Journal of Bacteriology

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J. Bacteriol. **2014, 196(8):1525. DOI: 10.1128/JB.01393-13. Published Ahead of Print 7 February 2014.**

JB Journals.ASM.org

Identification of an *O*-Acyltransferase Gene (*oacB*) That Mediates 3- and 4-O-Acetylation of Rhamnose III in *Shigella flexneri* O Antigens

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O antigen (O polysaccharide) is an important and highly variable cell component present on the surface of cells which defines the serospecificity of Gram-negative bacteria. Most O antigens of *Shigella flexneri*, a cause of shigellosis, share a 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 substituents, giving rise to 19 serotypes. The known modifications include glucosylation on various sugar residues, O-acetylation on Rha^{I}, and phosphorylation with phosphoethanolamine on Rha^{II} or/and Rha^{III}. Recently, two new O-antigen modifications, namely, O-acetylation at position 3 or 4 of Rha^{III} and position 6 of GlcNAc, have been identified in several *S. flexneri* serotypes. In this work, the genetic basis for the 3/4-O-acetylation on Rha^{III} was elucidated. Bioinformatic analysis of the genome of *S. flexneri* serotype 2a strain Sf301, which carries 3/4-O-acetylation on Rha^{III}, revealed an O-acyltransferase gene designated *oacB*. Genetic studies combined with O-antigen structure analysis demonstrated that this gene is responsible for the 3/4-O-acetylation in serotypes 1a, 1b, 2a, 5a, and Y but not serotype 6, which has a different O-antigen backbone structure. The *oacB* gene is carried by a transposon-like structure located in the *proA-adrA* region on the chromosome, which represents a novel mechanism of mobilization of O-antigen modification factors in *S. flexneri*. These findings enhance our knowledge of *S. flexneri* O-antigen modifications and shed light on the origin of new O-antigen variants.

Shigella flexneri is the major cause of shigellosis in developing countries. It is estimated that there are 125 million shigellosis cases annually in Asia, with 14,000 deaths, the majority of which are children under 5 years of age (1). The O-polysaccharide chain of the lipopolysaccharide, called O antigen, is an important and highly variable cell component present on the surface, and it provides the chemical basis for serotyping of *S. flexneri*. Except for serotype 6, all *S. flexneri* serotypes share a polysaccharide backbone composed of the tetrasaccharide repeats (O units) of three rhamnose (Rha^{I} to Rha^{III}) and one 2-acetamido-2-deoxy-D-glucose (GlcNAc) [→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). The backbone can be modified by adding various chemical groups to different sugars, giving rise to diverse O-antigen structures and, correspondingly, to various serotypes (3).

The first two O-antigen modification types identified in *S. flexneri* were O-acetylation at position 2 of Rha^{I} (2-O-acetylation) and glucosylation at different positions on each of the four sugar residues in the O-unit (3). 2-O-acetylation occurs in serotypes 1b, 3a, 3b, 4b, and 7b and confers on the host a group 6 antigenic determinant (O-factor 6) (4, 5). A single O-acetyltransferase-encoding gene (*oac*) carried by the temperate bacteriophage Sf6 mediates the 2-O-acetylation (6, 7). Glucosylation defines type I, IC, II, IV, and V antigenic determinants as well as the group 7,8 antigenic determinant in various serotypes (3, 8). Factors responsible for glucosylation are arranged in a single operon known as the *gtr* gene cluster, with 3 genes, including *gtrA*, *gtrB* (both conserved), and *gtr* (type specific), carried by bacteriophages SfI, SfIC, SfII, SfIV, SfV, and SfX (7–14). The Sf6 and SfIC genomes are integrated into the host chromosome at the *tRNA-argW* site adjacent to a conserved gene, *yfdC*, and at the site adjacent to the *yejO* locus, respectively (8, 15). The other serotype-converting phages map to *tRNA-thrW*, located between genes *proA* and *adrA* (3, 16).

A third known type of modification is the addition of phosphoethanolamine (PEtN) to position 3 of either Rha^{III} or Rha^{II} or both (17–20). The PEtN group confers the host with monoclonal antibody MASF IV-1 reactivity and defines the variant (v) factor in newly named serotypes Xv, 4av, and Yv (17–20). A single gene, *opt* (O-antigen phosphoethanolamine transferase gene), which is carried on a 6.8-kb plasmid (pSFxv_2 or pSFyv_2), mediates the PEtN modification (17, 18, 20).

