

Temperature-dependent expression of flagella in *Legionella*

MANFRED OTT,^{1*} PAUL MESSNER,² JÜRGEN HEESEMANN,³ REINHARD MARRE⁴ and JÖRG HACKER¹

¹Institut für Genetik und Mikrobiologie, Universität Würzburg, Röntgenring 11, W-8700 Würzburg, Germany

²Zentrum für Ultrastrukturforschung und Ludwig Boltzmann-Institut für Ultrastrukturforschung, Universität für Bodenkultur, A-1180 Wien, Austria

³Institut für Hygiene und Mikrobiologie, Universität Würzburg, Josef Schneider Strasse 2, W-8700 Würzburg, Germany

⁴Institut für Medizinische Mikrobiologie, Universität Lübeck, Ratzeburger Allee 160, W-2400 Lübeck, Germany

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Legionella pneumophila, the causative agent of Legionnaires' disease, was analysed by electron microscopy for production of surface structures. Crystalline surface (S-) layers and fimbriae were not detected, but monotrichous flagellation was seen. Polyclonal antibodies specific for the 47 kDa flagellin subunit of *L. pneumophila* Philadelphia I were used in Western blots to confirm the presence of flagella subunits in various *L. pneumophila* strains tested, but the antiserum also reacted with flagellin subunits of *L. micdadei*, *L. hackelia* [serogroup (SG) 1 and SG2] and *L. longbeachae* (SG2). Flagellation of *Legionellae* was shown to be temperature regulated. When the growth temperature of virulent and avirulent variants of strain *L. pneumophila* Philadelphia I was shifted from 30 °C to either 37 or 41 °C, a decrease in the percentage of flagellated bacteria within the population was observed.

Introduction

Legionella pneumophila, the aetiological agent of a severe pneumonia called Legionnaires' disease, is a Gram-negative rod-shaped bacterium which is able to multiply in lung macrophages (Winn, 1988; Cianciotto *et al.*, 1989). The environmental source of *L. pneumophila* is water, where an association with free-living amoebae has been reported (Fields *et al.*, 1989). Fourteen distinct serogroups (SGs) of the species *L. pneumophila* and another 33 *Legionella* species, also distinguishable by serotyping, have been described. *Legionella* species other than *L. pneumophila* are also found in aquatic habitats and some of them are associated with human disease (Winn, 1988).

Virulence of *Legionella* strains is characterized *in vitro* by multiplication of bacteria in human monocytes or macrophage-like cell lines at 37 °C (Cianciotto *et al.*, 1989). *In vivo* models comprise aerosol- and intraperitoneally-infected guinea pigs, and cultivation in embryonated hen eggs (Baskerville *et al.*, 1981; Cianciotto *et al.*, 1989; Catrenich & Johnson, 1988; Elliott & Johnson, 1982). *Legionella* isolates are also able to invade free-

living amoebae at lower temperatures which is considered to be a prerequisite for their survival in aquatic habitats (Winn, 1988). There are few reports about the mechanisms involved in pathogenicity of *Legionella* (Cianciotto *et al.*, 1989, 1990; Hoffman *et al.*, 1990; Keen & Hoffman, 1989) and on the survival mechanisms in the environment (Kilvington & Price, 1990).

Surface structures, which are often involved in bacterial pathogenicity, have not been thoroughly described in *Legionella*. The chemistry of lipopolysaccharide, determining the different serogroups (Conlan & Ashworth, 1986), has been elucidated for *L. pneumophila* (Sonesson *et al.*, 1989). Capsules have not been detected (Hebert *et al.*, 1984), but some authors report the presence of fimbriae and flagella (Chandler *et al.*, 1980; Rodgers *et al.*, 1980). In this report we further analysed the surface of *L. pneumophila* for crystalline surface (S) layers (Sleytr & Messner, 1988*a*) and focussed our studies on the expression of flagella in *L. pneumophila* and other *Legionella* species.

