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Regulation by a Novel Protein of the Bimodal Distribution of Lipopolysaccharide in the Outer Membrane of *Escherichia coli*

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We report on the cloning and characterization of the *rfb* gene cluster of the O75 lipopolysaccharide from a urinary tract isolate of *Escherichia coli*. Deletion cloning defined the minimum region of DNA that expressed the O75 antigen in *E. coli* host strains to be on a 12.4-kb insert. However, the *E. coli* strain expressing this region did not produce a polymerized O chain as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. A slightly larger DNA clone of 13.4 kb produced a polymerized O chain in *E. coli* SØ874 but was found to be abnormal in its distribution over the surface membrane. Normal wild-type *E. coli*, as with *Salmonella* spp., has a bimodal distribution of the lipopolysaccharide on the surface which is seen as an abundance of long and short O chains attached to the lipid A-core structure. We found in a region adjacent to the cloned *rfb* region, and on the opposite side from where the putative polymerase (*rfc*) is encoded, a novel protein of 35.5 kDa expressed from a 1.75-kb DNA fragment. This protein was shown to complement in *trans* the *E. coli* strains carrying plasmids that expressed abnormal, unregulated lipopolysaccharides. The expression of these complemented strains was bimodal in distribution. Mutation of the gene encoding this protein destroyed its ability to regulate O-chain distribution. We propose to call this regulator gene *rol*, for regulator of O length.

The lipopolysaccharide (LPS) antigen of *Escherichia coli* and *Salmonella* spp. consists of three different molecular species (lipid A, core oligosaccharide, and O polysaccharide) that are synthesized independently of each other and are ligated together in or on the inner membrane (17). The O-polysaccharide subunit typically consists of one to five sugar molecules linked together to form a macromolecular polymer of repeated units that vary from 1 to more than 40 in number (11). After assembly, the entire LPS molecule is translocated to the outer membrane by an unknown mechanism (11, 15). The lipid A and core units are embedded in the outer membrane, while the O antigen extends outward from the cell. The O antigen has been shown to be important in mediating the effects of serum resistance, infection by certain bacteriophages, and interaction with the microenvironment (4, 20, 22, 25). The presence of O antigen on the surface of these bacteria confers a smooth phenotype and has been correlated to increased virulence (18).

On sodium dodecyl sulfate (SDS)-polyacrylamide gels, silver-stained or radiolabeled LPS has a ladderlike appearance, with each rung or step of the ladder corresponding to a complete lipid A-core unit plus an O chain of a specific length. Several groups of investigators have noted a bimodal distribution of LPS from wild-type strains in stained SDS-polyacrylamide gels (3, 10, 21). High-molecular-weight O antigens with chain lengths of 19 to 34 make up to 77% of the total antigen, with low-molecular-weight O antigen (1 to 5 chain lengths) forming the bulk of the remainder. Intermediate sizes of O antigen are not easily seen on the gels unless they have been overloaded. Deviation from this bimodal pattern results in altered responses to phage infection, serum sensitivity, and decreased virulence of some strains (4, 19, 21, 24). The mechanism by which this distribution is achieved is unknown.

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The genetically defined loci known to be involved in forming the O-antigen oligosaccharide units for both *E. coli* and *Salmonella* spp. are in the *rfb* region or gene cluster. In *Salmonella* spp., additional loci code for a polymerase (*rfc*) which joins the O units together and a ligase (*rfaL* and *rfbT*) which joins the completed chain to the lipid A-core (15). Several O-antigen *rfb* regions have been cloned and characterized (5–7, 28). Here, we report the cloning and expression of the O75 antigen of *E. coli* from a urinary tract isolate. A protein that regulates the distribution of O antigen was found to be encoded in a region flanking the *rfb* gene cluster.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are described in Table 1. *E. coli* HB101, DH1, and SK2881 were used as initial cloning hosts for the *rfb* DNA and have been described by Kushner (12). Subsequently, the DNA was moved into an *rfb* mutant *E. coli* (HU1190 or SØ874) host for characterization of LPS expression. Bacteria were grown on L agar or L broth with 0.5% glucose and supplemented with ampicillin (100 μg/ml), tetracycline (20 μg/ml), or chloramphenicol (20 μg/ml). Cloning vectors used were pACYC184 (1), pUC19 (29), and pBluescript KSII (Stratagene Cloning Systems, La Jolla, Calif.).

