Serological identification and prevalence of a novel O-antigen epitope linked to 3- and 4-O-acetylated rhamnose III of LPS in *Shigella flexneri*

**Running Title:** A novel *Shigella flexneri* O-antigen epitope

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Shigella flexneri is the major cause of shigellosis in developing countries. Except for serotype 6, all serotypes share a common O-antigen backbone composed of \( \rightarrow 2\)-\( \alpha \)-L-Rhap\textsuperscript{III}\( \rightarrow 2\)-\( \alpha \)-L-Rhap\textsuperscript{II}\( \rightarrow 3\)-\( \alpha \)-L-Rhap\textsuperscript{I}\( \rightarrow 3\)-\( \beta \)-D-GlcNAc\( \rightarrow \) tetrasaccharide repeat. It can be modified by additions of glucosyl group to one or more sugar residues, and/or O-acetyl group to Rha\textsuperscript{I}, and/or phospheoethanolamine to Rha\textsuperscript{II} or/and Rha\textsuperscript{III}. These modifications give rise to type I, IC, II, IV and V as well as group 6, 7, 8 and MASF IV-1 specific antigenic determinants, which comprise the current serotyping scheme of S. flexneri. Recently, another O-antigen modification by adding an O-acetyl group to Rha\textsuperscript{III} at position either 3 or 4 (3/4-O-acetylation) has been found in S. flexneri serotypes 1a, 1b, 2a, 5a, Y and 6. A new O-acyltransferase gene named oacB has been shown to mediate the 3/4-O-acetylation in serotypes 1a, 1b, 2a, 5a and Y but not 6. In this work, we studied the distribution of the 3/4-O-acetylation in S. flexneri and the antigenicity that resulted from this modification. PCR screening of the oacB gene in clinical isolates of S. flexneri demonstrated that the oacB-mediated 3/4-O-acetylation is widespread in serotypes 1a, 1b, 2a, 5a and Y. Serological analysis indicated that this modification confers the host with a novel antigenic determinant provisionally named group O-factor 9. These findings enhance our understanding on the varieties of O-antigenic determinants related to O-antigen modification in S. flexneri, and will assist epidemiological studies and vaccine development.
Introduction

*Shigella flexneri* is the major cause of shigellosis in developing countries. Based on combinations of O-antigenic determinants (O-factors) on the cell-surface lipopolysaccharide (LPS), *S. flexneri* strains are subdivided into various serotypes. By now, a total of 19 serotypes [1a, 1b, 1c (7a), 1d, 2a, 2b, 3a, 3b, 4a, 4av, 4b, 5a, 5b, Y, Yv, X, Xv, 6 and 7b] have been reported by different research groups, using the commercially available monovalent antisera kit (Denka Seiken, Japan) and monoclonal antibody reagents MASF (Reagensia AB, Sweden) (1-14). Except for serotype 6, all serotypes share a common O-antigen backbone having a tetrasaccharide repeat (O-polysaccharide) composed of one N-acetylglucosamine (GlcNAc) and three rhamnose residues (Rha¹-Rha³III) (1). The basic O-antigen is referred to as serotype Y and is defined by group factors 3 and 4. Addition of various substituents, such as glucosyl or/and O-acetyl groups or/and phosphoethanolamine (PEtN), to one or more sugar residues in the O-unit forms the chemical basis for serotype variation in *S. flexneri* (1, 5, 15-17).

Glucosylation can occur on any of the monosaccharides in the O-unit giving rise to type I, IC, II, IV, V and group 7,8 specific antigenic determinants in serotypes 1-5, 7, X and Xv (1, 6). Adding of an O-acetyl group to Rha¹ at position 2 (2-O-acetylation) confers serotypes 1b, 3a, 3b, 4b and 7b with group factor 6 (6, 18, 19). Glucosylation is mediated by three genes (*gtr*A, *gtr*B and *gtr*type) that are arranged in a single operon known as the *gtr* cluster which is encoded by serotype-conversion bacteriophages with 6 such bacteriophages identified to date (SfI, SfIC, SfII, SfIV, SfV, SfV...
and SfX) (20-25). 2-O-Acetylation of Rha\(^{I}\) is performed by a specific O-acetyltransferase encoded by the gene \textit{oac} (18, 19), which is also carried by a serotype-conversion bacteriophage Sf6 (26).

