INFECTION AND IMMUNITY, June 1997, p. 2475–2479 Vol. 65, No. 6
0019-9567/97/$04.00+0

The Lipopolysaccharide O Side Chain of *Vibrio vulnificus* Serogroup E Is a Virulence Determinant for Eels

CARMEN AMARO,* BELÉN FOUZ, ELENA G. BIOSCA, ESTER MARCO-NOALES, AND ROSA COLLADO

Departamento de Microbiología, Facultad de Biología, Universidad de Valencia, Valencia 46100, Spain

Received 30 September 1996/Returned for modification 8 November 1996/Accepted 10 March 1997

Vibrio vulnificus **is a gram-negative bacterium capable of producing septicemic infections in eels and immunocompromised humans. Two biotypes are classically recognized, with the virulence for eels being specific to strains belonging to biotype 2, which constitutes a homogeneous lipopolysaccharide (LPS)-based O serogroup (which we have designated serogroup E). In the present study we demonstrated that the O side chain of this LPS determines the selective virulence of biotype 2 for eels: (i) biotype 1 strains (which do not belong to serogroup E) are destroyed by the bactericidal action of nonimmune eel serum (NIS) through activation of the alternative pathway of complement, (ii) biotype 2 strains (of serogroup E) are resistant to NIS, and (iii) rough mutants of biotype 2 lacking the O polysaccharide side chain are sensitive to NIS and avirulent for eels.**

Vibrio vulnificus is a gram-negative bacterial species that includes strains which are pathogenic for humans and eels and are grouped into two biotypes (41). Biotype 1 is an autochthonous member of marine ecosystems that can behave as an opportunistic human pathogen capable of causing a septicemic infection in immunocompromised hosts (36, 37). Biotype 2 has classically been considered an obligate eel pathogen, given that it is reported to have been isolated only from diseased eels (3, 10, 16). This biotype causes important economic losses in fisheries that maintain juvenile eels in brackish water over 25°C (4). Under these conditions, the inoculation of 10^5 cells/ml by bath or 10 cells/fish by intraperitoneal injection leads to the development of vibriosis in less than 24 h (4, 16). The disease is a septicemic infection, and bacteria are easily isolated from blood samples from moribund eels.

Recent evidence suggests that biotype 2 is also an opportunistic pathogen whose reservoir is probably seawater: (i) it survives in artificial seawater in a culturable state for months (31), (ii) it uses saline water as a route to infect healthy eels (4), and (iii) it is able to use humans as occasional hosts for developing infections (2). The pathogenic potential of biotype 2 for humans was recognized only after the recent identification of one human clinical American Type Culture Collection isolate belonging to biotype 2 (2).

In fact, strains of both biotypes are phenotypically and genotypically similar (3, 16, 41), they share immunologically related outer membrane proteins (14), and they seem to produce the same virulence factors, including (i) the capsule, a protective factor that allows cells to resist phagocytosis and lysis by human serum (6, 29, 43) but not by eel complement (15); (ii) various iron uptake systems, including siderophore production and the ability to use hemoglobin and hemin as iron sources (13, 24, 26, 39); and (iii) a cytotoxin, with hemolytic activity, together with potent proteases which are involved in the lesions produced in different organs (3, 16, 28, 36). Interestingly, the exotoxins produced by both biotypes seem to be equally lethal for eels; they produce the main symptoms of vibriosis when intraperitoneally inoculated as crude extracts of extracellular products (ECPs) (9).

* Corresponding author. Phone: 6-3864389. Fax: 6-3894372. E-mail: carmen.amaro@uv.es.

In spite of these homologies, the two biotypes can be distinguished by various tests: in the indole test, the majority of biotype 1 and biotype 2 strains are positive and negative, respectively (16); in serological tests, biotype 2 strains constitute a lipopolysaccharide (LPS)-based O serogroup (5, 16), whereas biotype 1 strains are serologically heterogeneous (5, 16, 32); and in eel virulence tests, only biotype 2 strains are virulent for eels (16, 41).

