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Serotype-Converting Bacteriophage SfII Encodes an Acyltransferase Protein That Mediates 6-O-Acetylation of GlcNAc in *Shigella flexneri* O-Antigens, Conferring on the Host a Novel O-Antigen Epitope

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***Shigella flexneri* O-antigen is an important and highly variable cell component presented on the outer leaflet of the outer membrane. Most *Shigella flexneri* bacteria share an O-antigen backbone composed of →2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ repeats, which can be modified by adding various chemical groups to different sugars, giving rise to diverse O-antigen structures and, correspondingly, to various serotypes. The known modifications include glucosylation on various sugar residues, O-acetylation on Rha^{I} or/and Rha^{III}, and phosphorylation with phosphoethanolamine on Rha^{II} or/and Rha^{III}. Recently, a new O-antigen modification, namely, O-acetylation at position 6 of *N*-acetylglucosamine (GlcNAc), has been identified in *S. flexneri* serotypes 2a, 3a, Y, and Yv. In this study, the genetic basis of the 6-O-acetylation of GlcNAc in *S. flexneri* was elucidated. An O-acyltransferase gene designated *oacD* was found to be responsible for this modification. The *oacD* gene is carried on serotype-converting bacteriophage SfII, which is integrated into the host chromosome by lysogeny to form a prophage responsible for the evolvement of serotype 2 of *S. flexneri*. The OacD-mediated 6-O-acetylation also occurs in some other *S. flexneri* serotypes that carry a cryptic SfII prophage with a dysfunctional *gtr* locus for type II glucosylation. The 6-O-acetylation on GlcNAc confers to the host a novel O-antigen epitope, provisionally named O-factor 10. These findings enhance our understanding of the mechanisms of the O-antigen variation and enable further studies to understand the contribution of the O-acetylation to the antigenicity and pathogenicity of *S. flexneri*.**

Shigella flexneri is the major pathogen mainly responsible for shigellosis, or bacterial dysentery, in developing countries. About 125 million shigellosis cases are estimated to occur annually in Asia, with 14,000 deaths, the majority of which are those of children under 5 years old (1). The O-polysaccharide chain of the lipopolysaccharide (LPS) called O-antigen plays an important role in the pathogenesis of *S. flexneri*; it protects the bacterium from the lytic action of serum complement and also enhances the adherence and internalization of the bacterium to intestinal epithelial cells (2–4). The fine structure of the O-antigen provides the chemical basis for serotyping of *S. flexneri*. All serotypes except for serotype 6 share a polysaccharide backbone, having a tetrasaccharide repeat (O-unit) of three L-rhamnose residues (Rha^{I} to Rha^{III}) and one *N*-acetylglucosamine (GlcNAc) residue: →2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ (5).

The basic O-antigen is referred to as serotype Y, and adding various chemical groups to different sugars gives rise to diverse O-antigen structures and, correspondingly, to various serotypes (6). The host immune response to *S. flexneri* infection is serotype specific, and antigenic diversity provided by O-antigen modifications enhances the survival of the pathogens as it helps them to escape the host defense (6). Furthermore, some modifications, such as glucosylation on GlcNAc, Rha^{I}, and Rha^{II}, promote bacterial invasion into host cells mediated by the type III secretion system (2). Therefore, elucidation of the O-antigen modification mechanisms is important for understanding the antigenicity and pathogenicity of *S. flexneri*.

The well-known O-antigen modification types in *S. flexneri* are 2-O-acetylation and glucosylation (6). 2-O-acetylation occurs on Rha^{I} in serotypes 1b, 3a, 3b, 4b, and 7b, conferring to the host group 6 and (in serotypes 3a and 3b) type III antigenic determinants (O-factors) (7, 8). An acetyltransferase protein encoded by the *oac* gene which is carried on prophage Sf6 mediates the 2-O-acetylation (9, 10). Glucosylation has been identified at different positions on one or two of the four sugar residues in the O-unit; it defines type I, IC, II, IV, and V as well as group 7,8 antigenic determinants in various serotypes (6, 11). Three genes, *gtrA*, *gtrB*, and *gtr* (type specific), are responsible for the glucosylation in *S. flexneri*; they are carried on six prophages or cryptic prophages (SfI, SfIC, SfII, SfIV, SfV, and SfX) and are arranged in a single operon known as the *gtr* locus (10–17). The first two genes are highly conserved and interchangeable between serotypes, whereas the third gene, *gtr* (type), encodes a serotype-specific glucosyltransferase responsible for the addition of a glucosyl group to a certain sugar residue in the O-unit (6). The Sf6 and SfIC pro-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
S. flexneri strains		
Sf301	Serotype 2a with 6-O-acetylation on GlcNAc used for *oacD* gene cloning and inactivation analysis, Ap^s Km^s	28
Sf301Δ*oacD*	Sf301 with the *oacD* gene replaced by the kanamycin resistance gene (*kan*) from pSR551, Km^r Ap^s	This work
Sf301Δ*oacD*_pSQZ6	Sf301Δ*oacD* transformed by plasmid pSQZ6	This work
51571	Serotype 1a with 4-glucosylation on GlcNAc used for *oacD* function analysis, Ap^s	24
51571_pSQZ6	51571 transformed by plasmid pSQZ6	This work
51577	Serotype 4b with 6-glucosylation on GlcNAc used for *oacD* function analysis, Ap^s	24
51577_pSQZ6	51577 transformed by plasmid pSQZ6	This work
E. coli JM109	*E. coli* strain used for plasmid propagation and gene cloning	TaKaRa
Plasmids		
pMD20T	T-A vector, Ap^r	TaKaRa
pSR551	Km^r, used for amplification of the *kan* gene	30
pKOBEG	A thermosensitive replicon that carries the λ phage *red*γβα operon expressed under the control of the arabinose-inducible pBAD promoter	31
pSQZ6	pMD20T carrying the whole sequence of the *oacD* gene from strain Sf301, Ap^r	This work

[a] Ap, ampicillin.

phages are located on the bacterial chromosome at the tRNA-*argW* site adjacent to the conserved *yfdC* gene and at the site adjacent to the *yejO* locus, respectively (11, 18), whereas the other prophages are integrated into the tRNA-*thrW* gene between the conserved *proA* and *adrA* genes (6, 19).

Recently, a third type of *S. flexneri* O-antigen modification has been identified, namely, that corresponding to the addition of phosphoethanolamine (PEtN) to position 3 of Rha^{III} or Rha^{II} or both, which confers to the host the MASF IV-1 (E1037) epitope called the "variant" (v) factor in newly proposed serotypes Xv, 4av, and Yv (20–23). An O-antigen PEtN transferase protein (Opt) encoded by a single dimorphic *opt* gene carried on a 6.8-kb plasmid (pSFxv_2 or pSFyv_2) mediates the PEtN modification (20, 21, 23). The pSFxv_2 plasmid can be transferred among *S. flexneri* serotypes by conjugation in the laboratory (24), demonstrating the potential for dissemination of this modification in nature.