Phosphorylation with PEtN of Rha\(^{III}\) or/and Rha\(^{II}\) at position 3 has been recently identified in the newly proposed \textit{S. flexneri} serotypes 4av, Xv and Yv (4, 5, 15, 27) and possibly also in atypical strains of serotypes 4, 6 and 4X (9-12), and demonstrated to confer the hosts with MASF IV-1 (E1037) antigenic determinant. A plasmid carrying gene \textit{opt} (O-antigen phosphoethanolamine transferase) mediates the PEtN modification in these serotypes (4, 5, 15, 17).

Recently, yet another O-antigen modification, namely adding of an O-acetyl group to Rha\(^{III}\) at position either 3 or 4 (3/4-O-acetylation), has been recognized in \textit{S. flexneri} serotypes 1a, 1b, 2a, 5a and Y as well as in serotype 6 having a different basal O-antigen structure (16, 28-30). A new O-acyltransferase gene named \textit{oacB} mediates the 3/4-O-acetylation in serotypes 1a, 1b, 2a, 5a and Y but not 6 (31). The \textit{oacB} gene is carried by a transposon-like structure located upstream of the \textit{adrA} gene on the chromosome (31). The 3/4-O-acetylation on Rha\(^{III}\) interferes with glucosylation (group factor 7,8) and PEtN phosphorylation (group factor IV-1) of the same sugar residue mediated by the \textit{gtrX} gene cluster and the \textit{opt} gene, respectively, resulting in the loss of the 7,8 determinant or a decrease of the level of the MASF IV-1 determinant manifestation (17).

In this work, we studied the distribution of the 3/4-O-acetylation in \textit{S. flexneri} by PCR screening of the \textit{oacB} gene and serological assay using a specific absorbed antiserum and found that this O-
antigen modification is widespread in serotypes 1a, 1b, 2a, 5a and Y, which confers the host with a novel antigenic determinant provisionally named factor 9.

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 CDC.

Strains and culturing conditions

*S. flexneri* 51251_pSQZ4 (strain 51251 carrying vector pSQZ4) (31), which possesses 3/4-O-acetylation on RhaIII in the O-antigen (Figure 1) was used as immunizing strain. *S. flexneri* 51251 (31), the parent strain of transformant 51251_pSQZ4, which lacked the 3/4-O-acetylation on RhaIII (Figure 1), was used as absorbing antigen, to remove unspecific cross-reacting antibodies. A total of 730 *S. flexneri* isolates representing 19 serotypes were used in the *oacB* gene PCR detection and antiserum 9 agglutination assay (Table 1). These strains were isolated from diarrheal patients in a surveillance program performed by China CDC during 2000–2012, or purchased from National Collection of Type Cultures (NCTC), or kindly donated by Dr B. Liu (Nankai University, Tianjin).

Twelve *Shigella dysenteriae* (serotypes 1 to 12), 18 *Shigella boydii* (serotypes 1 to 18), 31 *Shigella sonnei* and 10 *Escherichia coli* (O6, O8, O13, O42, O71, O78, O127, O128, O157, O159) were used for *oacB* gene PCR detection and antiserum 9 specificity evaluation. *S. flexneri* 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.

**oacB gene detection by PCR amplification**

DNA templates were prepared directly from bacterial colonies by the boiling method. Briefly, a single colony from an overnight culture at 37°C on LB agar was suspended in 30 µl distilled water and boiled at 100°C for 10 min. The sample was immediately cooled on ice for 5 min and centrifuged at 13,000×g at 4°C for 10 min. The supernatant was used as the template for PCR amplification. Primer pairs *oacB*-1F: FTCATCTGGAGTATGGGAAG and *oacB*-1R: CAAAGAATCAGTGGTAGCG, and *oacB*-2F: GGTGTGTCTCCGTGGTTTC and *oacB*-2R: CGACGTTGCTACTGGTTTC were used for the *oacB* gene detection and whole *oacB* gene sequencing, respectively (31). PCR amplifications were performed using TaKaRa PCR Amplification Kit (Takara, Japan) following the thermal cycling profile: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 50 s and 72°C for 5 min, on a SensoQuest LabCycler (SensoQuest, Germany). A portion (5 µl) of the reaction mixture was mixed with loading buffer, subjected to electrophoresis in 1.5% agarose gel, and visualized by ethidium bromide staining.