The main objective of the present work was to investigate the basis for the selective virulence of biotype 2 for eels. To our knowledge, the only phenotypic trait that could be related to host specificity is the possession of the serogroup E LPS. LPS is a complex outer membrane molecule that consists of three moieties: lipid A, a core, and an O side chain. Lipid A has been defined as toxic in some gram-negative bacterial species that produce septic shock (25), and the O side chain has been described as a virulence factor in other species, including fish pathogens (1, 8, 22, 30, 34). In the latter case, LPSs are responsible for resistance to nonspecific immune mechanisms, including complement-mediated killing and phagocytosis. Consequently, the aim of the present work was (i) to evaluate the toxic potential of the LPS as a specific endotoxin; (ii) to investigate whether both biotypes present differential susceptibility to nonspecific defense mechanisms, such as nonopsonic phagocytosis and/or lysis by complement; and, if so, (iii) to determine the role of LPS in specific resistance of biotype 2 by including in the experiments a mutant that lacked the O antigen (7).

We used strains of both biotypes, as listed in Table 1. Mutant M6 was previously obtained from strain E22 by UV irradiation (7). Strains were routinely cultured in Trypticase soy agar (TSA) or Trypticase soy broth (TSB) (Oxoid) supplemented with 0.5% (wt/vol) NaCl (TSA-1 or TSB-1) at 25°C (eel isolates) or 37°C (clinical isolate) for 24 h and were maintained as stock cultures in marine broth (Difco) plus 20% (vol/vol) glycerol at −80°C. Virulence testing of all isolates was performed with elvers (average weight, 10 g) by intraperitoneal inoculation (3), and the 50% lethal dose (LD_{50}) was calculated by the method of Reed and Münch (38).

Crude LPS from *V. vulnificus* biotype 2 strains was prepared from whole-cell lysates and from outer membranes as described by Amaro et al. (5). LPS was purified according to the method described by Westphal and Jann (42) and modified by Biosca et al. (12). Samples of crude and purified LPS were

TABLE 1. Characteristics of the strains used in this study

Biotype and strain	Source and origin	LD_{50} (CFU/fish)[a]	Resistance to phagocytosis (%)[b]
Biotype 2			
E22	Diseased European eel, Spain	7.5×10^2	1,160 ± 345
E86	Diseased European eel, Spain	1.2×10^2	1,800 ± 321
Biotype 1			
E109	European eel, Spain	−	1,011 ± 376
CDC 7184	Human blood, United States	−	995 ± 198

[a] −, LD_{50} of $>10^8$. Crude LPS of all strains tested was not lethal (after administration of 100 μg of crude LPS obtained from outer membrane samples per fish).

[b] Percent survival (mean ± standard deviation). All strains were resistant to phagocytosis as evidenced by total destruction of PEC after 18 to 24 h of incubation.

lyophilized and stored. The lethality of LPS was tested on elvers (average weight, 10 g) by intraperitoneal inoculation of 0.1 ml of O antigens, prepared as previously described (3), or crude LPSs. Experiments were performed in triplicate with groups of six animals, including a control set inoculated with 0.1 ml of saline solution (1.5% NaCl) (SS).

Phagocytosis was assessed by the procedure of Miyazaki and Kurata (35). After being collected, peritoneal exudate cells (PEC) from eels were resuspended at a concentration of 10^5 viable cells per ml (determined by the trypan blue exclusion test) in cold Hanks solution (pH 7.2) (Flow). Phagocytic activity was evaluated in triplicate as follows: a volume of 1.5 ml of PEC in Hanks solution was inoculated with viable bacteria (0.5×10^6 CFU per ml; ratio of bacteria to PEC, 5:1), and the mixtures were incubated at 25°C for 30 min, 1 h, 4 h, and 18 to 24 h. After being washed, samples of each mixture were smeared on glass slides, fixed with methanol, stained with Giemsa for 20 min, and observed under the microscope. Bacterium-laden cells per 300 total cells were counted under the microscope. Numbers of bacterial survivors (CFU per ml) were also evaluated by plate counting on TSA-1 at the end of each experiment. Controls for PEC viability as well as phagocytosis assays were made in parallel on microtiter plates. In this case, PEC suspensions (10^5 cells/ml) were disposed in 24-well culture plates and were inoculated with a volume of 0.1 ml of bacterial suspension (5×10^6 cells) per well (ratio of bacteria to PEC, 5:1) or SS. Plates were maintained at 25°C for a maximum period of 72 h, and phagocytosis was monitored by direct observation under an inverted microscope.

