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Characterization and Biological Role of the O-Polysaccharide Gene Cluster of *Yersinia enterocolitica* Serotype O:9[▽]

Mikael Skurnik,[1,2]* Marta Biedzka-Sarek,[1] Peter S. Lübeck,[3]† Tea Blom,[1]‡ José Antonio Bengoechea,[5] Camino Pérez-Gutiérrez,[5] Peter Ahrens,[4]§ and Jeffrey Hoorfar[3]

Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki,[1] and Helsinki University Central Hospital Laboratory Diagnostics, Helsinki,[2] Finland; National Food Institute[3] and National Veterinary Institute,[4] Technical University of Denmark, Copenhagen, Denmark; and Unidad de Investigacion, Hospital Son Dureta, Palma Mallorca, and Program Infection and Immunity, Fundación Caubet-Cimera Illes Balears, Bunyola, Spain[5]

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Yersinia enterocolitica **serotype O:9 is a gram-negative enteropathogen that infects animals and humans. The role of lipopolysaccharide (LPS) in *Y. enterocolitica* O:9 pathogenesis, however, remains unclear. The O:9 LPS consists of lipid A to which is linked the inner core oligosaccharide, serving as an attachment site for both the outer core (OC) hexasaccharide and the O-polysaccharide (OPS; a homopolymer of *N*-formylperosamine). In this work, we cloned the OPS gene cluster of O:9 and identified 12 genes organized into four operons upstream of the *gnd* gene. Ten genes were predicted to encode glycosyltransferases, the ATP-binding cassette polysaccharide translocators, or enzymes required for the biosynthesis of GDP-*N*-formylperosamine. The two remaining genes within the OPS gene cluster, *galF* and *galU*, were not ascribed a clear function in OPS biosynthesis; however, the latter gene appeared to be essential for O:9. The biological functions of O:9 OPS and OC were studied using isogenic mutants lacking one or both of these LPS parts. We showed that OPS and OC confer resistance to human complement and polymyxin B; the OPS effect on polymyxin B resistance could be observed only in the absence of OC.**

Yersinia enterocolitica serotypes O:3, O:5,27, O:8, and O:9 include strains that infect both humans and animals (16). Pathogenesis of these strains is associated with the presence of common virulence factors encoded on the chromosome and on pYV, the *Yersinia* virulence plasmid (73). Despite the genetic and phenotypic similarity, however, the serotypes display epidemiological and host range differences (17, 22). Biotyping and serotyping based on the antigenic O-polysaccharide (OPS or O-antigen) are important in studying *Y. enterocolitica* infections. OPS is a distal part of lipopolysaccharide (LPS), which is the major component of the outer membrane of gram-negative bacteria (55, 63). In addition to OPS, LPS comprises lipid A, a hydrophobic membrane anchor, and a core oligosaccharide, divided into a lipid A-proximal inner core and an outer core (OC). The latter typically provides the attachment site for the polymeric OPS (55). Heteropolymeric OPSs, such as that of *Y. enterocolitica* serotype O:8 (63), are the most frequent among gram-negative bacteria; they are made up of identical oligosaccharide repeat units comprising three to eight different monosaccharides (55). *Y. enterocolitica* serotypes O:3 and O:9, however, display a homopolymeric OPS composed of single-sugar repeating units of 6-deoxy-L-altrose or *N*-formylperosamine, respectively (24, 41). Furthermore, the structures of the O:3 and O:9 core oligosaccharides are identical (51, 63). They both have a branching hexasaccharide termed OC which in genetic and biosynthetic terms resembles more closely a nonpolymerized O unit than the classical hexose-containing OC of *Escherichia coli* or *Salmonella* species (67, 68). The linkage of both the OC and the homopolymeric OPS to the inner core is a unique feature shared between serotypes O:3 and O:9. The O:9 OPS is identical to that of *Brucella* species and thus gives false-positive reactions in serological assays for brucellosis in humans and animals (23, 28, 38, 53).

Homopolymeric OPSs are synthesized at the cytoplasmic face of the inner membrane. The OPS synthesis from nucleotide diphosphate-activated sugar precursors on the membrane-bound carrier undecaprenylphosphate is carried out by glycosyltransferases. Full-length polymer is then translocated to the periplasm by the ATP-binding cassette (ABC) transporter formed by Wzt and Wzm. Subsequently, the polymer is ligated to preformed lipid A-core compounds and further translocated to the outer membrane (55).

The genes encoding enzymes necessary for the OPS biosynthesis are usually clustered in the bacterial chromosome (60). In the genus *Yersinia* the locus between *hemH* and *gsk* contains the genes required for the biosynthesis of the heteropolymeric OPSs (57, 63, 64, 66). In serotypes O:9 and O:3, however, the *hemH-gsk* locus is occupied by the OC gene cluster, thereby indicating that the OPS gene cluster is located elsewhere in the genome (63, 65, 66).

In this work, we present the cloning and characterization of the *Y. enterocolitica* serotype O:9 OPS gene cluster. Due to the branched nature of the OC in LPS, mutants lacking OC but

* Corresponding author. Mailing address: Haartman Institute, P.O. Box 21, 00014 University of Helsinki, Finland. Phone: 358-9-191 26464. Fax: 358-9-191 26382. E-mail: mikael.skurnik@helsinki.fi.

† Present address: Bio Science and Technology, BioCentrum, Technical University of Denmark, Copenhagen, Denmark.

‡ Present address: Molecular Cancer Biology Program, 00014 University of Helsinki, Finland.

§ Present address: Statens Serum Institut, Copenhagen, Denmark.