Enzymes and buffers. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and New England BioLabs, Inc. (Beverly, Mass.). Conditions and buffers used were those recommended by the manufacturers. Klenow enzyme was purchased from Boehringer Mannheim, and T4 ligase was obtained from Bethesda Research Laboratories.

Recombinant DNA methods. Chromosomal DNA encompassing the *rfb* region of the O75 antigen from a wild-type urinary tract strain of *E. coli* (HU1124) was conjugally

TABLE 1. *E. coli* strains used in this study

Strain	Relevant properties[a]	Reference or source
SØ874	Δ(*sbc-rfb*)*86* O75$^-$	19
HU1190	SØ874 *hsdR4 recA56 srl*::TN*10* Tc^r O75$^-$	6
HU1124	A29 Rif^r F′ Kan^r O75$^+$	14
SCH1075	SØ874 *his*$^+$ O75$^+$	This study
HU1191	HB101(pRHU1191) Ap^r O75$^+$	This study
RAB1	HU1190(pRAB1) Cm^r O75$^+$	This study
RAB2	SØ874(pRAB2) Tc^r O75$^+$	This study
RAB3	HU1190(pRAB3) Ap^r O75$^+$	This study
RAB4	HU1190(pRAB4) Ap^r O75$^+$	This study
RAB5	HU1190(pRAB5) Ap^r O75$^-$	This study
RAB9	SØ874(pRAB9) Tc^r O75$^-$	This study
RAB10	SØ874(pRAB10) Tc^r O75$^-$	This study
RAB11	SØ874(pRAB11) Tc^r O75$^-$	This study
RAB12	SØ874(pRAB12) Tc^r O75$^-$	This study
RAB13	SØ874(pRAB13) Tc^r O75$^-$	This study
RAB14	SØ874(pRAB14) Tc^r O75$^-$	This study
RAB210	HU1190(pRAB210) Ap^r O75$^+$	This study
RAB240	HU1190(pRAB240) Ap^r O75$^+$	This study
RAB306	HU1190(pRAB306) Tc^r O75$^+$	This study
RAB310	HU1190(pRAB310) Ap^r O75$^+$	This study
RAB39	SØ874(pRAB3, pRAB9) Ap^r Tc^r O75$^+$	This study
RAB219	SØ874(pRAB210, pRAB9) Ap^r Tc^r O75$^+$	This study
RAB249	SØ874(pRAB240, pRAB9) Ap^r Tc^r O75$^+$	This study
RAB315	SØ874(pRAB306, pRAB9) Cm^r Tc^r O75$^+$	This study
RAB319	SØ874(pRAB310, pRAB9) Ap^r Tc^r O75$^+$	This study
RAB103	SØ874(pRAB10, pRAB3) Ap^r Tc^r O75$^+$	This study
RAB113	SØ874(pRAB11, pRAB3) Ap^r Tc^r O75$^+$	This study
RAB123	SØ874(pRAB12, pRAB3) Ap^r Tc^r O75$^+$	This study
RAB133	SØ874(pRAB13, pRAB3) Ap^r Tc^r O75$^+$	This study
RAB143	SØ874(pRAB14, pRAB3) Ap^r Tc^r O75$^+$	This study

[a] Tr^r, Ap^r, Cm^r, Rif^r, and Kan^r show resistance to tetracycline, ampicillin, chloramphenicol, rifampin, and kanamycin, respectively.

transferred to *E. coli* SØ874 to produce strain SCH1075. High-molecular-weight chromosomal DNA was isolated from this strain by the method of Hull et al. (9). This DNA was partially digested with *Sau*3A and subjected to NaCl gradient centrifugation and ethanol precipitation to select fragments of approximately 40 kb. These fragments were ligated into the *Bam*HI site of the cosmid vector pHC79 and subsequently packaged into lambda phage by using a kit (Boehringer Mannheim). These phage were used to infect *E. coli* HB101. Transductants harboring recombinant plasmids were initially screened by colony blot hybridization by the method of Tarr et al. (24), using a DNA probe made from the flanking region of the previously cloned O4 *rfb* region (5, 6). Colonies hybridizing with the probe were tested for agglutination in anti-O75 polyclonal rabbit antiserum (Difco Laboratories, Detroit, Mich.). Plasmid DNA was purified by the cleared lysate procedure (23).

