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Monoclonal antibodies against *Aeromonas salmonicida* lipopolysaccharide identify differences among strains

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ABSTRACT: Monoclonal antibodies (MAbs) directed against *Aeromonas salmonicida salmonicida* lipopolysaccharide (LPS) were produced and characterized. The specificity of the MAbs (N = 4) to LPS was determined by examination of western blots of proteinase K-treated whole cell preparations of A+ and A− strains, i.e. strains with and without a major surface protein layer, the A layer. The different MAbs identified different epitopes on *A. salmonicida* LPS. Three of the 4 MAbs (MAb 1, C, and D) reacted with all but a unique group of *A. salmonicida* strains tested. These MAbs also had binding properties that were insensitive to periodate oxidation of antigen. The fourth MAb (MAb 6) reacted with a more limited collection of bacterial isolates and the binding was sensitive to periodate treatment of antigen. These data demonstrated that heterogeneity is present in the LPS of this species. Lipopolysaccharide from all tested isolates of *A. salmonicida salmonicida* (N = 10) and *A. salmonicida masoucida* (N = 2) reacted with each MAb, while isolates of *A. salmonicida achromogenes* (N = 6) reacted with MAbs 1, C, and D but not with MAb 6. No patterns of similarity were apparent when unclassified isolates of *A. salmonicida* were examined.

INTRODUCTION

Aeromonas salmonicida, the etiologic agent of furunculosis and ulcer disease of salmonids and other fishes, causes major losses in cultured fish in many parts of the world (Snieszko et al. 1950, Bootsma et al. 1977, McCarthy & Roberts 1980). Considerable research effort has been expended to investigate means of preventing furunculosis in hatchery stocks. Efficacious vaccines have been reported, but their ability to stimulate a protective specific immune response is under debate (Ellis 1988). Therefore, these diseases are currently controlled by antibiotic therapy and pathogen avoidance.

Both cellular and secreted antigens of *Aeromonas salmonicida* have been investigated as possible immunogens (Munro 1984, Hastings & Ellis 1988). Immunologically important cellular antigens include the A layer (McCarthy et al. 1983) and lipopolysaccharide (LPS; Paterson & Fryer 1974). Uniformity of these 2 components was reported within the species (Chart et al. 1984, Kay et al. 1984).

Three subspecies of *Aeromonas salmonicida* are currently recognized. These are *A. salmonicida salmonicida*, *A. salmonicida achromogenes*, and *A. salmonicida masoucida* (Popoff 1984). However, many isolates do not fit into any of these categories and modifications to the current classification scheme have been proposed (McCarthy & Roberts 1980, Belland & Trust 1988, Austin et al. 1989). These proposals include the addition of a new subspecies, *A. salmonicida nova*, and combining most of the isolates from *A. salmonicida masoucida* and *A. salmonicida achromogenes* into a single subspecies with the latter epithet.

The present paper describes the production and characterization of monoclonal antibodies (MAbs) against *Aeromonas salmonicida* LPS. These MAbs were used to identify LPS heterogeneity within the species. Additionally, reactivity patterns were different among the different subspecies of *A. salmonicida*.

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MATERIALS AND METHODS

Bacterial isolates and antigen preparation. All isolates were grown on brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) agar for isolation and were stored at 4 °C on BHI agar slants. Isogenic A layer negative (A−) mutants were selected by growing wild type (A+) *Aeromonas salmonicida* strains on Coomassie brillant blue agar at 30 °C and recovering white colonies (Ishiguro et al. 1981, Wilson & Horne 1986). Whole cell extracts for electrophoresis and western blotting were prepared from cultures grown in BHI broth for 24 to 48 h at 17 °C. Cells were pelleted by centrifugation, washed in phosphate-buffered saline (PBS; 10 mM PO_4, 150 mM NaCl, pH 7.0) and resuspended in PBS at ca 10^9 cells ml^{-1}. The suspensions were kept on ice and sonically lysed with two 10 s bursts using a probe sonicator with power level at 60 W. Subsamples of these sonicates were mixed with equal volumes of sample buffer (Schleif & Wensink 1981), boiled for 2 min, and stored at −20 °C. The remaining sonicate was stored at −70 °C for future use or confirmatory analyses.