**Preparation of specific antiserum 9 against a 3/4-O-acetylated Rha\(^{III}\) epitope**

Immunization and antisera preparation were performed as described (32). Briefly, three New Zealand white rabbits (female, 1.5-2 kg) were immunized intravenously twice a week with heat-killed cells of *S. flexneri* strain 51251_pSQZ4 with increasing doses (1×10\(^9\), 2×10\(^9\), 4×10\(^9\), 8×10\(^9\),
16×10^9, 16×10^9 CFU). One week after the last of six immunizations, blood was drawn by cardiac
puncture, and the serum was separated by centrifugation and collected. To render the antiserum
specific to a 3/4-O-acetylated Rha\textsuperscript{III} epitope, the crude antiserum preparation was mixed with heat-
killed cells of \textit{S. flexneri} isolate 51251 to absorb non-specific antibodies that cross-reactive with
other O-antigenic epitopes as described (32). The antiserum agglutinated only with strain
51251_pSQZ4 but not 51251 was referred to as antiserum 9 below.

**Serotyping analysis**

Serological features of \textit{S. flexneri} strains were determined by slide agglutination test using the
commercially available monovalent antiserum kit (Denka Seiken, Japan) and monoclonal antibody
reagents MASF IV-1, MASF IV-2 and MASF 1C (Reagensia AB, Sweden). Antiserum 9 prepared
in this study was used for detection of the 3/4-O-acetylation by slide agglutination, and
agglutination apparent to the naked eye within 20 seconds was recorded as positive.

**LPS analysis**

LPS were prepared using the LPS extraction Kit (iNtRON, Korea) according to the manufacturer’s
instruction. LPS were electrophoresed on 15% polyacrylamide gels and detected by silver-staining
as described (33, 34). LPS Western blot assay was performed as described (3). The LPS separated
by SDS-PAGE was transferred onto a PVDF membrane and incubated with antiserum 9 prepared
in this study. After washed with PBS containing 0.0 5% Tween-20, the membrane was incubated
with anti-rabbit antibody labeled with the fluorescent IRDye™ 800 (Rockland), and the fluorescence was detected using the Odyssey Infrared Imaging System (LI-COR).

Isolation and NMR spectroscopy of O-polysaccharides

The LPS and O-polysaccharides of strains 05135 (serotype 1a), 07HN194 (2a) and M90T (5a) (Table 2, all factor 9 negative) were isolated as described previously (17). The O-polysaccharides were analyzed by NMR spectroscopy, including one-dimensional $^1$H and $^{13}$C NMR and two-dimensional $^1$H,$^{13}$C heteronuclear single-quantum coherence (HSQC) experiments. The spectra were run for solutions in 99.95 % D$_2$O at 30°C using an Avance II 600 MHz instrument (Bruker, Germany) and internal sodium 3-(trimethylsilyl) propanoate-2,2,3,3-d$_4$ ($\delta_{$H$} 0.00$) and acetone ($\delta_{$C$} 31.45$) as references.

Results and discussion

The oacB-positive genotype is predominant in S. flexneri serotypes 1a, 1b, 2a, 5a and Y

By now, a total of 16 S. flexneri strains belonging to serotypes 1a, 1b, 2a, 5a, Y and 6 have been structurally identified carrying the 3/4-O-acetylation on Rha$^{\text{III}}$ (15-17, 31). To determine the population distribution of this modification in nature, we screened 730 S. flexneri isolates by PCR amplification of the oacB gene (Table 1). These strains were isolated mainly from diarrheal patients and represented all the 19 serotypes known to date. Except for four strains isolated in USA, 6 in UK, 1 in Russia, and 14 in Australia, all were collected from China in a surveillance program performed by China CDC during 2000-2012.
A total of 349 strains belonging to 9 serotypes were found to be oacB gene positive, accounting for 47.80% of the 730 strains tested (Table 1). Among them, strains of 5 serotypes (1a, 1b, 2a, 5a and Y) have been reported to possess the 3/4-O-acetylation on RhaIII (16, 17, 31), and the oacB positive genotype was predominant within these serotypes with the rates of 95.33%, 100%, 96.45%, 64.29%, and 64.10%, respectively (Table 1).

