

Immunogenicity of various *Vibrio ordalii* lipopolysaccharide fractions in coho salmon *Oncorhynchus kisutch*

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ABSTRACT: The immunogenicity of intraperitoneally injected native *Vibrio ordalii* (Vo) lipopolysaccharide (LPS) and of various Vo LPS fractions obtained by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was examined in coho salmon *Oncorhynchus kisutch*. Immunogenicity was assessed using a challenge with live Vo cells, the challenge being sufficient to kill 85 to 87% of the control fish not receiving any Vo preparations. Native Vo LPS afforded the strongest protection, the relative percentage survival (RPS) being 85.3%. LPS fractions were found to contain 2 or more LPS components, and protective immunity associated with the fractions appeared to decline (from 51.4 to 5.9% RPS) as the molecular weight of the slowest migrating components in the fractions decreased. In fact, only those fractions containing LPS with molecular weights of 20 kD or above resulted in significant protection. We conclude that the higher molecular weight species of Vo LPS were the ones that were largely responsible for the anti-Vo immunity induced in coho by native LPS, and, based on the ladderlike pattern produced by the LPS on SDS-PAGE, we think it likely that the different LPS species represented molecules differing from one another largely in the number of O-polysaccharide repeat units present. Aggregation of the LPS molecules also occurred, and the possibility that aggregation may have contributed to the immunogenicity of the protective LPS fractions cannot be ruled out.

INTRODUCTION

Salmonids vaccinated with killed whole cells, cell wall extracts, or extracellular products of the fish pathogenic vibrios *Vibrio anguillarum* (Va) and *Vibrio ordalii* (Vo) have been shown to be protected from vibriosis caused by these organisms (Evelyn 1984). Using polyclonal antisera and immunoblotting, Chart & Trust (1984) showed that the major surface antigen of these vibrios was lipopolysaccharide (LPS), and the evidence is now convincing that LPS is, in fact, the protective antigen produced by each of these species (Evelyn 1988, Velji et al. 1990).

As judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), LPS produced by Vo appears to be considerably more heterogeneous than that produced by certain of the Va serotypes (Chart & Trust 1984). However, based on the ladderlike pattern of Vo LPS bands observed, and by analogy with the findings of others for such LPS banding

patterns (Goldman & Lieve 1980, Palva & Makela 1980), it is likely that the heterogeneity in Vo LPS is largely accounted for by the differing number of repeat units present in the various O-polysaccharide chains.

We have shown that LPS extracted from Vo cells or released to the culture medium by Vo during growth is immunogenic in extremely small quantities. The immunogenicity, measured in terms of anti-vibrio protection in juvenile coho salmon *Oncorhynchus kisutch*, was demonstrated in fish that had been vaccinated by either of 2 routes: by immersion or by injection (Velji et al. 1990).

The purpose of the present study was to determine whether all of the different molecular weight species of Vo LPS contribute to the protective immunity observed with native Vo LPS. We therefore fractionated Vo LPS by preparative SDS-PAGE (P-PAGE) and tested the resulting LPS fractions for their protectiveness in coho salmon.

MATERIALS AND METHODS

Bacterial isolate and preparation of LPS. The Vo isolate (# 74-48) used in this study as the source of LPS and for challenging the juvenile coho was originally isolated from a farmed sockeye salmon *Oncorhynchus nerka* that had died of vibriosis (Evelyn & Ketcheson 1980). The cells were grown for 32 h at 22 °C in Brain Heart Infusion Broth (Difco, Detroit, MI, USA) supplemented with 1% NaCl. The cell wall LPS was extracted from the Vo cells by the method of Darveau & Hancock (1983). LPS and LPS fractions were quantified by the chromogenic limulus amoebocyte lysate method (Whittaker Bio-Products, Walkersville, MD, USA).

