Acetylation of O-Specific Lipopolysaccharides from Shigella flexneri 3a and 2a Occurs in Escherichia coli K-12 Carrying Cloned S. flexneri 3a and 2a rfb Genes

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Most of the Shigella flexneri O-specific serotypes result from O-acetyl and/or glucosyl groups added to a common O-repeating unit of the lipopolysaccharide (LPS) molecule. The genes involved in acetylation and/or glucosylation of S. flexneri LPS are physically located on lysogenic bacteriophages, whereas the rfb cluster currently subtyped into 13 serotypes on the basis of antigenic determinants on the O-specific polysaccharide (see reference 38 for a review). This typing scheme arises from a variety of combinations of type- and group-specific antigens which have been identified chemically and immunologically (6, 12, 21–23, 38). In all S. flexneri serotypes except type 6, the O-antigen repeating unit is composed of a common tetrasaccharide with the following structure: -3β-D-GlcNAc-(1→2)-α-L-Rhap[-“RhaI”]-[1→2]-α-L-Rhap[-“RhaII”]-[1→3]-α-L-Rhap[-“RhaIII”]-[1→23]. This common structure has been associated with the group antigen 3,4 and is found in S. flexneri serotype Y (Fig. 1). The other S. flexneri serotypes, X, 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, and 5b, arise from the attachment of α-D-glucosyl and/or O-acetyl residues to different specific positions on the common repeating unit (38). Such substitutions appear to be the result of postpolymerization modifications of the O-specific LPS determined by various lysogenic bacteriophages (38).

Genetic mapping studies have revealed that S. flexneri rfb (rfb30), encoding the common tetrasaccharide structure (corresponding to serotype Y in Fig. 1), maps adjacent to the his locus (17), whereas the bacteriophages responsible for acetylations and glucosylations of the O-polysaccharide chain are integrated near the pro-lac region on the chromosome (36). Bacteriophage Sf6, a lysogenic phage identified in S. flexneri 3a, has been shown to encode a gene involved in the O-acetylation of the O-polysaccharide chain at the RhaIII (9, 18, 26, 47), thus leading to the expression of the group 6 antigen (Fig. 1). Group 6 antigen also occurs in S. flexneri serotypes 1b, 3b, and 4b but it is not found in the remaining serotypes (38).

Our laboratory is interested in the molecular study of genes involved in the biosynthesis of the O side chain of LPS in some enteropathogenic bacteria. In this article, we report the identification of a novel function encoded by the Escherichia coli K-12 chromosome involved in determining the acetylation of the S. flexneri O-specific LPS resulting in group 6-specific reactivity.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, chemicals, and antisera. The bacterial strains and plasmids used in this study are described in Table 1. pMAV3 was constructed as follows. First, a 1.4-kb Aval-Clal deletion eliminated the tetracycline resistance gene of pACYC184 (8), resulting in plasmid pMAV2. Then a 0.57-kb NaeI-PvuII fragment carrying a multiple cloning site sequence and the SP6 and T7 promoter regions of PGEM3 (Promega, Madison, Wis.) was cloned into the single HindII site of pMAV2, giving rise to pMAV3. Chemicals and antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim, Dorval, Quebec, Canada, and Pharmacia Canada Inc., Baie d’Urfe, Quebec, Canada, and used as recommended by the

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Serotype 2a  
(type II; Group 3,4)  
\[ \text{Glc} \quad \text{GlcNac-Rha-HalRha-Hal} \]

Serotype 3a  
(Group 6; Group 7,8)  
\[ \text{Glc} \quad \text{GlcNac-Rha-HalRha-Hal} \]

Serotype Y  
(Group 3,4)  
\[ \text{GlcNac-Rha-HalRha-Hal} \]

FIG. 1. Chemical structure of O-repeating units from S. flexneri serotypes 2a, 3a, and Y. Drawings are modified from those in reference 38. Abbreviations: Glc, glucose; GlcNac, N-acetylglucosamine; Rha, rhamnose; O-Ac, O-acetyl group. Roman numerals I to III after Rha indicate the position of the rhamnose residue relative to GlcNac.

antiserum) and goat anti-rabbit immunoglobulin G (secondary antiserum) coupled to horseradish peroxidase. Electrophoresis of plasmid DNA cleaved with restriction endonucleases was performed as previously described (28, 43, 46). Transformations were carried out by the calcium chloride method (10) and in some cases by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada), using 0.1-cm cuvettes and conditions described elsewhere (13).