Selected whole cell sonicates (0.5 mg ml^{-1} PBS) were treated with proteinase K (Sigma Chemical Co. St Louis, MO) for 4 h at room temperature. Digestion was confirmed using SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie brilliant blue staining. These proteinase K preparations were used as antigen in western blots.

Production of monoclonal antibodies. Viable strain RC 1 (*Aeromonas salmonicida salmonicida*) bacterial cells harvested from a BHI broth culture were washed in sterile PBS and adjusted to an optical density (A_{525}) of 1.0. These live cells were mixed with an equal volume of Freund's complete adjuvant (FCA) and 0.5 ml was injected subcutaneously into female Balb/C mice. One hundred microliters of an identical antigen preparation without FCA was injected intraperitoneally 21 d after the primary injection. Three days after the secondary injection, spleen cells were harvested and fused with Sp2/0 myeloma cells as described by Campbell (1984). Clones producing MAbs against wild type *Aeromonas salmonicida* cells were selected using an antibody capture ELISA with A+ *A. salmonicida* strain RC 1 cells as antigen (Campbell 1984), expanded, and cloned twice by limiting dilution. All MAbs were harvested from tissue culture. Heavy and light chain isotypes and subisotypes were determined using a commercial kit (Bio-Rad Laboratories, Richmond, CA).

Electrophoresis and western blots. Polyacrylamide gel electrophoresis was performed on each isolate used for antigenic analysis. Unless otherwise indicated, ca 3×10^6 sonically lysed bacteria were mixed with sample buffer (Schleif & Wensink 1981) and electrophoresed using a Bio-Rad Mini-Protean II electrophoresis apparatus. Gels were stained for total protein with Coomassie brilliant blue and for LPS using the silver stain technique of Tsai & Frasch (1982). Western blots of cell lysates were performed with a Bio-Rad Laboratories Mini-Transblot apparatus. After transfer, blots were blocked with 3 % bovine serum albumin (BSA) in tris-buffered saline (TBS; 150 mM NaCl, 50 mM tris pH 8.0) plus 0.1 % Tween-20 (TTBS). Blots were then washed once in TTBS and tissue culture supernatant of the appropriate hybridoma was applied. Blots at each step were incubated at room temperature for 1 h. After incubation, the blots were washed 3 times in TTBS and incubated in goat anti-mouse immunoglobulin antisera conjugated to horseradish peroxidase (Hyclone Laboratories, Logan, UT). Blots were washed 3 times in TTBS and once in TBS, and then developed with 4-chloro-naphthol (4CN) and hydrogen peroxide (0.5 mg ml^{-1} 4CN and 0.03 % H_2O_2).

Periodate treatment of blots to determine epitope sensitivity to oxidation was performed as described (Woodward et al. 1985). Briefly, blots were incubated in TTBS after transfer and then incubated in 10 mM periodic acid in 50 mM sodium acetate (pH 4.5) for 1 h in the dark. Blots were rinsed in 50 mM sodium acetate and incubated in 50 mM sodium borohydride in PBS. Control blots were incubated in 50 mM sodium acetate without periodate. After treatment with borohydride, the primary antibody was added to all blots and incubations proceeded as with untreated blots.

RESULTS

Characterization of monoclonal antibodies

Four MAbs were produced and characterized. Three were of the IgG_{2b} subisotype with κ light chains (MAb 1, C, and D). One was of the IgM isotype with a κ light chain (MAb 6). The MAbs were reacted with selected antigen preparations to examine their binding specificity. Western blots using proteinase K treated antigens and blots using A− derivatives of isolate RC1 showed that each MAb bound *Aeromonas salmonicida* LPS (Fig. 1).

Periodate treatment prior to western blotting demonstrated that the epitope bound by MAb D was periodate-insensitive and the epitope bound by MAb 6 was periodate-sensitive (Fig. 2). MAbs 1 and C reacted similarly to MAb D.