The oacB gene was detected by PCR in 25 strains of 3 other serotypes (21 serotype 2b, 3 serotype X and 1 serotype Xv isolates) but the oacB positive rates were significantly lower (34.43%, 6% and 0.79%, respectively) and no strains of these serotypes have been known to express the 3/4-O-acetylation on RhaIII (Table 1). To elucidate the mechanism underlying the gene inactivation in the 25 strains, as well as 4 other abnormal strains (3 serotypes 2a and 1 Y) as described below (Table 1), their oacB gene sequences were amplified and sequenced using the primer pair oacB-2. Except for one serotype 2b strain (04BJ04), whose oacB gene was identical to that of serotype 2a strain Sf301, all others carried a mutational oacB gene. Three serotype 2a strains (07HN111, 07HN117 and 07HN172) had two base substitution at position 1007 (A→G) and 1067 (C→T) resulting in two non-synonymous substitutions at amino acid residue 336 (Y→C) and 356 (S→L), respectively. These amino acid changes may have render the protein non-functional. Two serotype 2b strains (04BJ05, 04BJ26) possessed one IS element (IS1, 777 bp) insertion at position 948, and 18 other serotype 2b strains as well as 3 serotype X strains and one strain each of serotypes Xv and


187 Y (23 strains in total) had one base (T) deletion at position 668, which resulted in a stop codon at
amino acid 317 or 223, respectively, rendering the protein defective in these isolates.

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190 These data, combined with the serological features (see below), indicate that the oacB-mediating
191 3/4-O-acetylation on Rha\textsuperscript{III} is widespread in \textit{S. flexneri} strains belonging to serotypes 1a, 1b, 2a, 5a
and Y. Among them, 1a, 1b and 2a are the predominant serotypes in China and other Asian
countries (35), and the high 3/4-O-acetylation rates (>94%) found in these serotypes suggest that
3/4-O-acetylation may play a role in their preferable dissemination; further studies are necessary to
elucidate if this O-antigen modification contributes to virulence of \textit{S. flexneri}.

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3/4-O-acetylation confers the host with a new O-antigen epitope

198 In search for an O-antigen epitope associated with the 3/4-O-acetylation on Rha\textsuperscript{III}, a specific rabbit
antiserum was prepared. A recombinant strain of \textit{S. flexneri}, 51251_pSQZ4 (serotype 2b isolate
51251 harboring an \textit{oacB} expressing plasmid construct pSQZ4), carrying 3/4-O-acetylation on the
Rha\textsuperscript{III} of its LPS was used for immunization and its parent serotype 2b strain \textit{S. flexneri} 51251
lacking the 3/4-O-acetylation on Rha\textsuperscript{III} was used for absorption of crude polyclonal antiserum. As
both strains possess glucosylation on Rha\textsuperscript{I} and differ in replacement of glucosylation on Rha\textsuperscript{III} (in
51251) with 3/4-O-acetylation (in 51251_pSQZ4) on Rha\textsuperscript{III} (Figure 1), the absorption eliminated
all cross-reactive antibodies to Glc-linked epitopes and retained the desired antibodies to an
epitope(s) associated with the 3/4-O-acetylated Rha\textsuperscript{III}. After repeated absorptions, the antiserum
agglutinated only with strain 51251_pSQZ4 but not strain 51251.
To detect the serological specificity of the prepared antiserum, we performed immunoblotting assay with LPS of strain 51251_pSQZ4 and its host 51251 as well as strain Sf301 (serotype 2a, carrying 3/4-O-acetylation on Rha$^{III}$) and its oacB gene deletion mutant Sf301ΔoacB (31). The LPS samples were electrophoresed on SDS polyacrylamide (15%) gels and visualized by silver-staining. All samples showed the classical ladder-like pattern of an O-antigen composed of various number of repeating units, and no obvious difference was found between the parental and constructed strains (Figure 2A). Western blot showed that antiserum 9 bound to the ladder-like LPS bands of strains 51251_pSQZ4 and Sf301 that possessed the 3/4-O-acetylation on Rha$^{III}$ but did not recognize LPS of strains 51251 and Sf301ΔoacB lacking this modification (Figure 2B). Therefore, antiserum 9 is specific to a 3/4-O-acetylation-linked epitope(s) in the O-antigen.