Recently, two new O-antigen modifications have been identified: (i) O-acetylation at either position 3 or position 4 of Rha^{III} (3/4-O-acetylation) in serotypes 1a, 1b, 2a, 5a, Y, and 6 and (ii) O-acetylation at position 6 of GlcNAc (6-O-acetylation) in serotypes 2a, 3a, Y, and Yv (2, 21–23). The degree of the 3/4-O-acetylation varies not only between serotypes but also between strains of the same serotype in the range of 30 to 70% at position 3 and 15 to 30% at position 4 (2, 24). The latter variation likely is due to storage and/or cultivation conditions. Further studies indicated that the 3/4-O-acetylation on Rha^{III} interfered with the PEtN

Received 3 December 2013 **Accepted** 30 January 2014

Published ahead of print 7 February 2014

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.01393-13.

doi:10.1128/JB.01393-13

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
S. flexneri strains		
Sf301	Serotype 2a reference strain used for *oacB* gene cloning and inactivation analysis, Ap^s Km^s	NC_004337
301Δ*oacB*	Sf301 with the *oacB* gene replaced by the kanamycin resistance gene (*kan*) from pSR551, Km^r Ap^s	This work
301Δ*oacB*_pSQZ4	301Δ*oacB* transformed by plasmid pSQZ4	This work
51521	Serotype 2b strain used as host for the *oacB* gene function analysis, Ap^s	24
51521_pSQZ4	51251 transformed by plasmid pSQZ4	This work
51580	Serotype X strain used as host for the *oacB* gene function analysis, Ap^s	24
51580_pSQZ4	51580 transformed by plasmid pSQZ4	This work
E. coli DH5α	Used for plasmid propagation and gene cloning	TaKaRa
Plasmids		
pMD20T	TA vector, Ap^r	TaKaRa
pSR551	Km^r, used for *kan* gene cloning	31
pKOBEG	Thermosensitive replicon that carries the λ phage *red*γβα operon expressed under the control of the arabinose-inducible pBAD promoter	32
pSQZ4	pMD20T carrying the whole sequence of the *oacB* gene from strain Sf301, Ap^r	This work

phosphorylation at the same sugar residue and reduced the MASF IV-1 determinant manifestation (24). Until now, the genetic basis for the 3/4-O-acetylation on Rha^{III} and 6-O-acetylation on GlcNAc remained unknown.

O-antigen modifications are considered an important virulence factor. The host immune response has been shown to be serotype specific (3). Thus, antigenic diversity enhances the bacterium's survival, as it helps the bacterium escape the host defense (3). Furthermore, some modifications, such as glucosylation on GlcNAc, Rha^{I}, and Rha^{II}, have been demonstrated to promote bacterial invasion into host cells mediated by the type III secretion system (25). Therefore, elucidation of the 3/4-O-acetylation mechanism is important for understanding the *S. flexneri* antigenicity and pathogenicity. In this study, we identified a homolog of the *oac* gene for O-acyltransferase, designated *oacB*, and demonstrated that this gene is responsible for the 3/4-O-acetylation in *S. flexneri* serotypes 1a, 1b, 2a, 5a, and Y but not serotype 6. *oacB* was carried by a transposon-like structure located upstream of the *adrA* gene on the chromosome.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culturing conditions. Strains and plasmids used in this study are listed in Table 1. *S. flexneri* strain Sf301 (serotype 2a) with 3/4-O-acetylated Rha^{III} in the O antigen (Fig. 1) was used as the reference strain for *oacB* gene amplification and deletion analysis. *S. flexneri* strains 51251 (serotype 2b) and 51580 (serotype X), lacking O-acetylation on Rha^{III} (Fig. 1), were employed as hosts for the *oacB* gene transformation analysis. Thirty-one *S. flexneri* strains with known O-antigen structures (see Table S1 in the supplemental material) were used for *oacB* gene PCR detection analysis. They were either clinical isolates from our collection, purchased from the National Collection of Type Cultures (NCTC), or kindly donated by B. Liu (Nankai University, Tianjin, China). *Escherichia coli* JM109 was used for plasmid propagation. pMD20T vector (TaKaRa, Japan) was used for DNA sequencing and *oacB* gene function analysis. Plasmid pRS551 was used for kanamycin resistance gene amplification. pKOBEG, encoding a homologous recombination system, was used in *oacB* gene deletion analysis. Strains were grown in a 37°C incubator or orbital shaker in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg ml^{-1}), kanamycin (40 μg ml^{-1}), or chloramphenicol (50 μg ml^{-1}) when appropriate.

Bioinformatics analysis. The protein sequence of the O-acetyltransferase Oac (accession no. NP_958191.1) of bacteriophage Sf6 was compared to the genome of *S. flexneri* strain Sf301 using tBLASTn and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST). Searches for homologs to the gene *oacB* in the NCBI database were carried out using the BLASTP search engine.