Preparative SDS-Page and fractionation of LPS. Vo LPS (2.75 mg) was added to sample buffer and subjected to P-PAGE (Laemmli 1970) using a modified buffer system. For improved resolution of LPS in the gel, the sample buffer was modified to contain 4% SDS and 40 mM ethylene diamine tetraacetic acid (EDTA) (Angus 1986). Both the 4.5% stacking gel and the 12.5% separating gel contained 0.1% SDS. The separating gel measured 140 × 125 × 3 mm. The mixture of 275 µl of the LPS solution and 2.75 ml of sample buffer was boiled for 5 min, cooled, and 275 µl of the mixture was loaded per well. One additional lane was loaded with low molecular weight marker proteins (Bio-Rad Laboratories, Richmond, CA, USA). Electrophoresis was carried out for 9 h at a constant current of 30 mA. Immediately after electrophoresis, the low molecular weight lane was excised, the marker proteins in the gel stained with Coomassie Blue, and the unreacted stain in the gel removed using an electrophoretic destainer (Pharmacia Fine Chemicals, Uppsala, Sweden). Based on the molecular weights of the protein markers, and on the position of the silver-stained LPS bands (see below) detectable on a gel from a preliminary run, the LPS portion of the gel was cut into horizontal sections (fractions) corresponding to the following molecular weights: (1) 7 to 10 kD, (2) 10 to 15 kD, (3) 15 to 20 kD, (4) 20 to 25 kD, (5) 25 to 35 kD, and (6) 40 to 50 kD. A second preparative gel was run as a control. One lane of this gel was loaded with the low molecular weight marker proteins and the remaining lanes were loaded with a mixture of pyrogen-free water (PFW) (275 µl) and sample buffer (2.75 ml). Following electrophoresis (as above), a 3 cm wide horizontal strip was removed from the middle of the gel. All of the excised gel sections were freeze-dried overnight and then ground separately in a clean mortar and pestle in the presence of liquid nitrogen. Each ground gel sample was then suspended in extraction buffer (0.01% SDS and 50 mM Na₄EDTA in 10 mM Tris base buffer, pH 8.0) and extracted in a shaking water bath for 3 h at 60 °C. The gel particles were pelleted by

centrifugation and the supernatants were collected. The pelleted gel was re-extracted in the same manner and the homologous supernatants were combined and then passed through glass wool filters (treated with dimethyldichlorosilane, Sigma Chemical Co., St. Louis, MO, USA) to remove any gel particles present. The LPS present in each of the fraction supernatants was then precipitated with 3 volumes of 0.375M MgCl₂ in 95% ethanol using the procedure described for extracellular LPS by Velji et al. (1990). Each of the LPS fractions was then run on an analytical SDS gel (dimensions: 140 × 125 × 1.5 mm) for 14.5 h at a constant current of 20 mA. Resulting LPS bands in the gels were visualized using the silver staining procedure of Tsai & Frasch (1982) as modified by Hitchcock & Brown (1983).

Experimental fish. The juvenile coho salmon used in the experiment were from an east coast of Vancouver Island (Canada) stock (Big Qualicum River) and had been reared in well water at the Department of Fisheries and Oceans Rosewall Creek facility. The fish were transported to our laboratory at the Pacific Biological Station and held in an 800 l tank supplied with flowing dechlorinated city water. They were allowed to slowly acclimatize to a temperature of 13 ± 1 °C before the experiment was carried out.

Vaccination and challenge procedures. The native LPS and the LPS in 5 of the 6 LPS fractions (Fractions 1 to 5) obtained by P-PAGE were dissolved in PFW to a concentration of 100 ng ml⁻¹. Fraction 6 was not tested because in several trials it appeared to resist extraction from the gel using the extraction procedure just outlined. Each coho was injected intraperitoneally (IP) with 0.1 ml of one or other of the LPS preparations (10 ng LPS per fish). Fish in the control groups were injected IP with 0.1 ml of one or other of the following: PFW, an *Escherichia coli* 055:B5 (210-SE) LPS preparation (10 ng per fish) (Whittaker Bio-Products, Walkersville, MD, USA) or an extract of the control gel on which PFW had been run instead of the Vo LPS. Two lots of 20 coho each were used for each treatment and control group, the only exception to this being the groups treated with the *E. coli* LPS, where, because of a shortage of LPS, each replicate consisted of 12 fish. The various lots of fish were held in separate 35 l tanks, each of which was supplied with dechlorinated city water at 13 ± 1 °C. The fish were held for 21 d and then challenged IP with 0.1 ml of live, saline-washed, log phase Vo cells (1.7 × 10⁵ cells per fish). They were then observed for 14 d, by which time deaths resulting from the challenge had ceased to occur. Kidney samples from 10% of the fish that died following the challenge were cultured on Tryptic Soy Agar (Difco, Detroit, MI, USA) supplemented with 1% NaCl to verify that Vo was the cause of death. Identification of Vo growth on the plates was accomplished serologically (agglutina-

tion test) using a Vo-specific rabbit serum (Gibco, New York, NY, USA).