Hybridizations. Genetic and plasmid DNA fragments previously cleaved with restriction endonucleases were separated by electrophoresis and transferred to nitrocellulose filters as previously described (43, 46). Southern blot hybridization experiments were carried out by using undigested bacteriophage Sf6 DNA as a probe. The probe was labeled with [32P]dATP (Amersham, Arlington Heights, Ill.) by oligonucleotide synthesis (16), and hybridizations were carried out at 37°C for 16 to 18 h under conditions described previously (44, 45).

LPS analysis. LPS was extracted by the hot phenol-water method of Westphal and Jann (48) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (32). LPS was visualized by a silver-staining procedure (41) and also examined by immunoblotting under conditions described elsewhere (39). LPS immunodot blots were carried out by spotting 10 μl of LPS suspensions (containing 1 to 2 μg of LPS) on nitrocellulose paper. De-O-acetylated LPS for immunological analysis with group 6 antiserum was obtained by alkaline treatment as described by Carlin et al. (6). Acetate released from LPS samples was detected and quantitated by high-pressure liquid chromatography (HPLC) as described by Dupont and Clarke (14).

Conjugations. F' matings were done as described previously (33) with KL704(F'129) as the donor and the E. coli rfb-deleted strain CLM4 carrying the recombinant plasmids pYS1-5 or pEY5 as the recipient (Table 1). Exconjugants were selected on M9 agar medium containing ampicillin and the required supplements except histidine.

Immunoelectron microscopy. Protein A-collodial gold particles (diameter, ca. 15 to 20 nm) were provided by C.-S. Guo (Department of Vibrio cholerae, Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine). Bacterial cells were fixed at room temperature for 5 min in 1% glutaraldehyde diluted in 0.1 M phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 6,000 × g for 15 min. Washed bacterial pellets were resuspended in 2 ml of PBS. Bacterial suspension (50 μl) was mixed with 50 μl of S. flexneri polyvalent antiserum (1:100 dilution in PBS), and the mixture was incubated at 37°C for 60 min and washed once with PBS. Cells were resuspended in 100 μl of PBS, mixed with 50 μl of protein A-collodial gold (1:10 dilution in PBS), incubated at 37°C for 30 min, and given two washes with PBS. Finally, cells were resuspended in 50 μl of PBS. Copper grids were floated onto a drop of the bacterial suspension for 1 min and then onto a drop of 1% (wt/vol) uranyl acetate (pH 4.0) for 1 min and were then air dried. Grids were examined in a Philips 300 electron microscope at an operating voltage of 130 kV.

RESULTS AND DISCUSSION

Cloning of the S. flexneri 3a and 2a rfb regions. Chromosomal DNA fragments from S. flexneri 3a strain SF51575 and S. flexneri 2a strain SF51250 were cloned in the cosmid vector pHC79. Cosmids pY1214 and pEY5 were found to

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Suppliers. Except for KL704, all the strains were cultured in Luria broth (LB) or on LB agar. When appropriate, media were supplemented with ampicillin, chloramphenicol, or tetracycline at final concentrations of 100, 30, and 17.5 μg/ml, respectively. Strain KL704 was grown on M9 minimal medium containing the required amino acid supplements except histidine to maintain selective pressure for the F'129 carrying the his genes and surrounding sequences. S. flexneri polyvalent, group 3,4-, group 6-, group 7,8-, and type II specific antisera were purchased from the Beijing Institute of Biological Products, People's Republic of China. Antisera were adsorbed extensively with E. coli K-12 strain HB101 cells as described by Edwards and Ewing (15). The presence of S. flexneri O-specific LPS antigens was determined by slide agglutination.

Phage methods. A cell lysate containing bacteriophage Sf6 was obtained from strain FH10(Sf6) by induction with 1 μg of mitomycin C per ml added to a culture with an optical density at 600 nm of 0.3, followed by incubation for 3 h with vigorous shaking. The sensitivity of bacterial strains to Sf6 was determined by spot tests as described previously (18). For the isolation of Sf6 DNA, phage particles were concentrated by precipitation with 10% (wt/vol) polyethylene glycol and extracted with phenol-chloroform and the DNA was precipitated with ethanol.