Methods

Bacterial strains. Bacterial strains used are listed in Table 1. The avirulent derivative of *L. pneumophila* Philadelphia I, XXXV, has been described elsewhere (Bender *et al.*, 1990). The Philadelphia I strain

Abbreviations: BCYE, buffered charcoal yeast extract; S-layer, surface layer.

Table 1. *Analysis of flagellation of Legionella strains*

Strain	Flagellation*	Reaction with polyclonal antibodies†	Reference
<i>L. pneumophila</i> , SG1 Philadelphia I	+	+	ATCC 33152
<i>L. pneumophila</i> , SG1 XXXV avirulent	+	+	Bender <i>et al.</i> (1990)
<i>L. pneumophila</i> U ₁ SG1 water isolate	+	+	Bender <i>et al.</i> (1990)
<i>L. pneumophila</i> U ₂₁ SG1 water isolate	+	+	Bender <i>et al.</i> (1990)
<i>L. micdadei</i>	+	+	ATCC 33218
<i>L. longbeachae</i> , SG1	-	-	ATCC 33462
<i>L. longbeachae</i> , SG2	+	+	ATCC 33484
<i>L. hackelia</i> , SG1	+	+	ATCC 35250
<i>L. hackelia</i> , SG2	+	+	ATCC 35999
<i>L. dumoffii</i>	-	-	ATCC 33279
<i>L. oakridgensis</i>	-	-	ATCC 33761
<i>L. feeleii</i> , SG1	-	-	ATCC 35072
<i>L. feeleii</i> , SG2	+	-	ATCC 35849
<i>L. jordanis</i>	+	-	ATCC 33623

* Flagellation was determined by electron microscopy of bacteria grown at 30 °C.

† Determined by Western blot analysis using anti-*L. pneumophila* Philadelphia I flagellin antibodies (see text; cf. Fig. 4).

(ATCC 33152) has been passaged intraperitoneally in guinea pigs. Bacteria removed after the second passage from the peritoneum of the animal exhibiting symptomatic peritonitis were cultured once on buffered charcoal yeast extract (BCYE) agar and frozen. In this way fresh isolates were used for our studies. The avirulent derivative XXXV did not exhibit any infectivity after inoculation into the guinea pig peritoneum. The environmental *L. pneumophila* isolates originated from warm-water tanks. They were identified according to internationally accepted criteria (Harrison & Taylor, 1988) and subtyped by monoclonal antibodies for determining the serogroup according to Joly *et al.* (1983). All other strains were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). Strains were stored at -70 °C in 50% (v/v) glycerol.

Media and reagents. *Legionella* strains were cultured on BCYE at 37 °C with a 5% CO₂ atmosphere for 2-3 d (Edelstein, 1981) unless otherwise stated. Reagents for *Legionella* growth media were purchased from Oxoid. Peroxidase-conjugated anti-rabbit IgG antibodies were obtained from Dako. All other chemicals were a gift from Sigma.

Freeze etching. The bacteria were collected from the plates with a spatula loaded onto the specimen holder and frozen immediately by immersion into liquid Freon, cooled by liquid nitrogen. Freeze-etching was carried out in a Balzers BA 360M freeze-etching unit at -98 °C (175 K) and the samples were etched for 1.5 min. After unidirectional shadowing with platinum/carbon and backing with carbon, the replicas were cleaned in 30% chromium oxide for 2 d at room temperature. The micrographs were taken with a Philips EM 301 electron microscope (Messner *et al.*, 1984).

Electron microscopy. Bacteria grown on BCYE agar plates were carefully resuspended in distilled water on the agar plate and a drop of the suspension was directly applied to Formvar-coated copper grids. After sedimentation of the bacteria and removal of remaining fluid, the samples were shadowed with platinum/palladium and examined with a Zeiss 10A transmission electron microscope.

