temperature probe (which also provided a signal for temperature compensation of the pH controller) and an Ingold pH electrode (Mettler-Toledo, Leicester, UK). The pH was controlled to 6.9 by the addition of potassium hydroxide (1 M). After inoculation the culture was grown in batch until the OD_{540} reached 0.4. Continuous culture was initiated at a dilution rate (D) of 0.03 h^{-1} , and then increased to 0.08 h^{-1} (equivalent to a mean generation time (MGT) of 8.7 h) when the OD_{540} had increased to approximately 1.0. OD_{540} and cell dry weight were used to assess when the steady state had been achieved.

Electron microscopy. Cells obtained from the chemostat were left in 0.2% formaldehyde for 2 h, applied to formvar/carbon filmed, 400 mesh copper EM specimen grids, and negatively stained with 1% (w/v) sodium silicotungstate. Alternatively, cells were fixed in 2.5% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide for embedding, thin sectioning and staining by the silver proteinate method of Thiéry (1967). Specimens were examined in a Philips EM 400T transmission electron microscope operated at 80 kV.

Culture analyses. The optical density and biomass concentration of the cultures were monitored as described previously (Keevil *et al.*, 1988). Ammonia, amino acids and cations in the uninoculated medium and the clarified supernatants were analysed by the methods of Ellwood *et al.* (1974). Total viable counts were determined by plating on to BCYE agar and incubating at 37°C .

Cell analyses. The presence of intracellular lipid granules was confirmed using Sudan Black stain (Burdon, 1946). Polyhydroxybutyrate (PHB) was extracted from washed cells by a modification of the method of Findlay & White (1983). Briefly, 0.5 g lyophilized cells were placed in a cellulose thimble (Whatman) and extracted with chloroform for several hours in a Soxhlet apparatus. After cooling, the extract was transferred to a 100 ml flask and the chloroform removed by rotary evaporation. The PHB was purified by washing 4 times with 3 ml portions of ice cold ethanol, until the washings were colourless. This procedure was repeated with four 3 ml portions of cold diethyl ether, and the residue was dried by rotary evaporation, producing a white membrane-like solid. The material was dissolved in 25 ml chloroform and the PHB concentration in 20 μl aliquots was determined by a modification of the method of Slepecky & Law (1960), as follows: 5 ml concentrated sulphuric acid was added to the dry PHB in a screw cap tube and after thorough mixing the tubes were heated at 100°C for 30 min. This procedure dehydrated the PHB to crotonic acid, the UV absorption of which was determined at 235 nm in silica cuvettes using a SP1800 spectrophotometer (Pye Unicam). A standard curve was constructed using PHB purified from *Alcaligenes* sp. (Sigma); determinations were done in triplicate and the standard deviation was never more than 3% of the mean.

Fatty acid and polar lipid extraction. Cellular fatty acid samples were prepared as described by Wait (1988). Briefly, freeze-dried cells were incubated overnight at 60°C with 2 ml toluene/methanol (1:1, v/v), containing 50 μl concentrated sulphuric acid, in a 5 ml screw-capped tube. The tubes were cooled, 1 ml of saturated salt solution was added, and the fatty acids extracted into hexane/chloroform (4:1, v/v). The extracts were dried in a stream of nitrogen, and redissolved in 1 ml trimethylpentane for gas chromatography.

Phospholipids were extracted by a modification of the procedure of Bligh & Dyer (1959). Approximately 2 mg freeze-dried biomass was shaken with 1 ml of a monophasic mixture of chloroform, methanol and water (1:2:0.8, by vol.). Additional water and chloroform (0.3 ml of each) were then added so as to force a phase separation. The organic layer containing the phospholipids was removed, washed with water, concentrated under nitrogen, and redissolved in 100 μl chloroform/methanol prior to analysis by positive and negative fast atom bombardment (FAB) mass-spectrometry.

The position of fatty acid substituents in the phospholipids were determined by digestion with phospholipase A_2 . The samples were dissolved in 3 ml diethyl ether/methanol (98:2, v/v), and 10 units *Crotalus atrox* phospholipase A_2 (Sigma, product number P-3770) in 0.5 ml borate buffer (pH 8.9) were added. After incubation overnight at 37°C with constant shaking, the digestion products were recovered by Bligh-Dyer extraction and analysed by FAB-MS.

