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Dissemination and serotype modification potential of pSFxv_2, an O-antigen PEtN modification plasmid in *Shigella flexneri*

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The O-antigens of all *Shigella flexneri* serotypes, except serotype 6, share a linear tetrasaccharide repeat composed of one *N*-acetylglucosamine and three L-rhamnose residues, and differences between the serotypes are due to modification of various monosaccharide residues with glucosyl and/or O-acetyl and/or phosphoethanolamine (PEtN) groups. Plasmid-borne *opt* (formerly *lpt-O*) gene encoding a PEtN transferase which modifies the O-antigens of *S. flexneri* serotype X, 4a and Y strains and converts the hosts into MASF IV-1 (E1037) positive "variant" (v) Xv, 4av and Yv serotypes, respectively. In this study, we showed that the *opt*-carrying plasmid pSFxv_2 can transform strains of all *S. flexneri* serotypes (1–6) to confer them with the MASF IV-1 epitope recognized by monoclonal antibody MASF IV-1 and typing antiserum IV. The transformants possessed modified O-antigens with a PEtN group(s) at position 3 of one or two rhamnose residues. In some serotypes, the PEtN modification competed or/and interfered with glucosylation and O-acetylation at the same or its neighboring sugar residue. We also showed that the plasmid pSFxv_2 is mobilizable to other *S. flexneri* strains by conjugation. Although pSFxv_2-harboring *S. flexneri* strains found in clinical infections are restricted to serotypes Xv, 4av, Yv and, possibly, 6v, our results demonstrate a high potential of dissemination of this plasmid in *S. flexneri* and emergence of new *S. flexneri* serotypes.

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Introduction

Shigellosis or bacillary dysentery caused by *Shigella* spp. is an acute diarrheal disease common in developing countries. The annual number of shigellosis cases in Asia has been estimated to be 125 million, causing 14,000 deaths, mainly in children under 5 years of age (Bardhan et al. 2010). Among the four *Shigella* species, *Shigella flexneri* is predominant in developing countries and the second most common in industrialized countries (Kotloff et al. 1999; Shiferaw et al. 2004). By now, 19 *S. flexneri* serotypes [1a, 1b, 1c (or 7a), 1d, 2a, 2b, 3a, 3b, 4a, 4av, 4b, 5a, 5b, X, Xv, Y, Yv, 6 and 7b] have been recognized (El-Gendy et al. 1999; Stagg et al. 2008; Ye et al. 2010; Foster et al. 2011; Sun et al. 2011, 2012; Luo et al. 2012; Knirel et al. 2013).

The O-antigens (O-polysaccharide) of all *S. flexneri* serotypes are composed of linear tetrasaccharide repeats. All (serotypes 1–5, 7, X and Y) except serotype 6 share a linear tetrasaccharide repeat composed of one *N*-acetylglucosamine (GlcNAc) and three L-rhamnose residues (Rha^{I}-Rha^{III}): →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→. Serotype Y possesses the basic O-antigen structure and is characterized by a single group 3,4 antigenic determinant. Serotype 6 has a different linear tetrasaccharide repeat containing one *N*-acetylgalactosamine, one galacturonic acid (GalA) and two rhamnose residues, one of which is mono-O-acetylated at position 3 or 4 (Perepelov et al. 2012): →2)-α-L-Rha*p*3/4Ac-(1 → 2)-α-L-Rha*p*-(1 → 4)-β-D-Gal*p*A-(1 → 3)-β-D-Gal*p*NAc-(1→. Addition of glucosyl (Glc) and/or O-acetyl (OAc) and/or phosphoethanolamine (PEtN) groups to different sugars of the tetrasaccharide repeat gives rise to type (I, II, IV, V and 1C or VII)- and group (6; 7,8 and IV-1)-specific determinants in various serotypes (Perepelov et al. 2012; Sun et al. 2012; Knirel et al. 2013). The glucosylation and O-acetylation of *S. flexneri* O-antigens are mediated by a serotype conversion gene cluster of 3 *gtr* genes or a single gene (*oac*), respectively, which are carried by bacteriophages (Allison and Verma 2000). Seven serotype-converting bacteriophages or prophages (SfI, Sf1C, SfII, SfIV, SfV, SfX and Sf6) have been identified with the first six encoding the *gtr* gene clusters and the last encoding the *oac* gene (Clark et al. 1991; Mavris et al. 1997; Adhikari et al. 1999; Guan et al. 1999; Adams et al. 2001; Allison et al. 2002; Stagg et al. 2009).

