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Cloning and Expression of *rfb* Genes from *Vibrio anguillarum* Serotype O2 in *Escherichia coli*: Evidence for Cross-Reactive Epitopes

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***Vibrio ordalii* and *Vibrio anguillarum* O2 express lipopolysaccharide (LPS) O antigens containing both specific and cross-reactive epitopes. The localization of these epitopes on the O antigen is not known. We have cloned and expressed the *rfb* gene cluster for O-antigen synthesis from *V. anguillarum* O2 (rfb_{VaO2}) in *Escherichia coli*. *E. coli* DH5α containing the recombinant plasmid pAM86 expressed O antigens which reacted with polyclonal antisera to *V. ordalii* and to *V. anguillarum* O2 LPS and with monoclonal antibody (MAb) 7B4, which is specific for *V. anguillarum* O2 O antigens. The recombinant strains were also protected from bactericidal killing by normal fish serum. Surprisingly, the LPS expressed from the cloned rfb_{VaO2} genes also reacted with MAb A16, which is specific for *V. ordalii* O antigens. Western immunoblot analysis revealed that MAb 7B4 reacted with recombinant LPS bearing shorter O-antigen repeat units, while MAb A16 reacted with the longer O antigens. Similar results were obtained when pAM86 was transformed into *E. coli* CLM4, which has a deletion spanning the *sbcB-rfb* region, indicating that the changes in antigenic profiles of O antigens from the recombinant strains were not due to genes within the *E. coli rfb* cluster. These data suggest that the epitope recognized by the MAb A16 is expressed by *V. anguillarum* O2 strains but it is apparently not accessible to the antibody in the native O polysaccharide. Cloning of the rfb_{VaO2} gene cluster resulted in expression of a novel O antigen. The modification(s) which leads to the alterations in antigenic profile of these recombinant LPS remains to be determined.**

Vibrio ordalii and serotype O1 and O2 strains of *Vibrio anguillarum* are causative agents of vibriosis, a septicemic and often fatal disease of marine fish. These pathogens cause significant economic losses in salmonid marine aquaculture (20). Bacterins against fish vibriosis in Canada are traditionally composed of heat-inactivated preparations of *V. ordalii* and serotype O1 *V. anguillarum*. The major protective component of these bacterins is lipopolysaccharide (LPS) (19). LPS is composed of three major parts: the hydrophobic lipid A, the core oligosaccharide, and the antigenically variable O antigen. Antibodies against the O antigens give serotype-specific protection against strains bearing the homologous O antigens (15).

All *V. ordalii* strains are serotype O2. Polyclonal sera against *V. ordalii* or *V. anguillarum* O2 strains showed extensive cross-reactivity with LPS antigens from both species (12, 20). Chen et al. (3) described monoclonal antibodies (MAbs) that recognized cross-reactive determinants in LPS from both *V. ordalii* or *V. anguillarum* O2 strains. However, there are data suggesting heterogeneity in LPS O antigens within the serotype O2 group. For example, Rasmussen (16) identified subgroups O2a and O2b within the serotype O2 *V. anguillarum* strains. Airdrie et al. (1) reported outbreaks of vibriosis caused by *V. anguillarum* O2 strains in rainbow trout vaccinated with a *V. ordalii* bacterin. We have described MAbs that differentiated between *V. ordalii* and *V. anguillarum* O2 strains on the basis of species-specific epitopes in the O antigen (11). Recently, we found that when grown in the presence of rainbow trout blood, serotype O2 *V. anguillarum* produced an acidic polysaccharide capsular antigen which reacts with MAb 7B4, an antibody which normally detects an O-antigen-specific epitope (11, 18). The capsular and O polysaccharides are composed of a linear tetrasaccharide repeating unit containing a novel diamino uronic acid and an unusual *N*-L-alanylformamido group which has not been previously identified in bacterial polysaccharides (18). The compositions of O antigens from *V. ordalii* and *V. anguillarum* O2b strains have not been reported. LPS is known to contribute to the pathogenicity of enterobacteria (9), and it is likely to be important in virulence of *Vibrio* spp. pathogenic for fish. To elucidate the possible pathogenic role of LPS in *V. ordalii* and *V. anguillarum*, we initiated studies of the chemical composition and the genes for synthesis of the O antigens.