DNA techniques. Chromosomal DNA was isolated from *S. flexneri* strains using a DNA extraction kit according to the manufacturer's instructions (Qiagen, Germany). Primers used in this study are listed in Table 2. Primer pair *oacB*-1 was used for *oacB* gene detection. Primer pair *oacB*-2 was used for *oacB* gene function analysis. Primer pair *oac-adrA* was used to amplify regions downstream of *oacB* in *S. flexneri* strains 019 (serotype 1a), G1662 (1b), G1036 (5a), 51581, and G1040 (both Y). Primers *spr1*, *spr2*, and *spr3*, which are complementary to the reverse sequence of *oacB*, were used in genomic walking PCR to analyze the regions up-

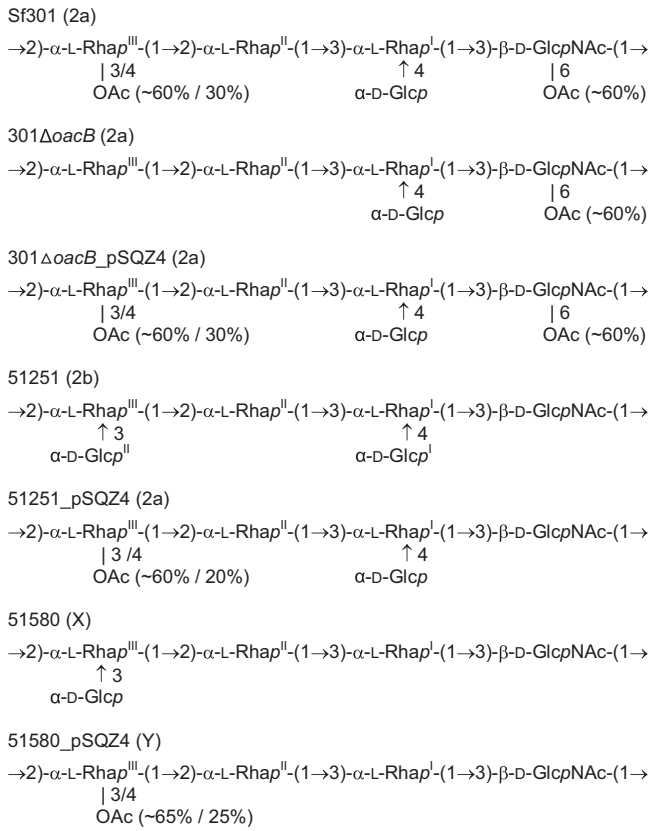

FIG 1 Structures of the O polysaccharides of *S. flexneri* strains studied. In addition to the major structure shown, a negligible amount (<5%) of glucosylated structure characteristic of serotype X was found in transformant 51580_pSQZ4, indicating incomplete blocking of glucosylation by O-acetylation on Rha^{III}.

TABLE 2 Primers used in this study

Primer	Primer sequence (5′–3′)	Target gene(s)
oacB-1		
Forward	TCATCTGGAGTATGGGAAG	*oacB*
Reverse	CAAAGAATCAGTGGTAGCG	
oacB-2		
Forward	GGTGTGTCTCCGTTTTGTTTC	*oacB*
Reverse	CGACGTTGCTACTGGTGTTTC	
kan		
Forward	GCAGGAATAATCAAATAGATGGAATGCG GGGGTTCTTAGCAATTTTCGTGCTTATTC ATCACGCACGTTGTGTCTCAAAATCT	*kan* and *oacB*
Reverse	CACGTCATTAGGCAATAAAGGAATATCC CATGCAGAAGGTAAACGCTGTAGGTAGT TTCTCCTAGCTTTCTGCGTCCCGTCAAGT CAGCGTA	
oacB-adrA		
Forward	ACCAGAAAGCTAGGAGAAACTAC	*oacB*, *adrA*
Reverse	GCAATCGGTAAGAACATGCCAG	
spr		
spr1	GCTAACAGATTTGATGAAGGTGCTTCCC	*oacB*
spr2	GCTAAGAACCCCCGCATTCCATC	
spr3	CCCCACAGCTAATAACGACATC	

stream of *oacB*. Oligonucleotide primers were synthesized by Sangon Biotech (Shanghai). PCR amplifications were performed using a TaKaRa PCR amplification kit (TaKaRa, Japan) by following a standard protocol. PCR products using primer pair *oacB*-2 were purified and cloned into the pMD20T vector (TaKaRa, Japan) to generate the *oacB* expression plasmid pSQZ4. The recombinant plasmid was first transformed into commercial *E. coli* DH5α competent cells (TaKaRa, Japan) and then into *S. flexneri* strains 51251 and 51580 using a standard chemical protocol (26). The transformants were selected on LB plates supplemented with ampicillin (100 μg ml^{-1}) and confirmed by PCR amplification of the *oacB* gene.