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TABLE 1. Bacterial strains, plasmids, bacteriophages, and bacteriocins used in this work

Bacterial strain, plasmid, or bacteriophage or bacteriocin	Comments	Reference(s) or source
Strains		
E. coli		
SY327λ_{pir}	λ(*lac pro*) *argE*(Am) *rif nalA recA56* (λpir)	50
Sm10λ_{pir}	*thi thr leuB tonA lacY supE recA*::RP4-2-Tc::Mu-Km (λpir)	61
S17-1	*thi pro hsdR hsdM*$^+$ *recA*::RP4-2-Tc::Mu-Km::Tn7 Str^r	61
S17-1λ_{pir}	*thi pro hsdR hsdM*$^+$ *recA*::RP4-2-Tc::Mu-Km::Tn7 Str^r (λpir)	29
HB101/pRK2013	Triparental conjugation helper strain; Km^r	31
C600	*thi thr leuB tonA lacY supE*	6
Y. enterocolitica		
Ruokola/71	Patient isolate	62
Ruokola/71-c	Spontaneous virulence plasmid-cured derivative of Ruokola/71	62
YeO9-OC-R	Phage ϕR1-37-resistant spontaneous OC-negative derivative of YeO9-R1	This work
YeO9-R1	Δ*per*::KmGB; rough (OPS negative); Km^r derivative of Ruokola/71	This work
YeO9-c-R3	Δ*per*::KmGB; rough (OPS negative); Km^r derivative of Ruokola/71-c	This work
YeO9-OC	Δ(*wzx-wbcL*)::KmGB; OC negative; derivative of Ruokola/71	This work
YeO9-c-OC	Δ(*wzx-wbcL*)::KmGB; OC negative; derivative of Ruokola/71-c	This work
YeO9-OC^P	Phage ϕR1-37-resistant spontaneous low-OC-expressing derivative of Ruokola/71	This work
YeO9-galF	Δ*galF*::KmGB; derivative of Ruokola/71; Km^r	This work
YeO9-c-galF	Δ*galF*::KmGB; derivative of Ruokola/71; Km^r	This work
Plasmids		
pRV1	Suicide vector; Clm^r	68
pRV1-galF	PvuII fragment of O:9 OPS gene cluster containing the *galF* gene cloned into pRV1	This work
pRV1-galF-Nsi	pRV1-galF with the *galF* internal NsiI fragment deleted	This work
pRV1-galF-Nsi::GB	pRV1-galF with the *galF* internal NsiI fragment replaced by KmGB	This work
pRV1-galU	MscI fragment of O:9 OPS gene cluster containing the *galU* gene cloned into pRV1	This work
pRV1-galU-del	pRV1-galU with a deletion in the *galU* gene	This work
p87/I	pHC79 carrying a genomic fragment of Ruokola/71-c containing the OPS gene cluster	This work
p46/III	pHC79 carrying a genomic fragment of Ruokola/71-c containing the OPS gene cluster	This work
p73/II	pHC79 carrying a genomic fragment of Ruokola/71-c containing the OPS gene cluster	This work
p77/II	pHC79 carrying a genomic fragment of Ruokola/71-c containing the OPS gene cluster	This work
pKK232-8	Promoter trapping vector; Amp^r	GE Healthcare
pPSL	10-kb PstI fragment of Ruokola/71 genomic DNA cloned into pUC18	This work
pWBO:9Sac	SacI fragment of pPSL	This work
pWBO:9SacGB	KmGB cloned into the EcoRV site within the *per* gene in pWBO:9Sac	This work
pRV1-WBO:9SacGB	4-kb PvuII fragment of pWBO:9SacGB cloned into the EcoRV site of suicide vector pRV1	This work
pHC79	Cosmid cloning vector; Amp^r	
pUC18	Plasmid cloning vector; Amp^r	
pUC4K	Source of the KmGB cassette; Amp^r Km^r	Pharmacia
pRV19-GB	Suicide vector to inactivate OC gene cluster; Δ(*wzx-wbcL*)::KmGB; Clm^r Km^r	68
Bacteriophages and bacteriocins		
ϕR1-37	OC-specific bacteriophage	46, 67
Enterocoliticin	OC-specific bacteriocin	69, 70

keeping the OPS and vice versa were constructed. We demonstrated that these mutants have both polymyxin B and serum resistance defects.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and culture conditions. Bacterial strains, plasmids, and bacteriophages used in this work are listed in Table 1. All the *Y. enterocolitica* O:9 derivatives used in this work originate from the wild-type strain Ruokola/71 (62).

Bacteria were routinely cultured in Luria-Bertani broth (LB) or on agar plates based on LB (LA plates). For bacteriophage cultures tryptic soy broth or agar plates were used. *Yersinia* selective agar plates (CIN agar; Oxoid, Basingstoke, England) supplemented with appropriate antibiotics were used for the selections of the transconjugants. Antibiotics were added to the growth media at following concentrations: ampicillin, 5 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 100 μg/ml in agar plates and 20 μg/ml in broth. *E. coli* strains were grown at 37°C and *Y. enterocolitica* strains at room temperature (RT; 22°C) unless otherwise indicated.

Recombinant DNA methods. DNA isolations, restriction enzyme digestions, and DNA ligations were performed as described previously (7, 58). For PCR screening reactions, bacteria resuspended in 100 μl of water were incubated for 5 min at 95°C. Subsequently, 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and samples were vortexed and centrifuged for 5 min at 13,000 × *g*. A 5-μl aliquot of the upper DNA-containing phase was used as a PCR template. *E. coli* was transformed by electroporation or by the Hanahan

TABLE 2. Primers used in this work

Primer name	Sequence	5′ position	Direction, accession no., use
Per-2	GGMGATGAAGTBATTGTWCCRAC	7148	Forward, AJ605741, degenerate primer for amplifying *per* gene fragment of Ruokola/71-c for a probe
Per-4	GTYTTRTTYCCAAARAARCTAAA	7482	Reverse, AJ605741, degenerate primer for amplifying *per* gene fragment of Ruokola/71-c for a probe
galU-f	GTTGTCATCGACGGGATATG	4700	Forward, AJ605741, plasmid PCR to delete part of *galU* gene
galU-r	ATTGAATCAGGGGCTTATCG	3919	Reverse, AJ605741, plasmid PCR to delete part of *galU* gene, cloning of the *galU* upstream promoter
PSL-F7a	TATCAACTGCAACTGCAAAG	3454	Forward, AJ605741, cloning of the *galU* upstream promoter
F7	CTGAATCATTACTTCGAGTG	3550	Forward, AJ605741, cloning of the *galU* upstream promoter
galF-F	ACCGGGCAATCCTATAACTG	5532	Forward, AJ605741, cloning of the *gmd* upstream promoter
pSL-7B	GTTGTAACATCATCCATTGC	6527	Reverse, AJ605741, cloning of the *gmd* upstream promoter
pSL-7600Ra	TGTTGACAATGGCCATATGA	8552	Forward, AJ605741, cloning of the *wzm* upstream promoter
pSL-6A	AATTTATGAAAAGGCATGCC	7528	Reverse, AJ605741, cloning of the *wzm* upstream promoter

method (36). Single-step screening of recombinant clones was performed as described earlier (13).