Slide agglutination with antisera. Bacterial colonies were tested for the presence of O75 antigen by reaction to polyclonal rabbit antiserum (Difco). Select colonies were suspended in 0.85% saline, and a 10:1 ratio of cells to antiserum was spread on a glass slide. Visible agglutination within 3 min was considered positive.

LPS profiles on acrylamide gels. LPS was isolated from bacterial strains by the method of Hitchcock and Brown (8). LPS samples were placed on an SDS-15% polyacrylamide resolving gel with a 6% stacking gel (0.8%:30%, bisacrylamide-acrylamide) as described by Laemmli (13) and electrophoresed at 30 mA for 4 h. The gels were fixed and silver stained by the method of Tsai and Frasch (27).

Immunoblot analysis of LPS gels. An SDS-15% polyacrylamide gel was prepared as described above but without fixation. LPS in this gel was transferred to nitrocellulose by the method of Towbin et al. (26). Electrophoretic transfer was done in a TE52 chamber (Hoefer Scientific Instruments, San Francisco, Calif.) at 0.5 A and 10°C overnight. O75 reactive antigen was visualized on the blots by using rabbit anti-O75 serum and an Immun-blot Goat Anti-rabbit Assay Kit (Bio-Rad Laboratories, Richmond, Calif.). A 1:200 dilution of antiserum was used, and all reagents and conditions were those described in the kit.

In vitro translation of cloned genes. Selected plasmids containing DNA fragments of interest were translated in vitro in the presence of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.), using a kit purchased from Amersham (Amersham Corp., Arlington Heights, Ill.). The resulting proteins were electrophoresed on an SDS-15% polyacrylamide resolving gel with 4% stacking gel (0.8:30, bisacrylamide-acrylamide) by the method of Laemmli (13). The gel was fixed overnight in 20% methanol–7% acetic acid, dried onto filter paper, and autoradiographed with X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.) after fluorographic treatment with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.).

RESULTS

Isolation and restriction mapping of recombinant DNA. A cosmid molecule designated pRHU1191 was identified by colony-blot hybridization with a DNA probe made from the flanking region of the O4 LPS antigen-coding region (5, 6). The strain containing this cosmid (HU1191) reacted positively with anti-O75 antiserum and was used for subsequent analysis. A 17.5-kb *Sal*I fragment was derived from this clone and inserted into the pACYC184 cloning vector (pRAB1). A map of restriction endonuclease recognition sites on pRAB1 and on several deletion clones is shown in Fig. 1.

Phenotypic analysis of deletion clones. As shown in Fig. 1, the reaction of bacterial strains harboring cloned DNA with anti-O75 rabbit antiserum revealed three basic phenotypes. Strains RAB1, RAB2, and RAB3 reacted strongly with the antiserum, while RAB4 produced a weak reaction. Deletion clones in strains RAB5 and RAB10 failed to express O75 antigen and defined the minimum O75-encoding region (*rfb*) as 12.4 kb of DNA (pRAB4). A silver-stained SDS-polyacrylamide gel of the deletion clones (Fig. 2A) displayed four different patterns of LPS distribution. The strain with the largest plasmid (RAB1) produced an LPS profile matching those produced by the parent recombinant strains, HU1191 and SCH1075. With these strains, the distribution of the LPS in the gel was typically bimodal. In contrast, the O antigen produced by RAB2 and RAB3 was not bimodal, although the LPS molecules of these strains were polymerized. Hence, a 13.4-kb region between *Eco*RI and *Xho*I was needed for expression of polymerized O75 antigen. The O-antigen chains produced by RAB3 ran the full length of the gel, and judging by the intensity of staining, the amount of LPS decreased as size increased. In contrast, strain RAB2 produced a more truncated ladder pattern. No polymerized O antigen was produced by RAB4. On immunoblot analysis (Fig. 2B), only the O antigen was seen to react with the polyclonal rabbit antiserum to O75 antigen. The pattern displayed by immunoblot analysis was very similar to that of the silver-stained preparations. The core region did not react. A small band was seen to react with LPS from RAB4