To determine whether an *oacB* homolog is present in serotype 6 strains, Southern hybridization was carried out. Genomic DNA extracted from serotype 6 strains was digested with the restriction enzyme NotI and subjected to agarose gel electrophoresis. The separated DNA was transferred onto a nylon membrane (Amersham Biosciences, Sweden) using a vacuum blotter (Bio-Rad, Hercules, CA). Hybridization was performed using an ECLTM direct nucleic acid labeling and detection system (Amersham) as recommended by the manufacturer. DNA product amplified from strain Sf301 using primer pair *oacB*-1 was prepared for a biotin-labeling DNA probe. The hybridization temperature was 42°C under 50% formamide.

***oacB* gene functional deletion and complementation analysis.** Deletion of the *oacB* gene was performed on *S. flexneri* strain Sf301 using a one-step method as described previously (27). The Km^r gene was PCR amplified from plasmid pRS551 using the *kan* primer pair (Table 2). 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}). The *oacB* gene deletion mutant 301Δ*oacB* was confirmed by PCR amplification of *oacB* using primer pairs *oacB*-1 and *oacB*-2 (Fig. 2). Plasmid pSQZ4 was transformed into mutant strain 301Δ*oacB*, giving rise to *oacB*-complemented strain 301Δ*oacB*_pSQZ4.

Serotyping. Serological features of *S. flexneri* wild-type strains and transformants were determined by slide agglutination test using a commercially available monovalent antiserum kit (Denka Seiken, Japan) specific for all known type and group O factors of *S. flexneri* O antigens.

O-polysaccharide isolation and structure analysis. Lipopolysaccharides of wild-type strains and transformants were isolated by the phenol-water extraction of dried bacterial cells (28). The crude extract without separation of the layers was dialyzed against tap water, nucleic acids and proteins were precipitated by adding aqueous 50% CCl_3CO_2H at 4°C to pH 2, the supernatant was dialyzed against distilled water, and the supernatant was freeze-dried. The purified lipopolysaccharide preparations were degraded with aqueous 2% acetic acid at 100°C, and the O polysaccharides were isolated in yields of 27 to 44% by gel permeation chromatography on Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 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 (20). Assignment of the ^1H and ^{13}C NMR spectra (see Table S2 in the supplemental material) was performed using correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and $^1H,^{13}C$ heteronuclear single-quantum coherence (HSQC) experiments. Positions of O-acetyl groups were determined by characteristic low-field displacements of the NMR signals for ^1H and ^{13}C atoms at the O-acetylation sites and by a comparison to the corresponding O-deacetylated polysaccharides studied earlier (2, 22). The degree of O-acetylation was determined by relative integral intensities of the ^1H NMR signals of the O-acetylated and non-O-acetylated Rha^{III} and GlcNAc residues. The O-polysaccharide structure of the parental strain Sf301 (2a) was essentially identical to that of another serotype 2a strain, G1663, studied earlier (22).

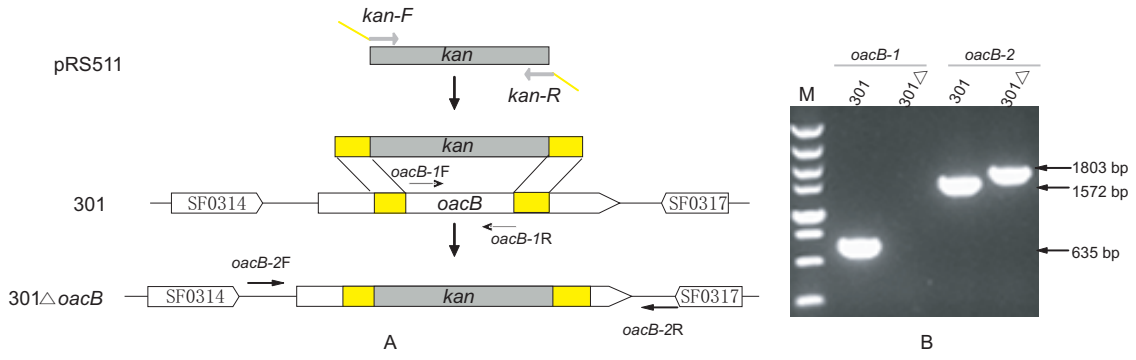

FIG 2 Schematic presentation of the *oacB* gene deletion inactivation using a one-step method and detection by PCR amplification. (A) Structural presentation of the *oacB* gene deletion by a one-step method. Open reading frames are shown as thick arrows, and primers used for detection are shown as thin arrows. The kanamycin resistance gene *kan* (gray) is amplified from plasmid pRS551 and flanked by DNA sequences complementary to the *oacB* gene (yellow). (B) PCR analysis of deletion mutants using primer pairs *oacB*-1 and *oacB*-2.

