Quantitative and Qualitative Studies of *Aeromonas salmonicida* Bacteriophage

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A comprehensive bacteriophage typing scheme for *Aeromonas salmonicida*, the causal agent of furunculosis in fish, is described. It distinguishes 27 bacterial groups based on sensitivity patterns to 18 bacteriophage isolates. In addition, the sensitivity patterns are shown to reflect the morphological characteristics of the host bacterium. The 'rough', 'smooth' and 'G-phase' forms possess different quantities of lipopolysaccharide in the cell wall and this influences bacteriophage attachment. The problems related to these morphological variations are discussed.

INTRODUCTION

The effectiveness of bacteriophage typing systems has been proved by the valuable information gained in epidemiological investigations of human pathogenic bacteria, e.g. *Salmonella* (Boyd *et al.*, 1951; Anderson, 1964; Gershman, 1976), *Shigella* (Pruneda & Farmer, 1977) and *Staphylococcus* (Asheshov, 1967). Moreover, the potential application of bacteriophage typing to fish pathogens, in particular *Aeromonas salmonicida*, the causal agent of furunculosis, has been indicated by the work of Paterson *et al.* (1969) and Popoff (1971). It is essential to locate the source of infection if meaningful control measures are to be adopted. However, all too often when furunculosis is diagnosed, mismanagement or the presence of carrier fish in farm stocks or wild populations is assumed to be the origin of the disease outbreak (McCarthy, 1978).

It has been recognized that *A. salmonicida* displays morphological variation in vitro insofar as the colonies may appear as 'rough', 'smooth' or 'intermediate' (G-phase) (Duff, 1937, 1939; McCarthy & Rawle, 1975) and much contention has centred around which of these forms is the most virulent. McCarthy (1978) found that the majority of strains from 'explosive' outbreaks exhibited a rough appearance, but Anderson (1972) reported that the virulent forms were predominantly smooth. The morphological appearance coincides with the quantity of lipopolysaccharide (LPS) in the cell wall (Anderson, 1972). However, the influence of LPS, or other cell wall components, on bacteriophage attachment and subsequent lysis of the host bacterium has not been determined. Indeed, the role of LPS in influencing bacteriophage groupings has not been considered.

Thus, to achieve an effective knowledge of the epidemiology of *A. salmonicida* infections, we have established a comprehensive bacteriophage typing scheme, thereby extending the earlier work of Paterson *et al.* (1969) and Popoff (1971). We have also examined the role of LPS in bacteriophage attachment.

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Table 1. *Aeromonas* strains studied