Recently, O-antigen modification with a PEtN group(s) attached at position 3 of Rha^{II} or Rha^{III} or both has been identified in "variant" (v) serotypes Xv, 4av and Yv of *S. flexneri*. It confers the three serotypes with the MASF IV-1 (E1037) antigenic determinant. A gene called *opt* (O-antigen PEtN transferase gene), formerly *lpt-O*, encoded on a 6.8 kb plasmid has been found to be responsible for this novel O-antigen modification (Sun et al. 2012; Knirel et al. 2013). Two forms of the gene, *optII* and *optIII*, carried by plasmids pSFxv_2 and pSFyv_2 mediate the preferential addition of PEtN onto Rha^{II} and Rha^{III}, respectively (Sun et al. 2012; Knirel et al. 2013). They differ by 11 base changes (243 A-G, 310 G-A, 379 G-A, 687 T-G, 691 T-C, 728 C-T, 772 C-T, 836 C-A, 1144 G-A, 1449 G-A and 1481 T-C), 7 of which resulted in amino acid changes at positions 104 (V-I), 127 (E-K), 231 (Y-H), 243 (A-V), 279 (A-D), 382 (V-I), 494 (I-T) (Knirel et al. 2013).

Considering that plasmids are generally highly mobile, plasmids pSFxv_2 and pSFyv_2 theoretically can spread among *S. flexneri* strains. However, apart from serotype Xv, Yv and 4av strains, no other *S. flexneri* strains harboring any of these plasmids have been found in nature. Whether the plasmids can transform other serotypes and give rise to novel v-serotypes remained unknown. In this study, we analyzed the host range of plasmid pSFxv_2 by transformation assays and demonstrated that this plasmid can be transferred into strains of all *S. flexneri* serotypes to convert them into MASF IV-1 positive variants. We also showed that this plasmid can be mobilized by conjugation.

Results and discussions

Plasmid pSFxv_2 can be efficiently transferred to, and stably maintained in, S. flexneri strains of various serotypes

optII-carrying plasmid pSFxv_2 was engineered to carry ampicillin resistance (pSFxv_2-amp) or kanamycin resistance (pSFxv_2-kan). pSFxv_2-amp was used to transform ampicillin-sensitive isolates of 10 different serotypes, 51571 (serotype 1a), 51572 (1b), 51250 (2a), 51251 (2b), 51575 (3a), NCTC 9725 (4a), 51577 (4b), 51247 (5a), 51580 (X), 51581 (Y) and 51579 (6), while pSFxv_2-kan was used to transform ampicillin-resistant but kanamycin-sensitive isolates X6 (1c) and HN153 (1d). After incubation on Luria-Bertani broth (LB) plates with ampicillin or kanamycin overnight at 37°C, visible growth was observed for all strains tested. The plasmid mixtures were extracted from the antibiotic resistant colonies and separated by agarose gel electrophoresis. Compared with its parental strain, each transformant acquired an additional plasmid band of approximately either 8.4 or 7.8 kb, corresponding to the expected sizes of plasmids pSFxv_2-amp and pSFxv_2-kan, respectively. PCR amplification of the *opt* gene from the plasmid extracts confirmed the presence of the recombinant plasmids in the transformants.

To determine whether *S. flexneri* isolates are generally transformable with pSFxv_2, we tested 32 more isolates from 10 serotypes [1a (5), 1b (3), 2a (4), 2b (2), 3a (1), 4a (3), 4b (2), 5a (3), X (4) and Y (5)] using either pSFxv_2-amp or pSFxv_2-kan and found that all isolates were transformed successfully. The transformation efficiency was $1.23–94.4 \times 10^4$ colony forming units (CFU) per mg of plasmid DNA, those of 51247 (5a) and 51579 (6) being the lowest and the highest, respectively (Table I).

To determine the stability of the recombinant plasmids pSFxv_2-amp and pSFxv_2-kan in *S. flexneri*, 100 transformants of each reference strain grown on LB plate with kanamycin or ampicillin were selected and transferred every 24 h to a fresh LB plate in the presence or absence of antibiotics. The number of colonies on the plate without antibiotics represented the quantity of total viable cells in a sample, whereas the number from a LB plate with kanamycin or ampicillin defined the abundance of resistant cells. It was found that 99 to ~100% transformants tested retained the acquired plasmid after 3 days, even without the selective pressure, suggesting that plasmid pSFxv_2 can be stably maintained in the transformants.