Isolation of flagella and SDS-PAGE. Bacteria grown on BCYE agar plates were resuspended in cold 50 mM-Tris/HCl (pH 6.8) 0.02% NaN₃. Cell appendages were removed by mixing the suspension in a Sorvall blender on ice to avoid autolysis of the bacteria. Mixing was carried out for 5 min three times with 5 min pauses between each procedure. Cells were removed by centrifugation and (NH₄)₂SO₄ was added to the supernatant to 15% saturation. Proteins were precipitated by stirring slowly overnight at 4 °C. After centrifugation at 30000 g the protein pellet was resuspended in Tris/HCl buffer and stored at 4 °C.

SDS-PAGE was performed according to the method of Laemmli (1970). Isolated flagella or whole bacterial cells were boiled for 10 min in Laemmli buffer and applied to the gels.

Preparation of polyclonal antibodies against whole bacteria. *L. pneumophila* ATCC 33152 cells grown on BCYE agar plates were suspended in 10 mM-PBS (pH 7.5) and immediately treated with 1% (v/v) formalin for 12 h. After washing, the bacteria (10⁹ ml⁻¹) were emulsified in equal volume of incomplete Freund's adjuvant (Difco). Two rabbits received approximately 1 ml emulsion subcutaneously in four to six sites in the scapular region of the back. After 4 weeks the procedure was repeated with live *L. pneumophila* suspended in PBS. Two weeks after the last injection, rabbits were exsanguinated. Serum was stored at -20 °C.

Affinity purification of anti-flagellin polyclonal antibodies. Anti-flagellin specific polyclonal antibodies were purified using the antiserum prepared against whole *L. pneumophila* Philadelphia I bacteria and the flagellin antigen was immobilized on nitrocellulose, as described by Sambrook *et al.* (1989).

Western blot analysis. Western blot analysis was performed according to the method of Towbin *et al.* (1979). Bacteria grown on BCYE were resuspended in distilled water. The OD₆₀₀ values of the suspensions were adjusted to 0.8. The suspension (1 ml) was centrifuged and the bacteria were resuspended in 100 µl of Laemmli buffer (Laemmli, 1970). After boiling for 5 min, 10 µl were loaded on to an SDS-PAGE

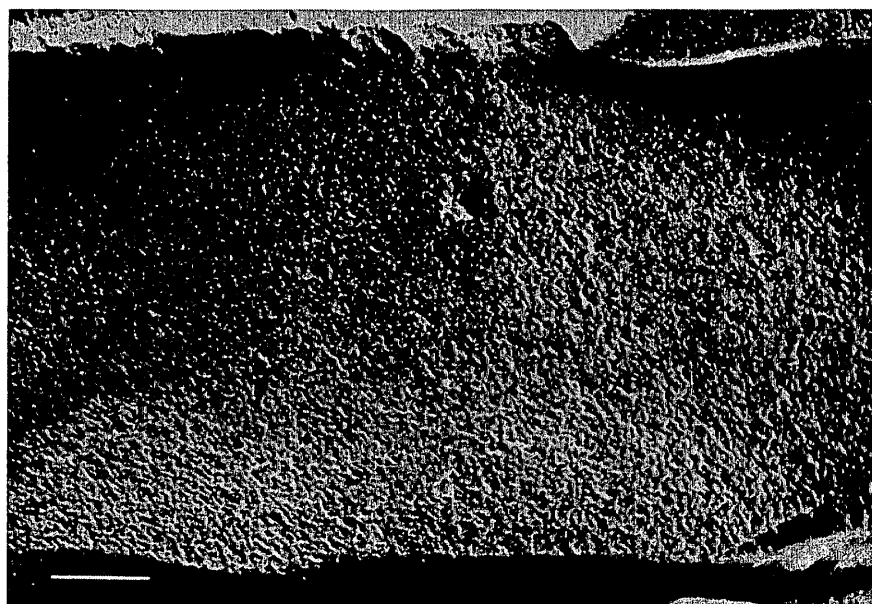


Fig. 1. Electron micrograph of a freeze-etched specimen of *L. pneumophila* Philadelphia I. Bar, 0.05 μm .

gel. As a control, approximately 0.5 μg of isolated flagella were applied. For the detection of bound antibodies, peroxidase-conjugated swine anti-rabbit IgG antibodies were used. The colour reaction was developed using 4,1-chloronaphthol.