Laboratory reference no.	Strain designation/ source*	Source of isolate	Bacteriophage typing group
<i>Aeromonas dourgesi</i>			
147/70	NCMB 1861	—	—
<i>Aeromonas hydrophila</i>			
7/67	NCMB 89	—	—
15/67	G. L. Bullock	(U.S.A.)	—
20/67	NCMB 1135	<i>Salmo gairdneri</i>	—
24/67	Laboratory isolate	(U.K.)	—
33/67	Laboratory isolate	(U.K.)	—
19/70	B. P. Eddy	—	—
66/70	Laboratory isolate	—	—
79/70	Laboratory isolate	—	—
103/70	E.F.D.L.	—	—
14/72	Laboratory isolate	<i>Rutilus rutilus</i> (U.K.)	—
37/75	Laboratory isolate	<i>Cyprinus carpio</i> (U.K.)	—
<i>Aeromonas liquefaciens</i>			
3/67	NCTC 9747	—	—
<i>Aeromonas punctata</i> subsp. <i>caviae</i>			
93/70	NCIB 9234	—	—
94/70	NCIB 9671	—	—
<i>Aeromonas salmonicida</i>			
21/67	NCMB 833	<i>Salvelinus fontinalis</i>	10
130/68	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
3/69	Laboratory isolate	<i>Esox lucius</i> (U.K.)	26
91/70	NCMB 1109	<i>Salmo trutta</i> (U.K.)	10
120/70	Laboratory isolate	<i>Tinca tinca</i> (U.K.)	6
121/70	Laboratory isolate	<i>Tinca tinca</i> (U.K.)	1, 10†
131/70	Laboratory isolate	<i>Salmo salar</i> (U.K.)	7
135/70	Laboratory isolate	<i>Salmo gairdneri</i> (U.K.)	1, 7†
143/70	NCMB 1110	<i>Salmo trutta</i> (U.K.)	10
144/70	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1, 2†
21/71	R. H. W. Schubert	<i>Oncorhynchus</i> sp.	10
26/71	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
9/72	Laboratory isolate	<i>Blicca bjoerkna</i> (U.K.)	13
10/72	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	7
15/72	N.R.C.	<i>Anaplopoma fimbria</i>	11
16/72	W.F.D.L.	<i>Salmo clarki</i> (U.S.A.)	1
17/72	W.F.D.L.	<i>Oncorhynchus kisutch</i> (U.S.A.)	1, 2†
18/72	W.F.D.L.	<i>Oncorhynchus kisutch</i> (U.S.A.)	10
7/73	T. Kimura	<i>Oncorhynchus</i> sp. (Japan)	1
13/73	Laboratory isolate	<i>Salmo namaylush</i> (Finland)	11
16/75	Laboratory isolate	<i>Rutilus rutilus</i> (U.K.)	15
18/75	Laboratory isolate	<i>Rutilus rutilus</i> (U.K.)	15
28/75	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
32/75	R. Bootsma	<i>Cyprinus carpio</i> (Netherlands)	18
34/75	R. Bootsma	<i>Cyprinus carpio</i> (Netherlands)	18
1/76	Laboratory isolate	<i>Blicca bjoerkna</i> (U.K.)	17
2/76	Laboratory isolate	<i>Blicca bjoerkna</i> (U.K.)	14
3/76	Laboratory isolate	<i>Blicca bjoerkna</i> (U.K.)	13
10/76	W. D. Paterson	(Canada)	1
16/76	I. W. Smith	<i>Salmo cordinius</i> (U.K.)	13
19/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	3
20/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	3
24/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
26/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
29/76	T. Hastein	<i>Salmo salar</i> (Norway)	25
43/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
49/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	4

Table 1. (continued)

Laboratory reference no.	Strain designation/ source*	Source of isolate	Bacteriophage typing group
<i>Aeromonas salmonicida</i> —continued			
50/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	10
62/76	T. Kimura	<i>Oncorhynchus masou</i> (Japan)	1
68/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
69/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
70/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
71/76	R. Sweeting	<i>Salmo trutta</i> (U.K.)	2
78/76	M. Popoff	<i>Salmo gairdneri</i> (France)	8
79/76	M. Popoff	<i>Salmo gairdneri</i> (France)	1
80/76	M. Popoff	<i>Salmo gairdneri</i> (Switzerland)	1
81/76	M. Popoff	(Norway)	7
82/76	M. Popoff	<i>Salmo gairdneri</i> (Denmark)	6
84/76	M. Popoff	(Japan)	1
85/76	M. Popoff	<i>Salmo gairdneri</i> (France)	2
89/76	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
90/76	T. Hastein	<i>Salmo salar</i> (Norway)	23
91/76	T. Hastein	<i>Salmo salar</i> (Norway)	24
92/76	T. Hastein	<i>Salmo salar</i> (Norway)	27
93/76	T. Hastein	<i>Salmo salar</i> (Norway)	27
103/76	P. de Kinkelin	<i>Salmo gairdneri</i> (France)	9
111/76	T. Hastein	<i>Salvelinus alpinus</i> (Norway)	22
125/76	T. Hastein	<i>Phoxinus phoxinus</i> (Norway)	15
126/76	T. Hastein	<i>Salmo trutta</i> (Norway)	20
127/76	M. Popoff	<i>Salmo gairdneri</i> (France)	1
128/76	M. Popoff	(Austria)	1
129/76	M. Popoff	(U.S.A.)	2
133/76	Laboratory isolate	(Denmark)	19
170/76	R. W. Gould	<i>Carassius auratus</i> (U.S.A.)	13
175/76	Laboratory isolate	(France)	1
178/76	O. Ljunberg	<i>Salmo trutta</i> (Sweden)	12
181/76	G. Giorgetti	<i>Salmo gairdneri</i> (Italy)	1
1/77	E.F.D.L.	<i>Salmo salar</i> (U.S.A.)	10
50/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1, 2†
51/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
52/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
53/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	4
64/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	5
65/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	4
66/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
67/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
70/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
75/77	T. Needham	<i>Salmo trutta</i> (U.K.)	9
77/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
78/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
82/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
83/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
84/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
85/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	10
86/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
87/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	10
88/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	10
89/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
91/77	Laboratory isolate	<i>Perca fluviatilis</i> (U.K.)	14, 16†
92/77	Laboratory isolate	<i>Cyprinus carpio</i> (U.K.)	21
121/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	2
123/77	Laboratory isolate	<i>Salmo trutta</i> (U.K.)	1
3/78	Laboratory isolate	<i>Rutilus rutilus</i> (U.K.)	26
<i>Aeromonas sobria</i>			
5/77	M. Popoff	(France)	—
6/77	M. Popoff	(France)	—