TABLE 3 Correlation between the 3/4-O-acetylation on Rha^{III} and the presence of the *oacB* gene in *S. flexneri* strains

S. flexneri strain	Serotype	3/4-O-acetylation on Rha^{III}	*oacB* gene PCR result	O-antigen structure reference[a]
51571	1a	+	+	24
G1661	1a	+	+	22
51572	1b	+	+	24
G1662	1b	+	+	22
X6	1c	−	−	24
HN153	1d	−	−	33
51250	2a	+	+	24
G1663	2a	+	+	22
51251	2b	−	−	This work
51575	3a	−	−	24
G1665	3a	−	−	2
G1666	3b	−	−	2
NCTC9725	4a	−	−	17
G1668	4av	−	−	19
51577	4b	−	−	24
G1669	4b	−	−	2
51247	5a	+	+	24
G1036	5a	+	+	21
51580	X	−	−	17
G1039	X	−	−	2
2002017	Xv	−	−	17
2003055	Xv	−	−	17
51581	Y	+	+	24
G1040	Y	+	+	2
036	Y	−	−	20
HN006	Yv	−	−	20
AH012	Yv	−	−	20
HN011	Yv	−	−	20
51579	6	+	−	24
G1038	6	+	−	2
G1671	6	+	−	2

[a] The O-antigen structures are shown in Table S1 in the supplemental material.

TABLE 4 Serological features of *S. flexneri* strains studied

Strain (serotype)[a]	Reactivity with type and group antisera								
	I	II	III	IV	V	VI	3,4	6	7,8
51251 (2b)	−	+	−	−	−	−	−	−	+
51521_pSQZ4 (2a)	−	+	−	−	−	−	+	−	−
51580 (X)	−	−	−	−	−	−	−	−	+
51580_pSQZ4 (Y)	−	−	−	−	−	−	+	−	−
301 (2a)	−	+	−	−	−	−	+	−	−
301Δ*oacB* (2a)	−	+	−	−	−	−	+	−	−
301Δ*oacB*_pSQZ4 (2a)	−	+	−	−	−	−	+	−	−

[a] Serotypes were determined using antisera of Denka Seiken, Japan, according to the current serotyping scheme of *S. flexneri*.

RESULTS

Identification of an O-acyltransferase gene that is present only in *S. flexneri* strains with 3/4-O-acetylation on Rha^{III}. 2-O-Acetylation of Rha^{I} in the O antigens of *S. flexneri* serotypes 1b, 3a, 3b, 4b, and 7b is mediated by Oac, an acyltransferase family protein encoded by the *oac* gene of bacteriophage Sf6 (6, 7). We hypothesized that the 3/4-O-acetylation of Rha^{III} in serotypes 1a, 1b, 2a, 5a, Y, and 6 is mediated by an Oac homolog. BLAST search using the Oac protein sequence of Sf6 against the genome of *S. flexneri* serotype 2a strain Sf301 that carries the 3/4-O-acetylation (Fig. 1) retrieved a hypothetical protein encoded by gene SF0315, which showed the highest homology to Oac with 26% identity, 51% coverage, and an E value of 3e−06 at the protein level. The protein contained 390 amino acids and possessed conserved domains of the acyltransferase family (COG1835 or acyl_trans_3).

BLAST search indicated that this hypothetical acetyltransferase is also present in completely or partially sequenced *S. flexneri* strains 2457T (WP_000570107), 4343-70 (WP_005094470), 2930-71 (WP_000570109), and 2747-71 (WP_005090992) with 99 to 100% identity at the protein or DNA level. It showed 72 to 74% identity to predicted acyltransferases of *S. flexneri* strains CDC 796-83 (WP_005054336) and CCH060 (WP_005114037) and 25 to 39% identity to predicted acyltransferases of *Pseudomonas* sp. strain S9 (WP_010486952.1), *Pseudomonas* sp. strain GM55 (WP_008016406.1), *Dechloromonas aromatica* (YP_285087.1), *Flavobacterium columnare* (YP_004943102.1), and some other species. We name the SF0315 protein 3/4-O-acyltransferase, or OacB, and the corresponding gene *oacB*, following the designation convention for *oac* of Sf6, which we suggest renaming as *oacA*. The function of *oacB* was confirmed as described below.

PCR screening of 31 strains with known O-antigen structures (see Table S1 in the supplemental material), using primer pair *oacB*-1 (Table 2), revealed a complete correlation between the 3/4-O-acetylation and the presence of the *oacB* gene. Except for three serotype 6 strains, the expected PCR product (653 bp) was amplified from all strains carrying 3/4-O-acetylation on Rha^{III}, including two strains each of serotypes 1a, 1b, 2a, 5a, and Y (Table 3). In contrast, strains lacking 3/4-O-acetylation did not carry the *oacB* gene (Table 3). The whole *oacB* gene sequence in the 11 *oacB*-positive strains was PCR amplified and sequenced using primer pair *oacB*-2. The *oacB* gene sequences in all of these strains were found to be identical to that of strain Sf301.