Construction of pUC and cosmid genomic libraries. Genomic libraries of strain Ruokola/71-c were constructed in *E. coli* strain C600 using pUC18 as a cloning vector. Both the genomic and the vector DNA were digested with BamHI, ClaI, EcoRI, HindIII, PstI, HindIII/BamHI, or HindIII/EcoRI or with HindIII/PstI prior to ligation. A cosmid library was constructed by cloning partially Sau3AI-digested genomic DNA of Ruokola/71-c into cosmid pHC79 using the lambda packaging system (GE Healthcare).

PCR strategies. Short-range PCR was performed using the thermostable DNA polymerase DynaZyme II (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. Reaction conditions for PCR cycles were adjusted according to the oligonucleotide primers and the length of the amplified fragment.

The previously sequenced perosamine synthetase (*per*) genes of *Vibrio cholerae* (accession no. X59554), *Vibrio anguillarum* (accession no. AF025396), *E. coli* O157:H7 (accession no. AF061251), and *Brucella melitensis* biovar 1 (accession no. AF047478) (50 to 60% identical to each other) served to design degenerate primers (Table 2). These were used to amplify a 335-bp fragment of the *per* gene of *Y. enterocolitica* O:9. This PCR fragment was used as a probe to screen the libraries by colony hybridization and also for Southern hybridization as described below.

Colony and Southern blotting. The 335-bp *per* fragment was digoxigenin labeled using the High Prime DNA Labeling Kit (Roche Molecular Biochemicals, Boehringer-Mannheim, Germany) according to the instructions of the manufacturer. Colonies replica plated onto nylon membranes were lysed and processed for hybridization as described earlier (58). Slot blotting was performed using the Minifold II Slot Blot Manifold (Schleicher & Schuell, Dassel, Germany). Denatured genomic DNA was applied to the slots as instructed by the manufacturer. Southern blotting was performed as described previously (58). Hybridization and probe detection were performed using the DIG Luminescence Detection Kit for Nucleic Acids (Roche Molecular Biochemicals).

Nucleotide sequencing and sequence analysis. Nucleotide sequencing reactions were performed on ABI373A and ABI377 automatic sequencers using the AmpliTaq FS dye terminator kit or the Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Nucleotide sequence analysis was performed using the computer programs of the Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI) and the EMBOSS programs and the HIBIO DNASIS program for Windows, Higgins and Sharp algorithm (CLUSTAL 4) (37). NCBI and EBI databases were searched using the BLAST programs (4). Similar amino acid sequences were aligned using the PILEUP program of the Genetics Computer Group package.

Construction of *per*, *galF*, and *galU* suicide vectors for allelic exchange. pRV1, a Clm^r derivative of the suicide vector pJM703.1, was used to construct genomic insertion derivatives by allelic exchange (68).

The suicide vector pRV1-WBO:9SacGB (Table 1) for the *per* gene inactivation was constructed by cloning the 2.6-kb SacI fragment containing the *per* gene from pPSL into pUC18. The resulting plasmid was named pWBO:9Sac and maintained in *E. coli* C600. The 1.2-kb kanamycin resistance gene block (KmGB) of pUC4K was cloned into the EcoRV site within the *per* gene of pWBO:9Sac to get plasmid pWBO:9SacGB. The 4-kb PvuII fragment of pWBO:9SacGB was cloned into the EcoRV site of the suicide vector pRV1 to get plasmid pRV1-WBO:9SacGB, which was maintained in *E. coli* SY327λ_{pir}.

The suicide vector pRV1-galF-Nsi:GB (Table 1) for the *galF* gene inactivation was constructed by cloning the *galF*-containing PvuII fragment of pPSL into pRV1. The resulting plasmid was named pRV1-galF. Deletion of the *galF*-internal 0.4-kb NsiI fragment resulted in plasmid pRV1-galF-Nsi. The KmGB was cloned into the NsiI site of the pRV1-galF-Nsi to get pRV1-galF-Nsi:GB, which was maintained in *E. coli* SY327λ_{pir}.

The suicide vector pRV1-galU-del (Table 1) for the *galU* gene inactivation was constructed by cloning the *galU*-containing MscI fragment of pPSL into pRV1. The resulting plasmid was named pRV1-galU. An 800-bp internal deletion in the *galU* gene of pRV1-galU was engineered by plasmid-PCR (20) using primers galU-f and galU-r (Table 2) to get plasmid pRV1-galU-del, which was maintained in *E. coli* SY327λ_{pir} and from there transformed into Sm10λ_{pir}.

The suicide vector pRV19-GB (Table 1) for the OC gene cluster inactivation has been described previously (68).

Inactivation of genes by allelic exchange. Direct or triparental conjugation to *Y. enterocolitica* O:9 strains Ruokola/71 and Ruokola/71-c was performed to mobilize the suicide vectors pRV1-galF-NsiGB, pRV1-galU-del, pRV19-GB, and pRV1-WBO:9SacGB from *E. coli* SY327$\lambda_{pir.}$ or Sm10λ_{pir}, as described earlier (68). Triparental conjugation was performed using the helper strain HB101/pRK2013 (Table 1). Transconjugants having the suicide vectors integrated by homologous recombination into the bacterial chromosome were subjected to cycloserine enrichment to select clones in which the second homologous recombination event had eliminated the suicide vector and the wild-type allele (34, 54). The constructed mutants were verified by PCR, Southern blotting, and/or sequencing. The LPS phenotype was analyzed by deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE; see below).

Isolation of spontaneous *Y. enterocolitica* O:9 OC mutants using ϕR1-37. Bacteriophage ϕR1-37 was used to isolate spontaneous OC mutants. An overnight LB culture of the *Y. enterocolitica* O:9 strain was spread as a lawn on LA plates. A few drops of ϕR1-37 were pipetted on the dried bacterial lawn. After 2 days of incubation, individual phage-resistant colonies were picked from the lysis zone and subjected to the same treatment several times. To verify the loss of OC, LPSs from ϕR1-37-resistant bacteria were analyzed by DOC-PAGE.

PCR-based promoter identification. PCR fragments containing selected intergenic regions, amplified using primers listed in Table 2, were cloned into the promoter trapping vector pKK232-8 (Table 1). The vector contains a promoterless *cat* gene encoding chloramphenicol acetyltransferase; thus, Clm^r clones can be obtained only when a fragment containing promoter activity is cloned upstream of the *cat* gene. Transformants displaying promoter activity were selected on chloramphenicol plates. The inserted PCR fragments in the recovered plasmids were analyzed by sequencing.