Table 1. (*continued*)

Laboratory reference no.	Strain designation/ source*	Source of isolate	Bacteriophage typing group
<i>Aeromonas</i> spp.			
6/69	—	<i>Salmo salar</i> (Ireland)	—
11/69	Laboratory isolate	<i>Cyprinus carpio</i> (U.K.)	—
15/69	Laboratory isolate	<i>Salmo salar</i> (U.K.)	—
35/70	B. P. Eddy	—	—
90/70	NCMB 86	—	—
110/70	G. L. Gilardi	(U.S.A.)	—
33/71	Laboratory isolate	(U.K.)	—
10/73	Laboratory isolate	<i>Perca fluviatilis</i> (U.K.)	—
55/77	N. J. Jensen	(Denmark)	—
142/77	Laboratory isolate	<i>Blicca bjoerkna</i> (U.K.)	—

* Sources: E.F.D.L., Eastern Fish Diseases Laboratory, Kearneysville, West Virginia, U.S.A.; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Colindale, London; N.R.C., National Research Council, Ottawa, Ontario, Canada; W.F.D.L., Western Fish Diseases Laboratory, Seattle, Washington, U.S.A.; Dr R. Bootsma, Department of Special Animal Pathology, University of Utrecht, The Netherlands; Dr G. L. Bullock, Eastern Fish Diseases Laboratory, Kearneysville, West Virginia, U.S.A.; Dr B. P. Eddy (deceased), Low Temperature Research Station, Cambridge, U.K.; Dr G. L. Gilardi, Mount Sinai Hospital, New York, U.S.A.; Dr G. Giorgetti, Istituto Zooprofilattico Sperimentale della Venezie, Italy; Dr R. W. Gould, Tavelek Inc., Redmond, Washington, U.S.A.; Dr T. Hastein, National Veterinary Institute, Oslo, Norway; Dr N. J. Jensen, Royal Veterinary and Agricultural College, Copenhagen, Denmark; Professor T. Kimura, Laboratory of Microbiology, Hokkaido University, Hakodate, Japan; Dr P. de Kinkelin, Laboratoire d'Ichtyopathologie, Institut National de la Recherche Agronomique, Paris, France; Dr O. Ljunberg, National Veterinary Institute, Stockholm, Sweden; Dr T. Needham, Unilever Research Laboratory, Aberdeen, Scotland; Dr W. D. Paterson, Environment Canada, Halifax Laboratory, Halifax, Canada; Dr M. Popoff, Institut Pasteur, Paris, France; Professor R. H. W. Schubert, Abteilung für Allgemeine und Umwelthygiene, Frankfurt, Germany; Dr I. W. Smith, Department of Bacteriology, University of Edinburgh, Scotland; Dr R. Sweeting, Thames Water Authority, Reading, Berkshire.

† Smooth and rough colony variants were recovered in different bacteriophage typing groups.

METHODS

Bacteria. The sources of the 1 strain of *Aeromonas dourgesi*, 11 strains of *A. hydrophila*, 2 strains of *A. punctata* subsp. *caviae*, 93 strains of *A. salmonicida*, 2 strains of *A. sobriæ* and 10 strains of *Aeromonas* spp. are shown in Table 1. The strains were maintained at 4 °C on slopes of tryptone soya agar (TSA; Oxoid). Subculturing was necessary every 6 months.