O-acetylation sites other than position 2 of Rha^{I} had been overlooked in early studies of *S. flexneri* O-antigen structures and have been identified only recently (5, 25–28). These are position 3 or 4 of Rha^{III} (3/4-O-acetylation) in serotypes 1a, 1b, 2a, 5a, Y, and 6 and position 6 of GlcNAc (6-O-acetylation) in serotypes 2a, 3a, Y, and Yv. The 3/4-O-acetylation on Rha^{III} interferes with the glucosylation (group O-factor 7,8) and PEtN phosphorylation (group O-factor IV-1) at the same sugar residue, thus abolishing the 7,8 determinant and reducing the IV-1 determinant manifestation (24). Further studies have indicated that an acyltransferase protein (OacB) which is encoded by gene *oacB* carried on a transposon-like element of the chromosome mediates the 3/4-O-acetylation in serotypes 1a, 1b, 2a, 5a, and Y (28), whereas an OacB homolog named OacC encoded by a phage-like structure is responsible for the 3/4-O-acetylation in serotype 6 (see the supplemental material). The *oacB* and *oacC* genes are widespread in serotypes 1a, 1b, 2a, 5a, Y, and 6 and confer to the host a novel antigenic determinant called group O-factor 9 (29). *oacB* and *oacC* are not involved with the 6-O-acetylation on GlcNAc as they could not be detected in strains carrying only this modification and their inactivation in strains carrying both 3/4-O-acetylation and 6-O-acetylation did not affect the 6-O-acetylation on GlcNAc (28) (see the supplemental material). Therefore, there is another mechanism involved with the 6-O-acetylation of *S. flexneri* O-antigens.

In this study, we identified yet another *oac* homolog, designated *oacD*, and demonstrated that this gene is responsible for the 6-O-acetylation on GlcNAc in *S. flexneri* O-antigens. *oacD* was carried on the serotype-converting bacteriophage SfII genome, which was integrated into the host chromosome by lysogeny to form a prophage present in serotype 2 strains. Some other serotypes of *S. flexneri* (3a, Y, and Yv) were found to possess a cryptic SfII prophage with a dysfunctional *gtr* locus. It was also shown that the OacD-mediated 6-O-acetylation confers to the host a novel antigenic determinant provisionally named O-factor 10.

MATERIALS AND METHODS

Ethics statement. This study was reviewed and approved by the ethics committee of National Institute for Communicable Disease Control and Prevention, China Center for Disease Control (CDC).

Bacterial strains, plasmids, and culturing conditions. Strains and plasmids used in this study are listed in Table 1. *S. flexneri* strain Sf301 (serotype 2a) (28) with the 6-O-acetylated GlcNAc in the O-antigen was used as the reference strain for *oacD* gene deletion and complementation analysis. *S. flexneri* 51571 (serotype 1a) and 51577 (serotype 4b) (24) were employed as hosts in function analysis of the *oacD* gene. *Escherichia coli* JM109 (TaKaRa, Japan) was used for plasmid propagation. pMD20T vector (TaKaRa, Japan) was used for *oacD* gene cloning and DNA sequencing analysis. Plasmid pRS551 was used for kanamycin resistance gene amplification. pKOBEG encoding a homologous recombination system was used in the *oacD* gene deletion analysis. Thirty-one *S. flexneri* strains with known O-antigen structures (listed in Table 2) were used for *oacD* PCR detection analyses. A total of 641 more *S. flexneri* isolates representing 18 serotypes were tested using the *oacD* gene PCR detection and antiserum 10 agglutination assay (Table 3). These strains were isolated from diarrheal patients in a surveillance program performed by China CDC during 2000 to 2012 or were purchased from the National Collection of Type Cultures (NCTC) or were kindly donated by B. Liu (Nankai University, Tianjin, China). Twelve strains of *Shigella dysenteriae* (one each of serotypes 1 to 12), 18 strains of *Shigella boydii* (one each of serotypes 1 to 18), 14 strains of *Shigella sonnei* (2 phase I and 12 phase II), and 10 strains of *E. coli* (one each of serotypes O6, O8, O13, O44, O71, O78, O127, O128,

TABLE 2 Correlation between the 6-O-acetylation on GlcNAc, the presence of the *oacD* gene, and the reactivity with grouping antiserum 10 of *S. flexneri* strains

Strain	Serotype	6-O-acetylation on GlcNAc	*oacD* gene PCR amplification	Antiserum 10 reactivity	Reference for the O-antigen structure
51571	1a	−	−	−	24
G1661	1a	−	−	−	26
51572	1b	−	−	−	24
G1662	1b	−	−	−	26
X6	1c	−	−	−	24
HN153	1d	−	−	−	32
51250	2a	+	+	+	24
G1663	2a	+	+	+	26
Sf301	2a	+	+	+	28
51251[a]	2b	−	+	−	24
51575	3a	+	+	+	24
G1665	3a	+	+	+	5
G1666	3b	−	−	−	5
NCTC9725	4a	−	−	−	20
G1668	4av	−	−	−	22
51577	4b	−	−	−	24
G1669	4b	−	−	−	5
51247	5a	−	−	−	24
G1036	5a	−	−	−	25
51580	X	−	−	−	20
G1039	X	−	−	−	5
2002017	Xv	−	−	−	20
2003055	Xv	−	−	−	20
51581[b]	Y	+	+	+	24
G1040[b]	Y	+	+	+	5
036	Y	−	−	−	23
HN006[b]	Yv	+	+	+	23
AH012[b]	Yv	+	+	+	23
HN011[b]	Yv	+	+	+	23
51579	6	−	−	−	24
G1038	6	−	−	−	5
G1671	6	−	−	−	5

[a] The *oacD* gene in serotype 2b strain 51251 has a one-base deletion (A) at position 191, which resulted in a stop codon at amino acid 64, rendering the gene defective.
[b] Strains HN006, AH012, and HN011 (all serotype Yv) and strains 51581 and G1040 (both serotype Y) carry a SfII prophage genome with a mutational *gtrII* gene.

TABLE 3 Distribution of the *oacD* gene in *S. flexneri* strains and their cross-reactivity with grouping antiserum 10

Serotype	No. of strains tested	No. of *oacD*-positive strains	No. of antiserum 10-reactive strains
1a	76	0	0
1b	22	0	0
1c	1	0	0
1d	5	0	0
2a	154	154	154
2b[a]	29	29	23
3a[b]	7	2	2
3b	2	0	0
4a	3	0	0
4av	3	0	0
4b	3	0	0
5a	2	0	0
X[c]	39	1	1
Xv[d]	189	1	0
Y[e]	37	14	13
Yv[f]	21	13	13
6	76	0	0
7b	2	0	0
Total	672	214	206

[a] Six serotype 2b strains (51251, 05BJ13, 08GS74, 08SX28, 2005049, and 2005001) are antiserum 10 negative. All carry a defective *oacD* gene, with strain 2005001 having a one-base (T) insertion at position 177 and the others having a one-base (A) deletion at position 191.
[b] Two serotype 3a strains (51575 and G1665) carry a cryptic SfII prophage lacking *gtrII*.
[c] One serotype X isolate (2001006) carries a cryptic SfII prophage with a defective *gtrII* gene having a single-base (T) deletion at base 1,031 resulting in a stop codon at amino acid 394.
[d] One serotype Xv strain (2003005) carries a cryptic SfII prophage with a defective *gtrII* gene having a single-base (T) deletion at base 1,291 resulting in a stop codon at amino acid 444; the *oacD* gene of 2003005 has a one-base (A) deletion at position 191 resulting in a stop codon at amino acid 64.
[e] Ten serotype Y strains carry a cryptic SfII prophage with a defective *gtrII* gene, with 5 strains (2000025, 2000026, 03HL08, 10HN064, and HN078) having a single-base (T) deletion at base 1,031 of *gtrII*, 2 strains (51581 and G1040) a two-base insertion (AT) at base 1,181, 2 strains (HN126 and XZ014) a four-base deletion (ACAT) at base 1,197 to base 1,282, and one isolate (XZ002) a one-base substitution (G to A) at base 87, all resulting in a stop codon at amino acid 28 or 344 or 394 or 445 in GtrII. Four serotype Y strains (HB06, 03HL32, AH104, and HN114) have one nonsynonymous mutation (A to C) in gene *gtrB* at position 560, resulting in an amino acid change (Q to P) at position 187; 06AH104 carries a dysfunctional *oacD* having a one-base (A) deletion at position 191, resulting in a stop codon at amino acid 64.
[f] Thirteen serotype Yv strains (HN006, AH012, HN068, HN069, AH028, HN171, HN182, HN049, AH029, HN011, HN033, HN116, and HN106) carry a dysfunctional *gtrII* locus (either one or both of the mutations in *gtrII* at position 1,222 and in *gtrB* at position 560).