The gene clusters determining expression of LPS O antigens in a number of gram-negative bacterial species are well characterized (9, 17). Analysis of these O-antigen genes, the *rfb* genes, has been pivotal in elucidating the molecular basis for the O-antigen heterogeneity in *Salmonella enterica* (17) and *Vibrio cholerae* (22). The genetics of LPS biosynthesis in *V. ordalii* and serotype O2 strains of *V. anguillarum* are not known. As a first step towards understanding the basis for the antigenic heterogeneity of LPS from the serotype O2 *Vibrio* pathogens, we have cloned the genes involved in biosynthesis and expression of O antigens from *V. anguillarum* ATCC 19264, the serotype O2 type strain. The antigenic properties of O polysaccharides expressed from the cloned rfb_{VaO2} genes in *Escherichia coli* are discussed.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth or agar. *E. coli* transformants were grown on 2× nutrient agar (1% NaCl, 1% nutrient broth, 0.5% yeast extract) containing 50 μg of ampicillin per ml. *V. anguillarum* and *V. ordalii* strains were grown in LB broth or LB agar containing 2% NaCl or on LB agar containing 2% NaCl and 10% fresh rainbow trout blood (11).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
V. anguillarum		
ATCC 19264	Serotype O2	2
NB122	Serotype O2	1
ATCC 43305	Serotype O1	20
ATCC 43307	Serotype O3	20
V. ordalii MT601	Serotype O2	11
E. coli		
DH1	F$^-$ *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* R$^-$	GIBCO/Bethesda Research Laboratories
DH5α	F$^-$ *supE44 hsdR17*($r_K^-m_K^+$) *recA1 endA1 gyrA96 thi-1 relA1* ϕ80*lacZ*M15 Δ(*argF lacZYA*)*U169*	GIBCO/Bethesda Research Laboratories
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	GIBCO/Bethesda Research Laboratories
HB101	*supE44 hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	GIBCO/Bethesda Research Laboratories
Sϕ874	*lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL*	13
CLM4	Sϕ874 Δ*recA*	24
Plasmids		
pcos4EMBL	Cosmid cloning vector; Tc^r Amp^r	4
pAM86	O2 LPS$^+$ cosmid clone in pcos4EMBL; Amp^r	This study

Polyclonal sera and MAbs. Rabbits were immunized with heat-killed *V. anguillarum* O2 cells to generate polyclonal antisera against LPS antigens as described previously (12). All polyclonal sera were absorbed extensively with *E. coli* DH5α cells to remove cross-reacting antibodies (12). MAbs A16 and 7B4, specific for LPS O antigens from *V. ordalii* and *V. anguillarum* O2, respectively, were described previously (11).

DNA methods. Plasmid DNA was purified by the alkaline lysis method as described by Maniatis et al. (10) and by using a commercial kit (Qiagen Inc., Chatsworth, Calif.). Genomic *V. anguillarum* ATCC 19264 DNA was purified as described by Maniatis et al. (10). Transformation of *E. coli* strains was done by the $CaCl_2$ method (10). For Southern hybridization, plasmid DNA from pAM86 or chromosomal DNA was digested with restriction enzymes; the chromosomal DNA was from *V. anguillarum* serotypes O1, O2, and O3; *V. ordalii*; *V. cholerae*; and *E. coli* DH5α. The DNA fragments were resolved on agarose gels and transferred by overnight capillary blotting onto nylon membranes. The DNA fragments were cross-linked to the membrane with a Stratagene UV Stratalinker 1800 and hybridized with digoxigenin-labeled pAM86. Labeling of the DNA with the digoxigenin system and detection of the hybridized probes with the chemiluminescence substrate system were performed with a kit from Boehringer Mannheim (Laval, Quebec, Canada) by following the manufacturer's specifications. Southern blots of the chromosomal DNA were also hybridized with a biotin-labeled oligonucleotide probe for detection of JUMPstart DNA sequences (6). The 18-bp oligonucleotide probe (5′-GCCAAGGGCGGTAGCG TG-3′) was based on the consensus sequence for the JUMPstart DNA sequence (6). The hybridized probe was detected with a streptavidin-alkaline phosphatase conjugate and by using a kit from Boehringer Mannheim. Hybridizations were performed under high-stringency conditions as described for the kits.

Construction of cosmid gene bank. Genomic DNA fragments of approximately 40 to 50 kilobase pairs (kb) were obtained by partial digestion of the *V. anguillarum* O2 chromosomal DNA with *Sau*3A. The cosmid vector pcos4EMBL (4) was linearized with *Pvu*II and then digested with *Bam*HI. The partial *Sau*3A fragments of the chromosomal DNA were ligated to the *Bam*HI site of the cosmid vector, packaged in lambda particles in vitro, and used to infect *E. coli* DH5α. Ampicillin-resistant colonies were transferred to nitrocellulose and screened by colony immunoblot analysis with *V. anguillarum* O2 polyclonal antiserum. Colonies that reacted with the antiserum were screened for LPS production by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis.