Gas chromatography and mass spectrometry. Gas chromatography was performed with a Carlo-Erba 4130 chromatograph fitted with a $25\text{ m} \times 0.2\text{ mm}$ fused silica column, coated with BP-5 (SGE) using helium as carrier gas at a linear velocity of 30 cm s^{-1} . The samples were introduced by splitless injection (splitless time 30 s) at an initial oven temperature of 80°C . After a one minute delay, the oven temperature was programmed at $4^{\circ}\text{C min}^{-1}$ to 205°C , and this temperature was held for 15 min. All peaks were identified by gas-chromatography/mass-spectrometry using a Kratos MS80 RFA mass-spectrometer, interfaced to a Carlo-Erba 5160 chromatograph. Chromatographic conditions were as above, except that a 25M BP-1 (SGE) column was used. The instrument was operated in electron-impact mode at an ionization energy of 70 eV, trap current of 100 μA , and a scan rate of 0.3 s per decade of mass. FAB mass spectra were obtained with a Kratos MS80 RFA equipped with an Ion-Tech saddle field gun using xenon (8 keV) as the bombarding gas. The accelerating voltage was 4 kV and the magnet was scanned downwards from mass 1500 at 30 s per decade of mass. The polar lipid extracts (1 μl samples) were applied

to the stainless steel FAB target which had been previously coated with liquid matrix [dithiothreitol/diethylerythritol (5:1, v/v) containing 1% (v/v) 5-crown-15]. Scans were acquired under the control of the DS90 data system which had been previously calibrated with caesium iodide clusters over the mass range of interest.

Results

Strains 74/81 and Corby grew well in ABCD broth which has been found suitable for the growth of *L. pneumophila* serogroup 1 (Knoxville 1), *L. bozemanii* and *L. dumoffii* in batch culture (Pine *et al.*, 1986). However, growth of both strains was critically affected by the dissolved oxygen tension (DOT) of the culture. Preliminary chemostat experiments using several different levels of DOT indicated that maximal biomass concentration occurred at a DOT of 4% (v/v) air saturation at 30 °C [equivalent to 0.31 (mg O₂) l⁻¹]. This is consistent with reports of oxygen toxicity in *L. pneumophila* (Locksley *et al.*, 1982; Pine *et al.*, 1986). Since the DOT is influenced by temperature, the oxygen concentration was maintained at 0.31 mg l⁻¹ during growth at 24 °C and 37 °C by adjusting the DOT to 3.6% (v/v) and 4.5% (v/v), respectively. To promote growth the medium was deoxygenated with nitrogen until the DOT decreased to 4% (v/v), prior to inoculation. The DOT was kept low by restricting agitator speed until growth was established. Steady state growth, defined by a constant culture turbidity and cell yield [(g dry wt) l⁻¹], was achieved within 10 generations.

Nutrient utilization and limitation

Analysis of the clarified culture supernatants of strain 74/81 showed that although serine was the principal source of carbon and energy, it remained in excess during steady state growth at all temperatures (Table 1), in agreement with the findings of George *et al.* (1980) for batch culture of *L. pneumophila* using a similar medium. Metabolism of approximately 18.5 mM-serine was accompanied by the production of approximately 15 mM-ammonia. Increasing the growth temperature from 30 °C to 37 °C did not cause any significant change in the metabolism of serine; ammonia production, however, did increase. Lowering the growth temperature to 24 °C slightly decreased serine utilization. Similar results were obtained with the clinical strain Corby (data not shown).

Only a few amino acids were completely depleted at 24 °C but several more could not be detected in the culture supernatants at the higher growth temperatures. To ascertain whether any of these were actually growth-limiting, the concentrations of all the amino acids except serine were doubled in the incoming medium. The biomass concentration increased immediately and the

Table 1. Concentration of nutrients (mM) in unused ABCD broth and in culture filtrate

L. pneumophila 74/81 was grown in a chemostat at $D = 0.08 \text{ h}^{-1}$ with dissolved oxygen concentration of 0.31 mg l⁻¹ and pH 6.9, at the temperatures shown. Figures for unused broth are means of 11 samples, filtrate figures are means of three samples. Standard deviations were all less than 15% of the mean.

Nutrient	Unused medium	Culture filtrate growth temperature (°C)		
		24	30	37
Iron*	0.16	0.16	0.16	0.16
Potassium	21.85	20.46	20.89	23.38
Zinc*	0.08	0.08	0.08	0.08
Ammonium	7.30	22.25	21.67	24.00
Phosphate	1.64	1.02	0.99	1.07
Alanine	0.93	1.16	0.60	0.60
Arginine	0.54	ND	ND	ND
Asparagine	0.66	ND	ND	ND
Aspartate	0.62	0.18	0.25	0.08
Cysteine	0.68	0.26	0.32	0.58
Cystine	0.12	0.09	0.02	0.22
Glutamate	0.59	0.10	ND	0.13
Glutamine	0.54	0.10	ND	ND
Glycine	0.97	0.88	0.74	0.86
Histidine	0.36	0.06	0.05	0.05
Isoleucine	0.70	0.06	ND	0.05
Leucine	0.66	0.02	ND	0.04
Lysine	0.44	0.06	ND	ND
Methionine	0.59	0.20	0.06	0.04
Phenylalanine	0.41	ND	ND	ND
Proline	0.82	ND	ND	ND
Serine	19.03	0.57	0.24	0.21
Threonine	0.63	0.04	ND	ND
Tryptophan	0.28	0.20	0.22	0.22
Tyrosine	0.12	ND	ND	ND
Valine	0.76	0.12	ND	0.08

ND, not detected.