The 730 strains used for PCR detection were tested by slide agglutination assay, and a total of 382 isolates (52.33%) agglutinated with the absorbed antiserum (Table 1). The overwhelming majority of the agglutination positive strains belonged to serotypes 1a (102 strains), 1b (25), 2a (160), 5a (9), Y (24) and 6 (59) (Table 1). Except for serotype 6, all were positive for the oacB gene by PCR; moreover, all oacB-carrying strains agglutinated with antiserum 9, except for 29 strains (21 serotype 2b, 3 X, 1 Xv, 3 2a and 1 Y) described above, which all but one serotype 2b strain carry dysfunctional mutations in the oacB gene. Therefore, a good correlation is observed between the presence of the oacB gene and the serological reactivity.
O-Antigens of all serotype 6 isolates studied chemically have been found to possess 3/4-O-acetylation on Rha\textsuperscript{III}. Our further studies showed that another acyltransferase encoded by a gene named \textit{oacC}, which presents 57.1\% similarity to \textit{oacB}, mediates the 3/4-O-acetylation in serotype 6 (authors’ unpublished data). It is not a surprise that serotype 6 strains with 3/4-O-acetylation on Rha\textsuperscript{III} reacted with the antiserum prepared for other \textit{S. flexneri} serotypes, since, in spite of a different O-antigen structure (36), they have the \textendash\textendash\textendash\textendash→2)-α-L-Rha\textsuperscript{III}\textendash\textendash\textendash\textendash→2)→2)-α-L-Rha\textsuperscript{II}\textendash\textendash\textendash\textendash→2)\textendash\textendash\textendash\textendash→2)→2)-α-L-Rha\textsuperscript{II} disaccharide fragment in common (Table 2).

The data obtained clearly demonstrate that the 3/4-O-acetylation on Rha\textsuperscript{III} in \textit{S. flexneri} confers the host with a novel O-antigen epitope, which has been neglected earlier owing to the absence of specific antiserum. We named this new antigenic determinant as group O-factor 9 and the absorbed antiserum as grouping serum 9, following the designations for group O-factors and antisera 3,4, 6 and 7,8. Combined with the current serotyping tools, grouping antiserum 9 can characterize serotype 1b and 6 as O-factor 9-positive, and each of serotypes 1a, 2a, 5a and Y as either O-factor 9-positive or -negative.

The presence of epitope 9 does not affect the antigenicity of other O-antigenic determinant within the host, with each O-factor 9-positive strain demonstrating the same serological pattern as that of the O-factor 9-negative counterpart. Although the majority of the O-factor 9-positive strains used in this study originated from China, the detection of this phenotype from strains from other part of
the world (9 from Australia and 1 from UK) indicates this O-antigenic determinant is likely to be distributed worldwide.

To further determine the antiserum 9 specificity, 71 strains of other species were tested by PCR amplification and slide agglutination. All strains tested carried no oacB gene and did not react with antiserum 9, thus confirming that antiserum 9 can be used for specific typing *S. flexneri* isolates.