Isolation of LPS, SDS-PAGE analysis, and Western immunoblotting. LPS was isolated by the proteinase K method of Hitchcock and Brown (5). LPS was analyzed by SDS-PAGE (12); analysis was followed by silver staining (23) and Western immunoblot analysis (11, 12).

Detection of cell surface LPS O antigens by immunoelectron microscopy. Transmission electron microscopy of cells of *E. coli* DH5α(pcos4EMBL) and the transformant *E. coli* DH5α(pAM86) treated with *V. anguillarum* O2 antiserum and protein A-colloidal gold was performed as described by Yao et al. (24).

RESULTS AND DISCUSSION

Cloning of LPS genes. A *V. anguillarum* O2 genomic library in *E. coli* DH5α was screened with polyclonal *V. anguillarum* O2 antisera. A single colony reacting with these polyclonal sera was recovered; the isolate contained a plasmid, designated pAM86, with a 45.5-kb chromosomal DNA insert (Fig. 1A). Digoxigenin-labeled pAM86 DNA was used in Southern blots to probe *Hin*dIII-digested chromosomal DNA from *V. ordalii* and *V. anguillarum* of several serotypes (Fig. 1B). *E. coli* DH5α with and without vector or recombinant plasmids were probed as controls. The probe hybridized with 13 restriction fragments ranging from 6.0 to 1 kb in both the recombinant plasmid pAM86 (Fig. 1C, lane 12) and the *Hin*dIII-digested chromosomal DNA from *V. anguillarum* O2 (Fig. 1C, lane 3). Three restriction fragments also hybridized with the probe in *V. anguillarum* O2 (Fig. 1C, lane 3) that did not hybridize with the recombinant plasmid (Fig. 1C, lane 12). These fragments may represent repeated DNA sequences in the *V. anguillarum* genome. Further studies are being performed to identify the *rfb* genes within the cloned DNA and to obtain information based on gene sequence analysis for understanding the heterogeneity of LPS O antigens of the serotype O2 strains and species.

A conserved 39-bp DNA sequence, termed JUMPstart, was recently identified in the noncoding region upstream of gene clusters directing synthesis of surface polysaccharides in gram-negative bacteria. The JUMPstart sequences were also proposed to play a significant role in regulation of polysaccharide gene expression (6). To determine whether pAM86 contained DNA homologous to the JUMPstart sequence, and to determine the orientation of the rfb_{VaO2} in pAM86, restriction fragments of pAM86 were hybridized with a biotin-labeled probe for the JUMPstart sequences (Fig. 2A). The probe hybridized to a 6.4-kb *Xho*I fragment of pAM86 (Fig. 2B, lane 1). Further studies showed that the probe hybridized to an internal 1.2-kb *Hin*dIII fragment within the 6.4-kb *Xho*I band (Fig. 1A). To investigate the distribution of the JUMPstart sequences in