Table I. Transformation efficiencies and copy numbers of plasmids pSFxv_2-amp and pSFxv_2-kan to the reference strains of *S. flexneri*

Strain (serotype)	Plasmid	Transfer efficiency ($\times 10^4$ CFU per mg DNA)	Copy number (X ± SD)[a]
51571 (1a)	pSFXv-2-amp	6.13	23.98 ± 6.59
51572 (1b)	pSFXv-2-amp	33.13	33.63 ± 2.19
X6 (1c)	pSFXv-2-kan	25.23	8.97 ± 2.36
HN153 (1d)	pSFXv-2-kan	4.59	7.40 ± 1.66
51250 (2a)	pSFXv-2-amp	9.82	27.66 ± 1.37
51251 (2b)	pSFXv-2-amp	4.91	40.54 ± 5.49
51575 (3a)	pSFXv-2-amp	3.68	22.07 ± 2.77
NCTC 9725 (4a)	pSFXv-2-amp	9.82	34.27 ± 3.96
51577 (4b)	pSFXv-2-amp	7.36	25.63 ± 2.53
51247 (5a)	pSFXv-2-amp	1.23	31.06 ± 2.75
51580 (X)	pSFXv-2-amp	30.67	25.41 ± 3.51
51581 (Y)	pSFXv-2-amp	62.58	34.34 ± 2.94
51579 (6)	pSFXv-2-amp	94.48	11.53 ± 0.99

[a]Result of three independent tests.

The copy number of plasmid pSFxv_2 in all transformants and the reference strain 2002017 (a serotype Xv clinical isolate) were also determined. The average number of plasmid pSFxv_2-amp or pSFxv_2-kan in the transformants ranged from 7.40 to 40.54/cell, those of HN153_1dv and 51251_2bv being the lowest and the highest, respectively (Table I). The copy number of pSFxv_2 in the transformants was higher than in strain 2002017 (6.69/cell). Therefore, plasmid pSFxv_2 can transform all serotypes of *S. flexneri* in the laboratory.

Transformation with pSFxv_2 confers the host with the MASF IV-1 antigenicity

All transformants were serotyped using monoclonal antibodies of the MASF scheme (Reagensia AB, Sweden) and monovalent antisera of the Seiken scheme (Denka Seiken, Japan), and serological features of the transformants and their parental strains are shown in Figure 1. Compared with their parents, all transformants, except 2 serotypes 4b and 5a, acquired additional reactivity with MASF IV-1, a specific monoclonal antibody associated with the PEtN modification in serotypes Xv, Yv and 4av (Figure 1). The MASF IV-1 antigenicity varied among the transformants, serotype 1a, Y, 1b and 2b transformants presenting a weak MASF IV-1 reaction, while others showing strong reactions (Figure 1). As what was found in serotypes Xv, Yv and 4av, all transformants also reacted with monovalent typing antiserum IV (Figure 1). Clearly, the acquisition of pSFxv_2 conferred its host with new serological features. Based on

serological data and O-antigen structure revealed below, we named the artificial v-serotypes of 1a, 1b, 1c, 1d, 2a, 2b, 3a and 6 as 1av, 1bv, 1cv, 1dv, 2av, 2bv, 3av and 6v, respectively, following the designation convention previously established for natural v-serotypes Xv, 4av and Yv. Although MASF IV-1 agglutination was not detected in the serotype 4b and 5a transformants, O-antigen PEtN modification revealed in them (see below) indicate the presence of the MASF IV-1 epitope, and hence they should be named 4bv and 5av, respectively.

It is noteworthy that transformation with pSFxv_2 affected reactivity with some other type- and group-specific antisera. The agglutination level decreased with Seiken monovalent antisera I for serotype 1 (1a and 1b); II for serotype 2b; III for serotype 3 (3a); 7,8 for serotypes X and 3a; 3,4 for serotypes 3a and