Survival experiments in nonimmune serum (NIS), immune serum (IS), inactivated serum, and treated serum from eels were performed in triplicate with stationary-phase bacteria by inoculating a volume of 0.45 ml of serum with 0.05 ml of a bacterial suspension in SS (10^5 to 10^6 cells per ml) and incubating at 25°C for 4 h. Viable-cell counts were determined by drop plating serial dilutions on TSA-1 (27). To obtain NIS, we used eels (average weight, 20 g) from a freshwater fish farm which had no history of *V. vulnificus* infection. NIS was obtained as previously described (15). IS was obtained from eels previously immunized with formalin-inactivated bacterial cells diluted in heat-inactivated ECPs (10^7 CFU per ml) from strain E86 (19). ECPs were obtained as previously described (3). The immunization schedule included two intraperitoneal injections (0.1 ml/fish) administered at 12-day intervals (19). Two weeks after the second injection, eels were bled by cardiac puncture. Antibody titers (both natural and induced) of NIS and IS against *V. vulnificus* were measured by an indirect dot blot assay by the method described by Burreson and Frizzell (17) and Cipriano et al. (18) with some modifications. Rabbit anti-eel serum was prepared as described previously (3) with heat-inactivated (56°C, 30 min) eel serum as the antigen. Dot blot assays were performed with immobilized whole-cell suspensions (10^6 CFU) or LPS samples (2 μl of crude extractions) on nitrocellulose papers. Membranes were incubated with serial twofold dilutions of NIS, and immunological reactions were revealed as previously described (5). Titers of antibody in sera were expressed as the reciprocal of the higher dilution that tested positive. Samples of NIS with antibody against *V. vulnificus* were discarded. Inactivated eel serum was obtained as previously described (20, 23, 33, 34, 40). Briefly, NIS was incubated with 20 mM EGTA plus 2 mM $MgCl_2$ for 30 min at 25°C for classical-pathway inactivation (23), heated for 20 min at 47 to 50°C for alternative-pathway inactivation (34, 40), or heated for 30 min at 56°C for inactivation of both pathways. Treated eel serum was obtained by incubation of NIS with whole cells or purified LPS from strain E22 (translucent variant) at 25°C. In the first case, serum was incubated with bacteria (10^8 CFU per ml) for 1 h, and cells were discarded by centrifugation for 5 min in an Eppendorf microcentrifuge and filtration (34). In the second case, samples of purified LPS were added to NIS at a final concentration of 0.1 or 0.5 mg/ml and incubated for 30 min (34).

Lethality of O antigens and crude LPS. Prior to any experiment, virulence of our strains for eels was confirmed (Table 1). No death was recorded after the injection of O antigen (10^9 heat-inactivated cells per fish) and crude LPS (Table 1), and no behavioral signs indicative of physical alterations were observed. The nontoxicity of the crude serogroup E LPS for eels suggested that this surface component would not act as a specific toxin of biotype 2 but as a protective factor against nonspecific immune mechanisms such as nonopsonic phagocytosis and/or bactericidal action of serum complement. Thus, the basis of the selective virulence of biotype 2 for eels would rely on the protective role of this molecule.

Resistance to nonspecific defense mechanisms. (i) Nonopsonic phagocytosis. We confirmed that all strains of both biotypes resisted nonopsonic phagocytosis by PEC of eels (Table 1). Samples of PEC contained mainly macrophages and a minor proportion of neutrophils (less than 5%). Slide preparations taken at 30 min and 1 h showed less than 10% PEC with phagocytosed bacteria (average of 4 bacteria per cell); at 4 h, PEC degeneration, especially evident in the case of biotype 2 samples, was observed; and at the end of the experiment, total PEC destruction was observed. Bacterial countings revealed that the number of CFU per ml increased by approximately 1 order of magnitude (Table 1). These results were confirmed by the tests performed in parallel on microtiter plates. In these assays, macrophages were identified as the predominant adherent cells and were successfully maintained in control wells for 3 days. The fact that *V. vulnificus* produces potent cytotoxins against different fish cell lines (3, 9) suggests that PEC were destroyed by the cytotoxic activity of cells. Similar findings have previously been found by other authors in experiments performed with virulent strains of *V. vulnificus* and PEC of unvaccinated Japanese eels (*Anguilla japonica*) (35).