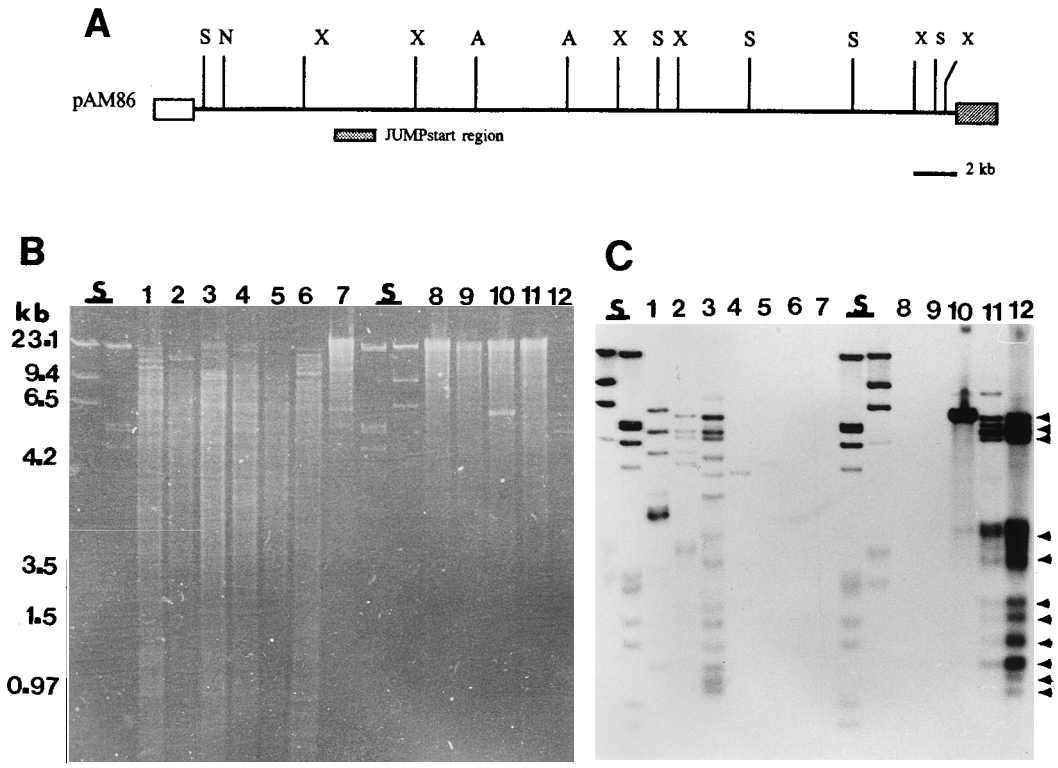

FIG. 1. (A) Physical map of the rfb_{VaO2} region of *V. anguillarum* O2. pAM86 contains approximately 45.5 kb of *V. anguillarum* O2 DNA cloned in cosmid vector pcos4EMBL. The shaded box beneath the 6.4-kb *Xho*I DNA fragment shows the position of the 1.2-kb *Hin*dIII fragment hybridizing with the JUMPstart probe. Vector DNA sequences are shown by the hatched boxes. The enzymes used were *Xho*I (X), *Sal*I (S), *Acc*651 (A), and *Nae*I (N). (B and C) Southern hybridization of plasmid and chromosomal DNA with digoxigenin-labeled recombinant pAM86 as a probe. (B) Ethidium bromide-stained agarose gel. (C) Corresponding hybridization blot. Chromosomal DNA was prepared from the following strains: *V. ordalii* (lane 1); *V. anguillarum* NB122 (lane 2); *V. anguillarum* ATCC 19264 (O2) (lane 3), ATCC 43305 (O1) (lane 4), and ATCC 43307 (O3) (lane 5); *V. cholerae* non-O1 (lanes 6 and 7); *E. coli* DH1 (lane 8), DH5α (lane 9), DH5α(pcos4EMBL) (lane 10), and DH5α(pAM86) (lane 11); and plasmid pAM86 (lane 12). S, molecular size markers (in kilobase pairs [kb]) of lambda DNA digested with *Hin*dIII and with *Eco*RI-*Hin*dIII. Arrowheads, fragments that hybridize with the probe in pAM86 and chromosomal DNA from *V. anguillarum* ATCC 19264.