O157, and O159) were used for the *oacD* gene PCR detection and antiserum 10 specificity evaluation (see Table S1 in the supplemental material). Strains were grown in a 37°C incubator or orbital shaker in Luria-Bertani broth (LB) supplemented with ampicillin (100 μg ml^{-1}), kanamycin (40 μg ml^{-1}), or chloramphenicol (50 μg ml^{-1}) when appropriate.

Bioinformatics analysis. The protein sequences of Oac of Sf6 (GenBank accession no. NP_958191.1), OacB of Sf301 (NP_706267.1), and OacC of CDC796-83 (WP_005054336.1) were compared to the protein database sequence of *S. flexneri* strain Sf301 (NC_004337.1) using the BLASTP web server (http://www.ncbi.nlm.nih.gov/BLAST). The candidates were further searched against the genomes of *S. flexneri* strains 2002017 (CP001383) and 036 (CP004056) (both lacking the 6-O-acetylation on GlcNAc) (20, 23) and 51581 (AZPG00000000) and HN006 (CP004057) (both with 6-O-acetylation) (23) using BLASTn. Searches for homologs to the OacD protein in the NCBI database were carried out using the BLASTP search engine.

DNA techniques. Genomic DNA and plasmid DNA were extracted using a DNA extraction kit according to the manufacturer's instructions (Qiagen, Germany). Primers used in this study were listed in Table 4. The *oacD-1* primer pair was used for *oacD* gene detection. The *oacD*-2 primer pair was used for *oacD* gene function analysis. The *oacD-3* primer pair was used to amplify regions flanking *oacD* in *S. flexneri* strains 51575 and G1665 (both serotype 3a). The *gtrII-1* primer pair was used to amplify SfII-specific gene *gtrII* for prophage SfII genome detection. The *gtrII-2* primer pair, whose product spans the whole *gtr* locus (*gtrA*, *gtrB*, and *gtrII*), was used for sequencing analysis. The PCR products of the *oacD*-2 primer pair were purified and cloned into the TA-pMD20T vector to generate the pSQZ6 expression plasmid. The recombinant plasmid was first transformed into commercial *E. coli* JM109 competent cells and then into *S. flexneri* strains, using a standard chemical protocol (33). The transformants were selected on LB plates supplemented with ampicillin (100 μg ml^{-1}) and confirmed by PCR amplification of the *oacD* gene. Oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). PCR amplifications were performed using a TaKaRa PCR amplification kit (TaKaRa, Japan) following a standard protocol.

TABLE 4 Primers used in this study

Primer	Primer sequence (5′–3′)	Length of the PCR fragment (bp)	Target gene(s) or reference sequence
oacD-1	F: GGGGCTGGCAAATTGTATCC R: GCCTATAATTGCTAAAGCCATAGG	822	*oacD*
oacD-2	F: GACCATGGTGCGAGAGTGGCAGG R: CTGGGCGAAGCATCAGGAAGGC	1,935	*oacD*
kan	F: GGTCACCTTGGGTTGGGGGCTGGCAAATTGTATCCTTTTGTTTTTAGTTAT CACGTCCCACTATTCTTTTTCGCTGCCACGTTGTGTCTCAAAATCT R: CAGGTATATATGATTGCGCAGATAGGATTTGGAATAGTCACGCTGA AGCCTATAATTGCTAAAGCCATAGGGCGTCCCGTCAAGTCAGCGTA	1,130	*kan* and *oacD*
oacD-3	F: GGATACAATTTGCCAGCCCCC R: CCCAGTACGCGGGTATCCCTCCC	3,775	*oacD*, *gtrB*
gtrII-1	F: ATTTATTGTTATTGGGGGTGGTTG R: ATTTGTTCTTTATTTGCTGGTT	1,268	*gtrII*
gtrII-2	F: TGAAAATTTTCTGGGGATCCCTCAG R: ATGGTGCCGATAATAGGAGTCGAAC	3,383	NC_004337

***oacD* gene functional deletion and complementation analysis.** Deletion of the *oacD* gene was performed on *S. flexneri* strain Sf301 using a one-step method as described previously (28, 34). The aminoglycoside 3′-phosphotransferase gene encoding kanamycin resistance (Km^r) was PCR amplified from plasmid pRS551 using the *kan* primer pair (Table 4). The PCR products were electroporated into strain Sf301 carrying plasmid pKOBEG and selected on an LB plate with chloramphenicol (50 μg ml^{-1}) and kanamycin (40 μg ml^{-1}). *oacD* gene deletion mutant Sf301Δ*oacD* was further confirmed by PCR amplification of *oacD* using the *oacD-1* and *oacD-2* primer pairs. Plasmid pSQZ6 was transformed into Sf301Δ*oacD*, 51571 (1a), and 51577 (4b), giving rise to *oacD*-complemented strains Sf301Δ*oacD*_pSQZ6, 51571_pSQZ6, and 51577_pSQZ6, respectively.

Preparation of specific antiserum 10 against a 6-O-acetylated GlcNAc-linked epitope. Immunization and preparation of antisera were performed as described previously (29, 35). Briefly, three New Zealand White rabbits (female, 1.5 to 2 kg body weight) were immunized intravenously with heat-killed cells of *S. flexneri* strain Sf301 twice a week at increasing doses (1×10^9, 2×10^9, 4×10^9, 8×10^9, 16×10^9, and 16×10^9 CFU). The serum was separated 1 week after the last immunization and mixed with heat-killed cells of *S. flexneri* isolate Sf301Δ*oacD* to absorb nonspecific antibodies that cross-react with other O-antigenic epitopes as described previously (35). The antiserum that agglutinated strain Sf301 but not Sf301Δ*oacD* is referred to as antiserum 10 below.

Serotyping analysis. The serological features of *S. flexneri* strains were revealed by a slide agglutination test using commercially available *Shigella* monovalent antisera (Denka Seiken, Japan) and monoclonal antibody (Reagensia AB, Sweden), as well as 3/4-O-acetylated Rha^{III}-specific antiserum 9 prepared previously (29). Antiserum 10 prepared in this study was used for detection of the 6-O-acetylated GlcNAc-linked epitope by slide agglutination, and agglutination apparent to the naked eye within 20 s was recorded as representing a positive result.

Western blot analysis. LPSs were prepared using an LPS extraction kit (iNtRON, South Korea) according to the manufacturer's instructions. The LPSs were electrophoresed on 15% polyacrylamide gels and detected by silver staining as described previously (36, 37). A Western blot assay of the LPS was performed as described previously (29). The LPS separated by SDS-PAGE was transferred onto a polyvinylidene difluoride (PVDF) membrane and incubated with antiserum 10. After washing, the membrane was incubated with anti-rabbit antibody labeled with fluorescent IRDye 800 (Rockland), and the fluorescence was detected using an Odyssey infrared imaging system (Li-COR).