Southern hybridization on the three serotype 6 strains 51579, G1038, and G1671 was performed using the *oacB* gene PCR product (653 bp) as the probe and showed no positive hybridization signal, indicating the absence of the *oacB* gene from these strains (data not shown).

Functional *oacB* gene mediates the 3/4-O-acetylation of Rha^{III} in *S. flexneri* O antigens. The entire *oacB* gene of 1,173 bp, together with 399-bp sequences upstream and downstream to cover its promoter and terminator sequences, was cloned from *S. flexneri* strain Sf301 (serotype 2a) into expression plasmid pMD20T to construct plasmid pSQZ4, which was then transformed into *S. flexneri* serotype 2b strain 51251 and serotype X strain 51580. Serological analysis of the 51521_pSQZ4 and 51580_pSQZ4 transformants using commercial *Shigella* antisera (Denka Seiken, Japan) showed that, compared to the parental strains, both transformants lost reactivity with group 7,8 antiserum and gained reactivity with group 3,4 antiserum (Table 4). Therefore, according to the current serotyping scheme, the 51521_pSQZ4 and 51580_pSQZ4 transformants converted from serotypes 2b and X into serotypes 2a and Y, respectively (Table 4), indicating that *oacB* affected another modification, namely, glucosylation on Rha^{III} that defines group O-factor 7,8.

The O polysaccharides of the transformants were isolated and analyzed using NMR spectroscopy as described in Materials and Methods (for assigned ^{1}H and ^{13}C NMR chemical shifts, see Table S2 in the supplemental material). Compared to the parental strains 51251 and 51580, both transformants acquired an O-acetyl

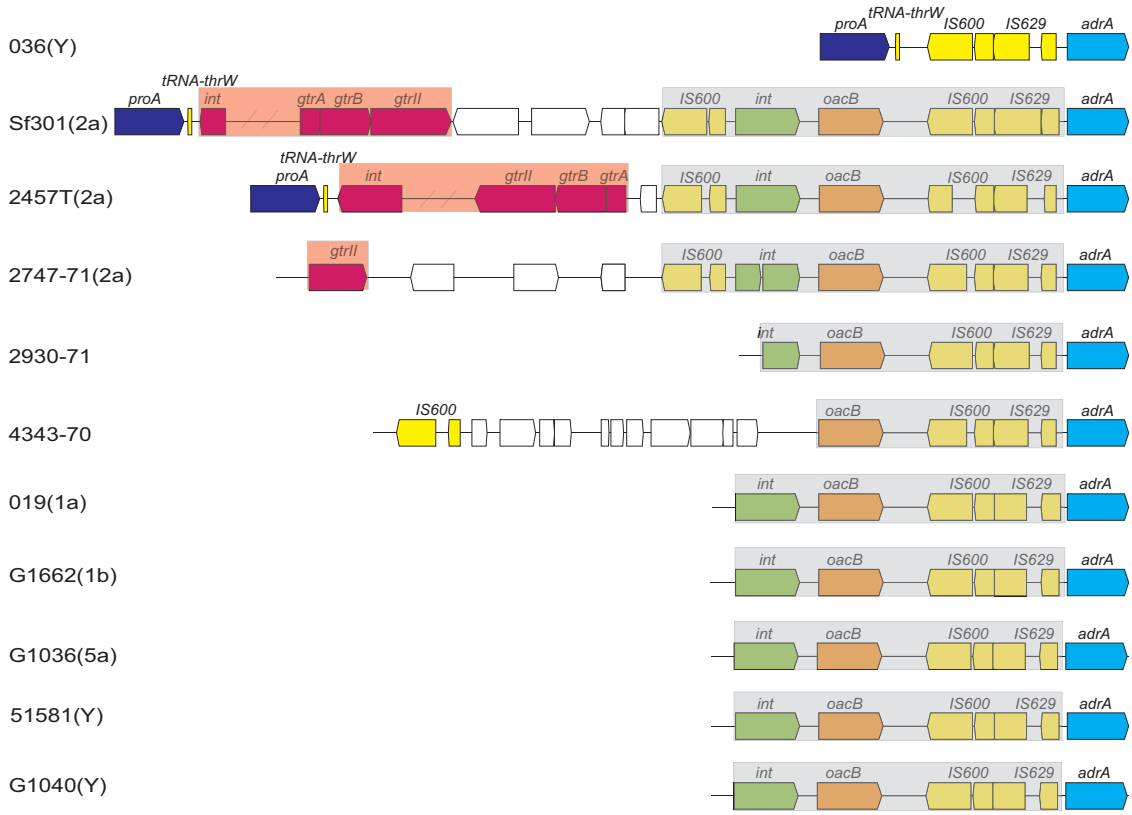

FIG 3 Genetic organizations of the genomic regions carrying the *oacB* gene in serotype 1a, 1b, 2a, 5a, and Y strains. Sequences of Sf301, 2457T, 2747-71, 2930-71, and 4343-70 were obtained from the NCBI database. The serotypes of 2930-71 and 4343-70 are unknown. Genomic sequences of 019 (1a), G1662 (1b), G1036 (5a), and 51581 and G1040 (both Y) were obtained by PCR amplification. The open reading frames are annotated as sequences in NCBI or predicted using ORF Finder and are shown as thick arrows. The conserved genes are shown in different colors: *proA*, dark blue; *adrA*, blue; IS600 and IS629, yellow; *oacB*, orange; integrase gene (*int*), green; prophage SfII genome genes, red. The serotype-converting prophage and *oacB*-carrying transposon are highlighted in red and gray, respectively.