Isolation and analysis of LPS. LPSs from OC and OPS mutant candidates were checked by DOC-PAGE. For small-scale LPS isolation, a modified version of the protocol devised by Hitchcock and Brown was used (11, 39). Overnight 5-ml bacterial cultures were diluted to obtain an optical density at 540 nm (OD_{540}) of <1. A 1.5-ml aliquot of the suspension was centrifuged in a microcentrifuge (13,000 × *g*, 3 min), and bacteria were resuspended in DOC lysis buffer (2% DOC, 4% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1 M Tris-HCl buffer, pH 6.8) in a volume adjusted according to the OD_{540} of the culture (100 μl of DOC lysis buffer/OD_{540} = 1.0). Samples were heated at 100°C for 10 min, and then 2 μl of proteinase K (20-mg/ml stock solution) was added. Samples were incubated at 55°C for ≥1 h. Samples were stored at −20°C until analyzed by DOC-PAGE as described previously (68).

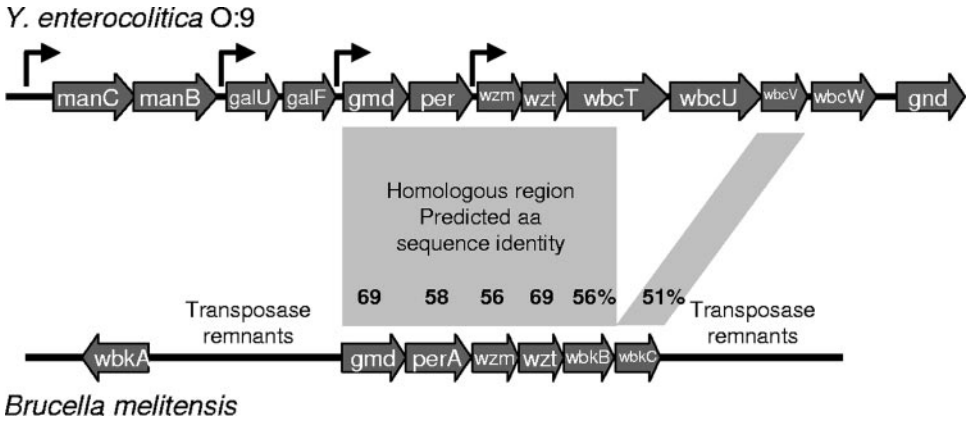

FIG. 1. OPS gene cluster of *Y. enterocolitica* O:9. The gene cluster figures were generated from the sequence files under the accession numbers AJ605741 for *Y. enterocolitica* O:9 and AF047478 for *B. melitensis* using the ggnVIEW sequence file viewer (http://colibase.bham.ac.uk/cgi-bin/fileprepare.cgi). The identified and predicted promoter locations of the O:9 gene cluster are indicated with bent arrows. The *orf1* gene (Table 3), present in the reverse direction upstream of the *manC* gene, is not shown. The regions of homology between the gene clusters are indicated with shadowed quadrangles, and the percentages of identity between the predicted amino acid sequences are given.

Anti-*Y. enterocolitica* O:9 antiserum and slide agglutination. Rabbit antiserum S-3 against formalin-killed Ruokola/71 bacteria was raised as described earlier (2). For the slide agglutination test the antiserum was diluted 1:100 in 0.9% NaCl.

Human serum and serum killing assay. Blood was obtained from healthy human donors who were devoid of anti-*Yersinia* antibodies. Sera were pooled and stored in aliquots at −70°C as described earlier (14). The killing assay was performed as described previously (14). Briefly, ~500 to 1,000 bacteria were incubated at 37°C for 30 and 120 min in 30 μl of 66.7% normal human serum (NHS), heat-inactivated serum, or EGTA-Mg serum. The latter contained 10 mM EGTA and 5 mM $MgCl_2$. Surviving bacteria were cultured and counted after growth on LA plates. The serum bactericidal effect was calculated as the survival percentage using the bacterial counts obtained with bacteria incubated in heat-inactivated serum as 100%. The killing experiment was repeated for each strain at least three times starting from independent cultures.

Polymyxin B resistance. *Yersinia* strains grown in 5 ml of LB either at 37°C or at 21°C were harvested (5,000 × *g*, 15 min, 5°C) in the exponential phase of growth. Bacteria were suspended in 1% (wt/vol) tryptone in phosphate-buffered saline (pH 7.4) to approximately 2.1×10^5 CFU/ml. Ten microliters of the suspension was mixed with various concentrations of polymyxin B in a volume of 200 μl and incubated at the original bacterial growth temperature for 30 min. Subsequently, 100 μl of the suspensions was directly plated on LB agar plates. The plates were incubated at 26°C, and colony counts were determined. The results were expressed as survival percentages, taking the colony counts of bacteria not exposed to antibacterial agents as 100%. The 50% inhibitory concentration of antimicrobial peptides (IC_{50}) was defined as the concentrations showing a 50% reduction in the colony count compared with bacteria not exposed to the antibacterial agent (21). All experiments were done in duplicate and on four independent occasions.

Statistical methods. Statistical analyses were performed using the analysis of variance or the two-sample *t* test or, when the requirements were not met, by the Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession number. The sequence data were annotated and submitted to the European Bioinformatics Institute (accession number AJ605741).

RESULTS

Identification, cloning, and characterization of the OPS gene cluster. To clone the OPS gene cluster from *Y. enterocolitica* O:9, several genomic libraries of strain Ruokola/71-c were constructed in pUC18. Southern blotting analysis was performed to select appropriate restriction enzymes for the cloning. Degenerate primers Per-2 and Per-4 (Table 2) based on the perosamine synthetase (*per*) genes of other bacteria were used to amplify a *per* gene fragment of *Y. enterocolitica* O:9. This PCR product served as a probe in Southern hybridization and in screening the libraries by colony hybridization (42, 48). From the pUC18 libraries a colony containing a 10-kb PstI fragment was identified and characterized. Sequencing data revealed that the PstI fragment did not carry all the genes required for the O:9 OPS biosynthesis. However, from the cosmid library of *Y. enterocolitica* O:9 we isolated four hybridization-positive clones (Table 1). These were also positive in the slide agglutination assay using the O:9 OPS-specific antiserum S-3. Thus, the cosmids carried the genes required for the O:9 OPS expression in *E. coli*. These four clones were subsequently used to complete the sequencing of the OPS gene cluster.

