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Genetic Analysis of the O7-Polysaccharide Biosynthesis Region from the *Escherichia coli* O7:K1 Strain VW187

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We recently cloned biosynthesis genes for the O7-lipopolysaccharide (O7-LPS) side chain from the *Escherichia coli* K-1 strain VW187 (M. A. Valvano, and J. H. Crosa, Infect. Immun. 57:937–943, 1989). To characterize the O7-LPS region, the recombinant cosmids pJHCV31 and pJHCV32 were mutagenized by transposon mutagenesis with Tn*3*HoHo1, which carries a promoterless *lac* operon and can therefore generate *lacZ* transcriptional fusions with target DNA sequences. Cells containing mutated plasmids were examined for their ability to react by coagglutination with O7 antiserum. The LPS pattern profiles of the insertion mutants were also investigated by electrophoresis of cell envelope fractions, followed by silver staining and immunoblotting analysis. These experiments identified three phenotypic classes of mutants and defined a region in the cloned DNA of about 14 kilobase pairs that is essential for O7-LPS expression. Analysis of β-galactosidase production by cells carrying plasmids with transposon insertions indicated that transcription occurs in only one direction along the O7-LPS region. In vitro transcription-translation experiments revealed that the O7-LPS region encodes at least 16 polypeptides with molecular masses ranging from 20 to 48 kilodaltons. Also, the O7-LPS region in VW187 was mutagenized by homologous recombination with subsets of the cloned O7-LPS genes subcloned into a suicide plasmid vector. O7-LPS-deficient mutants of VW187 were complemented with pJHCV31 and pJHCV32, confirming that these cosmids contain genetic information that is essential for the expression of the O7 polysaccharide.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria (21, 24). Its most external moiety, the O side chain, varies considerably among bacterial species in terms of chemical composition, structure, and antigenicity (19, 24). The O-side-chain genes in *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. are clustered in the *rfb* region of the bacterial chromosome next to the histidine operon (21). In *Salmonella typhimurium* the *rfb* region was cloned, and specific genes were localized by reference to known deletion mutants (4, 5, 37). In the case of *S. dysenteriae* type I, a plasmid-mediated function is required in addition to chromosomal genes for the biosynthesis of the O-side-chain polysaccharide (30). *E. coli* strains containing O7-LPS are found in cases of septicemic infections in neonates (1, 36). The O7 antigen is a component of the virulence repertoire in these strains, since it confers resistance to the lytic activity mediated by serum complement (1). We recently reported the cloning and expression in *E. coli* K-12 of the O7-LPS region from the strain VW187, an *E. coli* O7:K1 clinical isolate (35). Our data showed that the amount of O7-LPS expressed in *E. coli* K-12 by $O7^+$ recombinant plasmids was considerably lower than that produced by the wild-type strain, VW187. In this article, we describe the genetic characterization by transposition mutagenesis of the cloned O7-LPS gene cluster. We also present evidence that the cloned DNA contains the essential information for the expression of the O7 polysaccharide, since O7-deficient mutants of strain VW187 generated by site-directed mutagenesis can be complemented with the $O7^+$ recombinant plasmids.

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MATERIALS AND METHODS

Bacterial strains, plasmids, materials, and general procedures. The characteristics of the *E. coli* strains and plasmids used in this study are described in Table 1. Chemicals and antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim, Dorval, Quebec, and used according to the conditions suggested by the supplier. The presence of the O7-LPS antigen was determined by coagglutination as described previously (17, 35). Sensitivity to the rough-specific bacteriophages Ffm, BR60, BR2, and C21 was used as an indication of the presence of rough (lipid A-core oligosaccharide) LPS forms (35, 38). Cells carrying plasmids with transposon insertions within the O7-LPS region were examined for sensitivity to deoxycholate and sodium dodecyl sulfate (SDS) as described by Coleman and Leive (8). In the case of transposon insertions generated with Tn*3*HoHo1, mutants were examined for β-galactosidase expression by the method of Putnam and Koch (27). The units of β-galactosidase were determined as described by Miller (22).

Transposition mutagenesis of the cloned O7-LPS gene cluster. Insertions of Tn*3*HoHo1 in pJHCV31 and pJHCV32 were isolated in strain C2110 as described previously (35). Plasmids containing transposon insertions were then transformed into strain DH5α for further analysis. The locations of the transposon insertions were determined by restriction endonuclease analysis with single and double digestions with *Hin*dIII, *Xba*I, *Sal*I, and *Bam*HI. Recognition sites for the former three restriction enzymes are not present within the transposon sequences (29; M. A. Valvano, unpublished data). In contrast, there is a single *Bam*HI recognition site within Tn*3*HoHo1, whereas no *Bam*HI sites are found along the cloned DNA and cloning vector sequences (29, 35).

Mutagenesis of strain VW187. Mutants in the O7-LPS *rfb*

TABLE 1. *E. coli* strains, plasmids, and bacteriophages used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
E. coli		
DH5α	*E. coli* K-12 F⁻ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories
SM10 λ *pir*	*E. coli* K-12, λ *pir thi-1 thr-1 leuB6 supE44 tonA21 lacY1* *recA*::RP4-2-Tc::Mu Km^r	23
SY327 λ *pir*	*E. coli* K-12, λ *pir* F⁻ *araD* Δ(*lac pro*) *argE*(Am) *recA56* Rif^r *nalA*	23
C2110	*E. coli* C, *gyrA polA1 rha his*	18
VW187	*E. coli* O7:K1:H?, prototrophic	33
MV103	VW187, *gyrA*	34
MV121	MV103, pMAV001 inserted in the chromosome, Ap^r	This work
MV122	MV103, pMAV002 inserted in the chromosome, Ap^r	This work
MV123	MV103, pMAV003 inserted in the chromosome, Ap^r	This work
MV124	MV103, pMAV004 inserted in the chromosome, Ap^r	This work
MV126	MV103, pMAV006 inserted in the chromosome, Ap^r	This work
MV128	MV103, pMAV008 inserted in the chromosome, Ap^r	This work
MV129	MV103, pMAV009 inserted in the chromosome, Ap^r	This work
MV1211	MV103, pMAV011 inserted in the chromosome, Ap^r	This work
Plasmids		
pACYC184	Cloning vector, Cm^r Tc^r	6
pGP704	Cloning vector, *oriR6K* Ap^r *mobRP4*	23, J. Mekalanos
pHP45Ω	Cloning vector, Spc^r/Sm^r Ap^r	26
pJHCV31	*Hin*dIII cosmid clone in pVK102, O7$^+$ Tc^r	35
pJHCV32	*Hin*dIII cosmid clone in pVK102, O7$^+$ Tc^r	35
pJHCV64	14-kb *Bst*EII fragment of pJHCV32 cloned into pHP45Ω, O7$^+$	35
pCMV10	8.1-kb *Hin*dIII fragment J of pJHCV31 cloned into pACYC184, Cm^r O7$^-$	This work
pCMV21	8.5-kb *Cla*I fragment of pJHCV31 cloned into pACYC184, Cm^r O7$^-$	This work
pMAV001	1.8-kb *Eco*RI fragment of pJHCV31 cloned into pGP704, Ap^r	This work
pMAV002	3.4-kb *Eco*RI fragment of pJHCV32 cloned into pGP704, Ap^r	This work
pMAV003	1.2-kb *Eco*RI fragment of pJHCV31 cloned into pGP704, Ap^r	This work
pMAV004	2.2-kb *Eco*RI fragment of pJHCV31 cloned into pGP704, Ap^r	This work
pMAV006	1.9-kb *Eco*RV fragment of pJHCV32 cloned into pGP704, Ap^r	This work
pMAV008	1.4-kb *Eco*RV fragment of pJHCV32 cloned into pGP704, Ap^r	This work
pMAV009	4.0-kb *Xho*I fragment of pJHCV31 cloned into pGP704, Ap^r	This work
pMAV011	4.0-kb *Hin*dIII-*Pvu*I fragment of pJHCV31 cloned into pGP704, Ap^r	This work