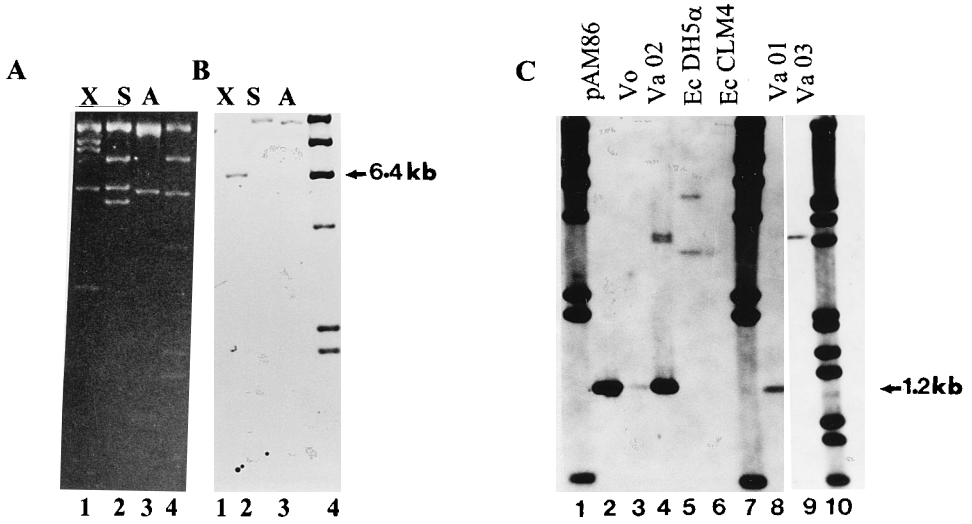

FIG. 2. Southern blot hybridization of the JUMPstart probe with plasmid pAM86 (A and B) and chromosomal DNA (C). pAM86 was digested with *Xho*I (X) (lane 1), *Sal*I (S) (lane 2), and *Acc*651 (A) (lane 3). The molecular size markers are lambda DNA digested with *Hin*dIII (lane 4). The agarose gel (A) and the corresponding autoradiogram of the Southern blot (B) show the position of the 6.4-kb *Xho*I fragment hybridizing with the JUMPstart probe. (C) Autoradiogram of Southern blots of *Hin*dIII-digested plasmid pAM86 (lane 2) and chromosomal DNA from *V. ordalii* (Vo) (lane 3); *V. anguillarum* (Va) serotypes O2, O1, and O3 (lanes 4, 8, and 9, respectively); and *E. coli* (Ec) DH5α and CLM4 (lanes 4 and 5). Molecular size markers are lambda DNA digested with *Hin*dIII (lanes 1 and 7) and with *Hin*dIII and *Eco*RI (lane 10). The sizes of the fragments hybridizing with the probe are indicated on the right.

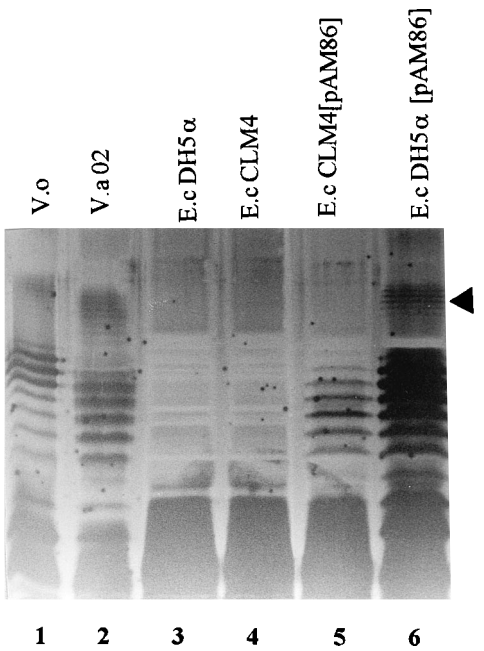

FIG. 3. Analysis of LPS by SDS-PAGE and silver staining. Lane 1, *V. ordalii* (V.o); lane 2, *V. anguillarum* (V.a) O2 ATCC 19264; lane 3, *E. coli* (E.c) DH5α; lane 4, CLM4; lane 5, CLM4(pAM86); lane 6, DH5α(pAM86). Arrowhead, DH5α(pAM86) transformants expressing LPS bearing the longer O-antigen repeat units.

Vibrio species pathogenic to fish, the probe was used in Southern hybridization of *Hin*dIII-digested chromosomal DNA from *V. ordalii* and *V. anguillarum* serotypes O1, O2, and O3. The probe hybridized to a 1.2-kb *Hin*dIII fragment in plasmid pAM86 (Fig. 2B, lane 2) and in chromosomal DNA digests of *V. ordalii* and *V. anguillarum* O2 and O1 (Fig. 2C, lanes 3, 4, and 8, respectively) and to a band of approximately 4.2 kb in *V. anguillarum* O3 (lane 9) and did not hybridize to chromosomal DNA from *E. coli* CLM4 (lane 7). The probe also hybridized to *Hin*dIII fragments of approximately 4.2 and 4.1 kb in *V. anguillarum* O2 (Fig. 2C, lane 3) and two fragments in *E. coli* DH5α (lane 6), suggesting homology to other DNA sequences in these strains. Stevens et al. (21) have recently identified DNA sequences with extensive homology to the JUMPstart sequences in the upstream regions of operons regulated by RfaH, including the *rfa* operon for synthesis of outer-core polysaccharides (21). These data suggest that pAM86 contains the entire rfb_{VaO2} gene cluster and the upstream region containing DNA sequences with homology to the JUMPstart sequences proposed to play a role in regulation of polysaccharide synthesis.