Assignment of putative functions to open reading frames. We identified 12 genes located upstream of the *gnd* gene. The genes were annotated based on similarity to sequences in the databases (Fig. 1; Table 3). The *manB*, *manC*, *gmd*, *per*, *wbcV*, and *wbcT* genes are predicted to encode enzymes involved in the biosynthesis of GDP-*N*-formylperosamine. The *wbcU* and *wbcW* genes are predicted to encode the *N*-formylperosaminyltransferases assembling the homopolymeric OPS onto undecaprenylphosphate carrier lipid while the *wzm* and *wzt* genes encode proteins for the transport of the OPS into the periplasm. The *gnd* gene encodes gluconate-6-phosphate dehydrogenase, which does not participate in OPS synthesis. The genes *galU* and *galF*, identified between the *manB* and *gmd* genes, however, were not assigned a clear function in the OPS biosynthesis.

Identification of promoters. Sequence analysis revealed several putative promoters in the O:9 OPS gene cluster, one upstream from the entire cluster, in front of *manC*, and three intergenic promoters within the cluster in front of the *galU*, *gmd*, or *wzm* gene (Fig. 1). The intergenic regions were PCR amplified using primers given in Table 2; the *manB-galU* intergenic region was in 369- and 465-bp fragments, the *galF-gmd* region in a 995-bp fragment, and the *per-wzm* region in a 1,024-bp fragment. The fragments were cloned into the promoter trapping vector pKK232-8. Sequence analysis of the plasmids isolated from the transformants showed that Clm^r

TABLE 3. OPS gene cluster of *Y. enterocolitica* O:9: genes, gene products, and similarities

Gene	Protein	Size (no. of amino acids)	Similarity	Putative function(s)
orf1	Orf1	>117	80–98% identity	N terminus: similar to TerC-family proteins, possibly involved in tellurite resistance
manC	ManC	472	>70% identity to several ManC proteins	Mannose-1-phosphate guanylytransferase; involved in biosynthesis of GDP-mannose and GDP-*N*-formylperosamine
manB	ManB	480	>70% identity to several ManB proteins	Phosphomannomutase; involved in biosynthesis of GDP-mannose and GDP-*N*-formylperosamine
galU	GalU	296	80–98% identity to several GalU proteins	UTP-glucose-1-phosphate uridylyltransferase
galF	GalF	297	80–98% identity to several GalU proteins	UTP-glucose-1-phosphate uridylyltransferase; 66% identical to GalU of same strain.
gmd	Gmd	373	>80% identity to several Gmd proteins	GDP-mannose-4,6-dehydratase; involved in biosynthesis of GDP-*N*-formylperosamine
per	Per	361	55–67% identity to several Per proteins	GDP-D-perosamine synthase; involved in biosynthesis of GDP-*N*-formylperosamine
wzm	Wzm	260	55–71% identity to several Wzm proteins	Putative ABC transporter system integral membrane protein; translocation of OPS
wzt	Wzt	251	45–78% identity to several Wzt proteins	Putative ATP binding protein involved in OPS export
wbcT	WbcT	581	37% identity to glycosyltransferase of *Aeromonas hydrophila*	Involved in biosynthesis of O:9 OPS, N-terminal half 55% identical to WbkB, a putative glycosyltransferase of *Brucella*
wbcU	WbcU	527	30–40% identity to mannosyltransferases	Putative *N*-formylperosaminyltransferase; amino acids 1 to 75 and 192 to 527 similar to *Brucella* mannosyltransferase
wbcV	WbcV	260	40–50% identity to several *N*-formyltransferase proteins	Putative *N*-formyltransferase; involved in biosynthesis of GDP-*N*-formylperosamine
wbcW	WbcW	370	45–55% identity to glycosyltransferases	Putative *N*-formylperosaminyltransferase
gnd	Gnd	390	85–98% identity to several Gnd proteins	Gluconate-6-phosphate dehydrogenase

colonies were obtained only when the fragments had been inserted into the vector in the correct orientation. This provided experimental evidence for the promoter activity upstream of the *galU*, *gmd*, and *wzm* genes.

Characterization of *Y. enterocolitica* O:9 *galF* and *galU* mutants. Two genes showing sequence similarity to the *galU* and *galF* genes were identified within the O:9 OPS gene cluster. The presence of these genes in the cluster was unexpected, as they both code for UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9; also called UDP-glucose pyrophosphorylase), which catalyzes the conversion of glucose-1-phosphate to UDP-glucose. UDP-glucose is a central nucleotide sugar in carbohydrate metabolism but redundant for the perosamine synthesis. We aimed to study the role of the *galU* and *galF* genes in the OPS gene cluster of *Y. enterocolitica* O:9. We thus inactivated the *galU* and *galF* genes and analyzed the LPS profiles and growth rates of the mutants. To inactivate the genes, suicide plasmids carrying the inactivated *galU* and *galF* genes were constructed and introduced into the *Y. enterocolitica* O:9 strains. No viable O:9 *galU* mutants were obtained. It suggested that there is only one copy of this gene in the chromosome and that it is essential for *Y. enterocolitica* O:9. On the other hand, we successfully constructed a *galF* mutant. The LPS profile of the *galF* mutant, analyzed by DOC-PAGE (Fig. 2), did not differ from that of the wild type. The growth of the mutant was not affected (data not shown).

Characterization of *Y. enterocolitica* O:9 *per* mutant. The *per* gene of the *Y. enterocolitica* serotype O:9 strain was inactivated by insertion of KmGB. The mutation was confirmed by Southern blotting, PCR, and sequencing. The LPS phenotype of the *per* mutant (YeO9-R1) was analyzed by DOC-PAGE, and as expected, the mutant did not express OPS (Fig. 2).