Three patterns were observed when the MAbs were reacted with different *Aeromonas salmonicida* isolates in western blots (Fig. 3). Isolates reacted with both MAb D and MAb 6 (+/+ reaction), or with MAb D but

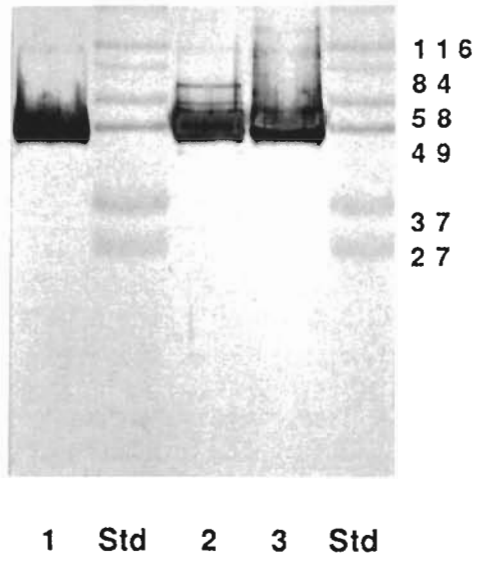

Fig. 1. Western blot of *Aeromonas salmonicida* isolate RC 1 cell preparations using MAb D as probe. Std: protein molecular weights, values in kilodaltons. (1) A layer − cells; (2) proteinase K treated A layer + cells; (3) untreated A layer + cells

not MAb 6 (+/− reaction), or the isolates reacted with neither antibody (−/− reaction). Several isolates were also tested with MAb 1 and MAb C and the pattern was identical to MAb D. All isolates that reacted with MAb 6 also reacted with MAb D. Isolates that did not react with MAb D (−/− strains) also did not react with rabbit antiserum directed against LPS from *A. salmonicida salmonicida* strain RC 1. Lipopolysaccharide from all isolates with MAb D were recognized by this rabbit antiserum. The Norwegian strains that were −/− (2013 and 2779) were shown by electrophoresis and subsequent silver staining to lack LPS O polysaccharide chains.

The results demonstrated that reactivity varied among isolates (Table 1). Comparisons among previously classified subspecies of *Aeromonas salmonicida* demonstrated that all tested *A. salmonicida salmonicida* (10 isolates) were +/+, strains of *A. salmonicida achromogenes* (6 isolates) were +/−, and the 2 *A. salmonicida masoucida* isolates were +/+. Reactivity patterns were not apparent in tested unclassified isolates.

The unclassified isolates harvested from fish in Norway were examined biochemically to determine their relationship to established taxonomic groups. Each of these isolates produced acid from sucrose and glucose. Pigment and indole production were variable and did not correlate directly with MAb reactivity (Table 2).

Antibodies were also used as probes in western blots of sonicates of other bacterial fish pathogens. This examination included strains of *Aeromonas hydrophila, Yersinia ruckeri, Pseudomonas fluorescens, Edwardsiella* sp., *Vibrio anguillarum, Carnobacterium piscicola* and *Renibacterium salmoninarum*. Neither MAb D nor MAb 6 reacted with these bacteria in western blots (data not shown).

DISCUSSION

We have produced MAbs against *Aeromonas salmonicida salmonicida* LPS which differentiate among isolates of *A. salmonicida*. These MAbs, produced against viable *A. salmonicida* emulsified in FCA, reacted specifically with *A. salmonicida* LPS and did not cross-react with other tested bacterial pathogens of fish. The MAbs were used exclusively for western blotting in the described research but they also can be used for enzyme-linked immunosorbent assays (ELISAs) and in 'dot-blot' immunoassays (not shown).

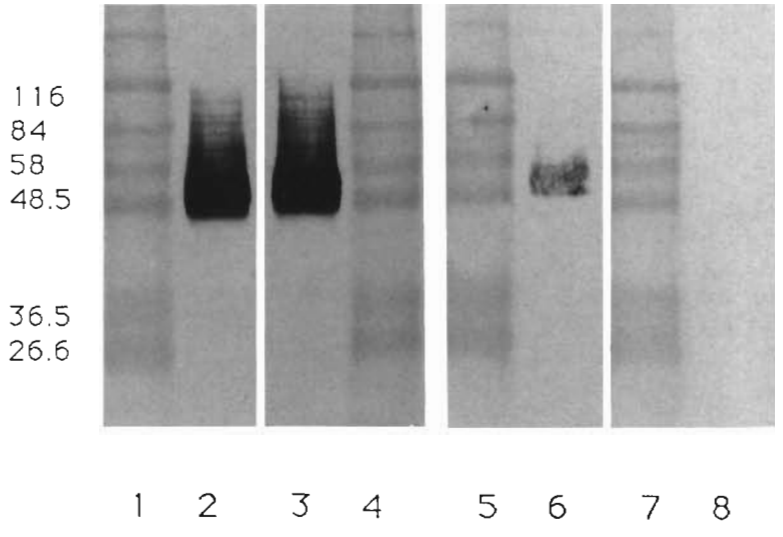

Fig. 2. Periodate treated and untreated western blots of *Aeromonas salmonicida salmonicida* isolate MLE-2 probed with MAb D or 6. Lanes: (1, 4, 5, 7) protein molecular weight standards for reference purposes; (2) untreated blot probed with MAb D; (3) treated blot probed with MAb D; (6) untreated blot probed with MAb 6; (8) treated blot probed with MAb 6