Results

Surface examination of L. pneumophila by electron microscopy

To determine if *L. pneumophila* had crystalline S-layers, samples of four *L. pneumophila* isolates, including strain Philadelphia I, grown at 37 °C were prepared by freeze-etching and examined by electron microscopy. All the strains investigated had a smooth surface, indicating the absence of S-layers (Fig. 1). Examination of further samples prepared by platinum/palladium shadowing for detection of cell appendages, revealed the presence of flagella (Fig. 2a), while other organelles such as fimbriae were not detected. Most bacteria carried one polar flagellum, but non-flagellated cells were also observed.

Flagellation of L. pneumophila isolates and other Legionella species

A 47 kDa band, which represents the flagellum subunit, was present after SDS-PAGE of flagella isolated from *L. pneumophila* Philadelphia I (Fig. 3a, lane 2; cf. Elliott & Johnson, 1981). To detect anti-flagellin antibodies in an anti-*L. pneumophila* antiserum prepared against whole bacteria, a Western blot was performed with whole-cell

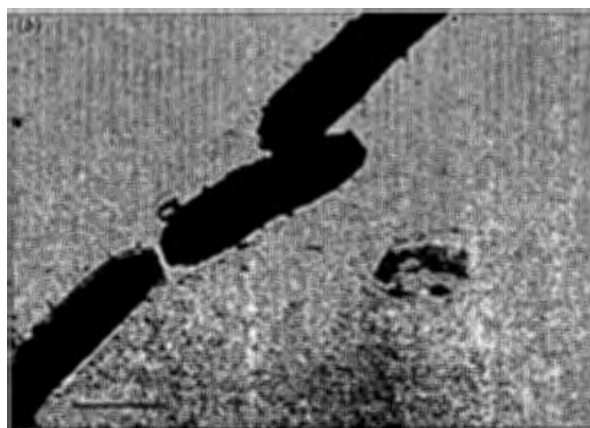
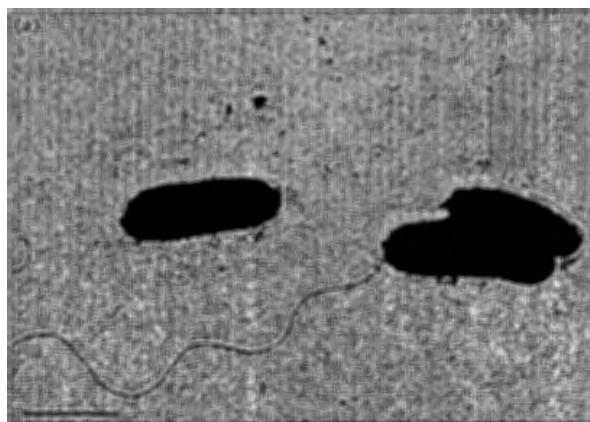


Fig. 2. Electron micrographs of *L. pneumophila* Philadelphia I grown at (a) 30 and (b) 41 °C. The samples were shadowed with platinum/palladium. Bars, 0.5 μm .