Plasmid pAM86 was transformed into *E. coli recA* mutant strains DH1, HB101, and CLM4, which has a deletion removing all of the *rfb* gene cluster (24). Although *rfb* expression was obtained in $recA^+$ strain LE392, pAM86 was unstable in the Rec^+ background and O antigen was lost with repeated subculture of the transformants. The loss of O-antigen expression was correlated with rearrangements of the plasmid (data not shown). *E. coli* CLM4 and DH5α transformed with pAM86 expressed LPS with the characteristic O-antigen ladderlike profile on silver-stained polyacrylamide gels (Fig. 3, lanes 5 and 6, respectively). The LPS banding profile of the *E. coli* transformants was similar to that of LPS from *V. anguillarum* O2 (Fig. 3, lane 2). The DH5α(pAM86) transformants expressed LPS bearing the longer O-antigen repeat units (Fig. 3). These higher-molecular-weight LPS bands were also detected when gels were loaded with increasing amounts of LPS preparations from CLM4(pAM86) (data not shown). Immunoelectron microscopy was used to show that *E. coli* DH5α transformed with pAM86 expressed O antigens on the cell surface (Fig. 4A); no

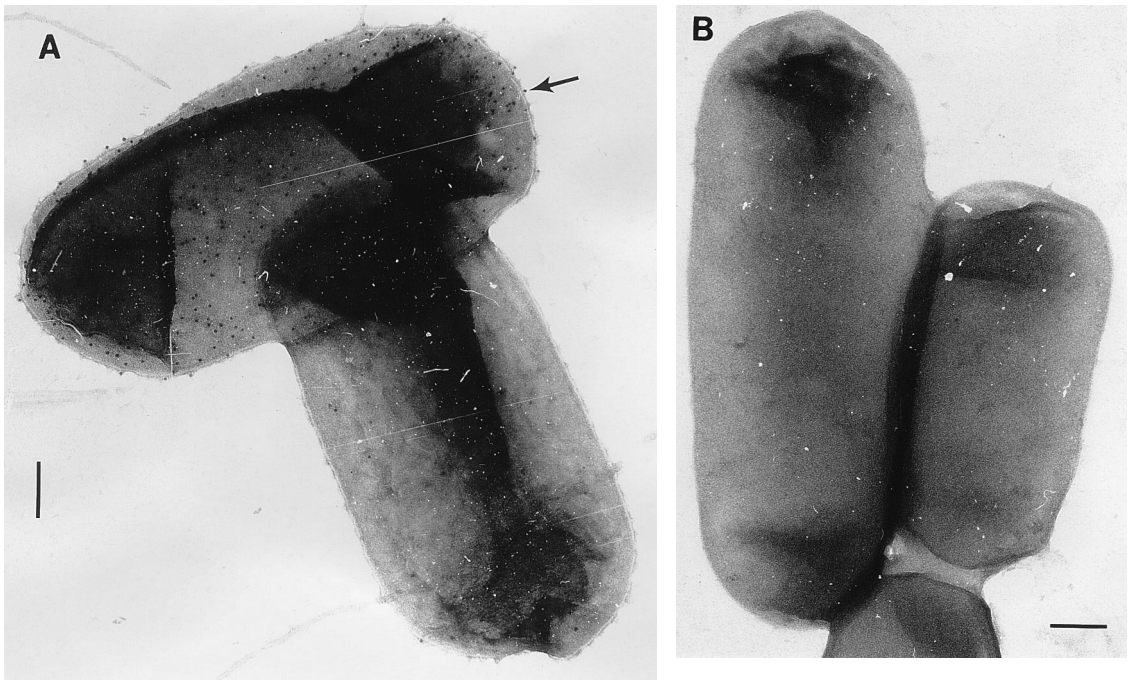

FIG. 4. Transmission electron microscopy of *E. coli* DH5α(pAM86) (A) and *E. coli* DH5α(pcos4EMBL) (B) cells reacted with polyclonal *V. anguillarum* O2 serum and protein A labeled with colloidal gold particles. The arrow (A) indicates gold particles on surfaces of *E. coli* DH5α(pAM86) cells expressing the cloned O antigens. Bar = 10 nm. Diameter of gold particles = 8 nm.