Since biotype 1 strains resisted nonopsonic phagocytosis, we focused our investigation on the role of serum complement.

(ii) Serum complement. In poikilothermic teleosts, in which both pathways of complement activation have been demonstrated, the direct bactericidal reaction of serum could be of particular importance in defense against infections since there are no appreciable C3 receptors on fish phagocytes (21). As we expected, only biotype 2 cells resisted the bactericidal action of

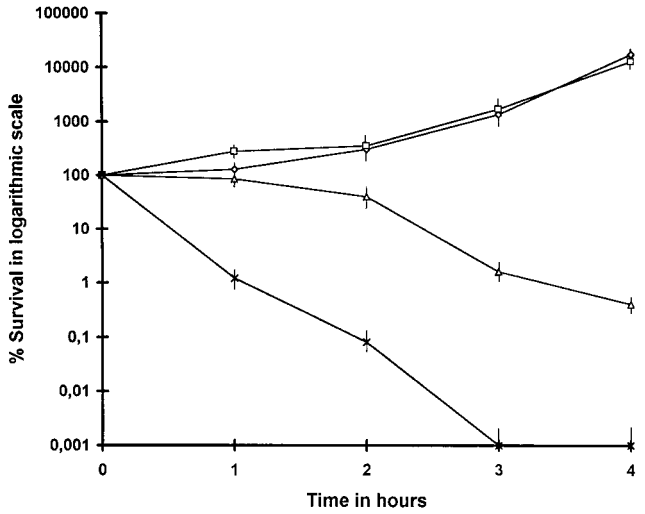

FIG. 1. Survival in NIS of *V. vulnificus* biotype 2 strains E22 (□) and E86 (◇) and biotype 1 strains E109 (×) and CDC7184 (△). Each point represents the mean for three different experiments.

eel serum. Survival curves of both biotypes in NIS are shown in Fig. 1. In all cases, biotype 2 cells were able to multiply, probably because they can use hemoglobin (liberated by hemolysis during blood extraction) as a nutrient source (24). Biotype 1 strains showed a large decrease in viable numbers after 4 h of incubation (Fig. 1). Thus, the susceptibility of biotype 1 to serum complement seems to explain its avirulent character for eels.

The complement activation mechanism displayed by biotype 1 strains was examined. Serum heated at 47 to 50°C for 20 min, with the alternative pathway inhibited, and serum heated at 56°C for 30 min, with both the classical and alternative pathways abolished, were nonlethal for both of the isolates tested. However, both strains were killed in serum treated with Mg^{2+}-EGTA, which selectively inhibits the classical complement pathway. Results for strain E109 are presented in Fig. 2. Thus, it seems that the alternative pathway of complement activation

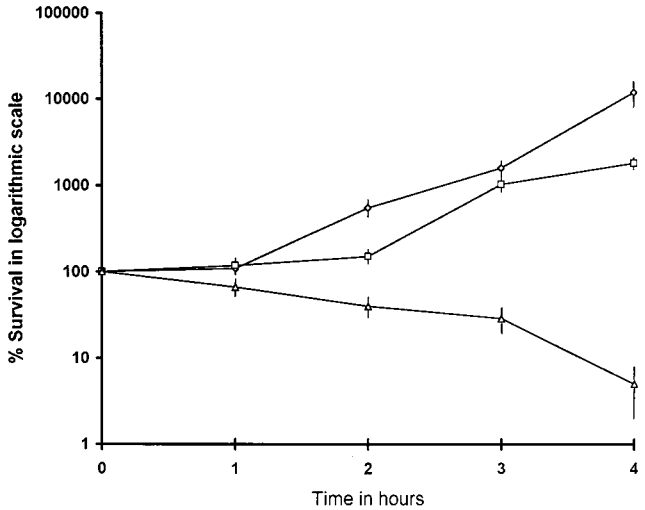

FIG. 2. Survival of *V. vulnificus* biotype 1 strain E109 in NIS heated at 56°C for 30 min (◇), NIS plus 20 mM EGTA plus 2 mM $MgCl_2$ (△), and NIS heated at 48°C for 20 min (□). Each point represents the mean for three different experiments.

