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Effect of Growth Temperature on Outer Membrane Components and Virulence of *Aeromonas hydrophila* Strains of Serotype O:34

SUSANA MERINO, SILVIA CAMPRUBÍ, AND JUAN M. TOMÁS*

Departamento de Microbiología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain

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Growth of *Aeromonas hydrophila* strains from serotype O:34 at 20 and 37°C in tryptic soy broth resulted in changes in the lipids, lipopolysaccharide (LPS), and virulence of the strains tested. Cells grown at 20°C contained, relative to those cultured at 37°C, increased levels of the phospholipid fatty acids hexadecanoate and octadecanoate and reduced levels of the corresponding saturated fatty acids. Furthermore, the lipid A fatty acids also showed thermoadaptation. In addition, LPS extracted from cells cultivated at 20°C was smooth, while the LPS extracted from the same cells cultivated at 37°C was rough. Finally, the strains were more virulent for fish and mice when they were grown at 20°C than when they were grown at 37°C and also showed increased different extracellular activities when they were grown at 20°C.

Aeromonas hydrophila is both an opportunistic and a primary pathogen of a variety of aquatic and terrestrial animals, including humans (31). The clinical manifestation of *A. hydrophila* infection ranges from gastroenteritis to soft-tissue infections, septicemia, and meningitis (11). Surface characteristics, such as the presence of an S-layer or the type of lipopolysaccharide (LPS), permit classification of *A. hydrophila* into different categories on the basis of their virulence (9, 14). We recently described a group of *A. hydrophila* strains belonging to serotype O:34 with heterogeneous O-polysaccharide chains in their LPS and without an S-layer, previously reported to be moderate in their virulence for fish (19, 23) and mice (23). Also, serotype O:34 has been recently reported as one of the most frequently encountered among mesophilic *Aeromonas* species (35).

Since *A. hydrophila* is ubiquitous in nature and also a pathogen, we are interested in the changes that temperature may have on its cellular chemistry, particularly as it affects membrane lipids, LPS, and outer membrane (OM) proteins, as well as their relationship with virulence. Studies on the effect of growth temperature on LPS chemistry have been largely restricted to members of the family *Enterobacteriaceae* and the genus *Pseudomonas*. Modifications of the composition of the lipid A component of LPS isolated from *Salmonella* species (41), *Proteus mirabilis* (34), *Escherichia coli* (38), and *Yersinia enterocolitica* (39) have been noted. Also, changes in the LPS have been observed at high temperatures on enterobacteria (20, 41) and *Pseudomonas* cells (16, 17), with the cells being smooth (S-form LPS) at low temperatures and rough (R-form LPS) at high temperatures.

In the present report, we describe the changes which occurred in the fatty acid content of total and LPS lipids, in the LPS, in the outer membrane proteins, and in the virulence of the strains and show that they change as a function of temperature.

* Corresponding author.

MATERIALS AND METHODS

Bacteria, bacteriophages, and media. The strains used are listed in Table 1. Strains AH-3 and Ba5 were isolated from moribund fish, and strains AH-101, AH-102, and AH-103 were human clinical isolates. Bacteriophages PM1, PM2, and 18 from *A. hydrophila* were previously described by us (24, 25, 27). *A. hydrophila* strains were usually cultured on tryptic soy broth (TSB). TSB-agar was obtained by adding 1.5% agar and 0.6% TSB soft agar.

Phage adsorption and sensitivity. Bacteriophage adsorption to bacterial cells was studied by incubating 10^3 PFU with 10^7 bacteria for 20 min at 37 or 20°C. Chloroform (2 to 3 drops) was added, and the mixture was vortexed for 1 min. After centrifugation at 12,000 × *g* for 10 min, the titers of the remaining unadsorbed phages in the supernatant were determined in *A. hydrophila* AH-3 grown at either 37 or 20°C. Phage sensitivity was assayed by a spot test.

Cell surface hydrophobicity. Bacterial adherence to hydrocarbons was measured as described by Rosenberg et al. (33) by using *n*-xylene. Cells were washed twice in phosphate-urea-magnesium buffer (pH 7.1), suspended in the same buffer at an optical density at 400 nm (OD_{400}) of 1.0, and vortexed with various volumes of *n*-xylene. The OD_{400} of the aqueous phase was expressed as a percentage of the OD_{400} of a standard volume of untreated cells.