* Differences in the concentrations of iron and zinc between the medium and culture filtrate are most probably below the detection of the procedure used.

oxygen concentration decreased to zero, becoming growth-limiting, suggesting that growth in the original ABCD medium was limited by one of the amino acids but not serine. Supplementing the medium with a cocktail of several of the amino acids which were completely depleted at all three temperatures (asparagine, proline, phenylalanine, tyrosine and threonine; threonine was detectable at 24 °C, but at a very low level) similarly increased the yield and also resulted in oxygen-limited growth, indicating that one of these was growth-limiting. Doubling the concentration of tyrosine in the medium (to 0.56 mM) gave an increase in OD₅₄₀ of 0.4 and an increase in dry weight of approximately 11%, confirming tyrosine as the growth-limiting nutrient. These findings would also suggest that the other amino acids in the cocktail are not essential. Further supplementation of the medium with serine resulted in an

Table 2. Growth characteristics of two different strains of *L. pneumophila*

Bacteria were grown in continuous culture at $D = 0.08 \text{ h}^{-1}$, $0.31 \text{ (mg dissolved oxygen)} \text{ l}^{-1}$ and pH 6.9, at the temperatures shown.

Characteristic	Growth temperature ($^{\circ}\text{C}$)				
	24		30	37	
	74/81	Corby	74/81	74/81	Corby
OD ₅₄₀	2.7	1.8	3.6	2.8	3.4
Biomass [(g dry wt) l ⁻¹]*	0.946	0.595	1.262	1.054	1.317
c.f.u. ml ⁻¹ ($\times 10^9$)†	3.8 ± 0.62	1.5 ± 0.39	7.2 ± 0.85	2.5 ± 0.50	6.2 ± 0.79
Y_{serine} (g mol ⁻¹)	51.2	45.7	67.2	56.0	70.2
q_{serine} (mmol g ⁻¹ h ⁻¹)	1.6	1.8	1.2	1.4	1.2
PHB (% of cell dry wt)	4.3	ND	2.9	1.5	ND

ND, not determined.

* Means of triplicate samples, standard deviations less than 5% of mean in all cases except Corby grown at 24°C (9.7% of mean).

† $\pm$ SEM.

additional increase in biomass concentration, indicating that serine was then limiting. The essential trace elements, including iron and zinc, remained in excess throughout the experiments.

Growth yield

The results of representative chemostat runs using each strain are given in Table 2. Similar results were produced on repeat experiments using fresh inocula. Three runs were carried out using strain 74/81 as the inoculum and two with strain Corby. The culture OD₅₄₀, biomass, molar growth yield for serine (Y_{serine}) and viable count indicated that 74/81 grew well at a temperature of 24°C (Table 2). This correlated with a high specific rate of serine metabolism (q_{serine}). Raising the temperature from 24°C to 30°C caused an increase in the culture turbidity, biomass, Y_{serine} and viable count, which was associated with a 25% decrease in q_{serine} as the growth efficiency improved. The $Y_{\text{potassium}}$ and $Y_{\text{phosphate}}$ also increased by 93% and 27% respectively. This temperature appeared optimal for growth of strain 74/81, since increasing the temperature to 37°C resulted in a lower OD₅₄₀ and poorer recovery of viable bacteria. The biomass, Y_{serine} and q_{serine} however, were less affected, suggesting that the mean weight of individual cells had increased. Strain Corby exhibited a much greater increase in yield when grown at 37°C rather than at 24°C , as indicated by an increase in cell dry weight of 121% and an increase of 54% in Y_{serine} (Table 2).