Statistical analysis. The G-test (log-likelihood ratio test) (Sokal & Rohlf 1981) was used to determine if there were significant differences in deaths between replicates. If the differences were not significant ($p > 0.05$), the deaths in the replicates were combined and deaths in the various treatment groups were then compared with those in the control groups. Yate correction was applied because of the small sample sizes used.

RESULTS AND DISCUSSION

The silver-stained profile of native cell wall Vo LPS obtained by analytical SDS-PAGE (Fig. 1, Lane 7) was similar to that obtained by Chart & Trust (1984) for Vo LPS. The profile consisted of a ladderlike pattern of bands reminiscent of those that occur with LPS from various members of the *Enterobacteriaceae*, and the various bands probably represented LPS molecules that differed from one another largely in the number of the O-polysaccharide repeat units present (Goldman & Lieve 1980, Palva & Makela 1980, Peterson & McGroarty 1985). However, as our results indicate, the bands or fractions tended to give rise to additional faster-migrating LPS bands when subjected to re-electrophoresis. The additional bands migrated in a similar manner to bands yielded by native LPS, and the high-

est molecular weight species detected in a given fraction always corresponded closely in its migration to that of the fraction from which it was derived (Fig. 1, Lanes 1 to 5). It appears, therefore, that aggregation of the various Vo LPS molecular species occurs, a phenomenon that has also been reported for LPS from a number of other Gram negative bacteria (Hurlbert & Hurlbert 1977, Logan & Trust 1984, Ohta et al. 1985) and that is favoured (and becomes evident) when heavily loaded gels are used. Native LPS isolated from certain bacteria may therefore consist of more than one molecular species, and the various molecular species can occur as aggregates. Hurlbert & Hurlbert (1977) found that the LPS fractions (aggregates) from P-PAGE were moderately stable: they migrated on re-electrophoresis to their original positions, showing no disaggregation, as long as electrophoresis occurred in a non-disaggregating system (i.e. one lacking SDS).

Protection against challenge with Vo was LPS-specific because it was obtained only when LPS derived from Vo was used in the vaccines (Table 1). Further, when the various Vo LPS fractions from the P-PAGE were compared for their protective properties in coho salmon, there appeared to be a relationship between the strength of the protection conferred and the apparent molecular weight of the LPS in the fraction (Table 1). The RPS declined from 51.4 to 5.9% as the molecular weight of the slowest migrating LPS components in the fractions decreased. On statistical analysis, however,

Fig. 1 Silver-stained lipopolysaccharide (LPS) patterns obtained on an analytical gel by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) using samples representing native *Vibrio ordalii* LPS (Lane 7) and various fractions of decreasing mobility (Lanes 1 to 6) obtained from Vo LPS using a preparative gel and SDS-PAGE. Note: The slowest migrating LPS fraction consistently appeared to resist elution from the preparative gel (see, however, 'Results and discussion') and thus it always failed to show up on analytical gels (Lane 6). The migration distances of various proteins of known molecular weights (in kilodaltons) are indicated by the horizontal lines on the left