Cell surface isolation and analyses. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected by centrifugation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium *N*-lauryl-sarcosinate (10), and the OM fraction was collected as described above. OM proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a modification (2) of the Laemmli procedure (18). Protein gels were fixed and stained with Coomassie blue. LPS was isolated and purified by using the methods of Westphal and Jann (40) as modified by Osborn (28). LPS was analyzed by SDS-PAGE and silver stained by the method of Tsai and Frasch (37). LPS monosaccharides were also analyzed to their alditol acetate derivatives by gas-liquid chromatography on a 3% SP-3840 column (Supelco Inc.,

TABLE 1. *A. hydrophila* strains, their relevant properties, phage sensitivities, and origins

Strain	Relevant properties	Growth temp (°C)	Sensitivity[a] to:			Origin (reference)
			PM1	PM2	18	
AH-3	Smooth, serotype O:34	20	S	R	R	23
		37	R	S	S	
AH-22	Isogenic rough mutant derived from AH-3	20	R	S	S	27
		37	R	S	S	
Ba5	Smooth, serotype O:34	20	S	R	R	23
		37	R	S	S	
AH-35	Isogenic rough mutant derived from Ba5	20	R	S	S	27
		37	R	S	S	
AH-101	Smooth, serotype O:34	20	S	R	R	23
		37	R	S	S	
AH-161	Isogenic rough mutant derived from AH-101	20	R	S	S	27
		37	R	S	S	
AH-102	Smooth, serotype O:34	20	S	R	R	27
		37	R	S	S	
AH-103	Smooth, serotype O:34	20	S	R	R	27
		37	R	S	S	

[a] S, sensitive; R, resistant.

Cras, Switzerland) as previously described (36). The LPS was subjected to chloroform-methanol extraction prior to assay of its fatty acid content in order to release the phospholipids from it.

Fatty acid analyses. Cells were grown in 200 ml of TSB in 500-ml flasks with shaking at 200 rpm to an OD_{650} of 1.0. Cells were harvested by centrifugation, washed twice with 0.1 M ammonium acetate, and lyophilized. The residue was reduced to a fine powder by grinding, and samples (25 mg) were added to small screw-cap test tubes together with 1.0 ml of 2 M HCl in methanol. The obtention of fatty acid methyl esters was performed according to the method of Kropinski et al. (16). The tubes were sealed under N_2 and hydrolyzed at 100°C for 16 h. After cooling and neutralization with silver carbonate, an internal standard (tetradecanoic acid methyl ester; 5 μl of a 40.97-mg/ml [wt/vol] solution in methyl octonate) was added, and the fatty acid methyl esters were resolved and quantitated by gas-liquid chromatography as previously described (17). The individual fatty acid peaks were identified with internal standards either purchased from Supelco Inc. or prepared by us.

Antiserum. Anti-LPS serum was obtained and assayed as previously described (23). This serum was rendered specifically anti-O:34 after extensive adsorption of the serum with the rough strain AH-22 (O-antigen-deficient mutant).

Hemolysin assays. Strains were evaluated for their hemolytic activity basically by the method of Asao et al. (3). Strains were grown in 50 ml of TSB with shaking at 200 rpm until an OD_{650} of 1.5 at either 20 or 37°C was reached. Cultures were centrifuged at 4°C, and supernatants were filtered through a 0.45-μm-pore-size filter. Then 50-μl samples of twofold serial dilutions of the filtrates in 0.01 M Tris hydrochloride buffer (pH 7.2) containing 0.9% NaCl were mixed with an equal volume of a 1% suspension of sheep erythrocytes in a microtiter plate and incubated at 37°C for 1 h. The hemolytic activity was expressed as the highest dilution of filtrate showing total hemolysis.

Cytotoxin assay. Supernatants obtained as described above were tested for the presence of cytotoxin on Vero cell monolayers as described by Blanco et al. (5). Plates were read after 24 h under an inverted microscope, and wells showing totally or partially destroyed monolayers were considered positive. Cytotoxic titers were expressed as the highest dilution giving a positive reaction.

Caseinase assay. The caseinase activity was quantified as previously described (29). Briefly, two serial dilutions (100 μl) of the culture supernatants in 0.1 M Tris (pH 7.2) buffer were placed in 8-mm wells made on a petri dish filled with 30 ml of 2% skim milk and 2% agar in the same buffer. Plates were incubated for 24 h at 37°C, and the caseinase activity was expressed as the highest dilution showing a halo of digestion.

Virulence for fish and mice. The virulence of the strains was measured by their mean lethal dose (LD_{50}), evaluated according to the method of Reed and Muench (32). The strains, prior to inoculation, were grown at the temperature indicated (20 or 37°C).

(i) Fish. Rainbow trout (12 to 18 g) were maintained in 20-liter static tanks at 17°C. Fish were injected intraperitoneally with 0.05 ml of the test samples. Mortality was recorded up to 2 weeks; all the deaths occurred within 2 to 7 days.

(ii) Mice. Albino Swiss female mice (5 to 6 weeks old) were injected intraperitoneally with 0.25 ml of the test samples. Mortality was recorded up to 7 days; all the deaths occurred within 1 to 4 days.

RESULTS

Growth. The most obvious effect of temperature on strains of *A. hydrophila* serotype O:34 was on the growth rate. The generation times at 20 and 37°C were 115 and 59 min, respectively. The cell pellets from cultures grown at 37°C appeared smaller and less transparent than those from cultures grown at 20°C.