Morphology

Changes in the growth temperature resulted in morphological differences (Figure 1). At 24°C , short fine rods,

approximately $1.3 \mu\text{m} \times 0.25 \mu\text{m}$ were observed, many of them bearing a single sub-polar flagellum ($3.6\text{--}5 \mu\text{m}$ long). Free flagella were also present, these having presumably been sheared off by the rapid agitation of the culture. No flagella were observed at either 30°C or 37°C , either attached to the cells or free in the culture. The absence of flagella on cells grown at 30°C and 37°C may be due to the increase in shear force caused by the higher agitator speeds required to maintain the oxygen concentration at these temperatures (due to lower oxygen solubility and increased demand for oxygen resulting from the greater biomass concentrations at these temperatures), and failure to observe free flagella in the electron microscope preparations. Some of the cells appeared slightly elongated at 30°C ($1.8 \mu\text{m} \times 0.4 \mu\text{m}$) and intracellular inclusions could be seen in the majority. The culture exhibited a high degree of pleomorphism at 37°C with long rods predominating. These were up to $8 \mu\text{m}$ in length and intracellular inclusions of up to $0.5 \mu\text{m}$ diameter were observed in a proportion of the cells. Although there are numerous examples of growth environment influencing cell morphology (e.g. Herbert, 1961; Brown & Hough, 1965) it should be noted that a direct relationship between agitator speed and mean cell volume was reported by Wase *et al.* (1982).

The negatively-stained appearance of inclusion granules either remaining inside or released from the cell envelope of lysed bacteria suggested that they were indeed solid granules, and not vacuoles (Fig. 2). Discrete granules were also observed with a light microscope after staining the cells with the lipophilic stain Sudan Black. Chemical analysis of the bacteria confirmed the presence of the well-known microbial carbon storage compound polyhydroxybutyrate (PHB). PHB comprised 4.3% of the dry weight of cells grown at 24°C but decreased to

Fig. 1. Transmission electron micrographs of *L. pneumophila* 74/81 grown under defined conditions in continuous culture at (a) 24 °C, showing short rods with a single polar flagellum (F); (b) at 30 °C, cells of similar size and shape, but without flagella; and (c) at 37 °C, the culture exhibiting pleomorphism, with the presence of both long and short rods. Bar, 1 µm.

1.5% at 37 °C (Table 2). The proportion of cells containing granules did not, however, correlate with the PHB content of the biomass, therefore other possibilities were investigated. Thin sections of bacteria were examined by electron microscopy after treatment with the polysaccharide-specific silver proteinate stain of Thiéry (1967). The intracellular granules did not take up the stain, which indicates that they are not composed of glycogen or other polysaccharides.

Extracellular material is frequently observed in Gram-negative bacteria, including *L. pneumophila* grown in complex media (Dennis, 1986) but none was observed in the present study.

Although the cultures were transiently brown-pigmented in the initial batch culture phase, no pigmentation was observed during continuous culture. However, when continuous cultures were pulsed with excess tyrosine brown, pigmentation was observed, probably due to melanin formation following tyrosinase activity.

Membrane fatty acid content

The fatty acid composition of *L. pneumophila* is unusual among Gram-negative bacteria because of the predominance of branched chain acids, of which 14-methyl pentadecanoic acid (i-16:0) was the most abundant

when the organism was grown on BCYE agar at 37 °C (Moss *et al.*, 1977). A similar fatty acid profile was obtained in continuous culture at 37 °C using defined medium (Table 3). However, as the temperature of the culture was lowered, there was a progressive reduction in the relative proportion of saturated acids, particularly i-16:0 and i-14:0, and a corresponding increase in unsaturated acids so that at 24 °C the most abundant acid was 9-hexadecenoic acid, and the proportion of i-16:0 had fallen to less than 20% of the total (Table 3). The 9,10 methylenehexadecanoic acid (cyclopropane 17), a significant constituent of legionellae grown on BCYE agar (Moss *et al.*, 1977) was barely detectable in bacteria grown at 37 °C in continuous culture and was absent at 24 °C.

Phospholipid analysis

FAB-MS was used to record profiles of intact phospholipids at the various temperatures. Two representative spectra are shown in Fig. 3. Signals in the region m/z 664–706 represent the protonated molecular ions of phosphatidylethanolamines and *N*-methylphosphatidylethanolamines, while those between m/z 718 and 748 correspond to phosphatidylcholines, all varying in their pattern of fatty acid substitution. The cluster of ions between 523 and 563 are fragments derived from the loss of the polar head group from phosphatidylethanolamines and phosphatidylglycerols, with charge retention on the glycerol backbone fragment.

Several differences are apparent between the 37 °C and the 24 °C samples. Firstly, as the temperature is reduced, the proportion of phospholipids containing an unsaturated fatty acid substituent is increased; thus the

Fig. 2. Transmission electron micrographs of *L. pneumophila* 74/81 grown under defined conditions in continuous culture at various temperatures, showing the presence of intracellular granules. (a) A field showing cells grown at 30 °C, most cells have at least one granule, and (b) 37 °C, showing the cell envelope of long cell containing several granules. Bar, 1 µm.