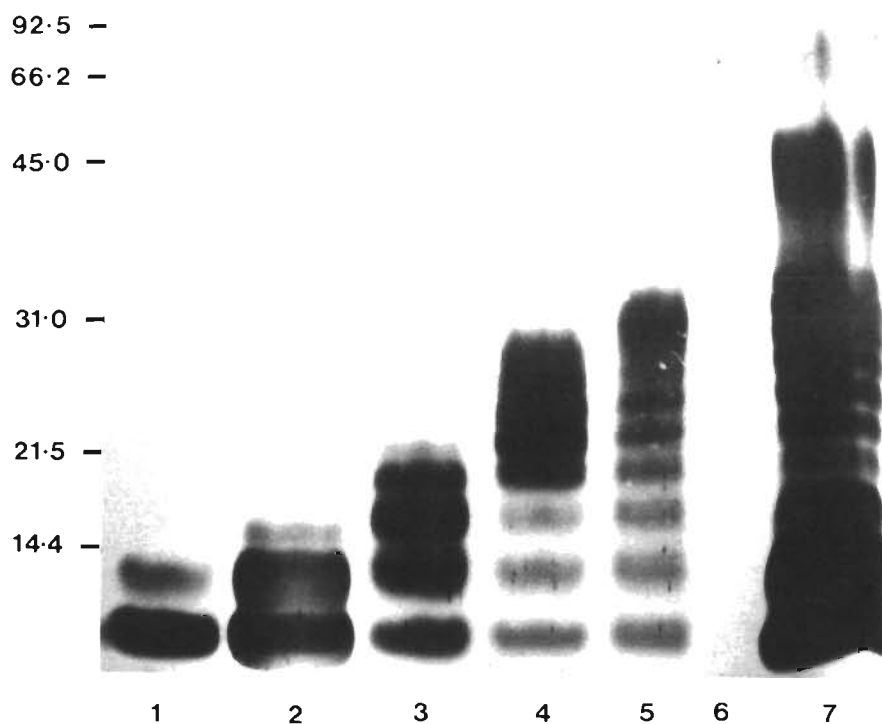


Table 1. *Oncorhynchus kisutch*. Mortalities following challenge with *Vibrio ordalii* (Vo) cells of juvenile coho salmon vaccinated with various Vo and *Escherichia coli* lipopolysaccharide (LPS) preparations and with various placebos. Experiment was carried out in fresh water at 13 ± 1 °C with fish averaging 14.1 ± 4.5 g; fish were challenged intraperitoneally with 1.7×10^5 viable Vo cells 21 d post vaccination; mortalities were monitored for 14 d post challenge

Type of preparation injected	Fraction no. (approximate size of LPS, kD)	Dose of LPS	No. dead ¹ tank ⁻¹ (t ₁ /t ₂)	Total no. dead/no. challenged ²	Total % mortality	RPS ³ (%)
PFW ⁴	–	–	15/19	34/40 ^a	85.0	0
Blank gel extract	0	–	18/17	35/40 ^a	87.5	–
<i>Vibrio ordalii</i>	N-LPS ⁵	10 ng	1/4	5/40 ^b	12.5	85.3
	5 (<35.0)	10 ng	12/5	17/40 ^c	42.5	51.4
	4 (<31.0)	10 ng	11/11	20/40 ^d	55.0	35.3
	3 (<22.0)	10 ng	16/12	28/40 ^a	70.0	17.6
	2 (<17.0)	10 ng	15/17	32/40 ^a	80.0	5.9
	1 (<14.4)	10 ng	12/20	32/40	80.0	5.9
<i>Escherichia coli</i>	N-LPS	10 ng	11/10	21/24	95.8	–

¹ Comparison of mortalities between replicate tanks of each treatment was shown to be non-significant ($p > 0.05$) except for Fraction 1 ($p < 0.05$)

² Combined mortalities of each treatment followed by different letters are significantly different at $p < 0.05$ from controls

³ Calculated as per Amend (1981): $RPS = [1 - (\% \text{ vaccinate mortality} / \% \text{ PFW control mortality})] \times 100 \%$

⁴ PFW: Pyrogen-free water

⁵ N-LPS: Native unfractionated LPS

only 2 fractions (Fractions 4 and 5) yielded significant protection ($p < 0.05$); those containing LPS of the highest molecular weight (20 to 35 kD). These findings indicate that the size of the O-polysaccharide component of the LPS molecule, which is dependent on the number of O-polysaccharide repeat units present, is an important factor determining immunogenicity. The basis for this conclusion is that the coho injected with the various fractions each received a constant amount of lipid A [lipid A is the LPS component actually measured by the LPS assay technique used in our study (Tanamoto et al. 1984)]. Variations in the strength of the protection elicited could thus not have been related to the lipid A portion of the LPS molecules.

Unfortunately, the immunogenicity of LPS in Fraction 6 was not tested because, in 3 attempts, we appeared unable to remove it from the preparative gel using the extraction procedures outlined earlier. In fact, however, subsequent tests indicated that our problem with this fraction was due to its occurrence in low concentration relative to that of the other Vo LPS fractions. (It was the first fraction to disappear when increasingly dilute Vo LPS preparations were electrophoresed; data not shown). Thus, when re-electrophoresed following extraction, its separated subcomponents yielded bands that were too weak to be seen. The net outcome is that we do not know the extent to which this fraction contri-

buted to the superior protection obtained with native LPS relative to that obtained with Fractions 4 and 5. Superior protection observed with native LPS may also have been partly a function of aggregation of the various LPS molecules produced by Vo. Our data indicate that aggregation of the various molecular species occurred, and aggregation would be expected to result in a complex that was larger, more persistent in fish, and thus more immunogenic than the individual LPS fractions. Aggregation may thus be another factor contributing to the immunogenicity of LPS preparations. It should be pointed out, however, that aggregation per se was obviously not sufficient to ensure immunogenicity of all of the LPS molecules. When, for example, the fastest migrating LPS fractions (Fractions 1 and 2), presumably representing the smallest LPS molecules, were injected into the coho under conditions that would have favoured aggregation (i.e. in the presence of Mg^{++} and absence of SDS or of EDTA), there was no evidence of protection (Table 1).