Recombinant DNA methods. Chromosomal DNA for the construction of genomic libraries was prepared as described previously (29). A rapid miniscale isolation of whole genomic DNA (35) was used for hybridization experiments (see below). Plasmids were extracted and purified by using a commercial kit (Quiagen Inc., Chatsworth, Calif.) and also in some cases by the method of Birnboim and Doly (4) followed by ultracentrifugation in cesium chloride-ethidium bromide density gradients (43). Small-scale plasmid preparations were carried out as described by Xu et al. (49). S. flexneri 3a and 2a genomic DNA libraries were constructed by using cosmid pHC79 linearized with BamHI, treated with alkaline phosphatase, and ligated with partially digested (Sau3AI) chromosomal DNA fragments from S. flexneri 3a strain SF51575 and S. flexneri 2a strain SF51250. The ligated DNA was in vitro packaged into λ phage particles by using a commercial kit (Packagene; Promega, Madison, Wis.) and transduced into E. coli K-12 HB101. Gene libraries were screened by colony immunoblots (29), using a 1:1,000 dilution of adsorbed S. flexneri polyvalent antiserum (primary
TABLE 1. Strains and plasmids used in this study

| Designation | Relevant properties | Source or referencea |
|-------------|---------------------|----------------------|
| Strains     | Serotype            | Laboratory stocks    |
| S. flexneri |                     |                      |
| SF51575     | 3a (II-6, 7, 8)     | Laboratory stocks    |
| SF21250     | 2a (II-3, 4)        | Laboratory stocks    |
| SF51581     | Y (~ -3, 4)         | Laboratory stocks    |
| FH10(Sf6)   | Serotype Y with    | Laboratory stocks    |
|             | bacteria             | Laboratory stocks    |
| SF51572     | 1b (1:6)            | Laboratory stocks    |
| SF51574     | 3b (III-3, 4, 6)    | Laboratory stocks    |
| SF51577     | 4b (IV-6, 7)        | Laboratory stocks    |
| E. coli     |                     |                      |
| HB101       | hsdK serA ara proA | Laboratory stocks    |
|             | lacY galK rpsL     |                      |
|             | xyl mtl supE        |                      |
| DH1         | F- gyrA96 recA1 relA1 thi-1 hsdR17 supE44 R' | Laboratory stocks |
| DH5α        | F- endA hsdR17 (tK- mK+) supE44 thi-1 recA1 gyrA96 Δ80lacZM15 Δ(argF lacZYA)J169 | Bethesda Research |
|             |                     | Laboratories         |
| S0874       | lacZ trp Δ(bbcB-bb) upp rel rpsL | B. Bachmann |
| CLM4        | S0874 ΔrecA        | 31                   |
| SY327       | F' araD (lac-pro) | 34                   |
|             | argE(ΔAm) recA56    |                      |
|             | Rif' mnaA λnic      |                      |
| CC118       | araD139 (ara leu) | 30                   |
|             | 7697 ΔlacX74        |                      |
|             | aphA20 galE galK   |                      |
|             | thi rpsB recA(ΔAm) |                      |
|             | recA1               |                      |
| M8820       | araD139 (ara leu) | 7                    |
|             | 7697 Δ(pro lac)XIII rpsL |          |
| 3000X111    | relΔ1 spoT1 thi-1 λ- Δgpt-lac | B. Bachmann |
| KL704       | F'129 leuB6 histG1 | B. Bachmann          |
|             | argG6 metB1 lacY1 gal-6 malA1 (λ') xyl-7 mtl-2 rps-104 tonA2 λ- supE44 |          |

Plasmids

- pH7C9: Cosmid vector; Ap' (Cm')  
- pMAV3: Cloning vector; Cm'  
- pY1214: 3a rfb+ cosmid clone in pH7C9; Ap'  
- pEY5: 2a rfb+ cosmid clone in pH7C9; Ap'  
- pYS1-2: 3.4-kb HindIII fragment deletion from pY1214; Ap'  
- pYS1-4: 0.4-kb HindIII fragment deletion from pY1214; Ap'  
- pYS1-5: 14.2-kb HindIII fragment deletion from pY1214; Ap'  
- pYS1-8: 24.6-kb HindIII fragment deletion from pY1214; Ap'  
- pYS1-9: 11.2-kb HindIII fragment deletion from pY1214; Ap'  
- pMEY2: 11.8-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  
- pMEY3: 15.8-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  
- pMEY13: 3.5-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  
- pMEY31: 7.5-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  
- pMEY32: 4.0-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  
- pMEY38: 15.3-kb HindIII fragment of pEY5 cloned in pMAV3; Cm'  

a Strains provided by B. Bachmann are deposited in the E. coli Genetic Stock Center, Yale University, New Haven, Conn.  