We also noticed that cells from *A. hydrophila* serotype O:34 grown at 20°C showed a lower relative hydrophobicity (a higher percentage of bacteria in the aqueous phase after treatment with *n*-xylene) than the same cells grown at 37°C (Table 2). Similar results were obtained measuring the cell

TABLE 2. Surface hydrophobicity of *A. hydrophila* strains from serotype O:34

Strain	BATH[a] of cells grown at:	
	20°C	37°C
AH-3	72 ± 2	59 ± 2
Ba5	71 ± 3	58 ± 4
AH-101	73 ± 3	57 ± 2
AH-102	71 ± 4	58 ± 3
AH-103	74 ± 2	59 ± 3
AH-22	60 ± 3	58 ± 4
AH-35	58 ± 4	59 ± 2
AH-161	57 ± 3	55 ± 4

[a] BATH, bacterial adherence to hydrocarbons (*n*-xylene); percentage of OD of the aqueous phase after treatment with *n*-xylene (0.2 ml) relative to initial OD. The mean values are the averages from at least three independent experiments.

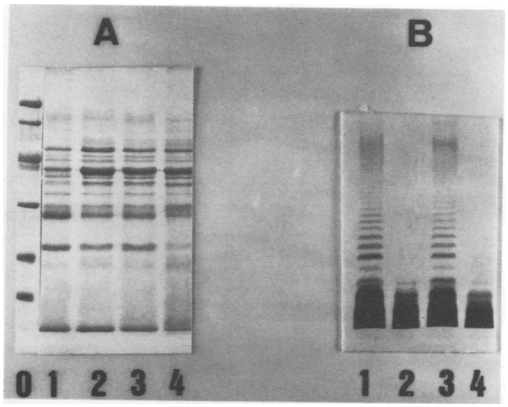

FIG. 1. SDS-PAGE of OM proteins and LPS from *A. hydrophila* strains (serotype O:34). (A) OM proteins were obtained as sodium lauryl sarcosinate-insoluble material (10). Lanes: 0, molecular size standards (14.4, 21.5, 31.0, 45.0, 66.2, and 91.4 kDa) from Bio-Rad Laboratories; 1, AH-3 grown at 20°C; 2, AH-3 grown at 37°C; 3, Ba5 grown at 20°C; 4, Ba5 grown at 37°C. (B) LPS was extracted by the method of Darveau et al. (7) and assayed by the method of Tsai and Frasch (37). Lanes 1 to 4 are as defined for panel A.

surface hydrophobicity by other methods (data not shown). The relative hydrophobicity of the wild-type cells grown at 37°C was similar to that observed for the O-antigen LPS-deficient mutants (AH-22, AH-35, and AH-161) grown at either 20 or 37°C.

Also, strains of *A. hydrophila* serotype O:34 grown at 20°C were sensitive to bacteriophage PM1 (a bacteriophage whose receptor is the O-antigen LPS of strains from serotype O:34 [28]) and resistant to bacteriophages PM2 and 18 (bacteriophages whose bacterial receptor is the LPS core [25, 26]). However, when we grew the cells of these strains at 37°C, they were sensitive to phages PM2 and 18 and resistant to phage PM1 (Table 1). Furthermore, whole cells of *A. hydrophila* strains from serotype O:34 grown at 20°C were able to inactivate PM1 bacteriophage but not PM2 or 18 bacteriophage, while the same strains grown at 37°C were able to inactivate bacteriophages PM2 and 18 but not bacteriophage PM1.

All these results prompted to us to examine the cell surface components of these strains grown at 20 and 37°C.

OM proteins. No major differences were observed on the OM protein profiles by SDS-PAGE of different *A. hydrophila* strains (serotype O:34) when the cells were grown at 20 or 37°C (Fig. 1A). However, a 41-kDa band (maybe the porin) appears to be decreased at 20°C (clearly for strain AH-3), and a 24-kDa band is increased at 20°C (clearly for strain Ba5) (Fig. 1A).

LPS. Either by using purified LPS from different *A. hydrophila* strains (serotype O:34) extracted from cells grown at 20 or 37°C or by the extraction method of Darveau and Hancock (8) using whole cells treated with proteinase K, it is clear that all the subpopulation of LPS molecules produced by the cells grown at 37°C are rough (R-form LPS) while the LPS molecules produced by the same cells grown at 20°C are rough (R-form LPS) and smooth (S-form LPS) (Fig. 1B). Also, chemical analyses of purified LPSs from these strains grown at 20°C showed a large amount of hexosamines (mainly *N*-acetylgalactosamine), which are components, as we previously described (26), of the O-antigen LPS of serotype O:34. No hexosamines were found on purified LPSs of the same strains grown at 37°C. Also, when we plot a growth curve at 37°C with an inoculum of these strains grown at 20°C, the ability to produce O-chains is lost just at the beginning of the exponential phase (data shown for LPS of strain AH-3 in Fig. 2). Also, as we indicated in Fig. 2, the cells during this growth at 37°C are unable to inactivate bacteriophage PM1, while the inoculum grown at 20°C was able to do it. Furthermore, purified LPS from cells grown at 20°C or whole cells grown at this temperature coated to a microtiter plate answered in a positive way (OD_{405} > 1.0) in an enzyme-linked immunosorbent assay (ELISA) against anti-O:34 serum. If we used the same serum in the same ELISA with either coated purified LPS from these strains grown at 37°C or whole cells grown at the same temperature, the answer was always negative (OD_{405} < 0.1) (Fig. 3 for results with purified LPS). All these results were in agreement with the ones obtained with bacteriophages PM1, PM2, and 18.