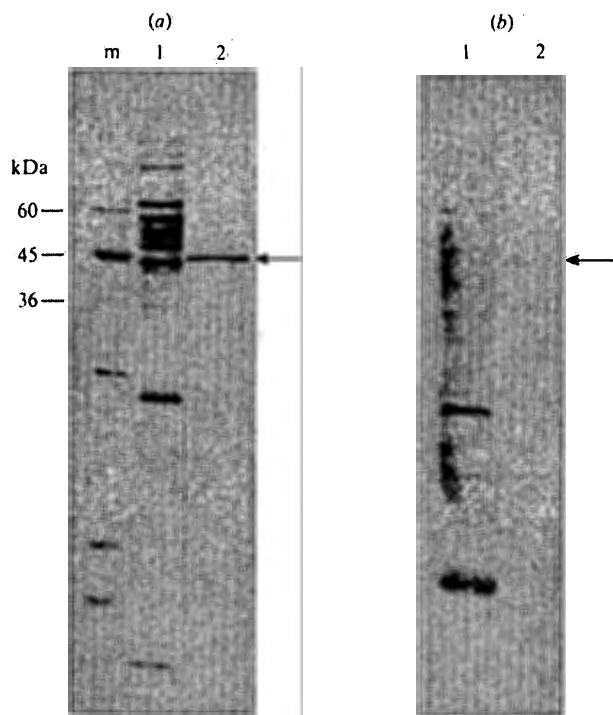


Fig. 3. (a) SDS-PAGE and (b) Western blot analysis of whole-cell extracts of *L. pneumophila* Philadelphia I (lane 1) and isolated flagella of this strain (lane 2), using antiserum prepared against whole *L. pneumophila* Philadelphia I bacteria. The arrows indicate the 47 kDa flagellin band. Molecular mass standards are shown in lane m.

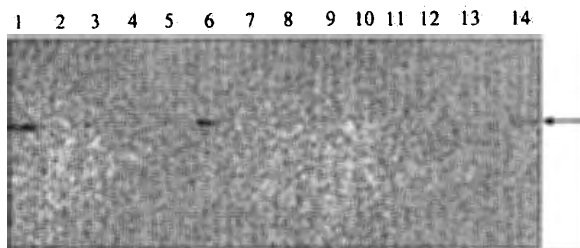


Fig. 4. Western blot analysis using affinity purified anti-Philadelphia I flagellin antibodies of whole-cell extracts of strains grown at 30 °C. Lanes: (1) *L. pneumophila* Philadelphia I; (2) *L. pneumophila* U₁ SG₁; (3) *L. pneumophila* U₂₁ SG₆; (4) *L. longbeachae* SG₁; (5) *L. longbeachae* SG₂; (6) *L. micdadei*; (7) *L. hackelia* SG₁; (8) *L. hackelia* SG₂; (9) *L. oakridgensis*; (10) *L. dumoffii*; (11) *L. jordanis*; (12) *L. feeleii* SG₁; (13) *L. feeleii* SG₂; (14) isolated flagella. The arrow indicates the 47 kDa flagellin band.

extracts and isolated flagella of the Philadelphia I strain. The flagellin subunits reacted with the serum (Fig. 3*b*, lane 2), indicating a high titre of anti-flagellin antibodies. The reaction with the cell extract (Fig. 3*a*, lane 1) appeared as a smear (Fig. 3*b*, lane 1). Starting with this serum, anti-flagellin antibodies were affinity purified using antigen immobilized on nitrocellulose. Flagellation was further analysed in various *L. pneumophila* isolates

Table 2. Percentage of flagellated *L. pneumophila* within a bacterial population grown at different temperatures

Percentage of flagellated bacteria was determined by electron microscopy. Approximately 400 bacteria were observed at each temperature.

Strain	Percentage of flagellated bacteria at (°C):		
	30	37	41
Philadelphia I virulent	60-70	20-30	<5
Philadelphia I XXXV, avirulent	60-70	20-30	<5

and other *Legionella* species. The strains were first examined by electron microscopy. All strains, with the exception of *L. longbeachae* SG₁, *L. dumoffii*, *L. oakridgensis* and *L. feeleii* SG₁, were flagellated (Table 1). The use of polyclonal anti-flagellin antibodies in Western blot analysis of whole-cell extracts of the strains grown at 30 °C (Fig. 4) produced a positive reaction with the *L. pneumophila* water isolates (U1SG₁ and U21SG₆; Fig. 4, lanes 2 and 3, respectively), *L. longbeachae* (SG₂; Fig. 4, lane 5), *L. micdadei* (Fig. 4, lane 6), *L. hackelia* (SG₁ and 2; Fig. 4, lanes 7 and 8, respectively) which all exhibited a band of approximately the same size as the Philadelphia I strain. For the remaining strains no reaction was observed (Fig. 4, lanes 4 and 9-13).