group at either position 3 or 4 of Rha^{III} and lost the original glucosylation at position 3 of the same sugar (Fig. 1). The degree of O-acetylation at positions 3 and 4 was ~60% and 20% in 51521_pSQZ4 and ~65% and 25% in 51580_pSQZ4, respectively. Like the parental serotype 2b and X strains, neither transformant contained the 6-O-acetyl group on the GlcNAc residue.

The function of the *oacB* gene was confirmed by deletion and complementation assay performed on strain Sf301 using the one-step method. A stretch of 740 bp of the *oacB* gene from bp 172 to 911 was replaced by the kanamycin resistance gene (Fig. 2). The deletion mutant was selected on a chloramphenicol and kanamycin resistance plate and confirmed by PCR amplification of the *oacB* gene using primer pairs *oacB*-1 and *oacB*-2. A PCR product of 1,803 bp was amplified from 301Δ*oacB* using primer pair *oacB*-2, which was the expected size of the product containing the kanamycin resistance gene (Fig. 2). No serological difference was observed between deletion mutant 301Δ*oacB* and parent strain Sf301 using *Shigella* antisera from Denka Seiken (Table 4).

Analysis of the O-polysaccharide structure by NMR spectroscopy (see Table S2 in the supplemental material) showed that the deletion mutant lost the 3/4-O-acetylation on Rha^{III} compared to its parental strain, and this modification was restored by transformation of the mutant with the *oacB*-carrying plasmid pSQZ4 (Fig. 1). The NMR data also indicated that the *oacB* gene deletion did not affect the 6-O-acetylation on GlcNAc in 301Δ*oacB*. Accordingly, the *oacB* gene could not be PCR amplified from strains 51575 and G1665 (both serotype 3a) or HN006, AH012, and HN011 (all serotype Yv), which carried 6-O-acetylation on GlcNAc but lacked 3/4-O-acetylation on Rha^{III} (see Table S1 in the supplemental material).

***oacB* gene is carried by a transposon-like structure located upstream of the *adrA* gene on the chromosome.** Analysis of the regions flanking the *oacB* gene in sequenced strains Sf301, 2457T, and 2747-71 (all 2a) as well as 4343-70 and 2930-71 (both of unknown serotype) showed that in all 5 strains, the *oacB* gene was located downstream of an integrase-encoding gene (*int*). Furthermore, in all strains studied except for strain 2930-71, whose DNA sequence upstream of *int* had not been sequenced, the *int-oacB* locus was flanked by insertion sequences (IS), with IS*600* upstream and IS*600* and IS*629* downstream, giving rise to a transposon-like structure (Fig. 3). In serotype 2a strains Sf301, 2457T, and 2747-71, this structure was located immediately downstream of the SfII prophage genome sequence and was followed by the conserved *adrA* gene (Fig. 3). In strains 4343-70 and 2930-71 of unknown serotype, the *oacB*-carrying transposon was also found upstream of the *adrA* gene (Fig. 3). This observation was confirmed by PCR amplification and sequencing of the DNA regions upstream and downstream of the *oacB* gene in strains 019 (1a), G1662 (1b), G1036 (5a), 51581, and G1040 (both Y), which revealed the same structure in all five strains (Fig. 3).

DISCUSSION

The present data revealed a clear correlation between the presence of the *oacB* gene and 3/4-O-acetylation on Rha^{III} and demonstrated the functional *oacB* gene to be responsible for this O-antigen modification in *S. flexneri* serotypes 1a, 1b, 2a, 5a, and Y. This conclusion was supported by the following pieces of evidence: (i) the OacB protein encoded by the *oacB* gene showed significant similarity to acyltransferase family proteins; (ii) the cloned *oacB* gene mediated the 3/4-O-acetylation of Rha^{III} upon transformation; and (iii) deletion of the functional *oacB* gene resulted in the loss of 3/4-O-acetyltion. The fact that one gene mediates modification at both positions 3 and 4 of Rha^{III} suggests that OacB is a bifunctional enzyme with respect to the substrate O-acetylation site. Such bifunctional catalysis is also found on the PEtN transferase Opt, which mediates the addition of a PEtN residue(s) to position 3 of either Rha^{III}, Rha^{II}, or both (17–20, 24).