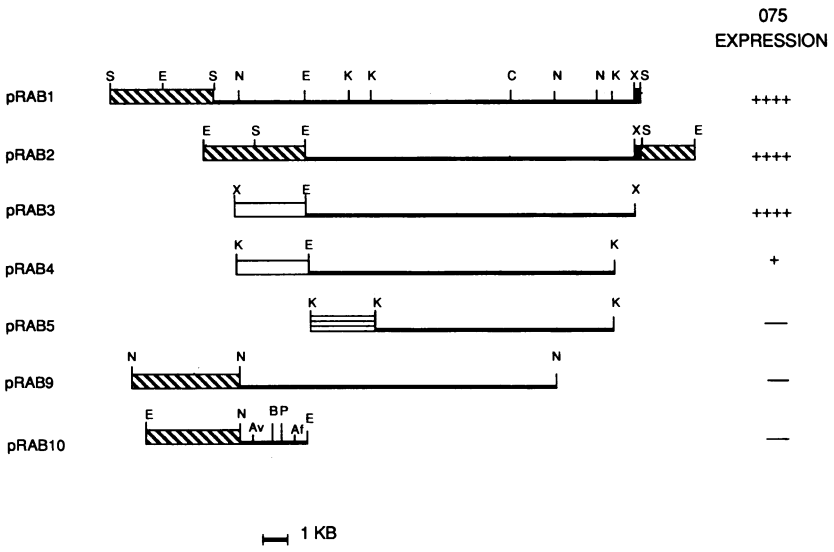

FIG. 1. Physical map of the insert DNA of pRAB1 and its deletion derivatives. Restriction endonuclease recognition sites are designated as follows: S, *Sal*I; E, *Eco*RI; N, *Nco*I; K, *Kpn*I; C, *Cla*I; X, *Xho*I; Av, *Ava*I; B, *Bgl*II; P, *Pst*I; Af, *Afl*II. The cloning vectors are represented as follows: diagonal bars, pACYC184; open box, pBluescript KSII; horizontal bars, pUC19; small black box, pHC79. A + or − under the O75 expression indicates the capability of the *E. coli* HU1190 or SØ874 containing the plasmid to agglutinate in O75 antiserum.

and presumably represents the core plus one O-antigen subunit. Strains RAB5 and HU1190 did not react with the antiserum (lanes 9 and 16).

Figure 3 shows constructs that were made to determine whether the difference in ladder length between RAB2 and RAB3 was due to the possible effects of cloning vectors or residual vector DNA. The silver-stained LPS preparations from these strains are shown in Fig. 4. In general, the ladder size was higher in the strains harboring the high-copy-number plasmid (pBluescript KSII) than in strains harboring the lower-copy-number plasmid (pACYC184). However, it was observed that these differences were also influenced by culture conditions such as temperature of incubation (data not shown).

Complementation of O75 expression. The following experiments were done to determine the possible contribution to O75 expression of gene products from the flanking region left of the *Eco*RI site of pRAB1. The plasmid pRAB9 has a 13-kb *Nco*I insert from pRAB1 that does not express O75 antigen in SØ874. A strain containing this plasmid, RAB9, was transformed with various constructs containing the 13-kb *Eco*RI-*Xho*I fragment. The LPS ladder profiles of these doubly transformed strains (RAB39, RAB219, RAB249, RAB315, and RAB319) are shown in Fig. 5. The pRAB9-encoding region was found to complement in *trans* the expression of plasmids containing the 13-kb *Eco*RI-*Xho*I fragment. The resulting LPS profile was bimodal in distribution. The pRAB9 plasmid did not complement the unpolymerized O antigen of RAB4, however (data not shown). Thus, it was concluded that a protein that regulates the