Bacteriophage. Water and effluent samples, collected from seven fish farms, five rivers and two reservoirs in England and Wales, and one effluent sample sent from a goldfish farm in Australia, were examined for the presence of bacteriophage to *A. salmonicida*. Methods for the isolation, propagation and induction of temperate bacteriophage, bacteriophage typing and lysotyping have been described elsewhere (Paterson *et al.*, 1969; Popoff, 1971). Additional bacteriophage isolates were received from Professor M. Popoff (France) and Dr W. D. Paterson (Canada). Details of all the bacteriophage are given in Table 2.

Lipopolysaccharides. Cell wall LPS of rough and smooth strains was extracted and purified using the hot phenol technique outlined by Shaw & Hodder (1978). A modified technique, using 0.5% (w/v) MgCl₂ to precipitate LPS, was necessary for G-phase cells.

Inactivation of bacteriophage by lipopolysaccharide. The amount of LPS capable of inhibiting plaque formation by bacteriophage was assessed as described by Adams (1959).

Antisera. Antisera to *A. salmonicida* were produced in female New Zealand white rabbits as described by McCarthy & Rawle (1975).

Agglutination reactions. The indirect agglutination method of Hansen & Lingg (1976) was used to characterize soluble antigen. This method involved the use of latex particles (Difco; 0.8 µm diam.) suspended in phosphate-buffered saline (Dulbecco 'A'; Oxoid) and sensitized with an equal volume of antigen for 1 h at 37 °C. Sensitized latex particles were coated with 1% (w/v) bovine serum albumin (Koch-Light) to prevent non-specific agglutination.

Equal volumes of sensitized latex particles (50 µl) and antisera, containing 200 mg polyvinylpyrrolidone 1⁻¹ (BDH), were pipetted into micro-titre trays (Cooke, Billingshurst, Sussex) (Bereks & Querforth, 1971). Doubling dilutions were prepared to 1:65 536 using phosphate-buffered saline. These were thoroughly mixed by shaking for

Table 2. *Details of bacteriophage isolates*

Laboratory reference no.	Source*	Comment
A	Fish farm effluent (U.K.)	Virulent
B	Fish farm effluent (U.K.)	Virulent
C	Reservoir (U.K.)	Virulent
D	Fish farm effluent (Australia)	Virulent
E	Fish farm effluent (Canada; W. D. Paterson)	Virulent
F	Fish farm effluent (U.K.)	Virulent
G	Fish farm effluent (U.K.)	Temperate
H	Fish farm effluent (U.K.)	Temperate
I	Fish farm effluent (U.K.)	Temperate
J	Fish farm effluent (U.K.)	Temperate
K	Fish farm effluent (U.K.)	Temperate
L	Fish farm effluent (U.K.)	Temperate
M	Fish farm effluent (France; M. Popoff)	Virulent
N	Fish farm effluent (France; M. Popoff)	Temperate; u.v.-induced
O	Fish farm effluent (France; M. Popoff)	Virulent
P	Reservoir (France; M. Popoff)	Virulent
Q	Reservoir (France; M. Popoff)	Virulent
R	Reservoir (France; M. Popoff)	Virulent

* Sources: Dr W. D. Paterson, Environment Canada, Halifax Laboratory, Halifax, Canada; Dr M. Popoff, Institut Pasteur, Paris, France.

10 min at 20 °C on an oscillating plate (Luckham, London). Following overnight incubation at room temperature, the micro-titre trays were shaken to resuspend the contents, and agglutination was observed through a binocular microscope.

Electron microscopy. Cultures of *A. salmonicida* grown overnight at 26 °C in tryptone soya broth (Oxoid) were inoculated with high-titre bacteriophage suspension. After incubation at room temperature for 30 min, the cultures were centrifuged at low speed (750 rev. min⁻¹; 10 min) and the deposited cells were washed in phosphate-buffered saline.

Washed cells were double-fixed in glutaraldehyde (4%, v/v) and osmium tetroxide (1%, w/v) (Millonig, 1961) and dehydrated by serial transfer through graded ethanol solutions. Following embedding in epoxy resin adhesive (Araldite; Ciba-Geigy) (Luft, 1961) and curing at 60 °C for 72 h, they were sectioned on a Reichert OMU3 ultramicrotome. Ultra-thin sections were double-stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and studied in a JEOL 100 CX transmission electron microscope.