Polymyxin B resistance of *Y. enterocolitica* O:9 OPS and OC mutants. In several pathogens the LPS confers resistance to cationic antimicrobial peptides including polymyxin B (47, 67). We thus aimed to elucidate whether *Y. enterocolitica* O:9 LPS mediates resistance to polymyxin B. To this end we analyzed the polymyxin resistance phenotypes of the wild-type strain Ruokola/71 and OPS-negative (YeO9-R1), OC-negative

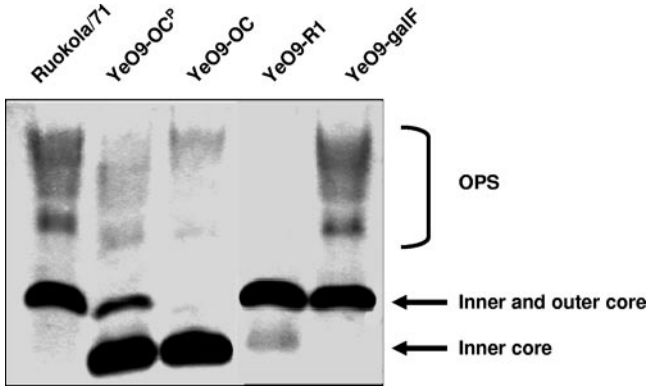

FIG. 2. DOC-PAGE analysis of the LPS of wild-type and mutant strains grown overnight at RT. Strains are indicated at the top, and the different parts of LPS are indicated at the right. The peculiar situation of the LPS of *Y. enterocolitica* O:9 is demonstrated here; the OC likely represents a nonpolymerized O unit attached to typical (inner) core oligosaccharide.

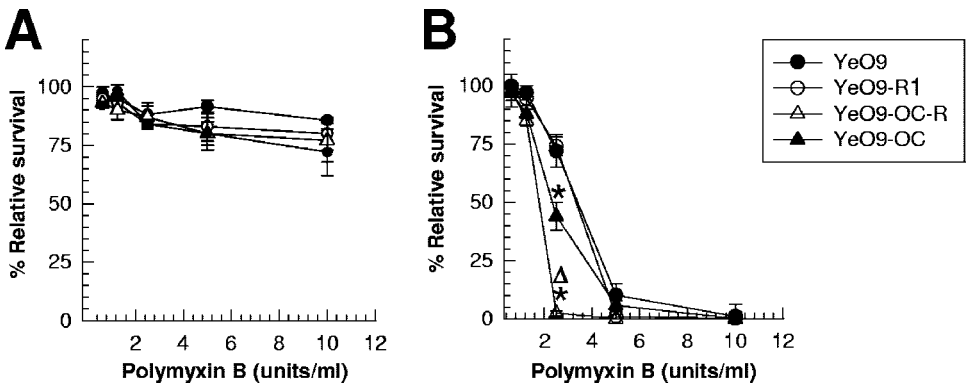

FIG. 3. Role of LPS phenotype in resistance of *Y. enterocolitica* O:9 to polymyxin B. Bacteria were grown either at RT (A) or at 37°C (B). *, $P < 0.05$, significant difference from wild-type strain; Δ, $P < 0.05$, significant difference from OC mutant.

(YeO9-OC), and OPS-negative and OC-negative (YeO9-OCR) strains in a survival assay. We also determined the IC_{50} values of the strains. In general, the resistance of the strains to polymyxin B was growth temperature dependent.

In the survival assay all strains grown at RT displayed high, wild-type-level resistance to polymyxin B (Fig. 3A). The IC_{50} values revealed, however, slight (less-than-twofold) differences between the RT-grown strains: the most sensitive was YeO9-OCR (IC_{50}, 50 ± 6 units/ml), followed by YeO9-OC (74 ± 7 units/ml), YeO9-R1 (84 ± 5 units/ml), and the wild-type strain

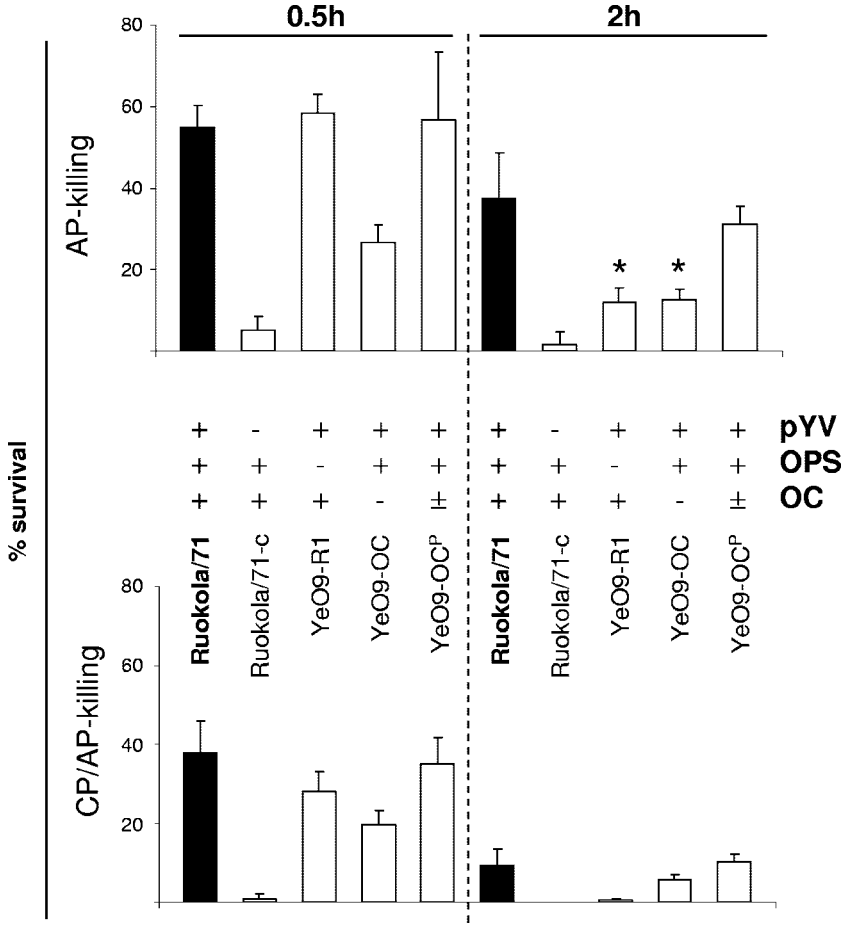

FIG. 4. Role of LPS phenotype in serum resistance of *Y. enterocolitica* O:9. Survival of bacteria in 66.7% NHS (CP/AP-killing bottom panel) and Mg-EGTA-treated serum (AP-killing, top panel) at 0.5- and 2-h time points. The columns indicate the mean survival percentage of the strain, and the bars indicate the ranges of standard errors. The filled columns show the results for the wild-type strain. In between the panels, the strains and their properties with respect to the presence of pYV (*Yersinia* virulence plasmid) and expression of OPS and OC are indicated. *, the AP killing results of YeO9-OC and YeO9-R1 at 2 h differed significantly ($P < 0.001$, Student's *t* test) from those of Ruokola/71.