In summary, native Vo LPS appears to be comprised of a number of different-sized LPS molecules, the higher molecular weight species apparently being the dominant immunogenic components. Experiments are underway to determine whether the immunogenic components are the only ones recognized by the immune system of the coho.

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LITERATURE CITED

- Amend, D. F. (1981). Potency testing of fish vaccines. *Develop. Biol. Standard.* 49: 447-454
- Angus, B. L. (1986). Structural and functional studies on the role of the outer membrane of *Pseudomonas aeruginosa* in resistance and permeability to antibiotics. Ph.D. thesis. University of British Columbia, Vancouver
- Chart, H., Trust, T. J. (1984). Characterization of the surface antigens of the marine fish pathogens *Vibrio anguillarum* and *Vibrio ordalii*. *Can. J. Microbiol.* 30: 703-710
- Darveau, R. P., Hancock, R. E. W. (1983). Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* 155: 831-838
- Evelyn, T. P. T. (1984). Immunization against pathogenic vibrios. In: de Kinkelin, P., Michel, C. (eds.) Symposium on fish vaccination. *Off. Intl. des Epizoot.*, Paris, p. 121-150
- Evelyn, T. P. T. (1988). *Vibrio* vaccines for salmonids. In: Congress Proceedings, Aquaculture International Congress and Exposition. Aquacult. Intl Congress, B.C. Corp., Vancouver, B.C., Canada, p. 459-469
- Evelyn, T. P. T., Ketcheson, J. E. (1980). Laboratory and field observations on antivibriosis vaccines. In: Ahne, W. (ed.) Fish diseases, Third COPRAQ Session. Springer-Verlag, Berlin, Heidelberg, New York, p. 45-52
- Goldman, R., Lieve, L. (1980). Heterogeneity of antigenic-side-chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. *Eur. J. Biochem.* 107: 145-153
- Hitchcock, P. J., Brown, T. M. (1983). Morphological heterogeneity among salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154: 269-277
- Hurlbert, R. E., Hurlbert, I. M. (1977). Biological and physicochemical properties of the lipopolysaccharide of *Chromatium vinosum*. *Infect. Immun.* 16: 983-994
- Ohta, M., Rothmann, J., Kovats, E., Pham, P. H., Nowotny, A. (1985). Biological activities of lipopolysaccharide fractionated by preparative acrylamide gel electrophoresis. *Microbiol. Immunol.* 29: 1-12
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head bacteriophage T4. *Nature, Lond.* 227: 680-685
- Logan, S., Trust, T. J. (1984). Structural and antigenic heterogeneity of lipopolysaccharides of *Campylobacter jejuni* and *Campylobacter coli*. *Infect. Immun.* 45: 210-216
- Palva, T., Makela, P. H. (1980). Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. *Eur. J. Biochem.* 107: 137-143
- Peterson, A. L., McGroarty, E. J. (1985). High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli*. *J. Bacteriol.* 162: 738-745
- Sokal, R. S., Rohlf, F. J. (1981). *Biometry. The principles and practice of statistics in biological research*, 2nd edn. W. H. Freeman and Co., New York
- Tanamomto, K., Zchinger, U., McKenzie, G. R., Galanos, C., Rietschel, E. T., Luderitz, O., Kusumoto, S., Shiba, T. (1984). Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity, anti-complement activity and induction of gelation of limulus amoebocyte lysate. *Infect. Immun.* 44: 421-426
- Tsai, C.-M., Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharide in acrylamide gels. *Analyt. Biochem.* 119: 115-119
- Velji, M. J., Albright, L. J., Evelyn, T. P. T. (1990). Protective immunity in juvenile coho salmon (*Oncorhynchus kisutch*) following immunization with *Vibrio ordalii* lipopolysaccharide or from exposure to live *V. ordalii* cells. *Dis. aquat. Org.* 9: 25-29

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