Cellular fatty acids. Whole-cell methanolysates prepared from *A. hydrophila* AH-3 revealed the presence of 11 predominant fatty acids (Table 3). As the temperature increased from 20 to 37°C, the absolute concentrations of saturated fatty acids ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{20:0}$) increased while those of the unsaturated acids ($C_{16:1}$ and $C_{18:1}$) decreased. The level of the hydroxylated fatty acids showed a minor decrease or a drastic decrease in the case of the 2-OH $C_{12:0}$. These results suggested that not only the predominant membrane lipids but also lipid A were modified in response to temperature. In addition, it was noted that the ratio of phospholipids to LPS appeared to change as assessed by quantitating their respective fatty acids. The ($C_{12:0}$ + 2-OH $C_{12:0}$ + 3-OH $C_{12:0}$)/($C_{16:0}$ + $C_{16:1}$ + $C_{18:0}$ + $C_{18:1}$) ratio declined from 1:12.5 (20°C) to 1:24.5 (37°C).

The results of the chemical analyses of the fatty acids of the two LPS preparations (20 and 37°C) are presented in Table 4. As the temperature increased, the total content of fatty acid increased from approximately 516 nmol/mg of LPS at 20°C to approximately 552 nmol/mg of LPS at 37°C. This was expected, since the column chromatography and electrophoretic data indicated that the LPS at 37°C was essentially rough. When the data were normalized with respect to the content of 3-OH $C_{12:0}$ (on the basis of the assumption that this fatty acid should remain unchanged), there was no significant change in the number of fatty acid residues per lipid A molecule, but considerable changes occurred in the levels of the other fatty acids. As the growth temperature increased, there were specific increases in the levels of 2-OH $C_{12:0}$ (maybe due to a more complete release of amide-linked

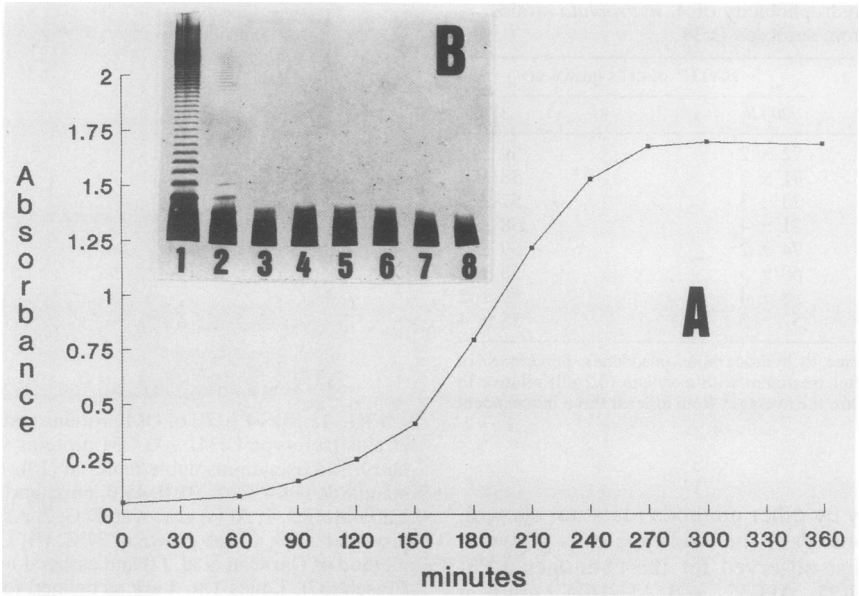

FIG. 2. (A) Growth curve of strain AH-3 at 37°C in TSB with an inoculum (1:1,000) grown at 20°C in the same medium measured by A_{650}; (B) LPS extracted by the method of Darveau et al. (7) and assayed by the method of Tsai and Frasch (37) from different points of the growth curve. Lanes 1 to 8 show the LPS extracted from cells at 0, 60, 90, 120, 150, 180, 210, and 240 min, respectively. Also, only cells from 0 min (the inoculum) were able to inactivate PM1 bacteriophage; the cells from 60 to 240 min were unable to do so. Similar results were obtained with other strains from serotype O:34, like Ba5 and AH-101.

fatty acids in purified LPS than in whole cells) and 2-OH $C_{14:0}$. On the other hand, the content of 3-OH $C_{10:0}$ decreased. We also observed a notable increase of $C_{12:0}$ and $C_{16:0}$.

Hemolytic, cytotoxic, and caseinolytic activities. Hemolytic, cytotoxic, and caseinolytic activities were a common feature among all the *A. hydrophila* strains from serotype O:34 tested. As is shown in Table 5, the titers of all these extracellular activities are higher when the wild-type strains are grown at 20°C than when they are grown at 37°C. Wild-type strains grown at 37°C showed hemolytic, cytotoxic, and caseinolytic activities similar to those of their isogenic rough mutants (AH-22, AH-35, and AH-161) grown at either 20 or 37°C.