Table 3. Influence of temperature on the membrane fatty acid composition (%) of *L. pneumophila* grown in continuous culture

Temperature	Fatty acid									
	i14:0	a15:0	i16:1	i16:0	16:1	16:0	a17:1	a17:0	17:0	18:0
44 °C	3	5	<1	36	12	12	<1	19	3	9
37 °C	2	9	1	31	23	11	<1	11	6	8
30 °C	1	7	2	32	30	10	1	6	2	6
24 °C	<1	7	4	17	30	10	3	15	4	4

Fig. 3. Part of the positive ion FAB mass spectra of *L. pneumophila* strain 74/81 grown under defined conditions in continuous culture at (a) 37 °C and (b) 24 °C.

signals m/z 690, 704 and 718 are significantly enhanced at low temperature. This tendency is particularly pronounced in the phosphatidylcholines, as the signals m/z 734 and 748 are virtually absent at 24 °C. In general, the unsaturated phospholipid species included only one double-bond-containing substituent. Digestion with phospholipase A₂ followed by FAB-MS demonstrated that these were almost exclusively located on position 2 of the glycerol. The reduced synthesis of tetradecanoic acid at low temperature is reflected in the low intensity of m/z 664 at 24 °C, while the slightly increased production of ai17:0 at this temperature (Table 3) results in an enhancement of m/z 678. Finally, there appears to be an overall reduction of the relative proportion of phosphatidylcholines as the temperature is lowered.

Discussion

The use of a chemically defined medium in conjunction with continuous culture has permitted a detailed investigation of how the growth environment modulates the physiology and morphology of *L. pneumophila*. Serine provided the principal source of carbon, nitrogen and energy, and tyrosine proved to be the growth-limiting nutrient. Only 15 mM-ammonia was produced from the 18.5 mM-serine metabolized at 24 °C, so the remainder was presumably incorporated into biomass, since the deamination of serine should result in a stoichiometric production of ammonia.

This study, in common with others, has shown that temperature has a pronounced effect on the cellular

morphology of *L. pneumophila*. Pine *et al.* (1979) used a similar medium supplemented with casein hydrolysate and, in batch culture, found coccobacillary *L. pneumophila* at 24 °C which became pleomorphic above 30 °C. Later, Berg *et al.* (1985) observed pleomorphism at 37 °C and 44 °C in continuous culture using a complex yeast extract medium supplemented with starch and cysteine. However, neither study commented on the presence or absence of flagella. Dennis (1986) noted induction of flagella in continuous culture at low temperature, using a complex yeast extract medium, and recently Ott *et al.* (1991) showed that in two strains of *L. pneumophila* the proportion of cells bearing flagella decreased with increasing growth temperature. The motility provided by flagella confers obvious benefits on aquatic micro-organisms; *in vivo*, however, flagella may not only be unnecessary, but may indeed have a detrimental effect on the survival of the bacteria due to their antigenicity.

Intracellular granules were discernible in electron micrographs and were observed by light microscopy after staining with the lipid stain Sudan Black. Although the presence of intracellular granules has not been commented on previously by physiologists and biochemists working with legionellae, such granules have been observed *in vivo* by microscopists. For example, Chandler *et al.* (1979) noted the presence of lipid granules in legionellae growing in alveolar macrophages at 37 °C. Similar observations were made for legionellae growing in yolk sac membranes (Rodgers, 1979), the protozoan *Tetrahymena pyriformis* (Fields *et al.*, 1986) and the amoeba *Acanthamoeba palestensis* at 35 °C (Anand *et al.*, 1983). By contrast, few granules were observed in

legionellae growing in *Acanthamoeba polyphaga* at a lower temperature of 30 °C (Rowbotham, 1984). The apparent influence of temperature on granule formation was confirmed by the present study, in which maximum granule formation was noted at 30 °C, significantly fewer being observed at 37 °C and very few at 24 °C. Since the abundance of granules did not correlate to the PHB content of the biomass, and the granules do not appear to be polysaccharide, their precise nature remains to be established. Chemical analysis confirmed that the cells contained the storage compound PHB (Mauchline & Keevil, 1991; R. Wait, W. S. Mauchline, R. Araujo and C. W. Keevil, unpublished results). PHB comprised 4.3% of the cell dry weight when bacteria were grown at 24 °C and decreased to 1.5% when the growth temperature was increased to 37 °C. PHB synthesis was not completely abolished, probably because the bacteria were not carbon-limited, as serine remained in excess at all temperatures. At 24 °C, *L. pneumophila* exhibited the lowest efficiency of biomass production for serine metabolized (q_{serine}), however, the PHB content of the cells was maximal at this temperature, suggesting that the accumulation of this storage compound is favoured at lower temperatures.