[a] The boundaries of DNA inserts from plasmids of the pMAV series and the locations of the plasmid insertions in the bacterial chromosome are shown in Fig. 3. *mob*, Bases of mobilization.

genes of VW187 were generated by homologous recombination with subsets of the cloned O7-LPS genes subcloned into the suicide mobilizable vector pGP704 as described by Miller and Mekalanos (23). This plasmid vector cannot replicate unless the replication protein is supplied in *trans* by a gene cloned into a lambda bacteriophage that is integrated in the bacterial chromosome (16). Thus, upon mobilization from strain SM10 λ *pir* to MV103, a Nal^r derivative of VW187, the recombinant plasmids integrated into the chromosome due to the homology between insert DNA and chromosomal sequences. The sites of integration were confirmed by DNA hybridization experiments (see below). Mutant strains were examined by coagglutination, sensitivity to rough bacteriophages, and LPS pattern profiles. Some of these mutants were also complemented with plasmids pJHCV31 and pJHCV32, which were mobilized by triparental matings carried out as previously described (35).

LPS analysis. LPS was extracted from cell envelopes by using a modification of current procedures (14, 32). Cell envelopes obtained from 20-ml cultures were prepared as described previously (35), suspended in 50 μl of a solution containing 10 mM Tris hydrochloride (pH 8.0) and 2% SDS, and boiled for 10 min. Proteinase K was added to a final concentration of 800 μg/ml, and the samples were incubated at 60°C for 1 h. Proteins were extracted with an equal volume of 90% phenol at 65°C for 15 min. The aqueous phase remaining after centrifugation was extracted once with 10 volumes of ethyl ether to remove traces of phenol, mixed in a 1:1 ratio with a tracking dye solution (125 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% [vol/vol] glycerol, 0.002% bromophenol blue, 10% mercaptoethanol), and boiled for 5 min before the gels were loaded. Gels (14% polyacrylamide; ratio acrylamide to bisacrylamide, 44:0.8) containing 4 M urea were eletrophoresed at 20 mA constant current (32). Immunoblot analysis was carried out as previously described (31, 35).

Recombinant DNA methods. Small- and intermediate-scale purifications of plasmid DNA (from 5- and 30-ml cultures, respectively) were carried out by the alkaline lysis method of Birnboim and Doly (2) with the following modifications: (i) the 3 M sodium acetate solution was replaced by 7.5 M ammonium acetate, (ii) ethanol precipitation was replaced by a precipitation with 0.54 volume of isopropanol at room temperature for 10 min, and (iii) the DNA pellets were washed three times with 1 ml of 70% ethanol. When needed, large-scale purifications of plasmid DNA (from 800-ml cultures) were carried out as described by Goto et al. (10). Genomic DNA was obtained by the miniscale procedure of Owen and Borman (25). Electrophoresis of plasmid DNA was performed as previously described (33). Transformations were carried out by the calcium chloride method (7), except that cells were grown in Luria broth containing 10 mM $MgCl_2$.