Virulence. Table 6 shows the LD_{50}s of different strains grown at 20 or 37°C for mice or fish. As could be observed,

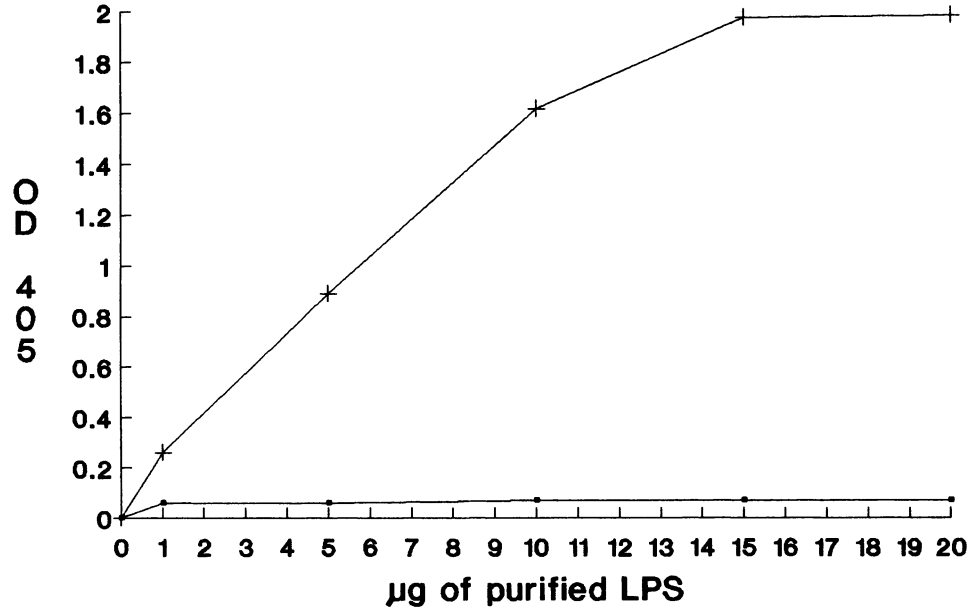

FIG. 3. Results of ELISA using anti-O:34 serum ($1:10^3$) (rendered specific as described in Materials and Methods) and purified LPS from strain AH-3 grown at 20°C (+) and 37°C (■). Similar results were obtained with purified LPSs from the other O:34 strains grown at 20 and 37°C. The values (OD_{405}s) are the averages from at least three independent experiments.

TABLE 3. Fatty acid composition of *A. hydrophila* AH-3 grown in TSB at 20 and 37°C[a]

Fatty acid	nmol of fatty acid/mg (dry wt) of cells at:	
	20°C	37°C
$C_{12:0}$	2.3	3.6
$C_{14:0}$	27.4	34.8
$C_{16:0}$	95.2	173.6
$C_{18:0}$	36.4	49.7
$C_{20:0}$	6.5	10.4
$C_{16:1}$	148.3	86.7
$C_{18:1}$	79.3	45.2
3-OH $C_{10:0}$	3.6	3.0
2-OH $C_{12:0}$	10.9	0.8
3-OH $C_{12:0}$	15.8	10.2
2-OH $C_{14:0}$	16.7	12.1

[a] Cell pellets washed with 0.1 M ammonium acetate were lyophilized and hydrolyzed with 2 M HCl in methanol at 100°C for 16 h. After neutralization with silver carbonate, the fatty acid methyl esters were identified and quantitated by gas-liquid chromatography.

wild-type strains grown at 20°C showed an LD_{50} for mice or fish about 1 log (or more) lower than when they were grown at 37°C. Also, the LD_{50} of the wild-type strains grown at 37°C for mice or fish was similar to the one observed for their isogenic rough mutants (AH-22 and AH-35) grown at either 20 or 37°C. All these temperatures were for the previous growth of the strains independent of the body temperatures of the animals. Also, the cells recovered from mice killed by rough strains are still rough (data not shown).

DISCUSSION

From the results of the present study it can be appreciated that thermal adaptation of *A. hydrophila* (serotype O:34) involves changes to the predominant phospholipids of the cells, to the LPS, and to the virulence of the strains tested to fish and mice.

A. hydrophila (serotype O:34) total lipids exhibit a classical type of thermoadaptation by increasing the content of unsaturated fatty acids, at the expense of saturated ones, with decreasing temperature. This type of homoviscous adaptation has also been observed with *E. coli* (20), *Serratia marcescens* (15), *P. mirabilis* (34), *Y. enterocolitica* (39), *Pseudomonas* spp. (12, 17), *Lactobacillus casei* (13), *Bacillus stearothermophilus* (22), and psychrophilic *Vibrio* species (4).