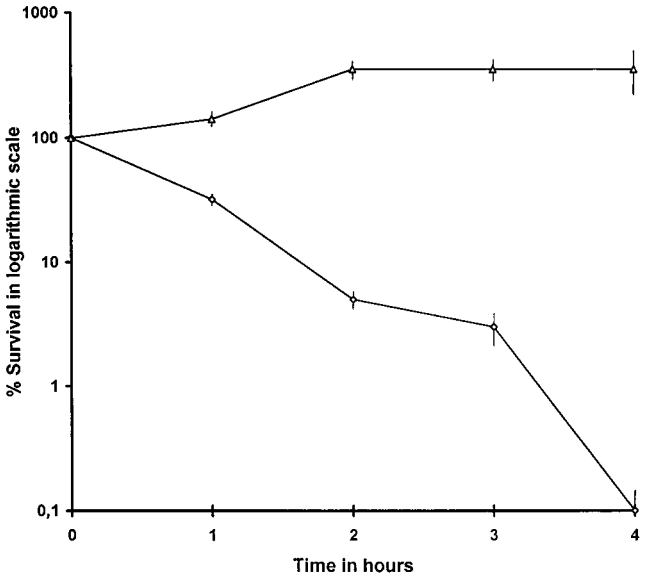

FIG. 3. Survival of mutant M6 in NIS (◇) and NIS heated at 56°C for 30 min (△). Each point represents the average for three different experiments.

is involved in serum killing of biotype 1 strains in nonimmunized eels. This finding is consistent with the results obtained with other bacteria and other species of teleosts (33, 40). In these cases, it has been postulated that in fish, as opposed to mammals, the alternative pathway plays a more important role than the classical one in the destruction of bacterial intruders. Nevertheless, we cannot discard the role of classical pathway in the clearance of this biotype from serum of nonimmune eels with natural antibodies against vibrios (members of the genus *Vibrio* are part of the normal microbiota of some fish, especially in marine habitats). From these results, it can be deduced that the basis for the selective virulence of biotype 2 for eels relies on the protective role of its LPS against serum complement.

Role of the O polysaccharide side chain in the resistance of biotype 2 to eel complement. We included in these experiments the rough mutant M6, which lacks the O polysaccharide side chain. This mutant was sensitive to NIS (Fig. 3) and avirulent for eels ($LD_{50} > 10^8$ CFU/fish). We treated NIS with biotype 2 cells (fresh and formalinized) and with purified LPS. This treatment totally or partially abolished the inhibitory effect of NIS against biotype 1 cells (Table 2). In the case of LPS, the result was dependent on the amount of LPS used (Table 2). The mutant acquired the ability to resist the bactericidal action of NIS alone (survival rate, approximately 100%), after incubation with whole cells (survival rate, 80% ± 14%), with 0.5 mg

TABLE 2. Inhibition of NIS bactericidal activity against *V. vulnificus* biotype 1 strains by treatment with whole cells, formalinized whole cells, and LPS

Strain	% Survival[a] after incubation in NIS:				
	Alone	Plus whole cells		Plus LPS[b]	
		Fresh	Formalinized	0.1 mg/ml	0.5 mg/ml
E109	0.0015 ± 0.001	90 ± 25	124 ± 9	43 ± 15	575 ± 50
CDC7184	0.30 ± 0.1	89 ± 11	102 ± 2	20 ± 9	371 ± 49

[a] Mean ± standard deviation from three different experiments.
[b] From biotype 2 strain E22 (translucent variant) (15).

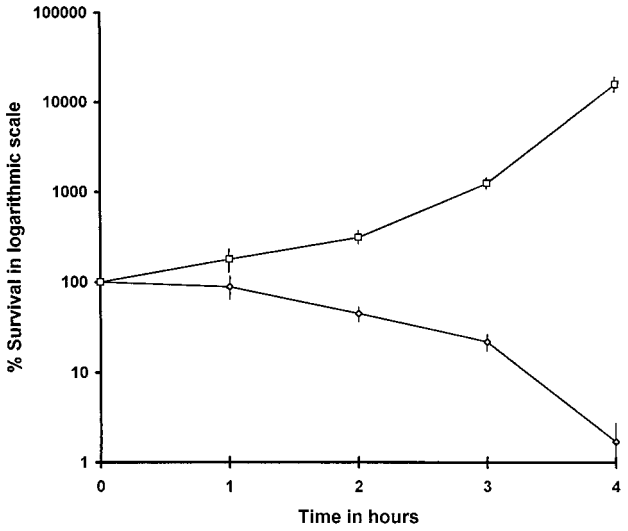

FIG. 4. Effect of immunization on the bactericidal activity of eel serum against *V. vulnificus* biotype 2. Survival of strain E86 in NIS (□) and IS (◇) is shown. Each point represents the average for three different experiments.