The assumed pathway for PHB synthesis from amino acids such as serine is via pyruvate and acetyl CoA (Dawes & Senior, 1973):

tions in this study. They may therefore need alternative electron acceptors to recycle cofactors.

A previous study has indicated that there was no significant difference between the fatty acid profiles of *L. pneumophila* grown on BCYE agar or in continuous culture using complex yeast extract medium at comparable temperatures, except that the production of 9,10-methylene hexadecanoic acid (cyclopropane 17) was much reduced in the chemostat (Wait, 1988). Similar results were obtained in the present study using a defined medium, except that at 24 °C the cyclopropane 17 acid is completely absent. In other micro-organisms (Halper & Norton, 1975), including *L. feeleeii* (Moss *et al.*, 1983), it has been observed that cyclopropane acids accumulate as the culture ages, the rate of their synthesis being lowest in the exponential phase. Hence it is unsurprising that concentrations are low in cells grown in continuous culture, where the majority of cells are by definition in the exponential phase.

Bacteria can only grow if their cell membranes are in a largely fluid state; i.e. if the temperature is above the gel to liquid-crystalline transition temperature of their membrane lipids so as to ensure the maintenance of essential transport functions. Thermotolerant organisms, such as legionellae, thus require adaptive mechanisms to ensure that their membranes are fluid across a wide temperature range. Generally, such adaptive

The synthesis of a carbon reserve such as PHB would provide an essential source of carbon and energy for survival in low-nutrient environments. Legionellae are able to metabolize exogenous 3-hydroxybutyric acid as a carbon source (Mauchline & Keevil, 1991) suggesting that they are able to grow following hydrolysis of the intracellular PHB reserve. This would explain their ability to maintain their ATP content for many months with no additional nutrient source (West *et al.*, 1989). It is also possible that PHB provides a carbon and energy supply for growth in macrophage and amoebae.

The PHB biosynthetic pathway also provides a mechanism to reoxidize NAD(P)H to NAD(P) to continue essential metabolic processes. Aerobes normally oxidize NAD(P)H via the respiratory chain with oxygen as the terminal electron acceptor. Legionellae, however, behave as microaerophiles (Hoffman *et al.*, 1983; Locksley *et al.*, 1982) and were inhibited by oxygen concentrations above 1 mg l⁻¹ under the condi-

strategies involve the synthesis of membrane constituents of lower melting point at reduced growth temperature. This can be achieved by switching fatty acids from saturated to unsaturated, from unbranched to branched, from iso- to ante-iso branched, by reducing acyl chain length, and by alteration of phospholipid type (Melchoir, 1982). The present data suggest that in *L. pneumophila* the major mechanism at work is increased production of unsaturated fatty acyl chains at low growth temperature. There is no evidence of any tendency towards acyl chain shortening. It is however significant that the reduction in saturated acid content is achieved almost entirely at the expense of straight chain and iso-branched acids, which have a significantly higher melting temperature than their ante-iso branched isomers (Rilfors *et al.*, 1978). The alterations in phospholipid composition are less easy to interpret. The reduced content of phosphatidylcholines at low temperature seems surprising, as phosphatidylcholines have a 20 °C lower melting temperature than

phosphatidylethanolamines with identical acyl substituents (Chapman *et al.*, 1967; Wilkinson & Nagle, 1981). However, since this is due to packing effects and the greater ability of the ethanolamine head group to form inter-molecular hydrogen bonds, the effect is likely to be less pronounced in mixed phospholipids. It would seem likely that increasing the proportion of unsaturated substituents in phospholipids of all types has a greater effect on phase transition temperatures than alterations to the polar head group.

In conclusion, the data obtained on the growth yields of *L. pneumophila*, and on its nutrient utilization are proving useful in our continuing investigation of the influence of environmental parameters such as temperature and iron-limitation on the virulence of *L. pneumophila*.

We should like to thank Dr C. Davies and his department for providing chemical analyses of culture filtrates and media samples, and Mr M. Riches and his section for media preparation. We gratefully acknowledge the Medical Research Council of Great Britain (Project grant G8823698SB) for funding this work.