Hybridizations. Hybridization experiments were carried

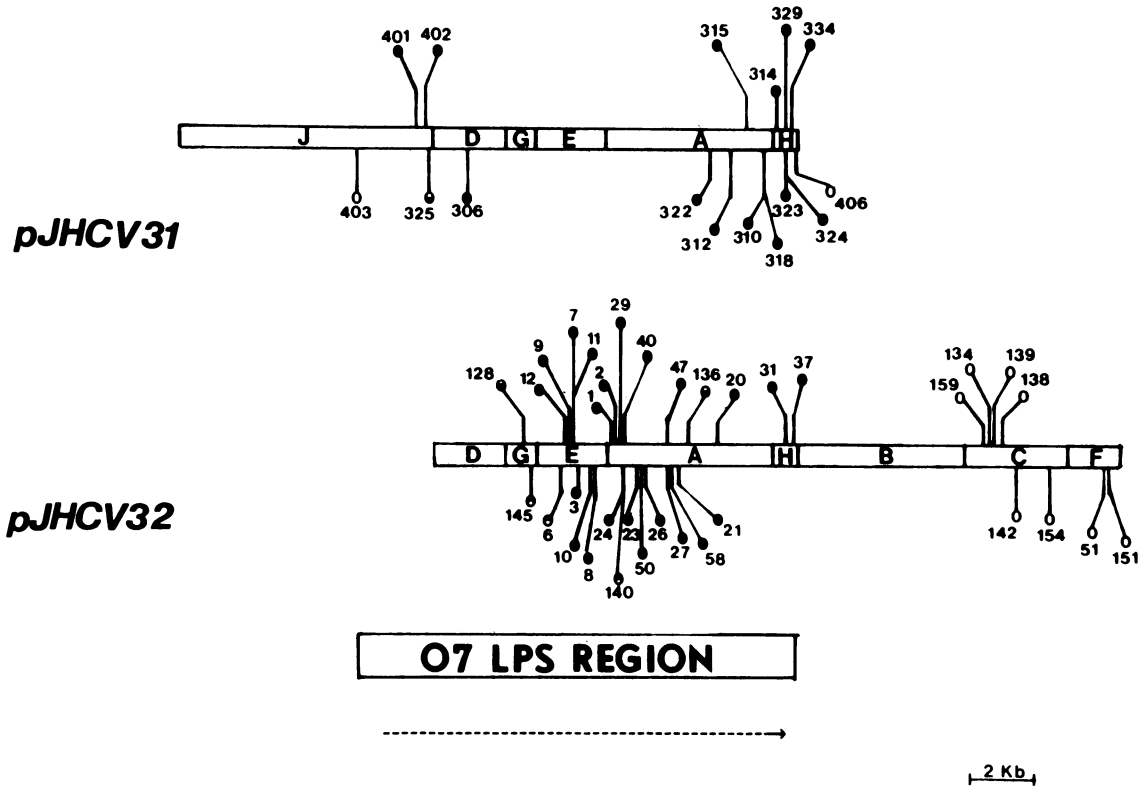

FIG. 1. Localization map of Tn*3*HoHo1 transposon mutants in pJHCV31 and pJHCV32. Letters J, D, G, E, A, H, B, C, and F indicate the *Hin*dIII restriction endonuclease fragments. Vector DNA sequences are not shown. Vertical lines placed above the maps of the plasmids indicate the locations of the transposon insertions in which Tn*3*HoHo1 inserted in the L orientation as described in Results. Vertical lines below the maps correspond to insertions in the R orientation. Symbols: ●, insertions causing class I mutants; ◓, insertions generating either class II or class III mutants; ○, insertions that do not cause any detectable changes in the phenotypes expressed by pJHCV31 and/or pJHCV32. The O7-LPS region denotes the boundaries of a 14-kb region containing the O7-specific genes. The dotted arrow indicates the predicted direction of transcription of mRNA along the O7-LPS region based on the production of β-galactosidase by the insertions placed above the maps of the plasmids.

out to confirm the location of plasmid insertions into the chromosomal O7-LPS region. The boundaries of the probe DNA fragments are shown in Fig. 3. DNA fragments were recovered by electrophoresis into a strip of DE-81 filter paper (Whatman, Inc., Clifton, N.J.) as previously described (35) and labeled with [^{32}P]ATP (Amersham Canada Ltd., Oakville, Ontario) by oligonucleotide synthesis (9). Southern blot hybridizations were carried out as described previously (33, 36).

Analysis of polypeptide gene products. The expression in vivo of polypeptides encoded by pJHCV64, pCMV10, and pCMV21, high-copy-number recombinant plasmids spanning the O7-LPS region (Table 1; see Fig. 3), were examined by the maxicell method (3). DH5α(pJHCV64), DH5α (pCMV10), and DH5α(pCMV21) were treated exactly as described by Boulnois and Timmis (3) and labeled with [^{35}S]methionine (Tran^{35}S-label; ICN Biomedicals Canada, St. Laurent, Quebec). DH5α(pHP45Ω), DH5α(pACYC184), and DH5α cells with no plasmids were used as controls. Also, purified plasmid DNAs were used as templates for in vitro transcription-translation with a commercial kit available from Amersham. For these experiments, [^{35}S] methionine was used as recommended by the supplier. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis followed by treatment with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.). Dried gels were exposed to a Kodak X-Omat film at −110°C for 24 to 48 h.

RESULTS

Analysis of the insertion mutations in the O7-LPS region of pJHCV31 and pJHCV32. Tn*3*HoHo1 transposon insertions in plasmids pJHCV31 and pJHCV32 were obtained and mapped as described in Materials and Methods. The mapping strategy not only localized the insertions to a given restriction endonuclease fragment but also made it possible to establish the orientation of the transposon insertion with respect to the target DNA sequences. A map of transposon insertions resulting from these experiments is shown in Fig. 1.

The distribution of Tn*3*HoHo1 insertions along the target DNA was not completely at random, a result expected from a transposon derived from the Tn*3* family (12). Most of the insertions in pJHCV32 were located within a region of about 12 kilobase pairs (kb) between *Hin*dIII fragments G and H and in fragments C and F (Fig. 1). *Hin*dIII fragments B and D did not receive any insertion, although over 300 insertion mutants were screened. This suggests that either the insertions in these regions determine a phenotype that is lethal for bacterial cells or, alternatively, transposition events within these DNA sequences occur at a very low frequency. In the case of pJHCV31, most of the insertions were located in fragments J, A, and H (Fig. 1). Approximately 30% of the insertions in pJHCV31 gave rise to deleted plasmids lacking fragments J, D, and G or only J and D (data not shown). The deletion plasmids were not further studied.

TABLE 2. Phenotypic characterization of transposon mutants in pJHCV31 and pJHCV32

Plasmid[a]	Location of insertion[b]	Coagglutination[c]	Class of mutant[d]	β-galactosidase (U)[e]
pJHCV31::Tn*3*HoHo1-403R	J	+	Wild type	12 (−)[f]
pJHCV31::Tn*3*HoHo1-401L	J	−	I	1,549 (+)
pJHCV31::Tn*3*HoHo1-402L	J	−	I	0 (−)
pJHCV31::Tn*3*HoHo1-325R	J	±	III	0 (−)
pJHCV31::Tn*3*HoHo1-306R	D	−	I	3 (−)
pJHCV31::Tn*3*HoHo1-322R	A	−	I	32 (−)
pJHCV31::Tn*3*HoHo1-312R	A	−	I	27 (−)
pJHCV31::Tn*3*HoHo1-315L	A	−	I	325 (+)
pJHCV31::Tn*3*HoHo1-310R	A	−	I	0 (−)
pJHCV31::Tn*3*HoHo1-318R	A	−	I	ND (−)
pJHCV31::Tn*3*HoHo1-314L	H	−	I	39 (−)
pJHCV31::Tn*3*HoHo1-323R	H	−	I	4 (−)
pJHCV31::Tn*3*HoHo1-324R	H	−	I	22 (−)
pJHCV31::Tn*3*HoHo1-329L	H	−	I	883 (+)
pJHCV31::Tn*3*HoHo1-334L	H	−	I	ND (−)
pJHCV31::Tn*3*HoHo1-406R	H	+	Wild type	72 (−)
pJHCV32::Tn*3*HoHo1-128L	G	±	III	13 (−)
pJHCV32::Tn*3*HoHo1-145R	G	±	III	25 (−)
pJHCV32::Tn*3*HoHo1-6R	E	±	II	25 (−)
pJHCV32::Tn*3*HoHo1-12L	E	−	I	203 (+)
pJHCV32::Tn*3*HoHo1-9L	E	−	I	127 (+)
pJHCV32::Tn*3*HoHo1-7L	E	−	I	88 (+)
pJHCV32::Tn*3*HoHo1-11L	E	−	I	109 (+)
pJHCV32::Tn*3*HoHo1-3R	E	−	I	30 (−)
pJHCV32::Tn*3*HoHo1-10R	E	−	I	31 (−)
pJHCV32::Tn*3*HoHo1-8R	E	−	I	41 (−)
pJHCV32::Tn*3*HoHo1-1L	A	−	I	45 (−)
pJHCV32::Tn*3*HoHo1-2L	A	−	I	23 (−)
pJHCV32::Tn*3*HoHo1-29L	A	−	I	40 (−)
pJHCV32::Tn*3*HoHo1-24R	A	−	I	8 (−)
pJHCV32::Tn*3*HoHo1-140R	A	±	II	40 (−)
pJHCV32::Tn*3*HoHo1-40L	A	−	I	0 (−)
pJHCV32::Tn*3*HoHo1-23R	A	−	I	10 (−)
pJHCV32::Tn*3*HoHo1-50R	A	−	I	0 (−)
pJHCV32::Tn*3*HoHo1-26R	A	−	I	32 (−)
pJHCV32::Tn*3*HoHo1-27R	A	−	I	ND (−)
pJHCV32::Tn*3*HoHo1-47L	A	−	I	0 (−)
pJHCV32::Tn*3*HoHo1-58R	A	−	I	0 (−)
pJHCV32::Tn*3*HoHo1-21R	A	−	I	12 (−)
pJHCV32::Tn*3*HoHo1-136L	A	±	II	128 (+)
pJHCV32::Tn*3*HoHo1-20L	A	−	I	78 (+)
pJHCV32::Tn*3*HoHo1-31L	H	−	I	45 (−)
pJHCV32::Tn*3*HoHo1-37L	H	−	I	40 (−)
pJHCV32::Tn*3*HoHo1-159L	C	+	Wild type	0 (−)
pJHCV32::Tn*3*HoHo1-134L	C	+	Wild type[g]	0 (−)
pJHCV32::Tn*3*HoHo1-139L	C	+	Wild type	11 (−)
pJHCV32::Tn*3*HoHo1-138L	C	+	Wild type	11 (−)
pJHCV32::Tn*3*HoHo1-142R	C	+	Wild type	37 (−)
pJHCV32::Tn*3*HoHo1-154R	C	+	Wild type	12 (−)
pJHCV32::Tn*3*HoHo1-51R	F	+	Wild type	0 (−)
pJHCV32::Tn*3*HoHo1-151R	F	+	Wild type	8 (−)