O-polysaccharide isolation and structure analysis. Lipopolysaccharides of wild-type strains, mutants, and transformants were isolated by phenol-water extraction of bacterial cells (38). The crude extract without separation of the layers was dialyzed against tap water to remove phenol, nucleic acids and proteins were removed by adding aqueous 50% CCl_3CO_2H to the dialysis retentate with stirring at 4°C for 5 min to reach pH 2, the precipitate was discarded, and the LPS-containing supernatant was dialyzed against distilled water with two water changes over the period of 48 h and freeze-dried to yield purified LPS preparations in yields of 7.6% to 9.6% of dry bacterial mass. Each LPS was degraded with aqueous 2% acetic acid (HOAc) at 100°C, and the O-polysaccharides were isolated in yields of 18% to 51% of the LPS mass by gel permeation chromatography on Sephadex G-50 Superfine medium (Amersham Biosciences, Sweden)–0.05 M pyridinium acetate buffer, pH 4.5, monitored with a differential refractometer (Knauer, Germany).

Structures of the O-polysaccharides were elucidated using two-dimensional nuclear magnetic resonance (NMR) spectroscopy essentially as described previously (23). Assignment of the ^1H and ^{13}C NMR spectra was performed using correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and ^1H,^{13}C heteronuclear single-quantum coherence (HSQC) experiments. The assigned ^1H and ^{13}C NMR chemical shifts were essentially identical to published data for the correspondingly substituted Rha and GlcNAc residues (5, 24, 26, 28). Positions of *O*-acetyl groups were determined by characteristic downfield displacements of the NMR signals for ^1H and ^{13}C atoms at the O-acetylation sites (see Table S2 in the supplemental material) compared with the corresponding O-polysaccharides lacking the O-acetylation (5, 24). In particular, the signals for H-3 and C-3 of Rha^{III} were shifted from δ_H 3.86 and δ_C 71.2 to δ_H 5.07 to 5.08 and δ_C 73.6 to 74.2, H-4 and C-4 of Rha^{III} from δ_H 3.33 to 3.35 and δ_C 73.8 to 73.9 to δ_H 4.79 to 4.82 and δ_C 75.3 to 75.6, and H-6a,6b and C-6 of GlcNAc from δ_H 3.77 to 3.98 and δ_C 61.9 to 62.6 to δ_H 4.30 to 4.42 and δ_C 64.1 to 64.5, respectively. The degree of 6-O-acetylation was determined by the relative integral intensities of the ^1H NMR signals of the O-acetylated and non-O-acetylated GlcNAc residues.

Nucleotide sequence accession numbers. The genome sequences of *S. flexneri* strains 036 and HN006 have been deposited in GenBank under accession numbers CP004056 and CP004057, respectively.

RESULTS

Identification of an O-acyltransferase gene, *oacD*, that is present only in *S. flexneri* strains with 6-O-acetylation on GlcNAc. Until

now, three acyltransferase-encoding genes responsible for O-acetylation of *S. flexneri* O-antigens had been identified, including *oac* (which we have renamed *oacA* [28]) for the 2-O-acetylation of Rha^{I} (9, 10) and *oacB* and *oacC* for the 3/4-O-acetylation of Rha^{III} (28, 29) (see the supplemental material). We hypothesized that the 6-O-acetylation of GlcNAc in serotypes 2a, 3a, Y, and Yv is mediated by an acyltransferase that is encoded by an *oac* homolog. To identify it, the OacA, OacB, and OacC protein sequences were searched against the genome of *S. flexneri* Sf301 (serotype 2a), which possesses the 6-O-acetylation on GlcNAc, using BLASTp and tBLASTn searches. Several proteins homologous to the Oac proteins (see Table S3 in the supplemental material) were retrieved, and further searches of the genomes of *S. flexneri* strains HN006 and 51581 (both having the 6-O-acetylation on GlcNAc) and strains 2002017 and 036 (both lacking the 6-O-acetlyation) were performed. As a result, a hypothetical protein encoded by a chromosomal gene, SF0309, was found in strains Sf301, 51581, and HN006 but not in strains 2002017 and 036. The predicted protein showed homology to OacC, with 27% identity and 45% similarity in the amino terminal domain (amino acids 13 to 93), suggesting that it is responsible for the 6-O-acetylation of GlcNAc.

The protein encoded by SF0309 contained 349 amino acids and possessed conserved domains of the acyltransferase or acetyltransferase family (Acyl_transf_3 or NoIL). A BLAST search also revealed the presence of this protein in completely or partially sequenced *S. flexneri* strains 2457T (NP_706261.1), SFL124 (AY900451.1), K-1770 (WP_000282634.1), and 2747-71 (WP_000613535.1), serotype-converting bacteriophage SfII (YP_008318503.1), and *E. coli* strains KTE33 (WP_016159269.1), 1-176-05_S3_C2 (EYD87781.1), KTE-18 (WP_001579928.1), and UMEA 3703-1 (WP_001579928.1) with 98% to 100% identity at the protein and DNA levels. It also showed 24% to 41% identity to predicted acyltransferase family proteins of *Pantoea ananatis* PA13 (YP_005993804.1), *Clostridium cellulovorans* 743Bv (YP_003844488.1), *Leptospira kmetyi* (WP_020986752.1), and some other species. The suspected SF0309-encoded acetyltransferase was named OacD and the corresponding gene *oacD*, following the designations for OacA to OacC (28, 29) (see the supplemental material) mediating the 2-O- and 3/4-O-acetylations.

PCR screening was performed on 31 other *S. flexneri* strains with known O-antigen structures (Table 2), using the *oacD-1* primer pair. The expected PCR product (822 bp) was amplified from strains 51250 and G1663 (both serotype 2a), 51251 (serotype 2b), 51575 and G1665 (both serotype 3a), 51581 and G1040 (both serotype Y), and HN006, AH012, and HN011 (all serotype Yv); except for strain 51251, all possessed 6-O-acetylation (5, 23, 24). In contrast, the other strains tested that lacked 6-O-acetylation were *oacD* negative (Table 2). The whole *oacD* gene in 10 *oacD*-positive strains was PCR amplified and sequenced using the *oacD-2* primer pair. It was found that the *oacD* gene in the strains tested was identical to that of Sf301, except for strain 51251, whose *oacD* carried a one-base (A) deletion at position 191, resulting in a stop codon at amino acid 64.

The *oacD* gene is responsible for the 6-O-acetylation of GlcNAc in *S. flexneri* O-antigens. To confirm the function of *oacD*, a deletion and complementation analysis was performed on strain Sf301. The *oacD* gene from 168 to 902 bp from the start site of translation was replaced with the aminoglycoside 3′-phosphotransferase-encoding gene (*kan*) sequence, resulting in a dysfunctional *oacD* gene in the deletion mutant.

To construct the *oacD* expression vector, the entire *oacD* gene of 1,050 bp, together with 885-bp sequences upstream and downstream to include its potential promoter and terminator sequences, was cloned from strain Sf301 into plasmid pMD20T to construct plasmid pSQZ6. The latter was transformed into the Sf301Δ*oacD* deletion mutant and two wild-type *S. flexneri* strains lacking *oacD* and 6-O-acetylation but carrying 4- or 6-glucosylation on GlcNAc (serotype 1a strain 51571 and serotype 4b strain 51577; for the O-polysaccharide structures, see Table 5). A serological assay using *Shigella* antisera of Denka Seiken, Japan, and antiserum 9 (29) revealed no differences among the Sf301 parental strain, its Sf301Δ*oacD* deletion mutant, and complementation mutant Sf301Δ*oacD*_pSQZ6 (Table 6). Similarly, the pSQZ6 complementation mutants of the wild-type strains were serologically identical to the parental strains, except that 51577_pSQZ6 had acquired the type III antigenic determinant (Table 6).