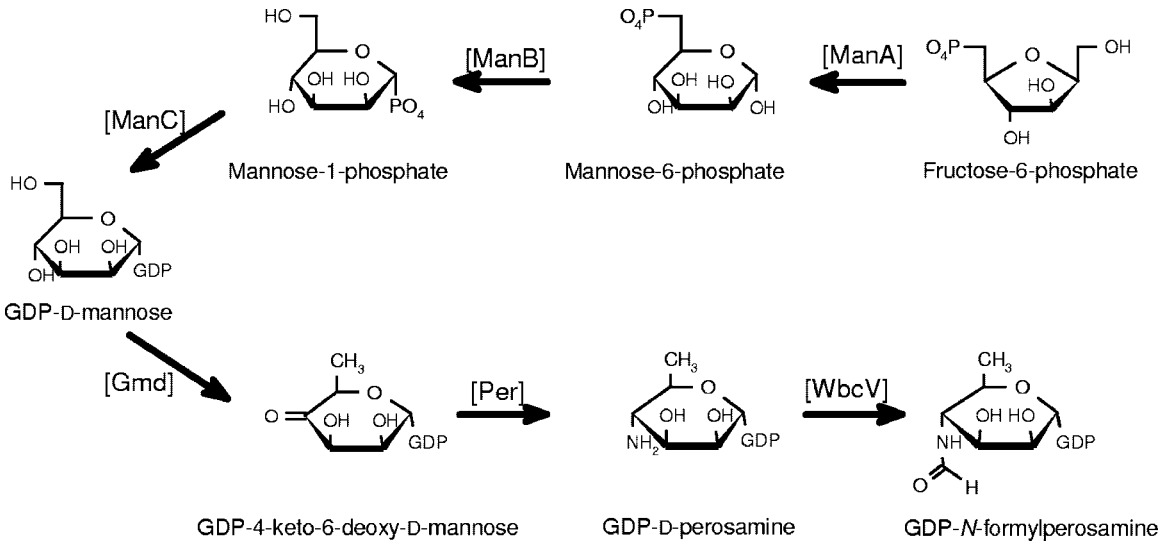

FIG. 5. Proposed biosynthesis pathway to GDP-*N*-formylperosamine in *Y. enterocolitica* O:9. The pathway up to GDP-4-keto-6-deoxy-D-mannose is identical to that of GDP-L-fucose biosynthesis; both Fcl (GDP-L-fucose synthetase) and Per (GDP-D-perosamine synthase) use this intermediate as a substrate.

(90 ± 9 units/ml). The value of YeO9-OCR was significantly different ($P < 0.05$) from that of YeO9-R1 and the wild-type strains. The other differences were not significant.

In contrast, the survival assay revealed that the strains grown at 37°C were more sensitive to polymyxin B (Fig. 3B) and that there were significant differences between the strains. The wild-type strain and YeO9-R1 displayed equal and highest resistance against polymyxin B. Significantly more sensitive were YeO9-OC and YeO9-OCR, the latter being the most sensitive (Fig. 3B). Accordingly, the IC_{50} values also demonstrated the same differences between the 37°C-grown bacteria: the most sensitive was YeO9-OCR (IC_{50}, 0.9 ± 0.6 units/ml), followed by YeO9-OC (2.1 ± 0.2 units/ml), YeO9-R1 (5.2 ± 0.4 units/ml), and the wild-type strain (5.6 ± 0.3 units/ml). The YeO9-OCR and YeO9-OC values differed significantly ($P < 0.05$) from those of YeO9-R1 and the wild type. On the other hand, the IC_{50} values of YeO9-R1 were not significantly different ($P > 0.05$) from those of the wild-type strain.

The role of *Y. enterocolitica* O:9 OPS and OC in serum resistance. To examine the role of *Y. enterocolitica* O:9 LPS in serum resistance, the wild-type strain Ruokola/71, the pYV-negative strain Ruokola/71-c, and the LPS mutants, missing the OPS (YeO9-R1) or the OC (YeO9-OC) or expressing a reduced amount of OC (YeO9-OC^P), were tested for the ability to resist alternative and classical complement pathway (AP and CP, respectively)-mediated killing (Table 1; Fig. 4).

In the bactericidal assay Ruokola/71-c was the most sensitive to both AP- and CP/AP-mediated killing, confirming the importance of pYV-encoded YadA (26) in protection of *Y. enterocolitica* O:9 against complement-mediated killing (Fig. 4). Both YeO9-OC and YeO9-R1 were less resistant to serum than the wild-type strain. They survived well; however, at the 0.5-h time point, YeO9-R1 was clearly more resistant than YeO9-OC. This suggested the role of OC in early resistance to complement (Fig. 4). At the 2-h time point the relationship was opposite and the YeO9-R1 mutant, especially in NHS, was more efficiently killed. Finally, YeO9-OC^P showed resistance intermediate between that of the wild-type strain and that of YeO9-OC at 2 h of incubation in both types of serum (Fig. 4). Thus, even partial reduction in OC expression (YeO9-OC^P, Fig. 2) resulted in reduced resistance to complement.

DISCUSSION

Cloning and sequencing of the *Y. enterocolitica* O:9 OPS gene cluster. In this work, we describe the cloning and characterization of the OPS gene cluster of *Y. enterocolitica* serotype O:9. The cluster directs the biosynthesis of the homopolymeric O:9 OPS composed of α1,2-linked *N*-formylperosamine (4,6-dideoxy-4-formamido-D-mannopyranose) (24). In *Yersinia* spp. expressing heteropolymeric OPSs the OPS gene cluster is located between the *hemH* and *gsk* genes (63, 65, 66). In *Y. enterocolitica* serotypes O:3 and O:9, both encoding homopolymeric OPSs, the genomic locus between *hemH* and *gsk* genes is occupied by the OC gene cluster (63). We identified the O:9 OPS gene cluster located upstream from the *gnd* gene, similar to the OPS gene clusters of *Salmonella* and *E. coli* (12, 59) (Fig. 1). The *gnd* gene encodes gluconate-6-phosphate dehydrogenase, an enzyme not involved in the OPS biosynthesis.