The type of thermoadaptation exhibited by the LPS fatty acids is complex and similar to the homoviscous adaptation seen with the phospholipid fatty acids and lipid A from certain members of the family *Enterobacteriaceae* (34, 38, 39, 41) but different from the one described for *Pseudomonas* spp. (17). High temperatures (37°C) were associated with increased levels of some hydroxylated and saturated fatty acids (mainly 2-OH $C_{12:0}$, 3-OH $C_{12:0}$, 2-OH $C_{14:0}$, $C_{12:0}$, and $C_{16:0}$). Also, there was a decreased level of 3-OH $C_{10:0}$ when the temperature increased (from 20 to 37°C).

However, the most significant change on the LPS was a lack of S-form molecules (O:34 antigen) from the cells grown at 37°C. This effect was easily observed with purified LPS extracted from cells grown at 20 and 37°C and also by the rapid method of Darveau and Hancock (8) for the analysis of LPS on SDS-PAGE from whole cells treated with proteinase K. In SDS-PAGE gels silver stained according to the method of Tsai and Frasch (37), by gas-liquid chromatography analyses of monosaccharides from the O:34 antigen, and also with specific antiserum against this antigen, it was clear that LPS extracted from cells grown at 20°C was smooth (subpopulation molecules of R and S forms) and the LPS

TABLE 4. Fatty acid content of *A. hydrophila* LPS prepared from cells grown at 20 and 37°C

Fatty acid	nmol/mg of LPS at[a]:	
	20°C	37°C
$C_{12:0}$	58.9 (0.78)	70.4 (0.86)
$C_{14:0}$	12.8 (0.16)	17.9 (0.22)
$C_{16:0}$	153.9 (2.01)	202.7 (2.49)
$C_{18:0}$	11.1 (0.14)	23.9 (0.25)
$C_{16:1}$	<1.0	<1.0
$C_{18:1}$	<1.0	<1.0
3-OH $C_{10:0}$	7.6 (0.10)	4.0 (0.04)
2-OH $C_{12:0}$	18.5 (0.24)	28.1 (0.34)
3-OH $C_{12:0}$	150.6 (2.00)	162.7 (2.00)
2-OH $C_{14:0}$	14.4 (0.19)	42.9 (0.52)

[a] Values in parentheses correspond to the molar ratio of the given fatty acid relative to 3-OH $C_{12:0}$. This fatty acid was assigned a value of 2 since there are 2 mol/mol of lipid A.

TABLE 5. Caseinolytic (CAS), hemolytic (HEM), and cytotoxic (CYT) activities of *A. hydrophila* strains from serotype O:34 grown at different temperatures

Strain	Titer of extracellular products at:					
	20°C			37°C		
	CAS	HEM	CYT	CAS	HEM	CYT
AH-3	1/1,024	1/64	1/64	1/32	1/16	1/16
Ba5	1/1,024	1/64	1/64	1/32	1/16	1/16
AH-101	1/512	1/32	1/32	1/16	1/8	1/4
AH-102	1/512	1/32	1/32	1/32	1/8	1/8
AH-103	1/1,024	1/64	1/64	1/32	1/16	1/16
AH-22	1/64	1/16	1/16	1/32	1/16	1/8
AH-35	1/32	1/8	1/8	1/32	1/8	1/8
AH-161	1/16	1/8	1/4	1/16	1/8	1/4

TABLE 6. Virulence for rainbow trout and mice of *A. hydrophila* strains from serotype O:34 by intraperitoneal injection at different growth temperatures (growth temperature of the inoculated strain)

Strain	Growth temp (°C)	LPS type	LD_{50}[a] for:	
			Rainbow trout	Mice
AH-3	20	Smooth	$10^{5.5}$	$10^{7.5}$
	37	Rough	$10^{6.9}$	$10^{8.7}$
Ba5	20	Smooth	$10^{5.3}$	$10^{7.2}$
	37	Rough	$10^{6.5}$	$10^{8.5}$
AH-101	20	Smooth	$10^{5.7}$	$10^{7.6}$
	37	Rough	$10^{6.7}$	$10^{8.8}$
AH-22	20	Rough	$10^{6.8}$	$10^{8.6}$
	37	Rough	$10^{6.9}$	$10^{8.6}$
AH-35	20	Rough	$10^{6.9}$	$10^{8.7}$
	37	Rough	$10^{6.9}$	$10^{8.6}$

[a] The values are the averages from three independent experiments, and the maximum deviation was always $<10^{0.3}$.

extracted from the same cells grown at 37°C was rough (only molecules of R form). While it is possible that the LPS extraction procedures used result in the extraction of an unrepresentative subpopulation of LPS molecules, other evidence in vivo supports the conclusions drawn here. The use of smooth (PM1)- and rough (PM2 and 18)-specific phages previously characterized by us (24, 25, 27) provides conclusive evidence that cells grown at 20°C are smooth and the same cells grown at 37°C rough. Wild-type cells grown at 20°C were sensitive and able to inactivate phage PM1, while the same cells grown at 37°C were resistant and unable to inactivate this phage (presence of O:34-antigen LPS at 20°C but not at 37°C). Furthermore, cells grown at 37°C were sensitive and able to inactivate phages PM2 and 18, while the same cells grown at 20°C were resistant and unable to inactivate these phages (the presence of the O:34-antigen LPS at 20°C impedes rough-specific phages from finding their bacterial surface receptor). Also, when we applied the specific anti-O:34 serum to whole cells, we obtained the same evidence as with the phages: cells grown at 20°C answered positively to the antiserum (presence of the O:34 antigen), and the same cells grown at 37°C were negative against this anti-O:34 serum (lack of the O:34 antigen). Changes in the degree of substitution of the R-form LPS have been observed at a high temperature with members of the *Enterobacteriaceae* (21, 41) and *Pseudomonas* spp. (17). For instance, when *Y. enterocolitica* or *Yersinia pestis* was cultivated at low temperatures, the cells were smooth, and they became rough when grown at high temperatures (1, 7).