[a] The number at the end of the plasmid designation has been used in Fig. 1 to identify the insertions. L indicates that the left end of Tn*3*HoHo1 (containing the *lac* genes) is located to the left end of the map as shown in Fig. 1. R indicate that the left end of the transposon is located to the right end of the map.

[b] Letters refer to the *Hin*dIII fragments of pJHCV31 and pJHCV32 as shown in Fig. 1.

[c] +, Positive result comparable to that of DH5α cells containing either pJHCV31 or pJHCV32; −, negative coagglutination; ±, weakly positive coagglutination.

[d] $O7^+$ phenotypes of DH5α cells containing each of the plasmids with Tn*3*HoHo1 insertions were assigned as described in the text and in Fig. 2 based on the result of silver-stained polyacrylamide gels and immunoblots.

[e] Production of β-galactosidase was determined by the lysis method of Putnam and Koch (27) and units calculated according to Miller (22). Values equal or lower than 30 were considered background activity. ND, Not done.

[f] (+) and (−) indicate the *lac* phenotype mediated by the transposon insertions as determined by plating onto MacConkey agar plates.

[g] Insertion 134 determined a class II mutant in the *E. coli* C2110 cell background.

The phenotypes encoded by the mutated plasmids in DH5α cells were initially investigated by coagglutination (Table 2). Insertions conferring a negative coagglutination were located in *Hin*dIII fragments J, D, E, A, and H. One insertion in fragment J, two insertions in fragment G, one insertion in fragment E, and two insertions in fragment A determined a weak positive coagglutination. One insertion in fragment J, one insertion in fragment H, and all of the insertions in fragments C and F gave positive coagglutinations comparable with those obtained with DH5α cells containing either pJHCV31 or pJHCV32. These experiments demonstrated that a 2.5-kb portion of fragment J contiguous to fragment D (Fig. 1) is needed for LPS biosynthesis. Therefore, the O7-LPS region spans a contiguous DNA

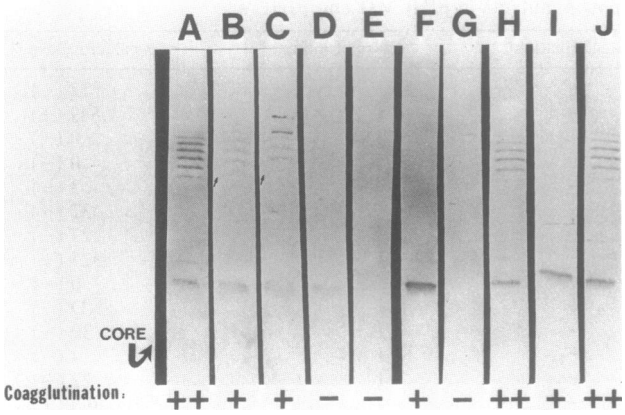

FIG. 2. Immunoblot analysis of DH5α cells containing pJHCV31 and/or pJHCV32 derivatives with insertion mutants. Cell envelope fractions extracted with hot phenol as described in Materials and Methods were separated by SDS-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (35). O7-specific LPS bands were detected with O7 rabbit polyclonal antiserum as described (35). Lanes: A and H, wild-type O7-LPS determined by pJHCV31 and pJHCV31::Tn*3*HoHo1-406R, respectively; B and C, class III mutants determined by pJHCV31::Tn*3*HoHo1-325R and pJHCV32::Tn*3*HoHo1-128L, respectively; D and F, class II mutants determined by pJHCV32::Tn*3*HoHo1-140R and pJHCV32::Tn*3*HoHo1-136L, respectively; E and G, class I mutants determined by pJHCV31::Tn*3*HoHo1-306R and pJHCV32::Tn*3* HoHo1-31L, respectively; I, class II mutant determined by pJHCV32::Tn*3*HoHo1-134L in strain C2110; J, O7-LPS determined by pJHCV32::Tn*3*HoHo1-134 in DH5α. The assignment of classes of mutants is described in the text. Small arrows in lanes B and C denote the O7-specific LPS bands, which are barely visible. Prominent bands in lane C correspond to the presence of residual outer membrane proteins in this preparation that cross-react with the O7 antiserum. CORE indicates the position in the gel of lipid A core molecules, which do not react with the O7-specific antiserum. The coagglutination results are indicated as follows: ++, positive; +, weakly positive; and −, no coagglutination.

segment of about 14 kb comprising *Hin*dIII fragments J, D, G, E, A, and H (Fig. 1). The limits of this region are defined by insertions 403 and 406, occurring in fragments J and H, respectively (Fig. 1).