of LPS/ml (survival rate, 90% ± 15%), and after inactivation by heating at 56°C for 30 min (Fig. 3).

In conclusion, among the different LPS components, the O side chain of serogroup E cells seems to be responsible for eel serum resistance since the rough mutant, which lacks this structure, is sensitive to NIS. In this respect, the serum resistance of biotype 2 (serogroup E) cells may be due either to a complete or nearly complete inability to activate complement or to the failure of activated complement to exert its effect. The fact that incubation with whole cells and LPS from wild biotype 2 strains inhibits serum bactericidal activity against sensitive strains suggests that this resistance may be due to the latter factor. Thus, although the cells are capsulated (15), the O side chains of LPS must be surface exposed and accessible to complement. A similar situation has been found with human serum and *Aeromonas salmonicida* (34), with the role of the capsule being performed by the A layer.

Effect of immunization on the resistance of biotype 2 to eel serum. In fish, the specific immune response is not so well developed. Fish produce antibodies of only the M class, which have low affinity, limited heterogeneity, and poor anamnestic qualities (21). Nevertheless, by analogy with homeotherms, in which this antibody class serves as the best complement-fixing antibody as well as the major bacteriolytic antibody, complement-mediated bacteriolysis might be the primary antibacterial role of fish antibodies. In the experiments performed with immunized animals, eel serum was bactericidal for biotype 2. As we expected, strains were killed by IS (survival rate of approximately 0.5% after 4 h of incubation) (Fig. 4). The IS contained high titers of antibody to whole cells (1:64,000) and crude extractions of LPS (1:64,000) as determined by dot blotting. These findings suggest that the binding of antibodies to cell surface components can abolish the resistance of biotype 2 to serum. In this respect, we have developed a vaccine and a vaccination procedure for this vibriosis, which are being used successfully in eel farms in our geographical area (19). The vaccinated animals possess antibodies against *V. vulnificus* biotype 2 and have been well protected against experimental infections with this bacterium (19).

In summary, two main conclusions can be derived from the present investigation. Firstly, the O side chain of the characteristic LPS of serogroup E seems to be the virulence factor responsible for the pathogenic potential of *V. vulnificus* for eels. This surface component determines, at least in part, the resistance to serum complement of serogroup E (biotype 2) strains, which are able to invade the animal body and cause septicemic infections. Secondly, the alternative pathway of complement activation must play an important role in the defense of nonimmunized eels against bacterial infections since it is the main factor responsible for killing biotype 1 strains. Therefore, we hypothesize that the natural niche of both biotypes of *V. vulnificus* species is the aquatic environment and that the strains possessing the O antigen of the serogroup E LPS are able to colonize eels. Of these strains, only those that express the adequate virulence factors (mainly exotoxins and iron uptake systems) cause septicemic infections.

This work was partially supported by grant AGF95-1085-CO2-O1 from the Comisión Interministerial de Ciencia y Tecnología (CICYT) and grant C412 from IMPIVA, Valencia, Spain. B. Fouz thanks Consellería de Educación, Xunta de Galicia, for a postdoctoral research fellowship, and E. Marco-Noales thanks Generalitat Valenciana for a predoctoral fellowship.