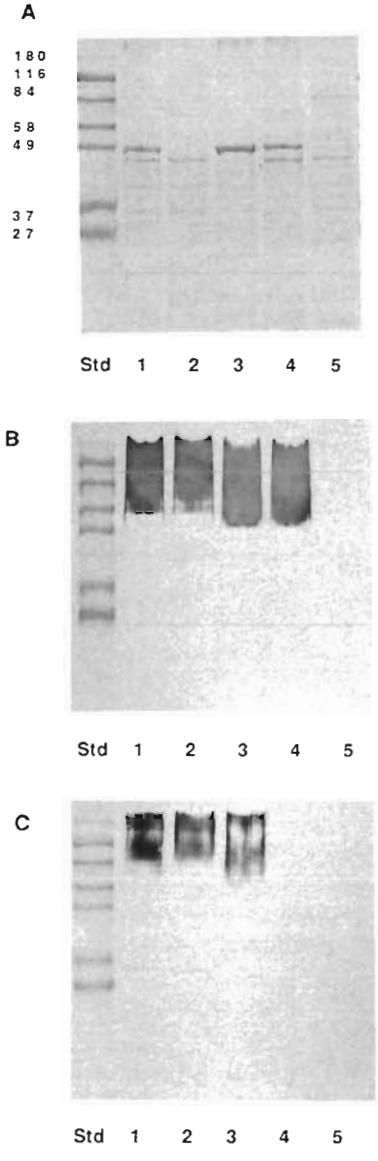

Fig. 3. (A) Polyacrylamide gel of selected *Aeromonas salmonicida* isolates; (B) western blot using MAb D as probe; (C) western blot using MAb 6 as probe. In each photograph the order is as follows: Std: molecular weight standards in kilodaltons; (1) A layer + RC 1; (2) A layer − RC 1; (3) 3173; (4) 2764; (5) 2779

The MAbs were differentiated on the basis of their isotype, binding sensitivity to periodate oxidation of antigen, and reactivity with selected *Aeromonas salmonicida* isolates. The binding of antigen by MAbs 1, C, and D (3 independent γ_{2b}, κ clones) was insensitive to periodate oxidation of antigen, whereas the binding of antigen by MAb 6 (μ, κ) was sensitive to this treatment. The reactivity of the MAbs in western blots with preparations of different *A. salmonicida* isolates as antigen showed that the majority of isolates reacted with MAbs 1, C, and D while a subset of these isolates reacted with MAb 6. All isolates which reacted with MAbs 1, C, and D also reacted with rabbit antiserum produced against *A. salmonicida salmonicida* strain RC 1. No isolates were identified that reacted only with MAb 6. Certain isolates did not react with any of the MAbs. Tested strains from this group did not have high molecular weight LPS chains.

Other MAbs against components of the *Aeromonas salmonicida* cell surface have been reported (Chart et al. 1984, Goerlich et al. 1984, Kay et al. 1984). Chart et al. (1984) used an anti-LPS MAb in their comparison of the structure and immunogenicity of different isolates of *A. salmonicida*. They showed that all strains with high molecular weight LPS reacted with the MAb. They used these and other data to support the conclusion that *A. salmonicida* LPS is structurally and immunochemically homogeneous. In this paper we demonstrated that LPS has antigenic variation among isolates. Such variability is common within Gram-negative bacterial species and the degree of identified heterogeneity within *A. salmonicida* is comparatively small (Ezura et al. 1980, Le Minor 1984). It is possible that future analyses will identify other variable epitopes within the species.

Western blots performed using different concentrations of antigen demonstrated that the appearance of the blot was dependent upon the concentration of antigen electrophoresed and transferred. This is shown by the differences between the banding patterns in certain blots (Figs. 1 & 3). Higher concentrations were used in the determination of reactivity (Fig. 3) because lower concentrations commonly resulted in questionable reactions.