The O-polysaccharides of the deletion and complemented mutants were isolated from the LPS and analyzed using NMR spectroscopy as described in Materials and Methods. The O-polysaccharide structures thus established are shown in Table 5. The NMR spectra of the Sf301Δ*oacD* deletion mutant lacked signals for the 6-*O*-acetyl group at δ_H 2.14 (Fig. 1A and B) and 6-O-acetylated GlcNAc, particularly those for C-6 and H-6a and H-6b, which were present in the spectra of the parental strain Sf301 at δ_C 64.8 and at δ_H 4.21 and 4.33. The signals for the 3- and 4-*O*-acetyl groups at δ_H 2.20 and 2.16, respectively, and the correspondingly acetylated Rha^{III} residues were present in the spectra of the mutant and were as intense as in the parental strain (~45% and ~25%, respectively). Therefore, the mutant O-antigen lost the 6-O-acetylation on GlcNAc, whereas the 3/4-O-acetylation on Rha^{III} was unaffected. Both *O*-acetyl modifications were present in Sf301Δ*oacD*_pSQZ6 (Fig. 1C) and, hence, the 6-O-acetylation was restored by transformation of Sf301Δ*oacD* with the *oacD*-carrying pSQZ6 plasmid. As in the parental strain, the 6-O-acetylation in the transformant was non-stoichiometric (~65%).

As judged by the appearance in the NMR spectra of minor signals for the 6-*O*-acetyl group at δ_H 2.14 (Fig. 1D to G), and 6-O-acetylated GlcNAc (δ_C 64.4 and δ_H 4.30 to 4.31 and 4.41 to 4.42), the pSQZ6 transformants of strains 51571 (1a) and 51577 (4b) acquired the 6-*O*-acetyl group on ~25% and ~30% GlcNAc residues, respectively (Table 5). In both cases, the degree of glucosylation on GlcNAc decreased correspondingly from 100% to ~75% and ~70%. In 51577_pSQZ6, the incomplete 6-glucosylation is the basis for the manifestation of the type III antigenic determinant, which is associated with the 2-O-acetylation on Rha^{I} but is blocked by glucosylation on GlcNAc in wild-type serotype 4b strains.

The *oacD* gene was carried on serotype-converting bacteriophage SfII, which is present as a prophage in serotype 2 (2a and 2b) or as a cryptic prophage with a dysfunctional *gtr* locus in other serotypes. The genomic regions flanking the *oacD* gene in sequenced strains of *S. flexneri* (Sf301, 2457T, 2747-71, HN006, and 51581) and *E. coli* (KTE33 and 1-176-05_S3_C2) were analyzed, and their genetic organizations are shown in Fig. 2. In all cases, the *oacD* was located downstream of the *gtr* locus (*gtrA*, *gtrB*, and *gtrII*) of the SfII prophage. One insertion sequence (IS_{sf13}) and two genes encoding phage tail fiber proteins were located upstream and downstream of *oacD*, respectively. This structure of *gtr*-IS-*oacD*-phage tail genes is identical in both genetic organization and DNA sequence to that of serotype-converting bacterio-

TABLE 5 Structures of the O-polysaccharides of *S. flexneri* strains studied

Strain (serotype)	O-polysaccharide structure	Reference and/or source
Sf301 (2a), Sf301Δ*oacD*_pSQZ6	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |3/4 ↑4 |6 ~45%/25% OAc α-D-Glc*p* ~65% OAc	28 and this work
Sf301Δ*oacD*	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |3/4 ↑4 ~45%/25% OAc α-D-Glc*p*	This work
51571 (1a)	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |3/4 ↑4 ~55%/25% OAc α-D-Glc*p*	24
51571_pSQZ6	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |3/4 ↑4/6 ~55%/30% OAc R R = 4-linked α-D-Glc*p* (~75%) or 6-linked OAc (~25%)	This work
51577 (4b)	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |2 ↑6 OAc α-D-Glc*p*	24
51577_pSQZ6	→2)-α-L-Rha*p*^{III}-(1→2)-α-L-Rha*p*^{II}-(1→3)-α-L-Rha*p*^{I}-(1→3)-β-D-Glc*p*NAc-(1→ |2 ↑6 OAc R R = α-D-Glc*p* (~70%) or OAc (~30%)	This work

phage SfII (NC_021857.1), a free particle that has been induced from serotype 2a strain NCTC4 (39) (Fig. 2).

The *oacD* region from serotype 3a strains 51575 and G1665 was PCR amplified and sequenced using complementation of the *oacD-3* primer pair to genes *gtrB* and *oacD* (Fig. 2). The 2,295-bp PCR products obtained were shorter than the 3,775-bp PCR product from reference strain Sf301. The two PCR products were identical and differed from that of Sf301 in the middle of the sequence, with a 1,675-bp insertion between *gtrB* and *oacD*, resulting in a shorter GtrB product and complete deletion of *gtrII* and the region up to IS$_{sf13}$ (Fig. 2). The 1,675-bp fragment encoded two putative proteins of unknown function and showed no homology to any sequence in the NCBI database. A similar genomic structure was found in partially sequenced *S. flexneri* strain K-1770 (Fig. 2). Therefore, a cryptic SfII prophage is present in these three strains.

The presence of the conserved *gtr*-IS$_{sf13}$-*oacD* structure in both the free SfII phage particle and *oacD*-positive strains suggested that *oacD* originated from phage SfII and therefore should occur in all prophage SfII-carrying strains. To confirm this, 183 serotype 2 strains (154 of 2a and 29 of 2b) were tested by PCR amplification of the *oacD* gene using the *oacD-1* primer pair, and all were found to be *oacD* positive (Table 3). Further screening of 488 *S. flexneri* isolates of various other serotypes showed that all were *oacD* negative, except for 31 strains belonging to serotypes 3a (2 strains), Xv (1), X (1), Y (14), and Yv (13) (Table 3).

Previously, we showed that all 13 *oacD*-positive strains of serotype Yv carry a dysfunctional *gtrII* locus with either one or both of the mutations in *gtrII* (position 1,222) and *gtrB* (position 560) (21). Therefore, it was concluded that these Yv strains originated from serotype 2a strains and, hence, should carry a cryptic SfII prophage. PCR detection of the SfII-specific *gtrII* gene using the *gtrII-1* primer pair showed that 18 other *oacD*-positive non-serotype 2 strains were *gtrII* positive, except for serotype 3a strains 51575 and G1665, which had lost the *gtrII* gene (see above). In 10 serotype Y strains and 1 strain each of serotypes X and Xv, the *gtrII* had frameshift mutations due to a base deletion, insertion, or substitution (see footnote to Table 3), rendering it defective. Four *gtrII*-positive serotype Y strains had no defect in *gtrII* but did have

TABLE 6 Serological features of *oacD* gene deletion and complementation mutants of *S. flexneri*

Strain	Reactivity with type and group antisera of Seiken										
	I	II	III	IV	V	VI	3,4	6	7,8	9[a]	10[b]
Sf301 (2a)	−	+	−	−	−	−	+	−	−	+	+
Sf301Δ*oacD*	−	+	−	−	−	−	+	−	−	+	−
Sf301Δ*oacD*_pSQZ6	−	+	−	−	−	−	+	−	−	+	+
51571 (1a)	+	−	−	−	−	−	+	−	−	+	−
51571_pSQZ6	+	−	−	−	−	−	+	−	−	+	+
51577 (4b)	−	−	−	+	−	−	−	+	−	−	−
51577_pSQZ6	−	−	+	+	−	−	−	+	−	−	+

[a] Antiserum 9 was prepared previously (29).
[b] Antiserum 10 was prepared in this work.