Further characterization of the insertion mutants within this region was achieved by silver-stained SDS-polyacrylamide gel electrophoresis and immunoblotting experiments (Fig. 2 and Table 2). Phenotypically, three classes of O7-LPS mutants were identified: (i) class I mutants, with no O7-specific LPS; (ii) class II mutants, showing a single O7-reacting LPS band with a molecular weight comparable to that of the lipid A core plus one O7 polysaccharide subunit in the wild type; and (iii) class III mutants, expressing multiple O7-specific LPS bands in very low amounts. Insertions in *Hin*dIII fragments C and F as well as insertion 403 in fragment J and insertion 406 in fragment H encoded an LPS banding pattern identical to that mediated by the control plasmids pJHCV31 and pJHCV32 (Fig. 2, lane H). However, the insertion 134 located in *Hin*dIII fragment C determined a class II mutant when pJHCV32::Tn*3*HoHo1-134 was present in strain C2110, a *polA* derivative of *E. coli* C used for the initial selection of the transposon mutants (Fig. 2, lane I) (35). In contrast, this insertion determined an LPS banding pattern comparable to that of pJHCV32 when examined in the *E. coli* K-12 strain DH5α cell background (Fig. 2, lane J). Insertion 134 was the only insertion mutant which differed in the phenotype expressed depending on the cell background. This suggests that a function provided by the *E. coli* K-12 host restores the defect created by the insertion mutant. No information was gained on the participation in the O7-LPS expression of sequences from the *Hin*dIII fragment B of pJHCV32, since no transposon insertions in this fragment were found. A site-directed mutagenesis strategy was utilized to establish whether this region plays any role in O7-LPS expression (see below).

Since Tn*3*HoHo1 carries a promoterless lactose operon located very close to one of the transposon ends, it can generate gene fusions under the control of promoter sequences located upstream from the insertion (29). In these cases, production of β-galactosidase serves as an indicator of the direction of transcription. Tn*3*HoHo1 can insert into sequences in either of two orientations with regard to its *lac* coding sequences. The letter L was assigned to all of the insertions with the left end of Tn*3*HoHo1 (containing the *lac* genes) located to the left end of the map shown in Fig. 1. Conversely, the letter R was given to insertions where the left end of the transposon is located to the right end of the map in Fig. 1. All of the Tn*3*HoHo1 insertions in pJHCV31 and pJHCV32 were screened for production of β-galactosidase. The results demonstrated that the insertions giving a significant amount of β-galactosidase were all located in the L orientation with respect to target DNA sequences in both pJHCV31 and pJHCV32 (Fig. 1 and Table 2). These insertions expressed β-galactosidase levels that were 3- to 100-fold higher than the background activity of the enzyme, indicating that transcription occurs from fragments J through H. Furthermore, insertions in pJHCV31 expressed approximately 5 to 6 times more β-galactosidase than those in pJHCV32.

It has been suggested by a number of investigators (21) that mutations in glycosyltransferases can compromise the integrity of the bacterial outer membrane and therefore these mutants show an increased sensitivity to dyes and detergents. Transposon insertions in plasmids pJHCV31 and pJHCV32 did not determine any significant changes in the sensitivity of DH5α cells to sodium deoxycholate and SDS as compared with controls containing either pJHCV31 and pJHCV32 or just DH5α cells with no plasmid (data not shown).

Site-directed mutagenesis in the wild-type strain VW187. We previously reported that the O7-LPS expression mediated by pJHCV31 and pJHCV32 in *E. coli* K-12 cells is very low when compared with wild-type levels of O7-LPS produced by the strain VW187 (35). Thus, to determine whether the genetic region defined by transposition mutagenesis in the cloned DNA correlates with the wild-type DNA chromosomal sequences, we constructed O7-deficient derivatives of the wild-type strain VW187. Site-directed mutations were introduced in the wild-type O7-LPS region by using the suicide plasmid vector pGP704 as described in Materials and Methods. Restriction endonuclease fragments from pJHCV31 or pJHCV32 (Fig. 3) were cloned in pGP704 and mobilized by conjugation to MV103, a nalidixic acid-resistant derivative of VW187. Since pGP704 and its derivatives cannot replicate unless the replication protein is provided in *trans* (23), these experiments resulted in the stable integration of the recombinant plasmids (vector and insert DNA) by homologous recombination between the cloned sequences and chromosomal genes. As a result of the integration the cloned region is then duplicated (23). Since VW187 possesses various indigenous plasmids, control experiments were done to confirm that insertion of suicide clones did not occur in any of these plasmids. Plasmid DNA preparations