For scanning electron microscopy, washed cell suspensions were fixed in osmium tetroxide for 1 h, washed with buffer, dehydrated by serial transfer through graded ethanol solutions and dried in a Polaron critical point drying apparatus. The dried material was mounted on stubs, coated with gold on a Polaron sputter coater and examined in a JEOL 100 CX Temscan electron microscope.

Pathogenicity for fish. Five 6-month-old brown trout (*Salmo trutta*) were each injected intramuscularly with 1 ml of washed cell suspension, containing about 10⁸ cells ml⁻¹. Strains of *A. salmonicida* in rough, smooth and G-phase forms were examined. The fish were maintained in fibre-glass tanks containing 15 l of circulating, oxygenated fresh water at 16 °C. Feeding was not carried out during the course of these experiments. All fish were examined daily, for up to a maximum of 14 d. Dead fish were removed and subjected to post-mortem examination. Samples of kidney, spleen, and muscle from the injection site were removed aseptically and plated on to TSA. Following incubation of these plates at 22 °C for 72 h, colonies of *A. salmonicida* were identified by a latex agglutination reaction (McCarthy, 1975a, b) and bacteriophage sensitivity patterns were determined.

RESULTS

The 18 bacteriophage strains examined in this study were specific to *Aeromonas salmonicida* (Table 1). Thus, the 93 isolates of *A. salmonicida* were divided into 27 bacteriophage sensitivity groups (Table 3), with the majority of the isolates, totalling 57, recovered in only three groups, namely 1, 2 and 10. These groups contained the majority of the U.K. isolates (Table 1). Indeed, there was a pronounced geographical distribution among the bacteriophage typing groups. The Scandinavian isolates were recovered exclusively in

Table 3. *Bacteriophage typing patterns for A. salmonicida* strains

Phage typing group	No. of strains	Bacteriophage																	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	24*	+	v	+	+	+	—	—	—	—	—	—	+	—	+	—	+	—	—
2	22*	v	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	+
3	2	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+	—	+	+
4	3	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+	—	—	+
5	1	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	—
6	2	+	+	+	+	+	—	+	+	—	—	+	v	+	+	+	+	+	+
7	4*	+	+	+	+	+	—	+	+	—	+	—	+	+	+	+	+	+	+
8	1	+	+	+	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+
9	2	+	+	+	+	+	—	+	—	—	—	—	—	+	+	+	v	+	+
10	11*	+	—	—	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—
11	2	+	+	—	+	v	+	—	—	—	—	—	—	—	—	—	—	—	—
12	1	—	—	—	+	+	+	—	—	—	—	—	—	—	—	+	—	+	—
13	4	—	—	+	v	—	—	+	+	+	+	+	+	—	+	—	+	—	+
14	2*	—	—	—	—	—	—	+	+	+	+	+	+	+	+	—	+	—	+
15	3	—	—	—	—	—	—	+	—	+	—	+	—	—	+	—	+	—	+
16	1*	—	—	—	—	—	—	+	—	+	+	+	—	+	+	—	+	—	+
17	1	—	—	—	—	—	—	+	—	+	+	+	+	—	+	—	+	—	+
18	2	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
19	1	—	+	—	+	—	—	—	+	—	+	—	—	—	—	—	—	—	+
20	1	—	—	—	—	—	—	—	—	+	—	+	—	—	—	—	—	—	—
21	1	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—
22	1	+	—	—	+	+	—	+	—	—	—	—	+	+	—	+	—	—	—
23	1	—	—	+	+	+	—	+	—	+	—	—	+	+	—	+	—	—	—
24	1	—	—	+	+	+	—	+	—	—	—	—	+	+	—	—	—	—	—
25	1	+	—	—	+	+	—	+	—	—	—	—	+	+	—	+	—	+	—
26	2	—	—	+	—	+	—	—	+	—	+	+	+	+	+	—	—	—	+
27	2	—	—	—	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—

+, Lysis; —, no lysis; v, variable.

* Contains cultures with rough and smooth colonies which fall into two bacteriophage typing groups.

groups 7, 12, 15, 20, 22, 23, 24, 25 and 27. Similarly, the Dutch isolates belonged in group 18. However, the pattern of lysis was sometimes affected by colony morphology, with smooth and rough types differing in their bacteriophage sensitivities. Hence, a single isolate could belong to more than one bacteriophage typing group, depending on its morphology, viz. rough or smooth (Table 1). Clearly, this is a serious problem, and therefore a detailed examination was made of the effect of colony morphology on bacteriophage sensitivity.