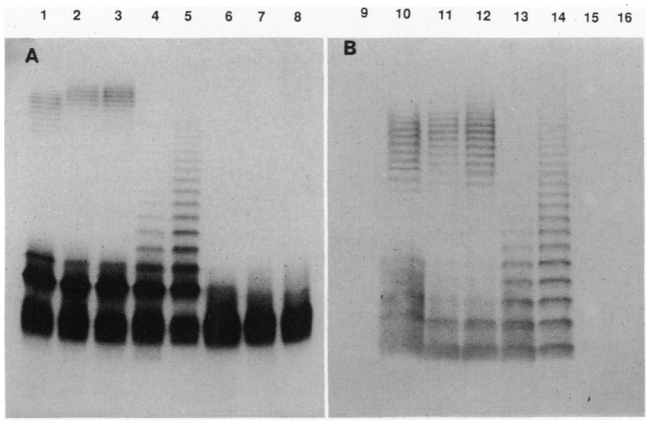

FIG. 2. SDS-PAGE of lipopolysaccharide from *E. coli* strains. (A) Silver-stained gels; (B) immunoblots with *E. coli* O75 antiserum. Lanes: 1, SCH1075; 2, HU1191; 3, RAB1; 4, RAB2; 5, RAB3; 6, RAB4; 7, RAB5; 8, HU1190; 9, HU1190; 10, SCH1075; 11, HU1191; 12, RAB1; 13, RAB2; 14, RAB3; 15, RAB4; 16, RAB5.

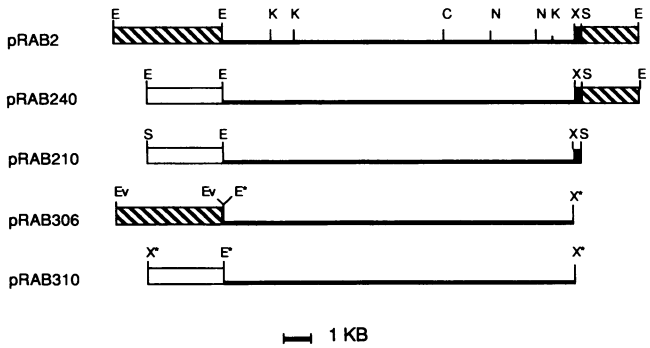

FIG. 3. Physical map of insert DNA coding for the polymerized O75 antigen in two different vectors. Restriction endonuclease sites are coded as follows: E, *Eco*RI; K, *Kpn*I; C, *Cla*I; N, *Nco*I; X, *Xho*I; S, *Sal*I; Ev, *Eco*RV. Restriction sites with an asterisk represent sites that have been opened with the respective enzyme, filled with Klenow enzyme, and ligated. The vectors are represented on the left side of the picture as an open box (pBluescript KSII) and diagonal bars (pACYC184). The solid black box and diagonal bars on the right side of the picture represent residual vector DNA from pHC79 and pACYC184, respectively, as described in the text.

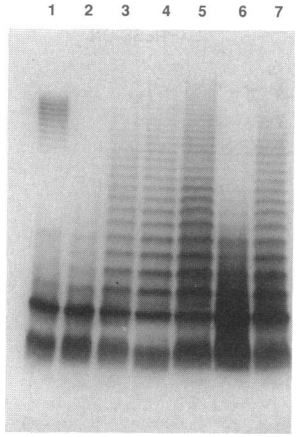

FIG. 4. Silver-stained SDS-polyacrylamide gel of LPS preparations from *E. coli* harboring identical or similar O75-encoding regions in two different vectors. Lanes: 1, RAB1; 2, RAB2; 3, RAB3; 4, RAB240; 5, RAB210; 6, RAB306; 7, RAB310.

length of the O75 antigen in *E. coli* was encoded in this region.

Genetic analysis of complementing region. To further delineate the size of the DNA encoding the complementary function, we moved the 2.8-kb *Nco*I-*Eco*RI fragment into pACYC184 to produce strain RAB10 (Fig. 1). Four restriction enzyme sites were mapped within this region: *Ava*I, *Bgl*II, *Pst*I, and *Afl*II. These sites were used to generate the contructs shown in Fig. 6. *E. coli* strains harboring deletion clone pRAB11 or pRAB14 together with pRAB3 (RAB113 and RAB143, respectively) displayed bimodal distribution (Fig. 7). These results defined the minimum encoding region to be on a 1.75-kb *Ava*I-*Afl*II fragment. The restriction enzyme sites of *Bgl*II and *Pst*I were mutated by digestion with the appropriate enzyme and filling with Klenow enzyme in the presence of all four nucleotide triphosphates. As shown in Fig. 7, these mutations destroyed the complementation function.