REFERENCES

1. **Al-Hendy, A., P. Toivanen, and M. Skurnik.** 1992. Lipopolysaccharide O side chain of *Yersinia enterocolitica* O:3 is an essential virulence factor in an orally infected murine model. Infect. Immun. **60:**870–875.
2. **Amaro, C., and E. G. Biosca.** 1996. *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Appl. Environ. Microbiol. **62:**1454–1457.
3. **Amaro, C., E. G. Biosca, C. Esteve, B. Fouz, and A. E. Toranzo.** 1992. Comparative study of phenotypic and virulence properties in *Vibrio vulnificus* biotypes 1 and 2 obtained from a European eel farm experiencing mortalities. Dis. Aquat. Org. **13:**29–35.
4. **Amaro, C., E. G. Biosca, B. Fouz, E. Alcaide, and C. Esteve.** 1995. Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. Appl. Environ. Microbiol. **61:**1133–1137.
5. **Amaro, C., E. G. Biosca, B. Fouz, and E. Garay.** 1992. Electrophoretic analysis of heterogeneous lipopolysaccharides from various strains of *Vibrio vulnificus* biotypes 1 and 2 using silver staining and immunoblotting. Curr. Microbiol. **25:**99–104.
6. **Amaro, C., E. G. Biosca, B. Fouz, A. E. Toranzo, and E. Garay.** 1994. Role of iron, capsule, and toxins in the pathogenicity of *Vibrio vulnificus* biotype 2 for mice. Infect. Immun. **62:**759–763.
7. **Amaro, C., B. Fouz, and E. G. Biosca.** 1995. El lipopolisacárido de *Vibrio vulnificus* determina el potencial patógeno para anguila de la especie, Abstr. XV, p. 96. Congreso Nacional de Microbiología, Sociedad Española de Microbiología, Madrid, Spain.
8. **Binns, M. M., S. Vaughan, and K. N. Timmis.** 1985. "O"-antigens are essential virulence factors of *Shigella sonnei* and *Shigella dysenteriae* 1. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **181:**197–205.
9. **Biosca, E. G., and C. Amaro.** 1996. Toxic and enzymatic activities of *Vibrio vulnificus* biotype 2 with respect to host specificity. Appl. Environ. Microbiol. **62:**2331–2337.
10. **Biosca, E. G., C. Amaro, C. Esteve, E. Alcaide, and E. Garay.** 1991. First record of *Vibrio vulnificus* biotype 2 from diseased European eels (*Anguilla anguilla*). J. Fish Dis. **14:**103–109.
11. **Biosca, E. G., C. Amaro, J. L. Larsen, and K. Pedersen.** 1997. Phenotypic and genotypic characterization of *Vibrio vulnificus*: proposal for the substitution of the subspecific taxon biotype for serovar. Appl. Environ. Microbiol. **63:**1460–1466.
12. **Biosca, E. G., R. M. Collado, J. D. Oliver, and C. Amaro.** 1995. Comparative study of biological properties of lipopolysaccharide from *Vibrio vulnificus* biotypes 1 and 2, p. 83. Abstracts of the Seventh International Conference "Diseases of Fish and Shellfish" of the European Association of Fish Pathologists, Palma de Mallorca, Spain.
13. **Biosca, E. G., B. Fouz, E. Alcaide, and C. Amaro.** 1996. Siderophore-mediated iron acquisition mechanisms in *Vibrio vulnificus* biotype 2. Appl. Environ. Microbiol. **62:**928–935.
14. **Biosca, E. G., E. Garay, A. E. Toranzo, and C. Amaro.** 1993. Comparison of the outer membrane protein profile of *Vibrio vulnificus* biotypes 1 and 2. FEMS Microbiol. Lett. **107:**217–222.
15. **Biosca, E. G., H. Llorens, E. Garay, and C. Amaro.** 1993. Presence of a capsule in *Vibrio vulnificus* biotype 2 and its relationship to virulence for eels. Infect. Immun. **61:**1611–1618.
16. **Biosca, E. G., J. D. Oliver, and C. Amaro.** 1996. Phenotypic characterization of *Vibrio vulnificus* biotype 2, a lipopolysaccharide-based homogeneous O