References

- ANAND, C. M., SKINNER, A. R., MALIC, A. & KURTZ, J. B. (1983). Interaction of *L. pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*). *Journal of Hygiene, Cambridge* **91**, 167–178.
- BERG, J. D., HOFF, J. C., ROBERTS, P. V. & MATIN, A. (1985). Growth of *Legionella pneumophila* in continuous culture. *Applied and Environmental Microbiology* **49**, 1534–1537.
- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- BROWN, C. M. & HOUGH, J. S. (1965). Elongation of yeast cells in continuous culture. *Nature, London* **206**, 676–678.
- BURDON, K. L. (1946). Fatty acid material in bacteria revealed by staining dried, fixed slide preparations. *Journal of Bacteriology* **52**, 665.
- CHANDLER, F. W., BLACKMON, J. A., HICKLIN, M. D., COLE, R. M. & CALLAWAY, C. S. (1979). Ultrastructure of the agent of Legionnaires' disease in the human lung. *American Journal of Clinical Pathology* **71**, 43–50.
- CHAPMAN, D., WILLIAMS, R. M. & LADBROOK, B. D. (1967). Physical studies of phospholipids. *Chemistry and Physics of Lipids* **1**, 445–476.
- DAWES, E. A. & SENIOR, P. J. (1973). The role and regulation of energy reserve polymers in microorganisms. *Advances in Microbial Physiology* **10**, 135–266.
- DENNIS, P. J. (1986). *Environmental factors affecting the survival and the pathogenicity of Legionella pneumophila*. PhD thesis, University of Warwick, UK.
- DENNIS, P. J., TAYLOR, J. A., FITZGEORGE, R. B., BARLETT, C. L. R. & BARROW, G. I. (1982). *Legionella pneumophila* in water plumbing systems. *Lancet* **1**, 949–951.
- DENNIS, P. J., GREEN, I. & JONES, B. P. C. (1984). A note on the temperature tolerance of *Legionella*. *Journal of Applied Bacteriology* **56**, 349–350.
- EDELSTEIN, P. H. (1981). Improved semi-selective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *Journal of Clinical Microbiology* **14**, 298–303.
- FEELEY, J. C., GORMAN, G. W., WEAVER, R. E., MACKEL, D. C. & SMITH, H. W. (1978). Primary isolation media for Legionnaires' disease bacterium. *Journal of Clinical Microbiology* **8**, 320–325.
- FIELDS, B. S., BARBAREE, J. M., SHOTTS, E. B., FEELEY, J. C., MORILL, W. E., SANDEN, G. N. & DYKSTRA, M. J. (1986). Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. *Infection and Immunity* **53**, 553–559.
- FINDLAY, R. H. & WHITE, D. C. (1983). Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Applied and Environmental Microbiology* **45**, 71–78.
- FLIERMANS C. B., CHERRY, W. B., ORRISON, L. H., SMITH, S. J., TISON, D. L. & POPE, D. H. (1981). Ecological distribution of *Legionella pneumophila*. *Applied and Environmental Microbiology* **41**, 9–16.
- GEORGE, J. R., PINE, L., REEVES, M. W. & HARRELL, W. K. (1980). Amino acid requirements of *Legionella pneumophila*. *Journal of Clinical Microbiology* **11**, 286–291.
- HALPER, L. A. & NORTON, S. J. (1975). Regulation of cyclopropane fatty acid biosynthesis by variation in enzyme activities. *Biochemical and Biophysical Research Communications* **62**, 683–688.
- HERBERT, D. (1961). The chemical composition of micro-organisms as a function of their environment. *Symposia of the Society for General Microbiology* **11**, 391–416.
- HERBERT, D., ELSWORTH, R. & TELLING, R. C. (1956). The continuous culture of bacteria: a theoretical and experimental study. *Journal of General Microbiology* **14**, 601–622.
- HOFFMAN, P. (1984). Bacterial physiology. In *Proceedings of the 2nd International Symposium on Legionella*, pp. 61–67. Edited by C. Thornsberry, A. Balows, J. C. Feeley & W. Jakubowski. Washington, DC: American Society for Microbiology.
- KEEVIL, C. W., GLENISTER, D. A., SALAMON, K. E., DENNIS, P. J. & WEST, A. A. (1988). A continuous culture biofilm model for the study of medical and industrial corrosion. In *Biofilms*, pp. 48–62. Edited by L. H. G. Morton & A. H. L. Chamberlain. Kew: Biodeterioration Society Press.
- LOCKSLEY, R. M., JACOBS, R. F., WILSON, C. B., WEAVER, W. M. & KLEBANOFF, S. J. (1982). Susceptibility of *Legionella pneumophila* to oxygen-dependent microbiocidal systems. *Journal of Immunology* **129**, 2192–2197.
- MAUCHLINE, W. S. & KEEVIL, C. W. (1991). Development of the BIOLOG substrate utilisation system for identification of *Legionella* spp. *Applied and Environmental Microbiology* **57**, 3345–3349.
- MELCHOIR, D. L. (1982). Lipid phase transitions and regulation of membrane fluidity in prokaryotes. *Current Topics in Membranes and Transport* **17**, 263–316.
- MONOD, J. (1950). La technique de culture continue; théorie et applications. *Annales de l'Institut Pasteur* **79**, 390–410.
- MOSS, C. W., WEAVER, R. E., DEES, S. B. & CHERRY, W. B. (1977). Cellular fatty acid compositions of isolates from Legionnaires' disease. *Journal of Clinical Microbiology* **6**, 140–143.
- MOSS, C. W., BIBB, W. F., KARR, D. E., GUERRANT, G. O. & LAMBERT, M. A. (1983). Cellular fatty acid composition and ubiquinone content of *Legionella feeleyi* sp. nov. *Journal of Clinical Microbiology* **18**, 917–919.
- NOVICK, A. & SZILARD, L. (1950). Experiments with the Chemostat on spontaneous mutations of bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **36**, 708–719.
- OTT, M., MESSNER, P., HEESEMANN, J., MARRE, R. & HACKER, J. (1991). Temperature-dependent expression of flagella in *Legionella*. *Journal of General Microbiology* **137**, 1955–1961.
- PINE, L., GEORGE, J. R., REEVES, M. W. & HARRELL, W. K. (1979). Development of a chemically defined medium for growth of *Legionella pneumophila*. *Journal of Clinical Microbiology* **9**, 615–626.
- PINE, L., HOFFMAN, P. S., MALCOM, G. S., BENSON, R. F. & FRANZUS, M. J. (1966). Role of keto acids and reduced oxygen scavenging enzymes in the growth of *Legionella* species. *Journal of Clinical Microbiology* **23**, 33–42.
- RILFORS, L., WIESLANDER, A. & STAHL, S. (1978). Lipid and protein composition of membranes of *Bacillus megaterium* variants in the temperature range 5–70 °C. *Journal of Bacteriology* **135**, 1043–1052.
- RISTROPH, J. D., HEDLUND, K. W. & GOWDA, S. (1981). Chemically defined medium for *Legionella pneumophila* growth. *Journal of Clinical Microbiology* **13**, 115–119.
- RODGERS, F. G. (1979). Ultrastructure of *Legionella pneumophila*. *Journal of Clinical Pathology* **32**, 1195–1202.