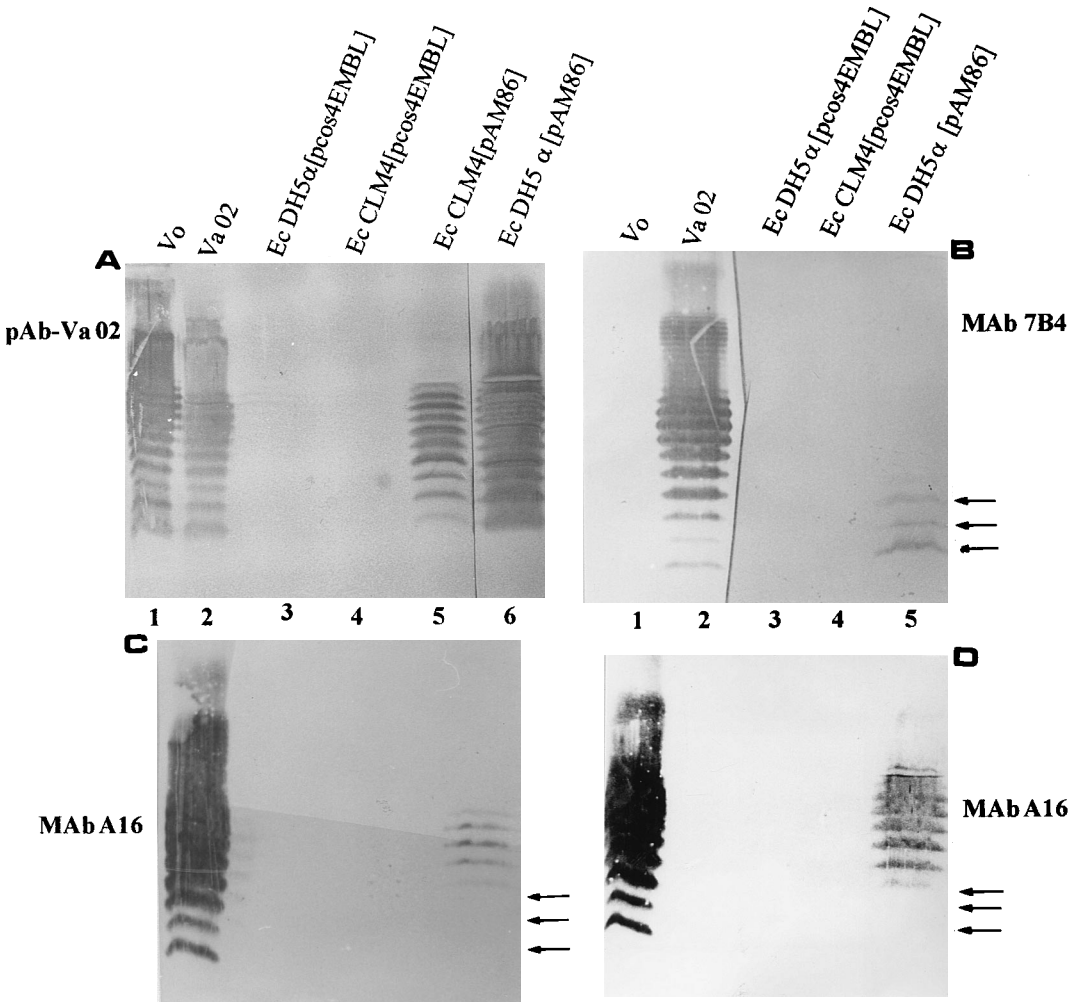

FIG. 5. Western immunoblot analysis of LPS with pAb-Va O2 (polyclonal *V. anguillarum* ATCC 19264 [O2] serum), MAb 7B4, and MAb A16. Lanes 1, *V. ordalii* (Vo); lanes 2, *V. anguillarum* (Va) O2 ATCC 19264; lanes 3, *E. coli* DH5α(pcos4EMBL); lanes 4, CLM4(pcos4EMBL); lanes 5 and 6, *E. coli* CLM4(pAM86) or DH5α(pAM86). Arrows, reactions of MAb 7B4 with low-molecular-weight LPS O antigens from *E. coli* DH5α(pAM86).

immunogold particles were deposited on the surface of *E. coli* DH5α containing only the vector pcos4EMBL (Fig. 4B). Therefore, pAM86 confers on *E. coli* strains, including strain CLM4, with a deletion of *rfb*, the ability to synthesize LPS O antigens, indicating that all the genes necessary for synthesis and polymerization of *V. anguillarum* O2 O-antigen backbone are present on the plasmid.

In previous studies, we observed alteration in the chain lengths of LPS O antigens when *V. anguillarum* or *V. ordalii* strains were grown in media containing fresh rainbow trout blood (11, 18). We did not detect any significant alterations in the profiles of O antigens when *E. coli* CLM4(pAM86) or DH5α(pAM86) was grown on media with fish blood (data not shown), which is unlike results obtained with wild-type *V. anguillarum* O2 strains. *E. coli* CLM4 or DH5α containing the vector pcos4EMBL alone failed to grow on the rainbow trout blood media, but transformation with pAM86 permitted growth on trout blood media. Although *E. coli* CLM4(pAM86) transformants expressed LPS antigens bearing mainly the medium-length O antigens (Fig. 3, lane 5), these antigens were sufficient to confer serum resistance and protected the transformants from the bactericidal effects of fresh rainbow trout blood. LPSs bearing long O side chains have been shown to hinder insertion into the bacterial membrane of activated complement factors, thus protecting cells from lysis (7, 14). However, the ability of O antigens to protect cells against complement lysis is a function of the length, conformation, and chemical composition of the molecules (14).