b In the nomenclature of the S. flexneri serotypes, the type antigen is indicated in roman numerals. The other numbers indicate the group antigens.

carry rfb sequences of S. flexneri 3a and 2a, respectively, as revealed by colony immunoblotting, slide agglutination, and immunoelectron microscopy with an S. flexneri polyvalent antiserum (Fig. 2; data not shown). pY1214 and pEY5 were transformed into E. coli HB101 and the E. coli rfb-deleted strain CLM4. Transformants in both strains gave a positive slide agglutination with the S. flexneri polyvalent antiserum. These results indicated not only that the antigens recognized by the antiserum were mediated by these cosmids but also that the rfb region from E. coli K-12 did not contribute to the expression of S. flexneri O antigen.

LPS expression was examined by SDS-PAGE followed by silver staining and immunoblotting with the S. flexneri polyvalent antiserum (Fig. 3). LPS extracted from HB101 (pY1214) and HB101(pEY5) revealed a typical ladder-like banding pattern with a bimodal distribution of O-specific polysaccharide chains similar to that seen in the parent S. flexneri SF51575 (Fig. 3, lanes A, B, D, and E), whereas no O-specific polysaccharide chains were detected in the host strain, HB101 (data not shown). O-specific LPS was also detected in CLM4(pY1214) and CLM4(pEY5); however, the polysaccharide chains showed a unimodal distribution (Fig. 3, lanes C and F). These experiments demonstrated that pY1214 (carrying the rfbSerA region) and pEY5 (carrying the rfbSerA region) directed the biosynthesis of O-specific LPS in E. coli K-12 strains, which normally do not express any O-specific antigen. O-specific LPS determined by pY1214 and pEY5 was also detected in E. coli CLM4, thus confirming that the entire rfb regions of S. flexneri 3a and 2a were present in these two plasmids.

Previous studies have shown the existence of a gene determinant involved in O-specific polysaccharide length distribution which maps on a region adjacent to the rfb genes in E. coli O75, E. coli O111, E. coli O7, Salmonella enterica group C2, and Yersinia pseudotuberculosis (1-3, 5, 24, 42). This gene, designated rol by one group (3), has recently been subcloned and sequenced and shown to map immediately upstream of the last gene of the histidine operon in an E. coli O75-K-12 hybrid as well as in Salmonella enterica (2). rol appears to be absent in the rfb-deleted strain S0874 and its recA derivative CLM4. Since both pY1214 and pEY5 directed the expression of unimodal O-specific LPS in CLM4 and bimodal O-specific LPS in HB101, we conclude that a rol-like gene is not present in these plasmids. It is possible that this gene has not been included in the cloned DNA or, alternatively, that the rol gene in S. flexneri is at a different
The restriction endonuclease maps of recombinant cosmid pY1214 and pEY5 are shown in Fig. 4. To localize the rfb<sub>S</sub> region in pY1214, various deletion derivatives from this plasmid were obtained by partial digestion of pY1214 DNA with HindIII followed by self-ligation. Deletion plasmids pYS1-2, pYS1-4, pYS1-8, and pYS1-9 lost the ability to direct the synthesis of O-specific LPS, as revealed by slide agglutination and SDS-PAGE followed by silver staining (Fig. 4a and 5, lanes B, C, E, and F). In contrast, pYS1-5 retained the ability to synthesize the O-specific LPS in E. coli HB101 and CLM4 (Fig. 4a and 5, lane D; data not shown). To localize the rfb<sub>S</sub> region in pEY5, DNA fragments from this plasmid obtained by partial cleavage with HindIII were subcloned in the vector pMAV3. Plasmids pMEY2, pMEY13, pMEY31, pMEY32, and pMEY38 did not express O-specific LPS, as shown by slide agglutination and SDS-PAGE (Fig. 4b and 5, lanes H and J; data not shown), whereas pMEY3 encoded a positive O-specific phenotype in E. coli HB101 and CLM4 (Fig. 4b and 5, lane I; data not shown).

The restriction maps of pYS1-5 and pEY5 showed a common region of approximately 9.5 kb flanked by a PstI and a SphI site, as indicated by the solid arrows in Fig. 4b. The restriction map of this region was also similar to that of the rfb<sub>S</sub> region reported by Macpherson et al. (27). These investigators documented by a hybridization experiment the close relationship at the DNA level among S. flexneri serotypes Y, X, 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, and 5b (27). Therefore, it was assumed that this common region in pYS1-5 and pEY5 should contain most of the rfb<sub>S</sub> gene.