- RODGERS F. G., GREAVES, P. W., MACRAE, A. D. & LEWIS, M. J. (1980). Electron microscopy evidence of flagella and pili on *Legionella pneumophila*. *Journal of Clinical Pathology* **33**, 1184–1188.
- ROWBOTHAM, T. C. (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *Journal of Clinical Pathology* **33**, 1179–1183.
- ROWBOTHAM, T. C. (1984) Legionellae and amoebae. In *Proceedings of the 2nd International Symposium on Legionellae*, pp. 325–327. Edited by C. Thornsberry, A. Balows, J. C. Feeley & W. Jakubowski. Washington DC: American Society of Microbiology.
- SLEPECKY, R. A. & LAW, J. H. (1960). A rapid spectrophotometric assay of alpha, beta-unsaturated acids and beta-hydroxy acids. *Analytical Chemistry* **32**, 1697–1699.
- TESH, M. J. & MILLER, R. D. (1981). Amino acids requirements for *Legionella pneumophila* growth. *Journal of Clinical Microbiology* **13**, 865–869.
- THIÉRY, J. P. (1967). Role de l'appareil de golgi dans la synthèse des mucopolysaccharides étude cytochimique. *Journal de Microscopie (Paris)* **8**, 689–708.
- WADOWSKY, R. M., WOLFORD, R., MCNAMARA, A. M. & YEE, R. B. (1986). Effect of temperature, pH and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology* **49**, 1197–1205.
- WAIT, R. (1988). Confirmation of the identity of legionellae by whole cell fatty-acid and isoprenoid quinone profiles. In *A Laboratory Manual for Legionella*, pp. 69–102. Edited by T. G. Harrison and A. G. Taylor. Chichester: John Wiley and Sons.
- WASE, D. A. J., NESARATAM, S. T. & BLAKEBROUGH, N. (1982). Variation of cell wall strength in *Klebsiella pneumoniae* NCTC 418 with change in agitation speed in a chemostat system. *Journal of Chemical Technology and Biotechnology* **32**, 553–555.
- WEST, A. A., ARAUJO, R., DENNIS, P. J. L., LEE, J. V. & KEEVIL, C. W. (1989). Chemostat models of *Legionella pneumophila*. In *Airborne Deteriogens and Pathogens*, pp. 107–116. Edited by B. Flannigan. Kew: Biodeterioration Society Press.
- WILKINSON, D. A. & NAGLE, J. F. (1981). Dilatometry and calorimetry of saturated phosphatidylethanolamine. *Biochemistry* **20**, 187–192.