The O:9 gene cluster contains 12 genes. Based on sequence similarity and the predicted GDP-*N*-formylperosamine biosynthetic pathway (27, 33), *manB*, *manC*, *gmd*, *per*, and *wbcV* genes code for enzymes involved in the biosynthesis of GDP-*N*-formylperosamine, *wbcU* and *wbcW* code for *N*-formylperosaminyltransferases assembling the homopolymer onto undecaprenylphosphate carrier lipid, and *wzm* and *wzt* code for proteins for transporting the OPS homopolymer into periplasm. The putative pathway for the biosynthesis of the GDP-*N*-formylperosamine is given in Fig. 5. The WbcT protein is left without a function; therefore, it is likely that the functional assignments still need adjustments after biochemical evidence of the last steps of the pathway is found. The last step in the biosynthesis is shown to be

the transfer of the formyl group by WbcV. However, it is not certain that the transfer of the formyl group takes place at the nucleotide sugar level; it could also take place after polymerization of OPS. Related to this, there is evidence for only one of the sugar nucleotides containing a formamido group, i.e., UDP-4-deoxy-4-formamido-L-arabinose (18, 72). In *Y. enterocolitica* O:9 the pathway could be verified either by identification of GDP-*N*-formylperosamine directly from bacterial cells or by characterization of the formyltransferase activity of WbcV in vitro.

The organization of the *Y. enterocolitica* O:9 OPS gene cluster is identical to that of *B. melitensis* over the region of the *gmd*, *per*, *wzm*, and *wzt* genes and the 5′ end of *wbcT* genes (Fig. 1); the gene products are 50 to 70% identical. Apparently the region of homology has originally extended further to the putative *N*-formyltransferase-encoding genes *wbcV* and *wbkC*. In *Y. enterocolitica* O:9, however, a DNA fragment containing the 3′ end of the *wbcT* gene and the *wbcU* gene was inserted upstream from the *wbcV* gene (Fig. 1). Otherwise there is no similarity between the clusters of these two organisms. Nucleotide sequence differences in the perosamine synthase (*per*) genes of *Brucella* and *Y. enterocolitica* O:9 were used to develop real-time PCR methods for identification of these bacteria (15, 42).

The OPS gene cluster also contains genes similar to *galU* and *galF*. These genes are involved in the synthesis of UDP-glucose and have no clear role in the OPS biosynthesis. Their presence in the OPS gene cluster was thus surprising. On the other hand, in *Salmonella* and *E. coli* the OPS gene clusters are usually located between the *galF* and *gnd* genes (12, 59). Our results showed that the *galF* mutation does not affect either the OPS synthesis or the viability of the bacterium. The *galU* gene, however, is essential for *Y. enterocolitica* O:9. Interestingly, in some bacteria *galU* mutants have been successfully constructed and displayed reduced virulence and resistance to antimicrobial agents (25, 32, 52).

Localization of the promoters of the OPS gene cluster. Sequence analysis revealed several putative promoters in the OPS gene cluster, one upstream of the entire cluster, in front of *manC*, and three intergenic promoters inside the cluster in front of the *galU*, *gmd*, and *wzm* genes. Experimental data identified promoter activity upstream of the *galU*, *gmd* and *wzm* genes. The presence of an active promoter upstream of the *manC* gene is also very likely. The upstream region of the *manC* gene in *Y. enterocolitica* O:9 (Fig. 1) contains a 346-bp fragment more than 92% identical to the fragment containing the experimentally verified tandem promoters of the OPS gene cluster in *Y. enterocolitica* O:3 (74) (nucleotides 440 to 786, accession no AJ605741, versus nucleotides 1497 to 1844, accession no. Z18920, respectively). In fact, this is the only region of significant sequence similarity between the O:9 and O:3 OPS gene clusters. This region contains the JUMP start sequence (40) typical for all surface polysaccharide biosynthesis gene clusters and thus also present in all OPS gene clusters of *Yersinia* studied to date (63). Although more-detailed characterization of the promoters and regulation of transcription is warranted, our data indicate that the O:9 OPS gene cluster is organized into four transcriptional units.

Serum resistance. The serum killing experiments confirmed the importance of pYV-encoded YadA in serum resistance of *Y. enterocolitica* O:9 (8). In contrast to serotype O:3 (14), however, O:9 LPS appears to play a clear role in serum resistance. Both OPS and OC protected *Y. enterocolitica* O:9 against complement killing (Fig. 4). The OPS seemed to provide long-term resistance to complement-mediated killing while OC was clearly more important at the early time point. The importance of long OPS chains in serum resistance has been shown in several studies (19, 35, 43). Long OPSs protect *Salmonella* and *E. coli* strains against insertion of the membrane attack complex into the membrane (44, 45). In *Y. enterocolitica* O:9, however, both OPS and OC seem to assist and strengthen the mainly YadA-dependent resistance against complement-mediated killing. Further work is warranted to elucidate the molecular mechanisms of this resistance.

Polymyxin B resistance. The OPSs provide a steric hindrance for the access of antimicrobial peptides to inner LPS targets (9, 30, 47, 67). The serotype O:9 OPS, however, similarly to that of serotype O:3 (67), plays a minor role in the polymyxin B resistance. Moreover, the resistance is apparent only in the absence of the OC. On the other hand, as rough mutants of other bacterial species containing perosamine in their OPSs are more sensitive to antimicrobial peptides than the wild-type strains (49, 52, 71), the lack of contribution of serotype O:9 OPS to polymyxin B resistance is not likely due to its chemical composition. The available evidence, in fact, suggests that OPSs from yersiniae do not play a significant role in the resistance to antimicrobial peptides (11, 67; also unpublished data).

Temperature-dependent resistance to antimicrobial peptides, however, seems to be a common feature of yersiniae (5, 10, 56; also unpublished data). Even though both OPS expression and resistance to antimicrobial peptides in yersiniae are highest at RT (1, 11), our data suggest that OPS cannot account for this phenotype. We are currently investigating whether temperature-induced lipid A modifications affect the resistance to antimicrobial peptides.

Concluding remarks. Isogenic OPS and OC mutants displayed decreased resistance to human serum complement and polymyxin B compared to the wild-type O:9 strain. Interestingly, the serum resistance phenotype, but not the polymyxin B resistance phenotype, differs from that of analogous OPS and OC mutants of serotype O:3 (3, 67). This points to different biological functions of the OPS and OC in serotypes O:3 and O:9. It is very likely that the chemical nature of the OPS may dictate the biological role that it plays in virulence. Since serotype O:9 strains more frequently infect animals than do serotype O:3 strains, one could further speculate that the LPS structures also influence the host preferences of the serotypes. Further studies are needed to address these questions.

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