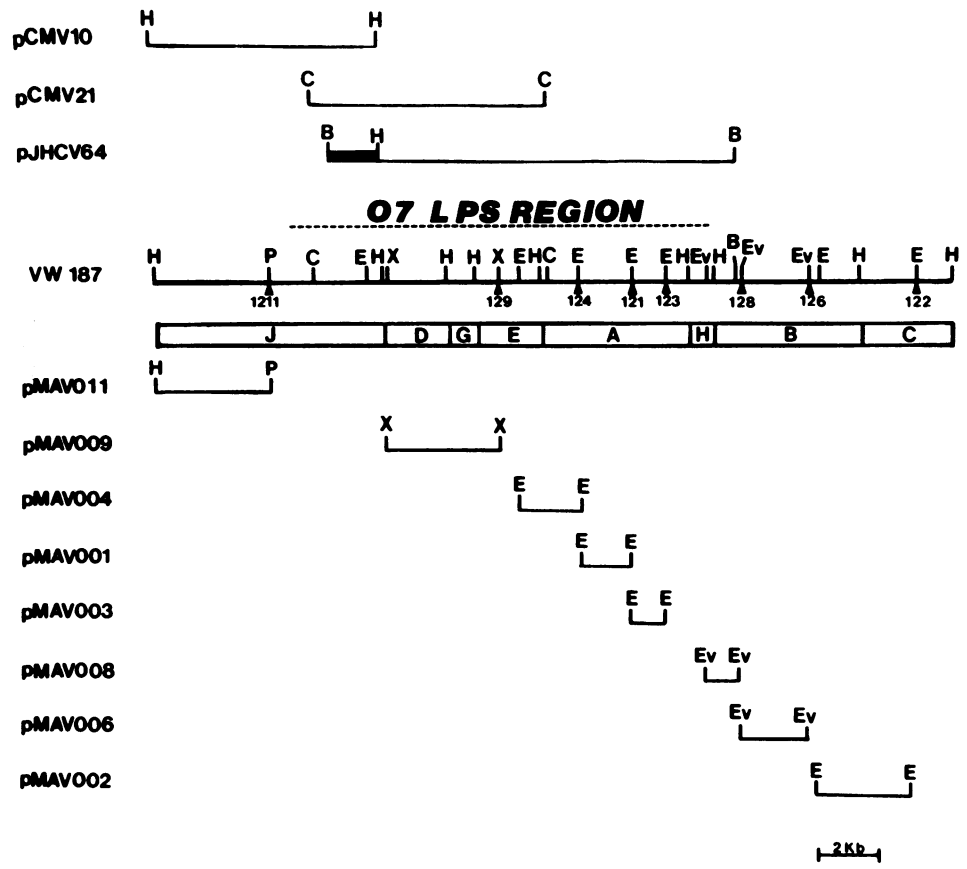

FIG. 3. Physical map of the chromosomal O7-LPS region containing insertions of suicide plasmids carrying subsets of the cloned O7-LPS genes. The dotted line indicates the O7-LPS region as defined by transposition mutagenesis of the cloned O7-LPS genes. Triangles (▲) denote the locations of the plasmid insertions in the chromosomal DNA. Numbers refer to the strain designation as shown in Tables 1 and 3. Rectangles with letters J, D, G, E, A, H, B, and C indicate the *Hin*dIII restriction endonuclease fragments shown in Fig. 1. A partial physical map of the O7-LPS region is shown: H, *Hin*dIII; P, *Pvu*I; C, *Cla*I; E, *Eco*RI; X, *Xho*I; Ev, *Eco*RV; B, *Bst*EII. Only the boundaries of insert DNA of the various subclones are shown. The bar (▬) in pJHCV64 denotes the 1.8-kb *Bst*EII-*Hin*dIII fragment containing sequences derived from the cosmid vector pVK102 (35). DNA inserts of the plasmids beneath the VW187 map were used as ^{32}P-radiolabeled probes to confirm the location of the insertions in the chromosomes of the respective mutant strains.

obtained from mutant strains and analyzed by agarose gel electrophoresis did not show any changes in plasmid pattern profiles as compared with that of strain VW187. Also, transformation experiments into the strain SY327 λ *pir* with the same plasmid preparations did not yield any Ap^r colonies, whereas pGP704 transformed this strain efficiently. Therefore, we concluded that the recombinant suicide plasmids were, indeed, integrated in the chromosome of strain VW187. The correct location of the plasmid insertions into the chromosomal DNA was verified by hybridization experiments with genomic DNA digested with restriction enzymes cleaving outside the regions spanned by the various probe DNAs (Fig. 3). These experiments demonstrated the absence of the wild-type DNA fragment in the region with the integrated plasmid (data not shown). Plasmid insertions 1211, 126 and 122 did not affect the expression of O7-LPS as determined by silver staining and immunoblots with O7-specific antibodies (Fig. 4, lanes B, G, and I, respectively). Also these strains were resistant to the lysis by rough-specific bacteriophages (Table 3). Plasmid insertion 1211 was located very close to the site of the transposon insertion 403 in pJHCV31 (Fig. 1 and 3). Plasmid insertions 126 and 122 were located in *Hin*dIII fragments B and C, respectively. These results are consistent with those obtained with transposon insertions in either pJHCV31 or pJHCV32 which do not cause any detectable change in the O7-LPS expression (Fig. 1 and Table 2), and therefore we conclude that these regions are likely not involved in O7-LPS biosynthesis. In contrast, plasmid insertions 129, 124, 123, and 121 lay on regions that are important for O7-LPS biosynthesis (Fig. 3) and, consequently, were sensitive to the lysis by rough-specific bacteriophages (Table 3). O7-deficient mutants of classes I and III were also found in these cases (Fig. 4, lanes C, D, E, and F). The results are then in agreement with the boundaries of the O7-LPS region defined by transposition mutagenesis in the cloned DNA (Fig. 1). However, the insertion of plasmid pMAV008 (insertion 128) located within *Hin*dIII fragment B caused an O7-deficient class I mutant phenotype (Fig. 3, Fig. 4, lanes H; Table 3). Thus, although Tn*3*HoHo1 transposon insertions in fragment B of pJHCV32 were not found (Fig. 1), the results obtained in VW187 with the plasmid insertion 128 demonstrate that at least part of the sequences from this fragment encode essential functions for O7-LPS biosynthesis.

The construction of O7-deficient mutants also served to demonstrate whether plasmids pJHCV31 and pJHCV32 can restore the wild-type O7$^+$ phenotype. For this purpose these two plasmids were mobilized by triparental matings into the

TABLE 3. Lysis by rough-specific bacteriophages

Strain	Chromosomal insertion[a]	Coagglutination[b]	Sensitivity to bacteriophage[c]			
			Ffm	BR2	BR60	C21
VW187	None	+	R	R	R	R
MV1211	pMAV011	+	R	R	R	R
MV129	pMAV009	−	S	S	S	S
MV124	pMAV004	±	S	S	S	S
MV123	pMAV003	±	S	S	S	S
MV121	pMAV001	±	S	S	S	S
MV128	pMAV008	−	S	S	S	S
MV126	pMAV006	+	R	R	R	R
MV122	pMAV002	+	R	R	R	R

[a] Suicide plasmids of the pMAV series are integrated by homologous recombination at various places in the chromosome of strain VW187 (see the text and Fig. 3 for details).

[b] +, Positive; ±, weakly positive; −, negative.

[c] Sensitivity to rough-specific bacteriophages was tested as previously described (35); S, sensitive to lysis; R, resistant to lysis.

O7-deficient strains MV121 and MV124 containing insertions of plasmids pMAV001 and pMAV004, respectively (Fig 3; Fig. 4, lanes D and F). These experiments resulted in restoration of the expression of O7-LPS to wild-type levels, and the mutants became resistant to the lysis by rough-specific bacteriophages (Fig. 5, lanes A through D; data not shown). Thus, it was demonstrated that both pJHCV31 and pJHCV32 must encode essential information for the O7 polysaccharide biosynthesis.

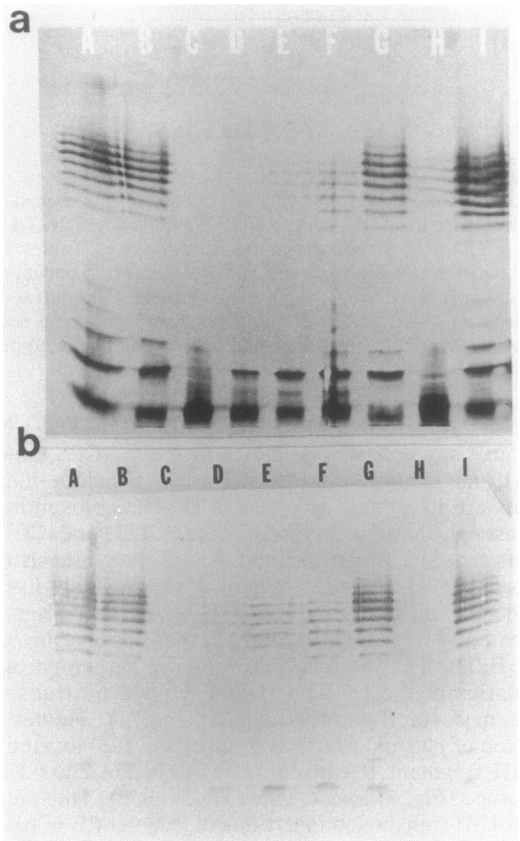

FIG. 4. Phenotypic analysis of O7-deficient mutants of strain VW187. (a) Silver-stained polyacrylamide gel showing the phenotypes resulting from the insertion by homologous recombination of various suicide plasmids in the chromosomal O7-LPS region (see Fig. 3 for the location of the insertions). Lanes: A, VW187; B, MV1211; C, MV129; D, MV124; E, MV123; F, MV121; G, MV126; H, MV128; I, MV122. (b) Immunoblot of a gel similar to that in panel a but treated with the O7-specific antiserum.