FIG. 2. Transmission electron micrographs of cells treated with S. flexneri polyvalent absorbed antiserum coupled to protein A-colloidal gold and stained with uranyl acetate. (a) S. flexneri 3a strain SF51575. Magnification, ×25,000. (b) E. coli HB101(pY1214). Magnification, ×17,000. (c) E. coli HB101. Magnification, ×20,000.

FIG. 3. Analysis of LPS by SDS-PAGE and Western immunoblotting. (a) Photograph of a 14% polyacrylamide gel stained with silver. Before electrophoresis, cell envelopes were extracted with hot phenol as described in the text. Lanes: A, SF51575 (S. flexneri 3a); B, HB101(pY1214); C, CLM4(pY1214); D, SF51250 (S. flexneri 2a); E, HB101(pEY5); F, CLM4(pEY5). (b) Western blot of a gel similar to that in panel a, with S. flexneri polyvalent antiserum absorbed extensively with E. coli K-12 strain HB101 whole cells.
cluster. Single XhoI restriction endonuclease sites were found within the common region in both pYS1-5 and pEY5 (Fig. 4b). These sites were mutated by digestion with XhoI followed by end fillings with the large fragment of DNA polymerase I (Klenow fragment) prior to ligation. Cells containing either pYS1-5 or pEY5 with the mutated XhoI sites lost the ability to express O-specific antigen as determined by slide agglutination. Therefore, it was confirmed that the XhoI sites in pYS1-5 and pEY5 were contained within rfbsf-specific sequences. Further evidence delineating the rfbsf region in these two plasmids was obtained by constructing a 3.1-kb HindIII deletion in pYS1-5 (Fig. 4a, pYS1-9) and a corresponding 4.2-kb HindIII deletion in pEY5 (Fig. 4b, pMEY2). These deleted plasmids lacked the 1.5-kb PstI-HindIII fragment located to the left of the common region in both parent plasmids (Fig. 4) and did not encode a positive slide agglutination phenotype.

![Diagram showing restriction endonuclease mapping, deletion analysis, subcloning of recombinant plasmids pY1214 and pEY5, and comparison of the physical maps of rfb regions from S. flexneri 3a and 2a.](https://example.com/diagram.png)

**FIG. 4.** Restriction endonuclease mapping, deletion analysis, subcloning of recombinant plasmids pY1214 and pEY5, and comparison of the physical maps of rfb regions from S. flexneri 3a and 2a. The map was determined for the enzymes shown in the figure. Abbreviations: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Hp, HpaI; K, KpnI; P, PstI; Ps, PsI; S, SacI; S, SalI; Sm, Smal; Sp, SphI; St, StuI; X, XhoI. Vector sequences are not shown. Expression of O antigen was assessed by slide agglutination: +, positive; −, negative. (a) Physical map of pY1214 carrying S. flexneri 3a rfb region and its HindIII deletion derivatives. (b) Physical maps of S. flexneri 3a and 2a rfb regions and adjacent sequences carried in pYS1-5 and pEY5, respectively, as well as the subclones derived from pEY5. “rfb region” denotes the boundaries of a 10-kb DNA segment carrying the O-specific biosynthesis genes. Dotted lines indicate that the precise limit of the rfb region is not yet determined. A region of the map common to S. flexneri 3a and 2a is flanked by solid arrows on PstI and SphI sites. Asterisks indicate the restriction sites which give rise to a loss of expression of S. flexneri O antigen upon modification (see text). The open arrow indicates the location of a SmaI site reported in reference 27 which is not present in both pEY5 and pYS1-5. Dots beneath certain areas of the pEY5 map denote the regions with restriction sites in a different relative order from that reported previously (27).
A single KpnI site unique to pYS1-5 was found at 0.3 kb to the right of the common region (Fig. 4b). To investigate whether this site was important for expression of O-specific LPS genes, a mutation was constructed as described above for the XhoI sites. Cells containing the resulting plasmid were not agglutinated by the O-specific antiserum, indicating that the mutation of the KpnI site affected the expression of an essential gene for the biosynthesis of the O-specific LPS. Therefore, the cloned rfbSfla cluster extended up to at least 0.3 kb to the right of the common SphI site (Fig. 4b). No KpnI site was found on pEY5 carrying rfbSfla. Also, pEY5 lacked a PsrI site located at 0.6 kb to the left of the 9.5-kb PstI-SphI common region in pYS1-5 (Fig. 4b). Thus, rfbSfla and rfbSfla regions showed heterogeneity in the restriction endonuclease sites located at the ends of the common sequences.