It has been described for some enterobacteria that smooth strains showed increased values of extracellular hemolytic activity compared with isogenic rough mutants (6, 30). When we tested different extracellular activities from whole cells of *A. hydrophila* (serotype O:34) at 20 and 37°C, we found the same effect. Also, Lallier et al. (19) always tested the different extracellular activities of *A. hydrophila* cells grown at a low temperature (22°C). The extracellular (hemolytic, cytotoxic, and caseinolytic) activities were higher from cells grown at 20°C than from cells grown at 37°C or from cells of isogenic rough mutants grown at both temperatures, this result being in agreement with the fact that cells grown at 20°C are smooth and those grown at 37°C are rough. Finally, the virulence assessed by the LD_{50} of the strains for fish and mice clearly indicated that the strains grown at 20°C were more virulent (lower LD_{50}) than the same strains grown at 37°C or isogenic rough mutants grown at both temperatures (higher LD_{50}). We cannot conclude whether the higher virulence at 20°C than at 37°C is due to the change in the subpopulation of LPS molecules or whether this LPS change allowed a higher extracellular activity of some products that renders the cells more virulent at 20°C.

Also, wild-type strains grown at 20°C were resistant to serum killing (26), while the same strains grown at 37°C were sensitive to serum killing (data not shown). These results backed up the fact that these strains are smooth at 20°C and rough at 37°C, since LPS phenotype can affect susceptibility to complement.

These studies clearly show that temperature plays a significant role in regulating the chemistry of the cell surface of *A. hydrophila* (serotype O:34) strains, and the changes on the cell surface explained the difference in virulence observed at different growth temperatures for the strains tested.

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REFERENCES

1. **Acker, G., K. Wartenberg, and W. Knapp.** 1980. Sugar composition of the lipopolysaccharide and ultrastructural study of the outer membrane of *Yersinia enterocolitica*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Abt. 1 Orig. Reihe A **247**:229–240.
2. **Ames, G. F. L., E. N. Spudich, and H. Nikaido.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. **117**:406–416.
3. **Asao, T., S. Kozaki, K. Kato, Y. Kinoshita, K. Otsu, T. Uemura, and G. Sakaguchi.** 1986. Purification and characterization of an *Aeromonas hydrophila* hemolysin. J. Clin. Microbiol. **24**:228–232.
4. **Bhakhoo, M., and R. A. Herbert.** 1979. The effects of temperature on the fatty acid and phospholipid composition of four obligated psychrophilic *Vibrio spp.* Arch. Microbiol. **121**:121–127.
5. **Blanco, J., M. Blanco, E. A. Gonzalez, M. P. Alonso, and J. I. Garabal.** 1990. Comparative evaluation of three tests for the detection of *Escherichia coli* cytotoxic necrotizing factors (CNF1 and CNF2) using filtrates of cultures treated with mitomycin C. FEMS Microbiol. Lett. **69**:311–316.
6. **Camprubí, S., J. M. Tomás, F. Muñoa, C. Madrid, and A. Juarez.** 1990. Influence of lipopolysaccharide on external hemolytic activity of *Salmonella typhimurium* and *Klebsiella pneumoniae*. Curr. Microbiol. **20**:1–3.
7. **Darveau, R. P., W. T. Charnetzky, R. E. Hurlbert, and R. E. W. Hancock.** 1983. Effects of growth temperature, 47-megadalton plasmid, and calcium deficiency on the outer membrane protein porin and lipopolysaccharide composition of *Yersinia pestis* EV76. Infect. Immun. **42**:1092–1101.
8. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for the isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
9. **Dooley, J. S. G., R. Lallier, D. H. Shaw, and T. J. Trust.** 1985. Electrophoretic and immunochemical analyses of the lipopolysaccharides from various strains of *Aeromonas hydrophila*. J. Bacteriol. **164**:263–269.
10. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. **115**:717–722.
11. **Freij, B. J.** 1984. *Aeromonas*: biology of the organism and diseases in children. Pediatr. Infect. Dis. **3**:164–175.
12. **Gill, C. O.** 1975. Effect of growth temperature on the lipids of *Pseudomonas fluorescens*. J. Gen. Microbiol. **89**:293–298.
13. **Gill, C. O., and J. R. Suisted.** 1978. The effects of temperature and growth rate on the proportion of unsaturated fatty acids in bacterial lipids. J. Gen. Microbiol. **104**:31–36.
14. **Janda, J. M., R. B. Clark, and R. Brenden.** 1985. Virulence of *Aeromonas* species as assessed through mouse lethality. Curr. Microbiol. **12**:163–168.
15. **Kates, M., and P. O. Hagen.** 1964. Influence of temperature on fatty acid composition of psychrophilic and mesophilic *Serratia* species. Can. J. Biochem. **42**:481–488.
16. **Kropinski, A. M., J. Kuzio, B. L. Angus, and R. E. W. Hancock.** 1982. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **21**:310–319.
17. **Kropinski, A. M. B., V. Lewis, and D. Berry.** 1987. Effect of growth temperature on the lipids, outer membrane proteins, and lipopolysaccharides of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **169**:1960–1966.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