Although serotype 6 strains also possess 3/4-O-acetylation on Rha^{III} (2) (see Table S1 in the supplemental material), they lack *oacB*. The serotype 6 O antigen has a linear tetrasaccharide repeat containing one residue each of *N*-acetylgalactosamine and galacturonic acid and two rhamnose residues (Rha^{II} and Rha^{III}) (29), and the structural difference from the O antigens of the other *S. flexneri* serotypes (1 to 5, 7, X, and Y) might require another mechanism of 3/4-O-acetylation on Rha^{III}. Indeed, in serotype 6 strains, the 3/4-O-acetylation was found to be mediated by another acyltransferase encoded by a gene named *oacC*, which presents 57.1% similarity to *oacB* and maps in a phage-like region on the chromosome as well (unpublished data). It is also evident that yet another unidentified acyltransferase gene is responsible for the 6-O-acetylation on GlcNAc in serotypes 2a, 3a, Y, and Yv.

Two known mechanisms of mobilization of *S. flexneri* O-antigen modification factors involve temperate bacteriophages (for 2-O-acetylation of Rha^{I} and glucosylation) or a plasmid (for PEtN phosphorylation). In this study, the *oacB* gene was found to be carried by a transposon-like structure, suggesting that it is transferred by a transposon mechanism among strains in nature. The serotype-converting bacteriophages for O-antigen glucosylation (SfI, SfII, SfIV, SfV, and SfX) are integrated into the host chromosome at the *tRNA-thrW* site located between genes *proA* and *adrA* (3, 8–14, 16). The transposon-like locus responsible for the 3/4-O-acetylation of Rha^{III} also maps upstream of the *adrA* gene. Therefore, one can speculate that the *proA-adrA* region is a conserved insertion site for both *oacB*-carrying transposons and serotype-converting bacteriophages in all serotypes involved.

The *oacB*-mediated addition of the O-acetyl group to positions 3 and 4 of Rha^{III} prevents this sugar from being glucosylated and results in serotype conversion of the host strains, as observed in the conversion of serotypes 2b and X into 2a and Y, respectively. Glucosylation at position 3 of Rha^{III} defines the group 7,8 antigenicity, and the loss of this modification upon transformation with an *oacB*-carrying plasmid pSQZ4 abolishes the reactivity with group 7,8 antiserum (Table 4). It is noteworthy that the 7,8 antiserum reactivity of transformants 51580_pSQZ4 and 51251_pSQZ4 could be recovered by curing the pSQZ4 plasmid (data not shown), suggesting that the potential glucosylation activity of the *gtrX* locus retained in the transformants and the loss of glucosylation upon transformation occurred by a competitive occupation with O-acetyl groups.

Group O-factor 3,4 is linked to the linear →3)-α-L-Rha*p*^{I}-(1→3)-β-D-Gl*cp*NAc-(1→2)-α-L-Rha*p*^{III}-(1→ trisaccharide fragment of the O-antigen backbone (30). This epitope is masked by glucosylation on Rha^{III}, whereas glucosylation on GlcNAc and 2-O-acetylation on Rha^{I} abolish the 3,4 reactivity only when they occur simultaneously (30). Our data (Table 4) and published data (2) indicate that, in contrast to glucosylation, 3/4-O-acetylation on Rha^{III} does not affect O-factor 3,4. Finally, the 3/4-O-acetylation also interferes with PEtN modification of Rha^{III} mediated by the plasmid-borne *opt* gene, and PEtN phosphorylation occurs at Rha^{II} only. As a result, the level of the reactivity with monoclonal antibody MASF IV-1 specific to PEtN-linked epitopes decreases significantly (24).

In conclusion, the genetic basis of the 3/4-O-acetylation of Rha^{III} was elucidated in the O antigens of *S. flexneri* serotypes 1a, 1b, 2a, 5a, and Y. A new O-acyltransferase responsible for this modification, OacB, is encoded by the *oacB* gene, which is carried by a transposon-like structure inserted into the *proA-adrA* region on the chromosome. The 3/4-O-acetylation of Rha^{III} interferes with glucosylation and PEtN phosphorylation at the same sugar residue. These findings enhance our understanding of the mechanisms of the O-antigen variation and enable further studies to understand the contribution of the 3/4-O-acetylation to the antigenicity and pathogenicity of *S. flexneri*.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (no. 81271788); the National Basic Research Priorities Program (2011CB504901); the Project of State Key Laboratory for Infectious Disease Prevention and Control (2011SKLID203); the National Key Program for Infectious Diseases of China (2013ZX10004221, 2013ZX10004216-001-002, 2012ZX10004215); and the Russian Foundation for Basic Research (12-04-00172).

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