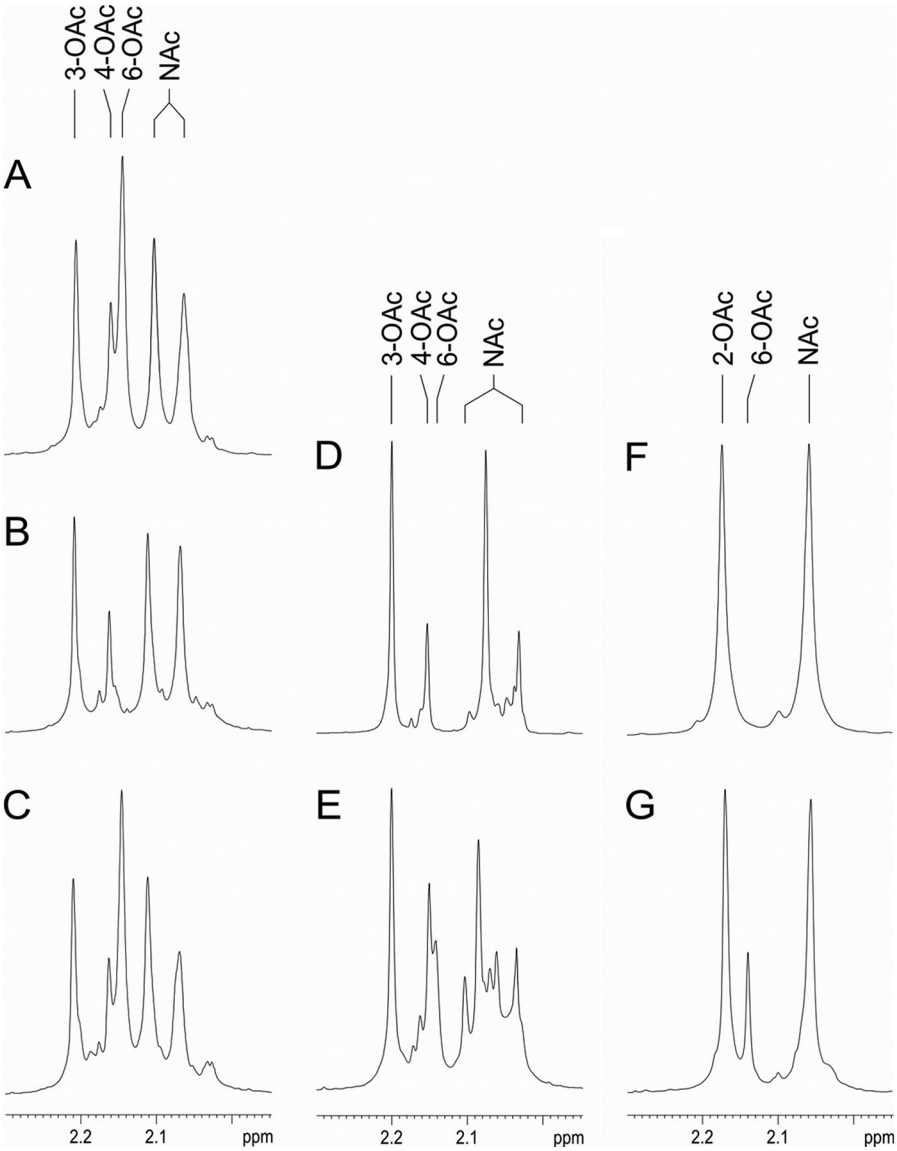

FIG 1 Parts of ^{1}H NMR spectra of the O-polysaccharides from wild-type strains and transformants showing signals for *O*-acetyl and *N*-acetyl groups. A, Sf301; B, Sf301Δ*oacD*; C, Sf301Δ*oacD*_pSQZ6; D, 51571; E, 51571_pSQZ6; F, 51577; G, 51577_pSQZ6. 2-OAc, 3-OAc, 4-OAc, and 6-OAc indicate *O*-acetyl groups at position 2 of Rha^{I}, position 3 of Rha^{III}, position 4 of Rha^{III}, and position 6 of GlcNAc, respectively. The presence of multiple signals for the *N*-acetyl group (NAc) reflects structural heterogeneity due to nonstoichiometric O-acetylation and (in strain 51571_pSQZ6) nonstoichiometric glucosylation.

a nonsynonymous mutation in the *gtrB* gene, which gave rise to an amino acid change at position 187 and might be responsible for the defective *gtrII*-based type II glucosylation.

Based on these data, we conclude that the *oacD* gene was carried on the phage SfII genome, which was integrated into the host chromosome by lysogeny to form a prophage. As a result, the *oacD* gene always coexists with the *gtr* locus in serotype 2 strains and is also present in strains of some other serotypes carrying a cryptic SfII prophage with a defective *gtr* locus. This conclusion was confirmed by serological studies described in the next section.

OacD-mediated 6-O-acetylation on GlcNAc confers to the host a new antigenic determinant. Rabbit polyclonal antiserum specific to a putative new epitope(s) associated with 6-O-acetylated GlcNAc was prepared using serotype 2a strain Sf301 carrying 6-O-acetylation on GlcNAc for immunization and its 6-O-acetylation-lacking *oacD* deletion mutant for absorption of the crude antiserum. The absorption eliminated cross-reactive antibodies to all O-antigen-linked epitopes except for the target one. After repeated absorptions, the antiserum obtained only agglutinated strain Sf301 but not the Sf301Δ*oacD* deletion mutant (Table 6). It was called grouping antiserum 10.

The specificity of antiserum 10 was characterized by an immunoblotting assay performed with the LPS of Sf301, its deletion mutant Sf301Δ*oacD*, and complementation mutant Sf301Δ*oacD*_pSQZ6 as well as serotype 1a strain 51571 and its complementation mutant 51571_pSQZ6. The LPS samples were resolved by SDS-PAGE on a 15% gel and visualized by silver staining (Fig. 3A). A typical ladder-like banding pattern of an LPS with an O-antigen composed of various numbers of O-units was observed for all strains (Fig. 3A). Differences were found in the LPS profiles of the