Differences were also found in the restriction maps of rfbSfla regions of strain SF21250 presented in this study (pEY5, Fig. 4b) and that of another S. flexneri 2a strain reported in a previous study (pPM2213 [27]). A SmaI site absent in pEY5 was identified in pPM2213 at 0.4 kb to the right of the PsrI site delineating the left end of the common region (Fig. 4b, open arrow). Some restriction sites within the common region were found to be present in pPM2213 but in a different order (Fig. 4b, dots), whereas other restriction sites were present in similar positions and in the same relative order in both cases. This indicates the existence of variations in rfbSfla of different strains of S. flexneri 2a.

Since the basic tetrasaccharide structure of the O-repeating unit in S. flexneri is common to all serotypes except type 6 (12, 38) and since the rfb regions in these strains are highly homologous (27), it is not surprising that the physical maps of rfbSfla clusters are similar. However, the differences in the restriction endonuclease maps of both the two cloned rfb regions found in this work and the cloned rfb region from S. flexneri 2a reported by Macpherson et al. (27) suggest that rfbSfla regions of strains with serotypes other than type 6 are not necessarily identical. The significance of these variations in the genetic structure of rfbSfla regions remains to be elucidated. It is likely that the rfbSfla clusters determining the tetrasaccharide O repeat have arisen from a common ancestor and that further evolution has taken place independently in strains of each individual type, resulting in the loss of a perfect conservation of the DNA sequence, possibly without alteration of gene functions.

**Identification of an O-acetylation activity encoded by the E. coli K-12 strains.** Since the chemical structures and antigenic composition of S. flexneri O-specific serotypes are known (38), we attempted to infer the structure of the cloned O-specific LPS by investigating its antigenic properties with type- and group-specific antisera. Table 2 shows that LPS preparations of HB101 cells containing pYS1-5 and pEY5 did not react with type II- or group 7,8-specific antisera. This suggests that glucosylation of the RhaII (determining serotype 2 specificity) and glucosylation of the RhaI (determining the group 7,8 specificity found in serotype 3a) are indeed not present in the LPS expressed by these plasmids (Fig. 1). The lack of expression of S. flexneri 2a-specific determinants by E. coli K-12 cells carrying pEY5 indicates that the gene determining this modification is not present near the rfbSfla cluster. Similarly, the lack of expression of group 7,8 antigen in the LPS expressed by pY1214 and pEY5 permitted us to conclude that the genes determining the group 7,8 antigen are not included in the cloned DNA and also cannot be supplied by E. coli K-12 strains.

In contrast, HB101(pYS1-5) and HB101(pEY5) were agglutinated by the S. flexneri group 6-specific polyclonal antiserum extensively adsorbed with HB101 (Table 2). Recombinant plasmids pYS1-5 and pEY5 transformed into other E. coli K-12 strains such as DH5α and DH1 conferred a polyclonal agglutination with group 6 antiserum (data not shown). These results were unexpected since group 6 reactivity denotes the O acetylation of the RhaIII of the O-subunit backbone (Fig. 1). The group 6 antiserum was specific since it also recognized epitopes in S. flexneri serotypes 1b, 3a, 3b, and 4b possessing similar O acetylations in the RhaIII of the O-repeating unit (Table 2) (38) but did not agglutinate S. flexneri strains representative of the other serotypes (data not shown). These results strongly suggested that the O-acetyl groups are attached to RhaIII, since our group 6 antiserum reacted only with LPS of the S. flexneri serotypes 1b, 3a, 3b, and 4b known to possess the group 6 antigen determinant (38), although a definitive proof will require chemical analysis of the LPS.