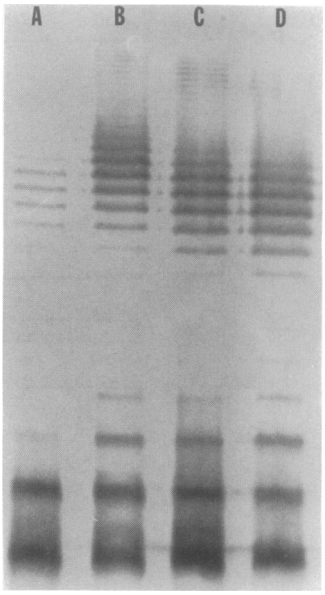

FIG. 5. Complementation of an O7-deficient mutant derived from strain VW187 with pJHCV31 and pJHCV32. Silver-stained polyacrylamide gel showing the LPS banding pattern of the following strains (lanes): A, MV121; B, MV121(pJHCV31); C, MV121 (pJHCV32); D, VW187.

Polypeptide analysis. Since the 14-kb region defined by transposition mutagenesis in pJHCV31 and pJHCV32 contains genetic information for O7-LPS biosynthesis, experiments were attempted to determine the number and species of polypeptide gene products encoded by this region. For this purpose we utilized the high-copy-number plasmid pJHCV64, a subclone of pJHCV32 that contains a 14-kb *Bst*EII fragment from which 12.3 kb correspond to the O7-LPS region (35). pJHCV64 confers a weak but consistent expression of O7-LPS and encompasses most of the O7-LPS region delineated by transposition mutagenesis except for the sequences contained in fragment J (Fig. 3) (35). To examine the contribution of these sequences, plasmids pCMV10 and pCMV21 were also utilized. pCMV10 contains the entire 8.1-kb *Hin*dIII fragment J, whereas pCMV21 contains a 8.5-kb *Cla*I fragment comprising part of the *Hin*dIII fragment J as well as sequences from fragments D, G, E, and A that are common to pJHCV31 and pJHCV32 (Fig. 1 and 3). Radiolabeled polypeptides encoded by pJHCV64, pCMV10, and pCMV21 were detected by the use of maxicells and also by in vitro transcription-translation. By the maxicell method it was apparent that pJHCV64 encoded a number of polypeptides with molecular masses ranging from 20 to 48 kilodaltons (kDa). However, plasmid-encoded polypeptides were very difficult to resolve in the gels due to the presence of background proteins of chromosomal origin that complicated the interpretation of the results (data not shown). In vitro transcription-translation gave more clear-cut results (Fig. 6). pJHCV64 encoded 13 polypeptides with molecular masses of 48, 37, 36.5, 33, 31.5, 29, 28.5, 26, 25, 24.5, 23.5, 23, and 20 kDa (Fig. 6, lane B). These polypeptides were also detected in a transcription-translation reaction with just the 14-kb *Bst*EII DNA insert from pJHCV64

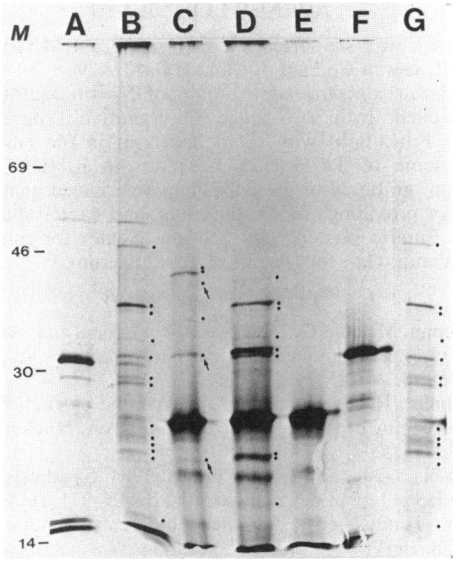

FIG. 6. Autoradiography of polypeptides expressed in an in vitro transcription-translation system. DNA templates were processed as described in Materials and Methods, and polypeptides were labeled with [^{35}S]methionine. The following DNA templates were used (lanes): A, pHP45Ω (cloning vector control for pJHCV64); B, pJHCV64; C, pCMV10; D, pCMV21; E, pACYC184 (cloning vector control for pCMV10 and pCMV21); F, pAT153 (internal control for the transcription-translation system); G, 14-kb *Bst*EII fragment from pJHCV64. Lanes A through F were obtained from the same gel, whereas lane G was added from a separate experiment. Dots indicate the polypeptides expressed only by the DNA inserts. Arrows in lane C point at common polypeptides encoded by pCMV10 and pCMV21. Positions of ^{14}C-labeled molecular mass markers (*M*): bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14 kDa.

(Fig. 6, lane G). In the case of the experiments with pCMV21 DNA, nine polypeptides of 41.5, 37, 36.5, 33, 31.8, 31.5, 23, 22.8, and 21.5 kDa were detected (Fig. 6, lane D). Six of these polypeptides were also encoded by pJHCV64, whereas polypeptides of 41.5, 31.8, and 22.8 kDa were unique to pCMV21. Also, the polypeptides of 41.5, 31.8, and 22.8 kDa were expressed by pCMV10 (Fig. 6, lane C, arrows). We therefore conclude that these three polypeptides must be encoded by the 2.8-kb DNA *Cla*I-*Hin*dIII segment from the fragment J shared by pCMV10 and pCMV21 and included within the O7-LPS region (Fig. 3).

The sum of the sizes of the 13 polypeptides expressed by pJHCV64 and the three proteins unique to pCMV21 accounts for a coding region of about 14 kb of DNA, assuming that there are no overlaps and noncoding regions between structural genes. This is in good agreement with the molecular size of the O7-LPS region.