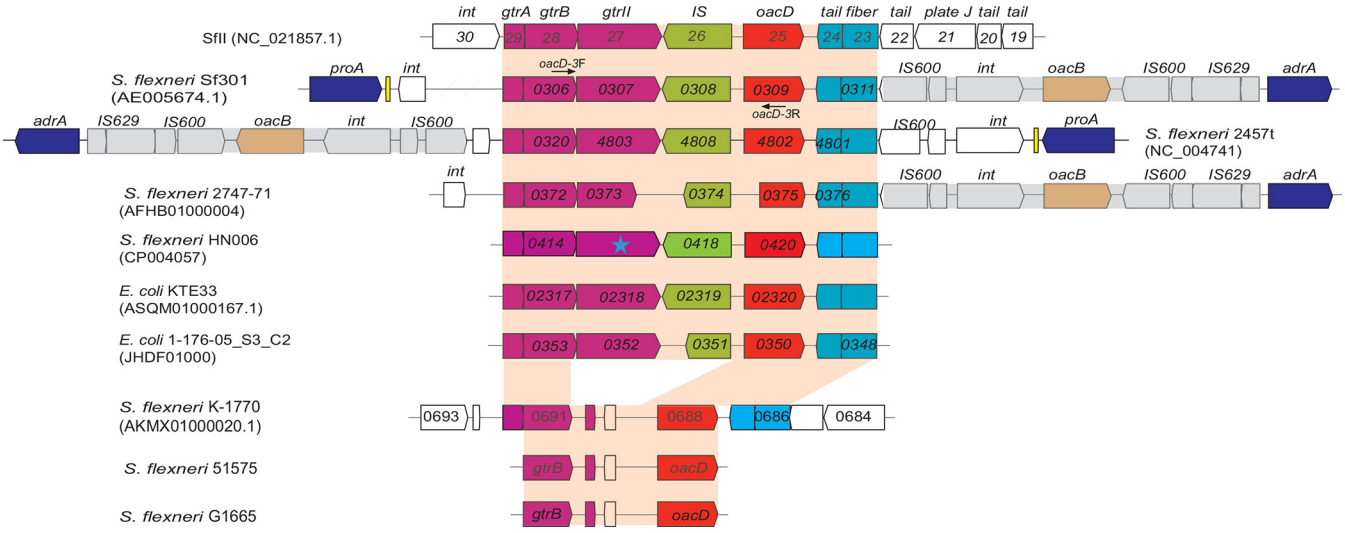

FIG 2 Genetic organizations of the genomic regions of SfII, *S. flexneri*, and *E. coli* strains carrying the *oacD* gene. Sequences of serotype-converting phage SfII, *S. flexneri* strains Sf301, 2457T, 2747-71, HN006, and K-1770, and *E. coli* strains KTE33 and 1-176-05_S3_C2 were obtained from the NCBI database. Genomic sequences of serotype 3a strains 51575 and G1665 were obtained by PCR amplification. The open reading frames (ORFs) were annotated as submitted in NCBI or predicted using ORF Finder and are shown as thick arrows. The locus tags are shown within arrows, and the encoding proteins are listed above. The conserved genes are shown in different colors: *proA* and *adrA*, dark blue; *gtr* locus, pink; IS_{sf13}, green; *oacD*, red; tail fiber light blue. The conserved *oacD*-carrying prophage genome and *oacB*-carrying transposon are highlighted in red and gray, respectively. The defective *gtrII* gene in strain HN006 is marked with a blue star.

parental strains and constructed mutants, with the complementation mutants (Sf301Δ*oacD*_pSQZ6 and 51571_pSQZ6) having more rough- and semirough LPS than the parental strains (Fig. 3A). We do not know the exact mechanism causing this difference, and further studies are needed. In Western blot analyses, antiserum 10 reacted only with the ladder-like LPS bands of Sf301, 51571_pSQZ6, and Sf301Δ*oacD*_pSQZ6 carrying the 6-O-acetylation on GlcNAc but did not recognize LPS of 51571 and Sf301Δ*oacD* lacking this modification (Fig. 3B). This serological specificity was further confirmed by an agglutination assay performed on other *oacD* transformants, which indicated that antiserum 10 reacted only with *oacD*-carrying transformants Sf301Δ*oacD*_pSQZ6, 51571_pSQZ6, and 51577_pSQZ6 but not with *oacD*-lacking strains 51571 and 51577 (Table 6). Therefore, antiserum 10 is specific to a 6-O-acetylated GlcNAc-linked epitope(s) on the O-antigen.

The 672 strains used for the *oacD* PCR detection were tested by slide agglutination, and a good correlation between the presence of the functional *oacD* gene and the antiserum 10 reactivity was observed. The agglutinated strains (206 in total, all *oacD* positive) belonged to serotypes 2a (154 strains), 2b (23), 3a (2), X (1), Y (13), and Yv (13) (Table 3). Except for 8 strains (6 of serotype 2b, 1 Xv, and 1 Y), all *oacD*-carrying strains were antiserum 10 positive, whereas all *oacD*-lacking strains were negative (Table 3). The *oacD* gene in the 8 aberrant strains was amplified and sequenced using the *oacD-3* primer pair, and all were found to carry a dysfunctional *oacD* gene, with serotype 2b strain 2005001 having a one-base (T) insertion at position 177 and the others having a one-base (A) deletion at position 191; all resulted in a stop codon at amino acid 59 or 64, rendering the protein defective in these isolates.

The data obtained demonstrate that the 6-O-acetylation on GlcNAc confers to the host a previously unknown O-antigen epitope(s). Following the designations for *S. flexneri* group O-factors 3,4, O-factor 6, O-factors 7,8, and O-factor 9, we name the new antigenic determinant group O-factor 10 and suggest that it should be included in the current serotyping scheme of *S. flexneri* for antiserum 10-positive strains carrying the 6-O-acetylation on GlcNAc.

A total of 54 strains of other species were tested by slide agglutination and found to be antiserum 10 negative, except for *E. coli* strains 042 (serotype O44) and G1237 (serotype O13) as well as 12 strains of *S. sonnei* phase II (see Table S1 in the supplemental material). *E. coli* O13 reacted with antiserum 10, evidently because it has a *S. flexneri* serotype 2a-like O-antigen structure with 6-O-acetylation on GlcNAc (25). The positive *oacD* gene PCR amplification confirmed that a similar *oacD*-mediating mechanism is involved in this modification in *E. coli* O13. In contrast, the O-antigen of *E. coli* O44 is devoid of 6-O-acetylation on GlcNAc (40), and *S. sonnei* phase II has no O-antigen; therefore, further studies are necessary to elucidate the structural basis for their reactivity with antiserum 10.

DISCUSSION

Modifications of the O-antigen of *S. flexneri* give rise to the expression of enormously diverse O-antigenic determinants on the same O-polysaccharide backbone. So far, the following molecular factors involved with these modifications have been revealed: (i) serotype-converting prophages carrying a *gtr* locus for glucosylation of various monosaccharides or the single *oac* (*oacA*) gene for 2-O-acetylation of Rha^{I} (6); (ii) transposon-like elements carrying *oacB* or *oacC* genes for 3/4-O-acetylation of Rha^{III} (28) (see the supplemental material); and (iii) plasmids carrying an *optII* or *optIII* gene for PEtN phosphorylation of Rha^{II} and/or Rha^{III} (20, 23). One can speculate that the coexistence of several O-antigen modification mechanisms allows *S. flexneri* to change rapidly the antigenic landscape to escape the serotype-specific host immunity and to promote the spread of shigellosis in the human population.