Effect of colony morphology on bacteriophage sensitivity

The morphological differences between smooth, rough and G-phase colonies (Fig. 1) were highlighted by examination of scanning electron micrographs of individual cells. Rough cells possessed an external granular layer (Fig. 2) which was absent in smooth cells (Fig. 3). G-phase cells appeared to have a very slight external layer (Fig. 4). Examination by transmission electron microscopy of thin sections from bacterial cells confirmed the presence of an extra layer around rough cells (Fig. 5), which was absent from smooth and G-phase cells (Figs 6, 7). The influence of these surface layers on bacteriophage attachment and subsequent lysis of the host cell was illustrated by the role of bacteriophage F and N, which were specific and lysed only G-phase cells and rough cells, respectively. In the small-scale pathogenicity experiments, rough cells were pathogenic to *Salmo trutta*, while smooth cells were less pathogenic and G-phase cells were completely avirulent.

Fig. 1. Morphology of rough (R), smooth (S) and G-phase (G) colonies of *Aeromonas salmonicida*. The bar marker represents 1 mm.

Figs 2–4. Scanning electron micrographs of rough cells (Fig. 2), smooth cells (Fig. 3) and G-phase cells (Fig. 4) of *A. salmonicida*. The bar markers represent 0.5 µm.

Lipopolysaccharide

Rough cells were rich in LPS: rough colonies of isolate 50/77 yielded about 2.4% cell weight of LPS compared with only 1.4% LPS in smooth cells of the same isolate. The importance of LPS in the processes leading to viral infection was indicated by the observation that LPS extracted from rough, smooth and G-phase cells inhibited the cycle of bacteriophage replication. However, it is noteworthy that there were marked differences between the morphological forms in the quantities of LPS needed to inactivate 50% of the bacteriophage. Thus, plaque formation by bacteriophage N, specific to rough cells of isolate 50/77, was inhibited by a small quantity of LPS extracted from the host cells (Table 4), but large quantities of the same LPS were needed to affect bacteriophage F, specific to G-phase cells. With bacteriophage C, which lyses rough and smooth cells but not G-phase cells, plaque formation was inhibited by small amounts of LPS from rough or smooth cells.

The antigenicity of LPS was confirmed by agglutination. Purified LPS extracted from rough cells of isolate 50/77 gave a higher titre (1:1024) with the homologous antiserum than that from the corresponding smooth cells (1:128). Furthermore, these serological reactions were non-specific, insofar as there was cross-agglutination with antisera prepared against smooth cells of 16/72 and rough cells of 17/72. Nevertheless, the titres were substantially lower than those for whole cells.

Figs 5–7. Transmission electron micrographs of thin sections through a rough cell (Fig. 5), a smooth cell (Fig. 6) and a G-phase cell (Fig. 7) of *A. salmonicida*. The rough cell shows the presence of an extra layer (E) around the cell wall (W); the smooth and G-phase cells lack an extracellular deposit. The bar markers represent 200 nm.

Fig. 8. Thin section through a rough cell experimentally infected with bacteriophage, showing the presence of virus particles attached to the surface of the host bacterium. The bar marker represents 200 nm.

Fig. 9. Negatively-stained preparation of temperate bacteriophage N, showing a hexagonal head and long tail. The bar marker represents 100 nm.

Fig. 10. Negatively-stained preparation of virulent bacteriophage F. The bar marker represents 50 nm.

Table 4. Quantities of purified LPS from different morphological forms of isolate 50/77 needed to inhibit plaque formation by bacteriophage C, F and N

Source of LPS	Amount of LPS (μ g hexose)		
	Bacteriophage:		
	C	F	N
Smooth	0.0024	>1000*	>1000
Rough	0.0024	>1000	0.009
G-phase	>1000	0.28	>1000

* No inhibition with LPS corresponding to 1000 μ g hexose.

Bacteriophage

Free bacteriophage were released into the aqueous media, following initial attachment to the specific bacterial cell (Fig. 8), penetration and replication. The morphology of the free virions, as examined by transmission electron microscopy, comprised icosahedral heads and contractile tails (Figs 9, 10). Attachment fibres were not observed.