**Confirmation of the 3/4-O-acetylation-negative phenotype of serotypes 1a, 2a and 5a**

Whereas the O-antigen structures of both 3/4-O-acetylation-positive and -negative phenotypes have been reported for serotype Y (Table 2) (1, 5, 15), no chemical substantiation has been obtained for the lack of O-factor 9 in serotypes 1a, 2a and 5a. Analysis by $^1$H and $^{13}$C NMR spectroscopy of the O-polysaccharides isolated from the LPS of O-factor 9-negative strains 05135 (1a), 07HN194 (2a) and M90T (5a) showed that, having the same carbohydrate backbone structures as those 3/4-O-acetylation-positive strains of serotypes 1a, 2a and 5a (1, 5, 15), respectively, they lack O-acetyl groups on Rha$^{III}$ (Table 2). The O-polysaccharides of strains 05135 (1a) and M90T (5a) were fully devoid of O-acetylation whereas the O-polysaccharide of strain 07HN194 (2a), as that of O-factor 9-positive serotype 2a strains (16, 17), possessed an O-acetyl group at position 6 of GlcNAc. This finding is in full agreement with the genetic and serological data of these strains, namely the absence of the oacB gene and the lack of agglutination with grouping antiserum 9. In addition, structure elucidation of the O-antigen of serotype 2a strain 07HN194 further confirmed that oacB is not responsible for the 6-O-acetylation on GlcNAc.
Conclusions

PCR screening showed that the \textit{oacB} gene responsible for the 3/4-O-acetylation on \textit{Rha}^{III} is widespread in \textit{S. flexneri} serotype 1a, 1b, 2a, 5a and Y strains. Serological assay indicated that in the overwhelming majority of these strains, the \textit{oacB} gene is functional and the modification on \textit{Rha}^{III} confers the host with a novel antigenic determinant, named group O-factor 9 and the antiserum for its specific recognition can potentially be used as a grouping antiserum for serotyping. Combined with the current serotyping tools, grouping antiserum 9 can further differentiate serotypes 1a, 2a, 5a, Y and 6 strains as O-factor 9-positive and -negative.

Accordingly, we suggest the current serotyping scheme of \textit{S. flexneri} be extended by defining O-factor 9-positive variants as distinctive serotypes, after evaluation by various laboratories worldwide. The findings of this work enhance our understanding of the varieties of O-antigenic determinants related to O-antigen modification in \textit{S. flexneri} and will assist epidemiological studies and vaccine development.

Acknowledgements

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); the Russian Scientific Foundation (14-14-01042).
References


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an epitope common to all *Shigella flexneri* and *Shigella dysenteriae* type 1 stains. Infect Immun *55*:1412-1420.


Table 1  Distribution of the *oacB* gene in *S. flexneri* and cross-reactivity with grouping antiserum 9

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number of strains tested</th>
<th>Number of <em>oacB</em>-positive strains (%)</th>
<th>Number of strains that cross-react with grouping antiserum 9 (%)</th>
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<tbody>
<tr>
<td>1a</td>
<td>107</td>
<td>102 (95.33)</td>
<td>102 (95.33)</td>
</tr>
<tr>
<td>1b</td>
<td>25</td>
<td>25 (100)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>1c</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1d</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2a&lt;sup&gt;a&lt;/sup&gt;</td>
<td>169</td>
<td>163 (96.45)</td>
<td>160 (94.75)</td>
</tr>
<tr>
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<td>61</td>
<td>21 (34.43)</td>
<td>0</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
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<td>4b</td>
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<td>9 (64.29)</td>
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<td>0</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>25 (64.10)</td>
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<td>59</td>
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<td>59 (100)</td>
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<td>7b</td>
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</tr>
<tr>
<td>Total</td>
<td>730</td>
<td>349 (47.80)</td>
<td>382 (52.33)</td>
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</table>
Three serotype 2a isolates (07HN111, 07HN117 and 07HN172) have two base substitutions at positions 1007 (A→G) and 1067 (C→T) resulting in two non-synonymous substitutions at amino acid residues 336 (Y→C) and 356 (S→L), respectively.

One serotype 2b isolate (04BJ04) carries the oacB gene identical to that of Sf301 (serotype 2a); two isolates (04BJ05, 04BJ26) have one IS element (IS1, 777 bp) insertion at position 948 resulting in a stop codon at amino acid 317; 18 isolates possess one base (T) deletion at position 668 resulting in a stop codon at amino acid 223.

Three serotype X isolates (06HN022, 2005130 and 05BJ13) and one isolate each of serotypes Xv (06HN012) and Y (06AH104) have one base (T) deletion at position 668 resulting in a stop codon at amino acid 223.
Table 2  O-Antigen structures of *S. flexneri* O-factor 9-positive and (if any) -negative serotypes 1a, 1b, 2a, 5a, Y and 6.