DISCUSSION

We report in this work the genetic characterization of the O7-LPS biosynthesis genes from a strain of *E. coli* O7:K1. Transposon insertions in the cosmids pJHCV31 and pJHCV32 identified a region of approximately 14 kb that is necessary for the expression of an O7$^+$ phenotype in *E. coli* K-12 cells. Insertions located within the 14-kb region expressed three distinct O7-deficient phenotypes based on the analysis of immunoblots and polyacrylamide gels stained with silver. The most common O7$^-$ phenotype was that of class I mutants that do not express O7-reacting material in immunoblots and do not show O-side-chain bands in silver-stained gels. Class I mutants denote the existence of genes most likely involved in early steps of the biosynthesis of the O7 oligosaccharide unit. Class II mutants, which express a single side-chain unit linked to lipid A core molecules, indicate lesions of genes involved in the polymerization of the O7 oligosaccharide subunit. This suggests that the gene(s) required for polymerization of the O7 subunit are located within the O7 *rfb* cluster. Alternatively, it is also possible that some of these mutants are affected in late steps of the biosynthesis of the O7 subunit, resulting in the formation of incomplete single oligosaccharide subunits that cannot be polymerized into a full-length side chain but are ligated to the lipid A core as demonstrated by Sturm et al. (30) in the case of *Shigella dysenteriae* type I O-side-chain genes. Chemical analysis will be required to distinguish among the two possibilities. Finally, class III mutants produce a reduced amount of O7-LPS, which may be due to mutations in biosynthesis genes resulting in a decreased enzyme activity or to mutations in regulatory components. In the case of the cloned *E. coli* O9 *rfb* genes, Kido et al. (15) have shown that a partial deletion of the cloned genes results in the synthesis of a new O-antigenic lipopolysaccharide. We did not find anything similar, since in all cases the results of silver staining and immunoblots gave comparable results.

The fact that one insertion mutant located outside of the 14-kb region mediates different phenotypes depending on the host cell background suggests that gene function(s) present in *E. coli* K-12 may contribute to the expression of O7-LPS. In the case of the cloned *rfb* region from *S. dysenteriae* type I, the *E. coli* K-12 chromosome provides some *rfb* functions that are able to substitute for analogous *S. dysenteriae* functions (30). The same appears to occur in the cloned O4 and O101 *rfb* regions (11, 13).

Also, our finding that sequences from the *Hin*dIII fragment J unique to pJHCV31 participate in the O7-LPS expression suggests that *E. coli* K-12 functions analogous to those provided by fragment J contribute to the expression of O7-LPS mediated by pJHCV32. However, these functions are likely not to be encoded by the *rfb* region from *E. coli* K-12, since pJHCV31 and pJHCV32 can express O7-LPS in strain SØ874, which harbors a deletion that eliminates the K-12 *rfb* region (35). We have also suggested that in *E. coli* K-12 there is a partial defect in the transfer of O7 polysaccharide chains to the lipid A core (35). Enzyme function(s) provided by the core oligosaccharide biosynthesis genes (*rfa* gene cluster) are needed for a proper translocation of O side chains onto the lipid A core (21). Five core chemotypes are known to occur in *E. coli* strains, among which the K-12 chemotype appears to be unique (21). It is then possible that translocation enzymes with different specificities may exist, depending on the chemical structure of the core oligosaccharide. This is supported by experiments carried out in the *E. coli* O8$^-$:K27$^-$ strain F470, which has an *rfb* mutation and expresses the R1 core chemotype (28). F470 containing pJHCV31 forms O7-LPS at levels comparable to those in the wild-type strain VW187 and becomes resistant to rough-specific bacteriophages (M. A. Valvano, unpublished observations).

When inserted in the proper orientation downstream from an indigenous promoter, Tn*3*HoHo1 can generate transcriptional fusions with *lac* operon sequences (29). Transposon insertions can therefore be screened for the production of β-galactosidase, and the direction of transcription of target genes can be predicted. Our results indicate that transcription occurs in only one direction along the 14-kb region

containing the O7-LPS genes. However, it is likely that the O7-LPS region consists of various genetic units. This is in part suggested by *trans*-complementation experiments in *E. coli* K-12 strains harboring plasmids containing Tn*3*HoHo1 insertions and subclones of the O7-LPS region (C. L. Marolda, L. Dafoe, and M. A. Valvano, manuscript in preparation). In addition, transposon insertions 402, 40, and 47 do not express β-galactosidase despite being inserted in the proper orientation, suggesting that these insertions may be located in the proximity of promoter regions. All of these observations support the idea that the O7-LPS region is organized as a gene cluster rather than a single operon.

The differential expression of O7-LPS between pJHCV31 and pJHCV32 (35) and the overall low expression by the two plasmids in *E. coli* K-12 prompted us to confirm the results of transposon mutagenesis by constructing site-directed mutants in the wild-type strain VW187. Suicide plasmids carrying subsets of the O7-LPS region became integrated into the chromosomal DNA by homologous recombination, thereby generating in some cases O7-deficient mutants from the wild-type strain VW187. The location of the plasmid insertions and the resulting phenotypes of the mutant strains parallel the results obtained by transposon mutagenesis in pJHCV31 and pJHCV32. These two plasmids complemented the O7-deficient mutants, confirming that they must contain essential sequences necessary for the O7-LPS biosynthesis. Furthermore, a locus located outside the 14-kb region not identified by transposition mutagenesis in the cloned DNA was found by this method. A mutation in this region determines a phenotype comparable with that of a class I mutant, suggesting an early blockage of the O7-LPS biosynthesis.

At least 16 proteins with molecular masses ranging from 20 to 48 kDa are encoded by the 14-kb O7-LPS region, as determined by in vitro transcription-translation experiments. This finding is in agreement with the DNA-coding capacity of the O7-LPS region and suggests that the polypeptide genes do not overlap. However, since [^{35}S]methionine was used, it is possible that other proteins lacking methionine or produced in very low amounts are encoded by the O7 region and remained undetected. From studies carried out with *S. typhimurium*, the *rfb* gene cluster contains 11 known genes and possibly some other additional, not yet identified genes (21). These genes are involved in the biosynthesis of a tetrasaccharide repeating unit containing the sugars abequose, mannose, rhamnose, and galactose (21). Also in *S. typhimurium*, rhamnose and mannose alone require five biosynthesis genes located in the *rfb* region (21). The O7 oligosaccharide repeating subunit consists of a pentasaccharide composed of *N*-acetylglucosamine, 4-acetamidodideoxyglucose, mannose, rhamnose, and galactose (20). It is then likely that the 16 proteins encoded by the O7-LPS region may be sufficient for the biosynthesis of the five O7-specific sugars and their transfer to the pentasaccharide chain. However, additional functions important for the overall expression of O7-LPS in *E. coli* K-12 may be located in chromosomal regions other than the O7-LPS region of strain VW187 (35).

In summary, our results demonstrate that the O7-LPS region is genetically complex and consists of a cluster of genes. Construction of a detailed genetic map of polypeptide genes, localization of promoters, and functional studies are under way in our laboratory to elucidate the fine molecular organization of the O7-LPS biosynthesis genes.

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