Temperature-dependent expression of flagella in L. pneumophila

The flagellation of *L. pneumophila* Philadelphia I and the avirulent variant XXXV was evaluated by electron microscopy after growing the strains at 30, 37 and 41 °C. In both strains the percentage of flagellated bacteria decreased with increasing growth temperature, i.e. at 30 °C nearly two-thirds of the bacteria were flagellated, while at 37 °C about one-third of the population displayed flagella and at 41 °C only a very small proportion of the bacterial cells (<5%) had flagella (Fig. 2*b*; Table 2). There was no marked difference between the virulent and avirulent variants.

The effect of temperature on flagellation was further analysed by Western blots. The antibodies were tested using whole-cell extracts of the Philadelphia I strain and of the avirulent derivative XXXV grown at 30, 37 and 41 °C. The purified antibodies reacted specifically with the 47 kDa flagellin protein subunit (cf. Fig. 3) in both strains when grown at 30 °C (Fig. 5*a*, lanes 3 and 4), but no reaction was observed with extracts obtained from bacteria cultivated at 37 °C (Fig. 5*a*, lanes 5 and 6). At a

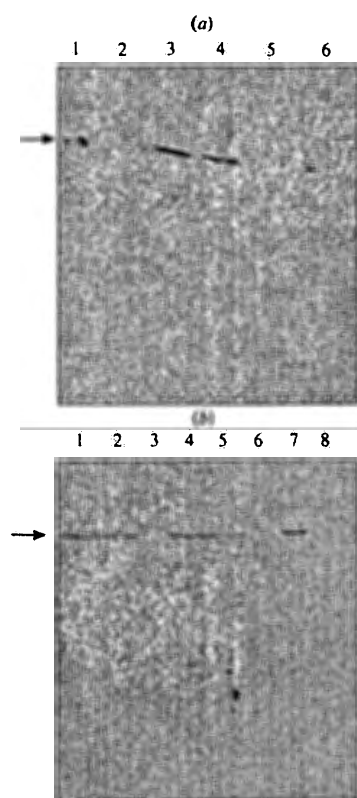


Fig. 5. Western blot analysis of whole cell extracts of *L. pneumophila* Philadelphia I and an avirulent derivative (XXXV) using purified anti-flagellin antibodies. Strains were grown at different temperatures as indicated. (a) Lanes: (1) isolated flagella; (2) *E. coli* 536 (37 °C); (3) *L. pneumophila* Philadelphia I (30 °C); (4) *L. pneumophila* XXXV (30 °C); (5) *L. pneumophila* Philadelphia I (37 °C); (6) *L. pneumophila* XXXV (37 °C). (b) Lanes: (1) *L. pneumophila* Philadelphia I (30 °C); (2) *L. pneumophila* Philadelphia I (37 °C); (3) *L. pneumophila* Philadelphia I (41 °C); (4) *L. pneumophila* XXXV (30 °C); (5) *L. pneumophila* XXXV (37 °C); (6) *L. pneumophila* XXXV (41 °C); (7) isolated flagella; (8) *E. coli* 536 (37 °C). Anti-flagellin antibody concentration in (b) was four times higher than in (a). Arrows indicate the 47 kDa flagellin band.

fourfold higher concentration of antibodies (Fig. 5b) flagella subunits could be detected in the strains grown at 37 °C, (Fig. 5b, lanes 2 and 5) but not in extracts obtained from cultures which were grown at 41 °C (Fig. 5b, lanes 3 and 6). These data are in good agreement with those of the electron microscopic examination (Table 2). Flagellated *Escherichia coli* strain 536 (O6:K15:H31, see Hacker & Goebel, 1987) used as control (Fig. 5a, lane 2 and Fig. 5b, lane 8) produced no reaction with the purified antibodies. Additionally, the other strains that reacted with the polyclonal antiserum, when grown at 30 °C (cf. Fig. 4), displayed a weaker or no reaction after cultivation at 37 °C (data not shown), arguing for a temperature-regulated expression of flagella also in these *Legionella* strains.