To demonstrate that reactivity with group 6 antiserum was due to O acetylation of the O-specific polysaccharides, we treated purified LPS with alkali, which causes the release of the O-acetyl groups. De-O-acetylated LPS from HB101 (pYS1-5), HB101(pEY5), and S. flexneri 3a strain SF51575 failed to react with the group 6 antiserum as determined by immunodot blotting experiments (Table 2). The presence of O-acetyl groups in the LPS was further confirmed by determining and quantifying the release of acetate by HPLC (Table 3). The results indicated that LPS purified from HB101(pYS1-5) and HB101(pEY5) contained 5 to 10 times more acetate groups than the LPS from the host HB101 strain. The small amount of acetate released from the HB101 LPS is probably present in the lipid A moiety, since this strain expresses only a lipid A core and lacks any O-specific LPS. The amounts of acetate detected in LPS from HB101 cells carrying the recombinant plasmids were smaller than that found in the LPS from the wild-type S. flexneri 3a strain SF51575 (Table 3). To investigate the possibility that the cosmids pYS1-5 and pEY5 encode a function involved in O acetylation of LPS, we transformed these plasmids by electroporation into S. flexneri serotype Y (strain SF51581). Transformants did not express any S. flexneri type or group

### Table 2. Results of slide agglutination tests

| Strain     | Species       | Plasmid* | Reaction with antiserum specific for: |
|------------|---------------|----------|--------------------------------------|
|            |               |          | Type II Group 3,4 Group 6 Group 7,8  |
| SF51575    | S. flexneri 3a| -        | -                                    |
| HB101      | E. coli K-12  | pYS1-5   | -                                    |
| CLM4       | E. coli K-12  | pYS1-5   | -                                    |
| SF51250    | S. flexneri 2a| +        | -                                    |
| HB101      | E. coli K-12  | pEY5     | -                                    |
| CLM4       | E. coli K-12  | pEY5     | -                                    |
| SF51572    | S. flexneri 1b| -        | -                                    |
| SF51574    | S. flexneri 3b| -        | -                                    |
| SF51577    | S. flexneri 4b| +        | -                                    |
| SF51581    | S. flexneri Y | -        | -                                    |
| SF51581    | S. flexneri Y | pYS1-5   | -                                    |
| SF51581    | S. flexneri Y | pEY5     | -                                    |

* Refers only to recombinant plasmids pYS1-5 and pEY5. S. flexneri strains can express several different indistinguishable plasmid-encoded antigenic determinants.

**LPS extracted from these strains lost the group 6 reactivity on treatment under alkaline conditions, causing de-O-acetylation of LPS (see text).**

**A weak positive reaction was found by immunodot blotting of LPS samples extracted from these strains.**
Table 3. Quantification of acetate released from purified LPS preparations.

| Source of LPS | Amt of acetic acid (nmol/mg) | Net amt of acetic acid (nmol/mg) |
|---------------|-----------------------------|---------------------------------|
| HB101         | 0.75                        |                                 |
| HB101(pEY5)   | 5.5                         | 4.75                            |
| HB101(pYS1-5)| 9.87                        | 9.12                            |
| CLM4          | 1.74                        |                                 |
| CLM4(pEY5)    | 2.72                        | 0.98                            |
| CLM4(pYS1-5) | 3.40                        | 1.66                            |
| SF51575       | 14.07                       | 14.07                           |

*The values (expressed as nanomoles of acetic acid per LPS weight) are the average of two independent determinations.

The background contents of acetate released from HB101 and CLM4 LPS samples were subtracted from the amounts detected in the LPS samples extracted from the same strains containing the recombinant plasmids pEY5 and pYS1-5.

antigens other than group 3,4 typical of serotype Y (Table 2). Plasmid DNA prepared from these strains was transformed back into E. coli HB101, and transformants were now agglutinated with group 6 antiserum (data not shown). These experiments suggest that the gene(s) responsible for O acetylation of the O-specific LPS purified from E. coli HB101(pYS1-5) and pEY5 is present in the E. coli K-12 chromosome.

Acetylation of recombinant O-specific LPS is not related to bacteriophage Sf6. O-acetylation of S. flexneri 3a O-specific LPS is associated with the presence of the lysogenic bacteriophage Sf6, which carries a gene believed to encode an O-acetyltransferase activity (18, 26). This gene has recently been cloned and sequenced by two different groups (9, 47). The Sf6 chromosomal integration site maps within the pro-lac region of the S. flexneri chromosome (36, 38). To investigate the possibility that Sf6 bacteriophage accidently infected HB101 cells used in this study and was integrated in the chromosome, we transformed pYS1-5 and pEY5 into E. coli K-12 strains DH5α, SY327, CC118, 3000X111, and M8820 containing various pro-lac deletions. Transformants from all these strains agglutinated with group 6 antiserum, suggesting that the acetylation function was not encoded in the pro-lac region of the E. coli K-12 chromosome.