19. **Lallier, R., F. Bernard, and G. Lalonde.** 1984. Difference in the extracellular products of two strains of *Aeromonas hydrophila* virulent and weakly virulent for fish. Can. J. Microbiol. **30:**900–904.
20. **Marr, A. G., and J. L. Ingraham.** 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. **84:**1260–1267.
21. **McConnell, M., and A. Wright.** 1979. Variation in the structure and bacteriophage-inactivating capacity of *Salmonella anatum* lipopolysaccharide as a function of growth temperature. J. Bacteriol. **137:**746–751.
22. **McElhaney, R. N., and S. A. Sousa.** 1976. The relationship between environmental temperature, cell growth and the fluidity and physical state of the membrane lipids in *Bacillus stearothermophilus*. Biochim. Biophys. Acta **443:**348–359.
23. **Merino, S., V. J. Benedí, and J. M. Tomás.** 1989. *Aeromonas hydrophila* strains with moderate virulence. Microbios **59:**165–173.
24. **Merino, S., S. Camprubí, and J. M. Tomás.** 1990. Identification of the cell surface receptor for 18 bacteriophage from *Aeromonas hydrophila*. Res. Microbiol. **141:**173–180.
25. **Merino, S., S. Camprubí, and J. M. Tomás.** 1990. Isolation and characterization of bacteriophage PM2 from *Aeromonas hydrophila*. FEMS Microbiol. Lett. **68:**239–244.
26. **Merino, S., S. Camprubí, and J. M. Tomás.** 1991. The role of lipopolysaccharide in complement-killing of *Aeromonas hydrophila* strains of serotype O:34. J. Gen. Microbiol. **137:**1583–1590.
27. **Merino, S., S. Camprubí, and J. M. Tomás.** 1992. Characterization of an O-antigen bacteriophage from *Aeromonas hydrophila*. Can. J. Microbiol. **38:**235–243.
28. **Osborn, M. J.** 1966. Preparation of lipopolysaccharide from mutant strains of *Salmonella*. Methods Enzymol. **8:**161–164.
29. **Paniagua, C., O. Rivero, J. Anguita, and G. Naharro.** 1990. Pathogenicity factors and virulence for rainbow trout (*Salmo gairdneri*) of motile *Aeromonas* spp. isolated from a river. J. Clin. Microbiol. **28:**350–355.
30. **Poole, K., and V. Braun.** 1988. Influence of growth temperature and lipopolysaccharide on hemolytic activity of *Serratia marcescens*. J. Bacteriol. **170:**5146–5152.
31. **Popoff, M.** 1984. Genus III. *Aeromonas* Kluyver and van Niel 1936, 398^{AL}, p. 545–548. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
32. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27:**493–497.
33. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measure cell surface hydrophobicity. FEMS Microbiol. Lett. **9:**29–33.
34. **Rottem, S., O. Markowitz, and S. Razin.** 1978. Thermal regulation of the fatty acid composition of lipopolysaccharides and phospholipids of *Proteus mirabilis*. Eur. J. Biochem. **85:**445–450.
35. **Thomas, L. V., R. J. Gross, T. Cheasty, and B. Rowe.** 1990. Extended serogrouping scheme for motile mesophilic *Aeromonas* species. J. Clin. Microbiol. **28:**980–984.
36. **Tomás, J. M., and J. T. Jofre.** 1985. Lipopolysaccharide-specific bacteriophage for *Klebsiella pneumoniae* C3. J. Bacteriol. **162:**1276–1279.
37. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119:**115–119.
38. **van Alphen, L., B. Lugtenberg, E. T. Reitschel, and C. Mombers.** 1979. Architecture of the outer membrane of *Escherichia coli* K12. Phase transition of the bacteriophage K3 receptor complex. Eur. J. Biochem. **101:**571–579.
39. **Wartenberg, K., W. Knapp, N. M. Ahamed, C. Widemann, and H. Mayer.** 1983. Temperature-dependent changes in the sugar and fatty acid composition of lipopolysaccharides from *Yersinia enterocolitica* strains. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Abt. 1 Orig. Reihe A **253:**523–530.
40. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
41. **Wollenweber, H. W., S. Schlecht, O. Luderitz, and E. T. Reitschel.** 1983. Fatty acid in lipopolysaccharide of *Salmonella* species grown at low temperature: identification and position. Eur. J. Biochem. **130:**167–171.