In this work, it was found that serotype-converting bacteriophage SfII, which possesses the *gtrII* locus responsible for the 4-glucosylation of Rha^{I}, also carries the *oacD* gene and that the

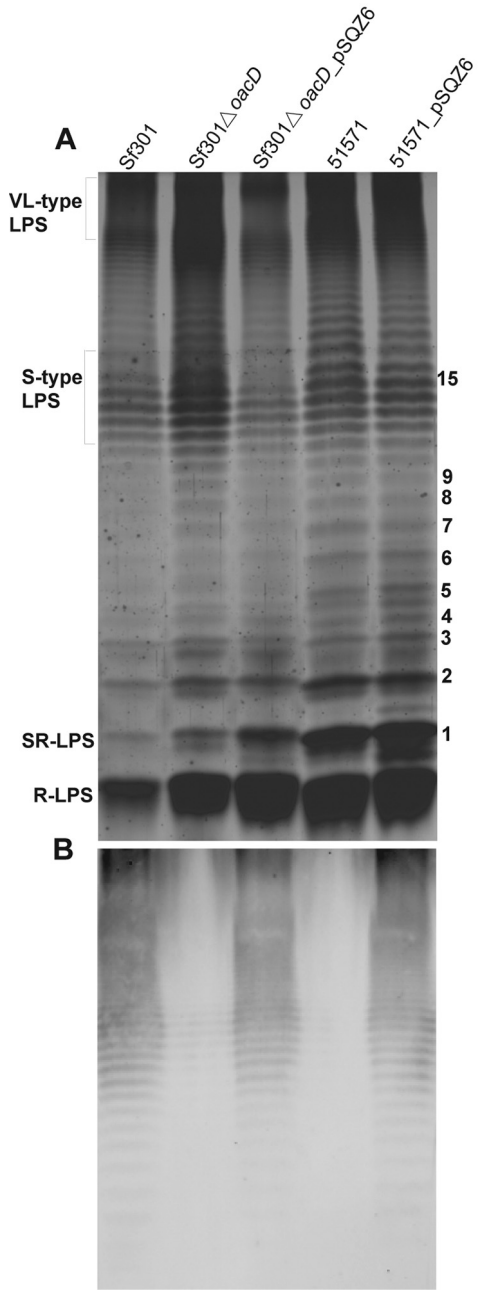

FIG 3 SDS-PAGE and Western blot analysis of the LPS of *S. flexneri* Sf301, its deletion mutant Sf301Δ*oacD* and complementation mutant Sf301Δ*oacD*_pSQZ6, and serotype 1a strain 51571 and its *oacD* transformant 51571_pSQZ6. (A) Silver-staining detection of LPS profiles on 15% polyacrylamide gels. The positions of LPSs of various types are indicated on the left. R-LPS, rough LPS; SR-LPS, semirough LPS; L-type LPS, long-type LPS; VL-type LPS, very-long-type LPS. The numbers of O-units are indicated on the right. (B) Immunoblotting detection of LPS with antiserum 10. The LPSs separated by SDS-PAGE were transferred onto a PVDF membrane and hybridized with grouping antiserum 10. An anti-rabbit antibody labeled with fluorescent IRDye 800 (Rockland) was used as the secondary antibody. Fluorescence was detected using an Odyssey infrared imaging system (Li-COR).

corresponding prophage is responsible for the 6-O-acetylation of GlcNAc of *S. flexneri*. The following data elucidated the role of *oacD* in modification of *S. flexneri* O-antigens: (i) the occurrence of conserved domains of the acyltransferase family (Acyl_transf_3) in the predicted OacD protein and its similarity to acyltransferases; (ii) a clear correlation between the presence of the functional *oacD* gene and the 6-O-acetylation on GlcNAc in the O-antigen; (iii) deletion of *oacD* resulting in the loss of the 6-O-acetylation and the finding that cloned *oacD* mediated the 6-O-acetylation upon transformation; and (iv) the finding that naturally occurring dysfunctional mutations in *oacD* in some strains abolished the 6-O-acetylation.

This is the first report showing that one serotype-converting phage carries two factors involved in different types of O-antigen modifications in *S. flexneri*. Being located in the SfII genome, the *oacD* gene may be transferred, together with the *gtrII* locus, by the SfII infection mechanism among *S. flexneri* strains in nature. The coexistence of the *gtrII* locus and *oacD* in SfII might indicate that the two factors are functionally dependent, but the presence of a dysfunctional *gtrII* locus in 6-O-acetylation-positive strains showed that this modification can be done without the assistance of the *gtr* locus. This is supported by the acquisition of the 6-O-acetylation-positive phenotype upon transformation with the *oacD* expression vector of serotype Y strain 036, which has no known serotype-converting phage within the genome (our unpublished data).

The serotype-converting SfII bacteriophage is integrated into the host chromosome at the tRNA-*thrW* site located between conserved genes *proA* and *adrA* by lysogeny (6, 13). Our previous work demonstrated that the *oacB*-carrying transposon responsible for the 3/4-O-acetylation of Rha^{III} maps upstream of the *adrA* gene (28). Therefore, the genetic elements involved in glucosylation and two types of O-acetylation are located in the *proA-adrA* region in serotype 2 strains, suggesting that this site is the hot spot for mobilization of O-antigen modification factors in *S. flexneri*.

In various *S. flexneri* serotypes, the same monosaccharide may carry different chemical groups; for instance, a glucosyl, *O*-acetyl, or PEtN group is attached at position 3 of Rha^{III} in serotypes X, 1, and 4av, giving rise to O-factors 7,8, O-factor 9, and O-factor IV-1, respectively (8, 20, 29). Earlier, we demonstrated that the 3-glycosyl group on Rha^{III} in serotype 2b and X strains can be completely or (in serotype X) almost completely replaced with the 3- or 4-*O*-acetyl group (28) upon transformation with the *oacB* gene for the specific acetyltransferase. In this work, a similar replacement of the glucosyl group on GlcNAc, whether it occurred at position 4 or 6, with the 6-*O*-acetyl group was observed upon transformation of serotype 1a and 4b strains with the *oacD* gene. However, the replacement was only partial and the degree of 6-O-acetylation (~25% to ~30%) was lower than in *S. flexneri* strains with the natural occurrence of this modification (40% to 65%) (5, 24). Therefore, the interrelations between O-acetylation and glucosylation are distinct on different monosaccharides mediated by different acetyltransferase proteins: in the case of GlcNAc glucosylation and OacD-mediated O-acetylation, the modifications proceeded competitively, whereas on Rha^{III}, the OacB-mediated O-acetylation suppressed glucosylation. This competitiveness or suppression may be due to a high expression level of the acetyltransferase proteins as, in both cases, the cloned *oac* gene dosage is much higher (>500) than that of the chromosomal *gtr* locus.

Like the 3/4-O-acetylation on Rha^{III}, the 6-O-acetylation on GlcNAc confers to the host an additional O-antigen epitope, which had been neglected earlier owing to the absence of specific antiserum. This new epitope, which we provisionally name O-factor 10, is expressed in all serotype 2 strains as well as in strains of

other serotypes that carry a cryptic SfII phage with a dysfunctional *gtr* locus. It should be noted that serotype 2 is one of the most predominant serotypes in China and other countries (41, 42), and the possibility is not excluded that the coexistence of 4-glucosylation on Rha^I and 6-O-acetylation on GlcNAc, and coexpression of the corresponding type II and group 10 antigenic determinants, contributes to the prevalence of this serotype in nature.

Conventional *Shigella* serotyping tools revealed the same LPS serological pattern for each O-factor 10-positive strain and its negative counterpart; hence, the 6-O-acetylation on GlcNAc does not affect other antigenic determinants. Therefore, it would be expedient to add the group O-factor 10 into the current serotyping scheme of *S. flexneri* and to use antiserum 10 for detection of strains carrying the functional *oacD* gene and, accordingly, the 6-O-acetylation on GlcNAc.

The findings of this work enhance our understanding of the serotype conversion mechanisms in *S. flexneri*, which will assist epidemiological monitoring of *S. flexneri* and development of vaccines against shigellosis. Further studies are necessary to gain an insight into the impact of the expression of the 6-O-acetylation-linked epitope on the pathogenicity of *S. flexneri*.

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