Southern blot hybridization analysis with whole Sf6 DNA as a radiolabeled probe demonstrated the presence of the Sf6 sequences only in chromosomal DNA from S. flexneri 3a strain SF51575, whereas the DNAs from S. flexneri Y strain SF51581 and from E. coli HB101 and CLM4 did not reveal any significant homologies with the probe (data not shown). These experiments ruled out the possibility that this bacteriophage or a similar one is responsible for the O acetylation of LPS observed in E. coli K-12 strains carrying the recombinant clones. Overall, all these results suggested that the acetylation of S. flexneri O-specific LPS expressed in E. coli K-12 strains containing pYS1-5 or pEY5 is due to a gene unrelated to that already identified in bacteriophage Sf6.

Early studies have reported that Sf6 has a narrow host range and that this is due mainly to its inability to infect strains of E. coli or S. flexneri carrying O-specific LPS with structures other than the basic tetrasaccharide unit corresponding to S. flexneri serotype Y (18, 26). Since Sf6 lysogens of E. coli and S. flexneri are resistant to infection with this bacteriophage, E. coli HB101(pYS1-5), E. coli HB101(pEY5), S. flexneri Y strain SF51581, and S. flexneri 3a strain SF51575 were tested for sensitivity to Sf6. E. coli HB101(pYS1-5), E. coli HB101(pEY5), and S. flexneri SF51581 were susceptible to Sf6, whereas S. flexneri 3a strain SF51575 was resistant to infection with this bacteriophage. Smaller and more turbid plaques were found in HB101(pYS1-5) and HB101(pEY5) compared with those in S. flexneri SF51581. Thus, our data demonstrate that Sf6 can, although to a lesser extent, infect E. coli K-12 expressing O-acetylated LPS. This could occur because the acetylation of the O-specific LPS in E. coli K-12 is less efficient or is not completely specific for rhamnose. A similar situation has been documented for the oafA gene harbored by strains of Salmonella enterica group C2, which is involved in the O acetylation of group C2 O-specific LPS but also can acetylate the O-specific LPS from S. enterica group B but to a lesser extent (19, 37).

Localization of an O-acetylation locus in the sbcB-rfb region of the E. coli K-12 chromosome. During the course of our studies involving group 6 antigen expression in different E. coli K-12 strains, we observed that cells from strain CLM4 containing either pYS1-5 or pEY5 failed to react with group 6 antiserum, although they were agglutinated with S. flexneri polyvalent antiserum. Detection and quantification of acetate released from purified LPS samples of CLM4 containing pYS1-5 and pEY5 indicated only one- to twofold larger amounts of acetate compared with the control values found in LPS extracted from the host strain with no plasmids (Table 3). This demonstrates that strain CLM4 failed to cause a significant O acetylation of the O antigen determined by the cloned S. flexneri rfb regions carried by these plasmids. Strain CLM4 carries a chromosomal deletion eliminating the sbcB-rfb region. To investigate whether the deleted region in CLM4 is involved in the expression of O acetylation, the F'his129, which carried part of the deleted region (40), was conjugated into CLM4(pEY5) and CLM4(pYS1-5). Ap' his' exconjugants were able to express the group 6 antigen, as determined by slide agglutination. It has been shown by other investigators that F'129 cannot complement mutations in the cps gene cluster located clockwise with respect to the sbcB-his-gnd-rfb chromosomal genes (40). Therefore, the finding that pYS1-5 and pEY5 cannot express LPS with group 6 reactivity in the rfb-deleted strain CLM4 but that this defect can be complemented with the F'129 containing part of the deleted region provides the basis for the demonstration that an O-acetylation locus is located near the his-rfb region of the E. coli K-12 chromosome. Recent attempts in our laboratory resulted in the isolation of a cosmids clone from E. coli K-12 strain W3110 containing the O-acetylation gene, which also can complement his-deficient mutants (50). This cosmid clone directs the expression of group 6 antigen in S. flexneri Y strain SF51581 (50).

The finding that cosmids carrying S. flexneri 3a and 2a rfb regions expressed an O-acetylated LPS in E. coli K-12 demonstrates for the first time the existence of a gene function involved in acetylation of LPS in this strain. A detailed analysis involving the cloning, fine genetic mapping of this gene(s), and characterization of gene product(s) is in progress in our laboratory (50). Our results are consistent with the view that although the cloning of rfb genes in E. coli K-12 is possible (see reference 42 for a review), genes involved in LPS biosynthesis already present in this microorganism may result in differences in the expression of recombinant O-specific LPS compared with the expression of the O-specific LPS in the wild-type parent isolate.
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