<table>
<thead>
<tr>
<th>Serotype (strain)</th>
<th>O-polsaccharide structure</th>
<th>Reference</th>
</tr>
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<tbody>
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<td><strong>O-factor 9-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a (G1661)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(16, 30)</td>
</tr>
<tr>
<td></td>
<td>~65%/25% OAc</td>
<td>α-D-GlcNAc</td>
</tr>
<tr>
<td>1b (G1662)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(16, 30)</td>
</tr>
<tr>
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<td>~70%/15% OAc</td>
<td>α-D-GlcNAc</td>
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<tr>
<td>2a (G1663)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(16, 28, 30)</td>
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<tr>
<td></td>
<td>~60%/25% OAc</td>
<td>α-D-GlcNAc</td>
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<tr>
<td>5a (G1036)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(16, 29)</td>
</tr>
<tr>
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<td>~35%/25% OAc</td>
<td>α-D-GlcNAc</td>
</tr>
<tr>
<td>Y (G1040)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(16)</td>
</tr>
<tr>
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<td>~30%/20% OAc</td>
<td>~40% OAc</td>
</tr>
<tr>
<td>6 (G1671)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→4)-β-D-GalpA-(1→3)-β-D-GalpNAc-(1→4)</td>
<td>(16, 36)</td>
</tr>
<tr>
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<td>~60%/30% OAc</td>
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<tr>
<td><strong>O-factor 9-negative</strong></td>
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<td>1a (05135)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>this work</td>
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<td></td>
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<td>α-D-GlcNAc</td>
</tr>
<tr>
<td>2a (07HN194)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
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<td>α-D-GlcNAc</td>
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<tr>
<td>5a (M90T)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>this work</td>
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<tr>
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<td>α-D-GlcNAc</td>
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<tr>
<td>Y (036)</td>
<td>→2)-α-L-Rhap&lt;sub&gt;III&lt;/sub&gt;-(1→2)-α-L-Rhap&lt;sub&gt;II&lt;/sub&gt;-(1→3)-α-L-Rhap&lt;sub&gt;I&lt;/sub&gt;-(1→3)-β-D-GlcNAc-(1→4)</td>
<td>(15)</td>
</tr>
</tbody>
</table>

<sup>a</sup> No O-factor 9-negative counterpart of this serotype has been found.
Legends to Figures

Figure 1 O-Antigen structures of immunizing strain *S. flexneri* 51251\_pSQZ4 and absorbing strain *S. flexneri* 51251 used for grouping antiserum 9 preparation.

Figure 2 LPS analysis of 3/4-O-acetylation carrying strains 51251\_pSQZ4 and Sf301 and lacking strains 51251 and 301ΔoacB. LPS were prepared as methods described in Materials and methods. A. Silver-staining detection of LPS profiles on 15% polyacrylamide gels. B. Immunoblotting detection of the specificity of antiserum 9. The LPS separated by SDS-PAGE were transferred onto a PVDF membrane and hybridized with grouping antiserum 9. An anti-rabbit antibody labeled with fluorescent IRDye™ 800 (Rockland) was used as the secondary antibody. Fluorescence was detected using the Odyssey Infrared Imaging System (LI-COR).
51251_pSQZ4

\[ \rightarrow 2) - \alpha-L-Rhap^{III} - (1 \rightarrow 2) - \alpha-L-Rhap^{II} - (1 \rightarrow 3) - \alpha-L-Rhap^{I} - (1 \rightarrow 3) - \beta-D-GlcNAc - (1 \rightarrow 3/4) \]

OAc(-60%/20%)

\[ \alpha-D-Glc \]

51251 (2b)

\[ \rightarrow 2) - \alpha-L-Rhap^{III} - (1 \rightarrow 2) - \alpha-L-Rhap^{II} - (1 \rightarrow 3) - \alpha-L-Rhap^{I} - (1 \rightarrow 3) - \beta-D-GlcNAc - (1 \rightarrow 3) \]

\[ \uparrow 3 \]

\[ \alpha-D-Glc \]

\[ \uparrow 4 \]

\[ \alpha-D-Glc \]