serogroup within *Vibrio vulnificus*. Appl. Environ. Microbiol. **62:**918–927.
17. **Burreson, E. M., and L. J. Frizzell.** 1986. The seasonal antibody response in juvenile summer flounder (*Paralichthys dentatus*) to the hemoflagellate *Tripanoplasma bullocki*. Vet. Immunol. Immunopathol. **12:**395–402.
18. **Cipriano, R. C., J. B. Pyle, C. E. Starliper, and S. W. Pyle.** 1985. Detection of *Vibrio anguillarum* antigen by the dot blot assay. J. Wildl. Dis. **21:**211–218.
19. **Collado, R., and C. Amaro.** 1995. Immune response of European eels to *Vibrio vulnificus* biotype 2 bacterins, p. 177. Abstracts of the Seventh International Conference "Diseases of Fish and Shellfish" of the European Association of Fish Pathologists, Palma de Mallorca, Spain.
20. **Eidinger, D., E. Bello, and A. Mates.** 1977. The heterocytotoxicity of human serum. I. Activation of the alternative complement pathway by heterologous target cells. Cell. Immunol. **29:**1–186.
21. **Ellis, A. E.** 1989. The immunology of teleosts, p. 135–152. *In* R. J. Roberts (ed.), Fish pathology. Bailliere-Tindall, London, England.
22. **Engels, W., J. Endert, M. A. F. Kamps, and C. P. A. van Boven.** 1985. Role of lipopolysaccharide in opsonization and phagocytosis of *Pseudomonas aeruginosa*. Infect. Immun. **49:**182–189.
23. **Fine, D. P., S. R. Marney, Jr., D. G. Colley, J. S. Sergent, and R. M. DesPrez.** 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109:**807–809.
24. **Fouz, B., R. Mazoy, M. L. Lemos, M. J. del Olmo, and C. Amaro.** 1996. Utilization of hemin and hemoglobin by *Vibrio vulnificus* biotype 2. Appl. Environ. Microbiol. **62:**2806–2810.
25. **Friedman, H., T. W. Klein, M. Nakano, and A. Nowotny (ed.).** 1990. Endotoxin. Plenum Press, New York, N.Y.
26. **Helms, S. D., J. D. Oliver, and J. C. Travis.** 1984. Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. Infect. Immun. **45:**345–349.
27. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44:**1246–1247.
28. **Kreger, A., and D. Lockwood.** 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. Infect. Immun. **33:**583–590.
29. **Kreger, A. S., L. DeChatelet, and P. Shirley.** 1981. Interaction of *Vibrio vulnificus* with human polymorphonuclear leukocytes: association of virulence with resistance to phagocytosis. J. Infect. Dis. **144:**244–248.
30. **Mäkelä, P. H., M. Hovi, H. Saxen, M. Valtonen, and V. Valtonen.** 1988. *Salmonella*, complement, and mouse macrophages. Immunol. Lett. **19:**217–222.
31. **Marco-Noales, E., E. G. Biosca, and C. Amaro.** 1995. Long-term survival of *Vibrio vulnificus* biotype 2 at different salinities, p. 75. Abstracts of the Seventh International Conference "Diseases of Fish and Shellfish" of the European Association of Fish Pathologists, Palma de Mallorca, Spain.
32. **Martin, S. J., and R. J. Siebeling.** 1991. Identification of *Vibrio vulnificus* O serovars with antilipopolysaccharide monoclonal antibody. J. Clin. Microbiol. **29:**1684–1688.
33. **Matsuyama, H., K. Tanaka, M. Nakao, and T. Yano.** 1988. Characterization of the alternative complement pathway of carp. Dev. Comp. Immunol. **12:**403–408.
34. **Merino, S., S. Albertí, and J. M. Tomás.** 1994. *Aeromonas salmonicida* resistance to complement-mediated killing. Infect. Immun. **62:**5483–5490.
35. **Miyakazi, T., and K. Kurata.** 1987. Phagocytic response of peritoneal exudate cells of the Japanese eel to *Vibrio vulnificus*. Bull. Fac. Fish. Mie Univ. **14:**33–40.
36. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 570–600. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York, N.Y.
37. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1982. Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. Appl. Environ. Microbiol. **44:**1404–1414.
38. **Reed, M. J., and M. Münch.** 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
39. **Simpson, L. M., and J. D. Oliver.** 1983. Siderophore production by *Vibrio vulnificus*. Infect. Immun. **41:**644–649.
40. **Sunyer, J. O., and L. Tort.** 1995. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. Vet. Immunol. Immunopathol. **45:**335–345.
41. **Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler.** 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. **44:**640–646.
42. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Mol. Immunol. **24:**319–331.
43. **Yoshida, S.-I., M. Ogawa, and Y. Mizuguchi.** 1985. Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. Infect. Immun. **47:**446–451.

Editor: R. E. McCallum