The classification of isolates into specific subspecies of *Aeromonas salmonicida* may be facilitated by examination with the MAbs. In our analyses,all isolates of the subspecies *A. salmonicida salmonicida* were +/+. These included isolates from North America, Europe, and Korea. Strains identified as *A. salmonicida achromogenes* strains, including type strain NCMB 1110, were +/−, and the 2 *A. salmonicida masoucida* strains (including the type strain) were +/+. Isolates referred to as unclassified had no consistent reactivity pattern. These isolates were taken from fish in Europe, Japan, North America, and Australia. Other authors have recently performed rigorous analyses of the systematics of this organism and each has discussed the creation of new subspecies within the species (McCarthy & Roberts 1980, Belland & Trust 1988, Austin et al. 1989). Analyses are continuing to determine the utility of these MAbs for accurately assessing the validity of the current and proposed classification schemes for *A. salmonicida*.

Table 1 Reaction of MAbs D and 6 by western blotting with selected isolates of *Aeromonas salmonicida*

Isolate	Subspecies[a]	Host[b]	D	6	Reference or source
ATCC 14174	*sal* T[c]	BT	+	+	A.T.C.C.
RC 1	*sal*	Ch	+	+	Rockey et al. (1988)
SS 70	*sal*	Ch	+	+	Udey & Fryer (1978)
MLE-2	*sal*	MS	+	+	Fryer et al. (1988)
567	*sal*	AS	+	+	N.V.I.[d]
2683	*sal*	AS	+	+	N.V.I.
3173	*sal*	AS	+	+	N.V.I.
77/88	*sal*	Br	+	+	E. M. Bernoth
MT 518	*sal*	AS	+	+	T Hastings
MT 194	*sal*	AS	+	+	T Hastings
NCMB 1110	*ach* T	Br	+	−	N.V.I.
MT 533	*ach*	AS	+	−	T Hastings
362	*ach*	AS	+	−	N.V.I.
937	*ach*	AS	+	−	N.V.I.
797	*ach*	AS	+	−	N.V.I.
2778	*ach*	AS	+	−	N.V.I.
ATCC 27013	*mas* T	MS	+	+	Kimura (1969)
2b1	*mas*	MS	+	+	M. Yoshimizu
865	Uncl.	Br	+	+	N.V.I.
1391	Uncl.	WF	+	+	N.V.I.
2221	Uncl.	BT	+	+	N.V.I.
1977	Uncl.	AS	+	+	N.V.I.
2120	Uncl.	Br	+	−	N.V.I.
2764	Uncl.	AS	+	−	N.V.I.
O2	Uncl.	Ayu	+	−	M. Yoshimizu
As 7	Uncl.	GF	+	−	Elliott & Shotts (1980)
Ar 57	Uncl.	GF	−	−	Tajima et al. (1987)
2013	Uncl.	AS	−	−	N.V.I.
2779	Uncl.	AS	−	−	N.V.I.
5602 (A405)[e]	Uncl.	GF	−	−	Trust et al. (1980)
5603 (A404)	Uncl.	GF	−	−	Trust et al. (1980)

[a] Subspecies codes: *sal, A. salmonicida salmonicida; ach, A. salmonicida achromogenes; mas, A. salmonicida masoucida;* uncl., unclassified *A. salmonicida*

[b] Host codes: Ch, chinook salmon *Oncorhynchus tschawytscha*; MS, Masou salmon *O. masou;* BT, brook trout *Salvelinus fontinalis*; AS, Atlantic salmon *Salmo salar*; Br, brown trout *Salmo trutta*; WF, whitefish *Coregonus* sp.; ayu, *Plecoglossus altivelis*; GF, goldfish *Carasius auratus*

[c] Indicates type strain

[d] National Veterinary Institute, Oslo, Norway

[e] Other common designations are indicated in parentheses

Table 2. Biochemical characteristics of selected unclassified isolates

	Strain									
	865	1391	2221	1977	2120	2764	2778	2013	2779	As 7[a]
MAb group	+/+	+/+	+/+	+/+	+/−	+/−	+/−	−/−	−/−	+/−
Acid from sucrose	+	+	+	+	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+
Pigment	−	−	−	−	+	+	+	+	−	−
Indole	+	+	+	−	+	+	+	+	+	+

[a] As reported by Elliott & Shotts (1980)

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