In vitro translation of encoding regions. Two plasmids encoding the O75 *rfb* region, pRAB1 and pRAB3, were

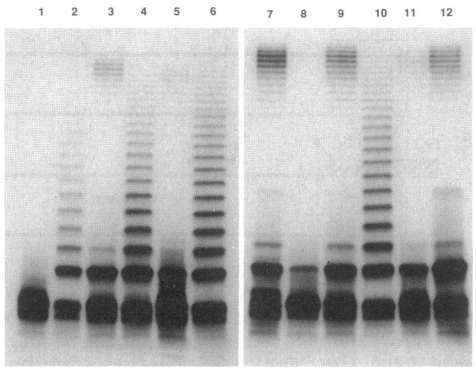

FIG. 5. Silver-stained SDS-polyacrylamide gel of LPS preparations from *E. coli* SØ874 carrying plasmids constructed with O75-encoding DNA inserts that express polymerized and unregulated O antigen alone or in combination with pRAB9. A composite of two gels. Lanes: 1, RAB9; 2, RAB3; 3, RAB39; 4, RAB240; 5, RAB249; 6, RAB210; 7, RAB219; 8, RAB306; 9, RAB315; 10, RAB310; 11, RAB319; 12, RAB1.

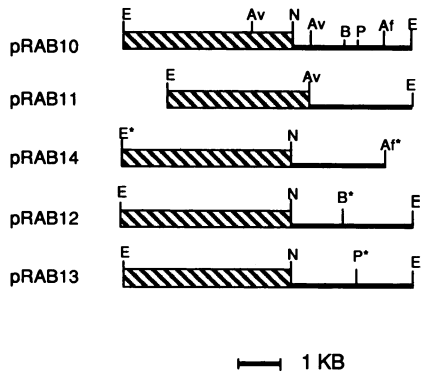

FIG. 6. Physical map of pRAB10 and derived deletion-mutation clones. Restriction endonuclease codes are as follows: E, *Eco*RI; Av, *Ava*I; N, *Nco*I; B, *Bgl*II; P, *Pst*I; Af, *Afl*II. The restriction sites marked with an asterisk are sites that have been digested, filled by Klenow enzyme, and ligated.

translated in vitro along with pRAB14, pRAB12, and pRAB13 expressing the regulatory component or mutated forms of it. The resulting radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Figure 8 shows the resulting autoradiograph. A 35.5-kDa protein was identified that was produced by pRAB14 and pRAB1, which express the bimodal distribution of LPS, but not by pRAB3, which does not express the bimodal distribution. The mutated strains, RAB12 and RAB13, did not produce this protein. In addition, 11 other bands were seen in the pRAB1 and pRAB3 lanes that were not present in the vector control lanes; the functions of these proteins cannot be assigned at this time.

DISCUSSION

The possible mechanisms for the heterogeneity and size distributions of the LPS patterns in SDS-polyacrylamide gels were recently studied by Goldman and Hunt (2). These investigators concluded from their data that enzyme speci-

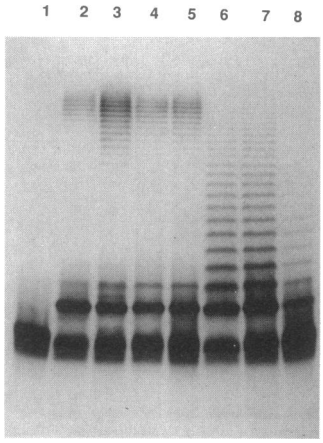

FIG. 7. Silver-stained SDS-polyacrylamide gel of LPS expressed from *E. coli* SØ874 carrying plasmid constructs as shown in Fig. 6 alone or in combination with pRAB3, which expresses polymerized and unregulated LPS. Strains RAB1 and RAB3 were run as controls for comparison. Lanes: 1, RAB10; 2, RAB1; 3, RAB103; 4, RAB113; 5, RAB143; 6, RAB3; 7, RAB123; 8, RAB133.