Discussion

Surface structures of Gram-negative cells, such as capsules, lipopolysaccharides and cell appendages including fimbriae and flagella play a major role in bacterial pathogenicity and in the ability of strains to survive in the environment (Isenberg, 1988; Finlay & Falkow, 1989). In *L. pneumophila* the prominent cell-surface structures were flagella consisting of protein subunits of 47 kDa and organized as monotrichous polar appendages. Freeze-etching studies did not reveal a crystalline S-layer, although such proteinaceous surface structures can be found in a variety of bacteria (Sleytr & Messner, 1988b). We were also unable to detect fimbriae which have been described by Chandler *et al.* (1980) and Rodgers *et al.* (1980). The failure to detect fimbriae might be due to differences in the growth conditions used, as Rodgers *et al.* (1980) reported the presence of fimbriae after cultivation in broth or on enriched blood agar.

The first reports on flagella in *L. pneumophila* came from Chandler *et al.* (1980) and from Elliott & Johnson (1981, 1982). The latter authors further described a difference in the expression of flagella in virulent and avirulent variants of *L. pneumophila* Philadelphia 2 and an influence of the growth medium on flagella production. In contrast to these findings, we could not detect any difference between virulent and avirulent derivatives of the Philadelphia I strain, when grown on BCYE agar. Using polyclonal anti-flagellin antibodies, we demonstrated that *L. pneumophila* water isolates and some other *Legionella* species produce proteins of approximately the same size as the *L. pneumophila* Philadelphia I flagellin subunit. This suggests that flagella may be conserved among some species of the genus *Legionella*, which are distantly related on the DNA level (Brenner, 1986). Other *Legionella* species did not display proteins reacting with the anti-flagellin antibodies of *L. pneumophila*, although flagellation could be demonstrated by electron microscopy.

Surface structures, e.g. fimbriae and capsules often show a strong temperature-dependent expression (Göransson & Uhlin, 1984; Schmoll *et al.*, 1990; for review see Maurelli, 1989). We therefore investigated the influence of growth temperature on flagella expression in *L. pneumophila*. At higher temperatures, production of flagella was reduced or nearly abolished. Using electron microscopy, we observed that the overall decrease in the number of flagella within a population was due to a reduced percentage of flagellated bacteria. Temperature-dependent expression of flagella has also been reported for *Listeria monocytogenes* (Peel *et al.*, 1988), and transition from Flag⁺ to Flag⁻ has been described for *Campylobacter jejuni* (Aguero-Rosenfeld *et al.*, 1990).

It is interesting to speculate whether flagellation of *Legionella* has an influence on virulence or survival ability in the environment. Flagella have been shown to contribute to the pathogenicity of *Salmonella* (Finlay & Falkow, 1989) and *Pseudomonas aeruginosa* (Drake & Montie, 1988). The fact that avirulent variants of *L. pneumophila* do not differ according to this phenotype suggests, that flagella are not important for virulence as also suggested by Elliott & Johnson (1982). Properties of pathogenic relevance are generally expressed at 37 °C while penetration into amoebae and other processes important for the survival of *Legionella* in the environment may occur at lower temperatures. Flagella, however, were expressed to a greater extent at lower temperatures, suggesting that flagellation might contribute to environmental survival processes rather than to pathogenicity. The precise role of flagellation in the biology of *Legionella* remains to be evaluated.

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