DISCUSSION

With 18 bacteriophage strains specific to *A. salmonicida*, and corresponding to the T-even morphology type of Paterson *et al.* (1969), a comprehensive typing scheme was devised which should be of value in epidemiological and ecological studies. The scheme has extended the work of Popoff (1971), who described only 14 'lysotypes' of *A. salmonicida* with 8 bacteriophage strains, and highlighted some of the hitherto unassociated problems that cell morphology confers on bacteriophage sensitivity patterns. LPS, considered primarily as an endotoxin of *A. salmonicida* (Ross, 1966; Anderson, 1973; Paterson & Fryer, 1974), was shown to be important in the infection processes, with the LPS-rich rough cells isolated most commonly from epizootics (Popoff & Vieu, 1970; McCarthy, 1978) and found in this study to be the most pathogenic form of the organism for *Salmo trutta*. However, it has been shown that small numbers of smooth colony types may be isolated on TSA along with the numerically dominant rough colonies from diseased fish (C. Rodgers, unpublished data). The significance of these observations is not known at present.

The results of phage typing, LPS extraction, serological studies and electron microscopy show there are differences in outer membrane and surface LPS characteristics between the rough, smooth and G-phase forms of *A. salmonicida*. With phage receptors located in the LPS cell fractions (Lindberg, 1973, 1977), the results show that the three morphological forms have different amounts of surface LPS which lead to differences in phage typing patterns and the difficulty experienced in extracting LPS from G-phase cells.

LPS from other Gram-negative bacteria (*Salmonella*, *Shigella* and *Escherichia*) generally consists of an O-specific side-chain and an inner core polysaccharide covalently bound to a lipid (Kabir *et al.*, 1978). The O-specific side-chains carry the serological specificity of each LPS and the sequence of carbohydrate molecules in this region is species specific, with the core polysaccharide being group specific (Kabir *et al.*, 1978).

Further work on the biochemical nature of *A. salmonicida* LPS would need to be undertaken to show whether or not fractions from the different morphological forms differ because of loss of polysaccharide side-chains, leaving an inner core region. In fact, the LPS inner core region, composed of 2-keto-3-deoxyoctonic acid (KDO) and lipid A, differs between *A. hydrophila* subsp. *liquefaciens* and *A. hydrophila* subsp. *punctata* (Ellwood, 1970). It would be interesting to know if the same is true between *A. hydrophila* and *A. salmonicida* or even between different strains of *A. salmonicida*, and whether any difference in composition between rough, smooth, G-phase and atypical strains corresponds to differences in phage attachment and typing patterns.

The biochemical nature of *Aeromonas* LPS remains largely unknown, but if the LPS layers lose side-chain units then it follows that, with phage receptors in the LPS, different phage typing patterns would be inevitable with typical and atypical strains of *A. salmonicida* possessing various ratios of outer side-chain units to inner core KDO-lipid A regions. Both scanning and transmission electron micrographs confirm that the surface characteristics of the three morphological forms are quite distinct.

Despite widespread interest in furunculosis, the ecology of *A. salmonicida* has been neglected, and the natural reservoir of the organism has not been established, although the involvement of carrier fish in native populations has been tentatively suggested (McCarthy, 1978). It could be argued that the presence, and hence isolation, of bacteriophage implies the presence of the host bacterium. This has been suggested by Zachary's work with *Vibrio natriegens* (Zachary, 1978), but the question of how long the virus particles remain viable in the aquatic environment remains unanswered. This would be an important consideration if bacteriophage were to be exploited in meaningful ecological studies.

The role of bacteriophage typing in bacterial taxonomy should be treated cautiously, although the technique has provided useful information in identification. For example, Evelyn (1971) isolated a bacterial culture from marine sablefish which was susceptible to *A. salmonicida* bacteriophage. This information formed a useful pointer in the identification of environmental isolates. However, the problem of varying sensitivity to bacteriophage between rough and smooth cells, ignored to date, could result in mis-identification.

In conclusion, the comprehensive bacteriophage typing system for *A. salmonicida* described in this study should prove useful in epidemiological and ecological studies providing the shortcomings, i.e. different sensitivity patterns between rough, smooth and G-phase cells of the same strain, are recognized.

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