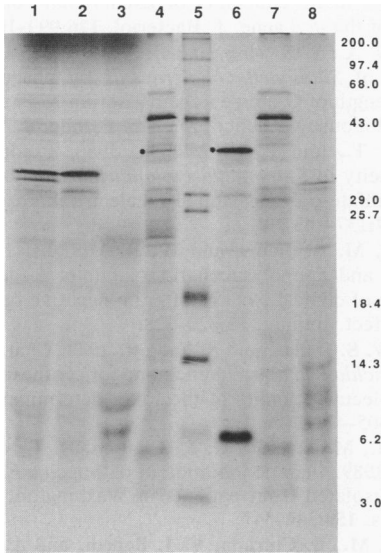

FIG. 8. SDS-PAGE and autoradiography of proteins expressed by in vitro translation of plasmids with the O75-encoding region and plasmids with the regulatory gene alone. Lanes: 1, pRAB13; 2, pRAB12; 3, pACYC184; 4, pRAB1; 5, size standards (as described on right side in kilodaltons); 6, pRAB14; 7, pRAB3; 8, pBluescript KSII.

ficity could best account for the bimodal distribution of LPS on the outer membrane. Random or stochastic events were less likely to be involved since perturbation of one or more of the enzyme concentrations (polymerase or ligase) had little effect on the distribution. They suggested that the polymerase has the ability to select or create certain sizes of O antigen before its translocation to the outer membrane. Our data support their basic conclusions. Enzyme specificity is responsible for the distribution of preferred sizes of O antigen on the surface. However, the protein in this case is not the polymerase since the strains RAB2 and RAB3 produce polymerized O75 antigen, yet are unregulated. In addition, the deletion strain RAB4 produces an O antigen; however, it is not polymerized, suggesting that the putative polymerase is near the opposite end of the *rfb* region from the gene that regulates the length of the O75 antigen. The function of the regulator protein appears to be separate from the polymerase, although it may act in concert with it. McGrath and Osborn (17) observed that monomer O subunits as well as polymers of O chain accumulated in the periplasmic face of the cytoplasmic membrane of temperature-sensitive mutants which were deficient in ligase production. Upon incubation at a lower temperature, the O subunits were rapidly ligated to the core and distributed in a normal pattern. Their results suggest that the ligase does not have a direct role in determining size distribution of the O chain by competing with the polymerase.

The length of O antigen on the outer membrane may be determined by factors independent of concentration or density. Haraguchi et al. (6), Valvano and Crosa (28), Marolda et al. (16), and Heuzenroeder et al. (7) have observed that the amount of O antigen expressed by cloned *rfb* regions was different from that expressed by the wild type. *E. coli* strains harboring these regions had less O antigen expressed on their surface than the wild type, although the distribution of it appeared bimodal. These differences were probably also related to expression of genes outside of the *rfb* region. The type of core structure exhibited by the cloning host may affect the efficiency with which O antigen is ligated to the core. Five different core types are found in *E. coli* (11). Initial observations that pRAB2 expressed a bimodal distribution in the *E. coli* cloning host DH1 but not in SØ874 led to our present experiments (data not shown). The DH1 strain, like RAB1 (pRAB1 in SØ874), apparently expressed a protein(s) outside the *rfb* encoding region that complemented those expressed by the pRAB2 plasmid. No apparent differences in density of O antigen were found in our pRAB1 clone or in the other clones expressed in situ with pRAB10. These results suggest that the O75 antigen is efficiently ligated to the core structures of the *E. coli* strains used in this study. Our results provide further evidence negating the role of ligase in determining O-chain length.

In this work, we showed that a protein encoded in a region adjacent to the *rfb* gene cluster of O75 antigen regulates the bimodal distribution of the O75 chain on the outer membrane of *E. coli*. This protein is not a polymerase or a ligase since strains lacking the expression of this protein continued to produce LPS with polymerized O antigen. In the absence of this protein, the size of the O antigen was unregulated. Since this protein is not needed for synthesis or polymerization of O antigen or ligation to the core-lipid A structure, we propose to call the gene encoding it *rol*, regulator of O length. We also noted differences in the lengths of O-antigen chains on SDS-polyacrylamide gels in strains harboring O75-encoding regions in different cloning vectors. In the absence of this regulatory protein (Rol), the size of the O chain may indeed be dependent on the concentration of available O antigen or on competing enzymes (i.e., polymerase or ligase) and may be more susceptible to different conditions of growth.

ACKNOWLEDGMENTS

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