Western immunoblot analysis of the cloned LPS O antigens. LPS antigens from *E. coli* transformants carrying pAM86 reacted with polyclonal sera against *V. anguillarum* O2 (Fig. 5A) and *V. ordalii* (data not shown), indicating that the recombinant O antigens contained epitopes that are common to both *V. anguillarum* O2 and *V. ordalii*. These observations were confirmed by the reaction of polyclonal *E. coli*(pAM86) serum with LPS from both *V. ordalii* and *V. anguillarum* O2 (data not shown). Surprisingly, MAb 7B4, which is specific for *V. anguillarum* O2 O antigens, reacted with only the low-molecular-weight LPS O antigens from *E. coli* DH5α(pAM86) (Fig. 5B, lane 5) and CLM4[pAM86] (data not shown), while MAb A16, which is specific for *V. ordalii* O antigens, reacted only with the higher-molecular-weight O antigens from the CLM4 and DH5α transformants (Fig. 5C and D, lanes 5, respectively). In contrast, MAbs 7B4 and A16 reacted with the entire O-antigen

ladder in *V. anguillarum* O2 and *V. ordalii* (Fig. 5B, lane 2, and Fig. 5C and D, lanes 1, respectively). In contrast to results with the wild-type strains, MAb 7B4 appeared to bind to recombinant LPS molecules with between one and three O-antigen repeat units (Fig. 5A, lanes 2 and 5), and the A16 epitope was detected in molecules with more than three O-antigen repeat units (Fig. 5C and D, lanes 1 and 5, respectively).

It is not uncommon to see modifications in LPS O antigens induced by cloning of *rfb* genes in *E. coli* K-12. Kogan et al. (8) reported that *E. coli* K-12 carrying O4 *rfb* genes expressed an LPS which reacted with anti-O4 serum but the recombinant antigens failed to react with anti-O18 serum, which differs from results with the wild-type strain. The alterations in serological reactivity of LPS from the recombinant *E. coli* strains was attributed to alterations in the position of a glucosyl side chain substitution and to changes in the anomeric configurations of the main-chain polysaccharides. Yao et al. (24) also showed acetylation of *Shigella flexneri* 3a and 2a O antigens when the cloned *rfb* genes were expressed in *E. coli* K-12, and these acetylated recombinant molecules then reacted with group 6 antiserum. No acetylation was detected when the *S. flexneri rfb* genes were expressed in *E. coli* CLM4, which has an *sbcB-rfb* chromosomal deletion. In this study, the O antigens expressed from the cloned rfb_{VaO2} reacted with MAb 7B4, which is specific for the native *V. anguillarum* O2 O antigens, and with MAb A16, which is specific for *V. ordalii* O antigens. These novel O polysaccharides were expressed in both DH5α (pAM86) and CLM4(pAM86), indicating that the changes in serological properties were not due to modifications of the O antigens by genes within the *E. coli rfb* cluster.

The data from our studies suggest that the epitopes recognized by MAbs 7B4 and A16 are expressed in the native *V. anguillarum* O2 LPS. It is probable that expression of the 7B4 epitope masks or modifies the epitope recognized by MAb A16 in the native O antigen. It is likely that cloning of the rfb_{VaO2} genes resulted in alterations of the O-specific polysaccharide in the recombinant strains, causing differential reactivity with MAb 7B4 and the exposure or accessibility of the A16 epitope. A lot more work, including structural and compositional analyses of LPS from the recombinant *E. coli* strains and from *V. ordalii*, is needed to determine the nature of the modifications in the novel recombinant O polysaccharides.

In conclusion, our study shows that we have cloned the entire gene cluster for expression of *V. anguillarum* O antigens. The expression of these antigens in *E. coli* transformants was sufficient to confer protection against lysis by fresh serum in rainbow trout blood. Chemical analysis of the O antigens from *E. coli* CLM4(pAM86) and comparison to the structure of the native O antigen will allow us to determine the alterations which allow binding of the *V. ordalii* species-specific MAb A16. These studies are the first step in the examination of the genetic and structural bases of antigenic heterogeneity between *V. ordalii* and *V. anguillarum* O2 (including O2a and O2b) strains.

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