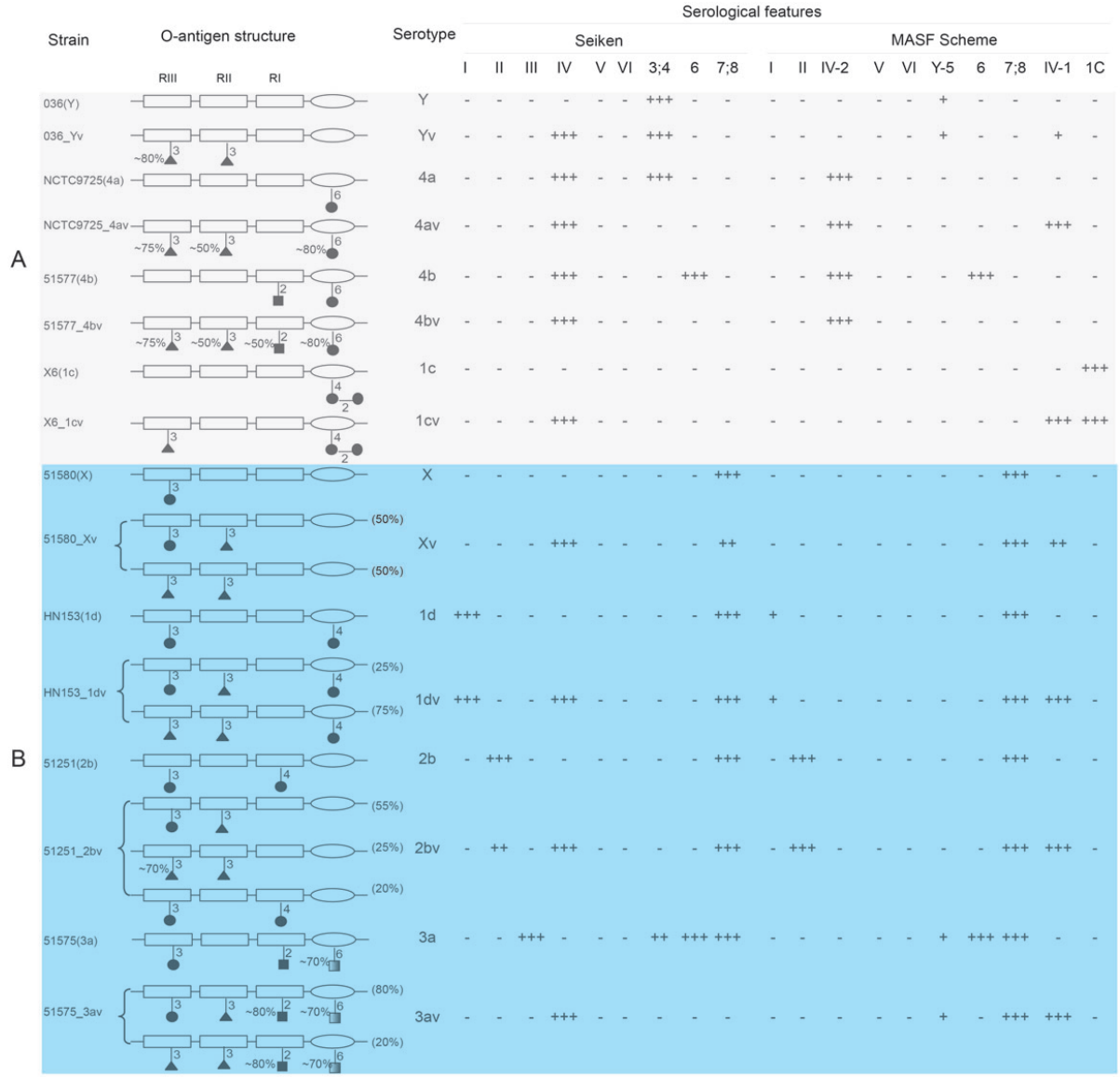

Fig. 1. O-polysaccharide structures and serological features of *S. flexneri* reference strains and their pSFxv_2 transformants. The basic O-antigen of serotypes 1–5, X and Y comprises one *N*-acetylglucosamine and three rhamnose residues (Rha^{I}-Rha^{III}). Serotype 6 shares the Rha^{III}-(1 → 2)-Rha^{II} disaccharide with the other *S. flexneri* serotypes, but the rest of the tetrasaccharide repeat is different. Serotypes differ by the addition of Glc and/or O-acetyl and/or PEtN groups to different sugars within the tetrasaccharide repeat unit via the linkages indicated. A modification degree of <100% is specified. Based on the backbone structure and substitution at Rha^{III}, the serotypes studied are divided into four groups (**A**–**D**), which are shadowed by different colors. The serotypes were determined by slide agglutination test using two commercial serotyping kits: monovalent antisera of Denka Seiken and monoclonal antibody reagents of Reagensia AB according to the manufacturer's instructions. The agglutination was scored according to the following scale: +++, 100% agglutination of the cells; ++, >50% agglutination; +, <50% agglutination; −, no agglutination detected.

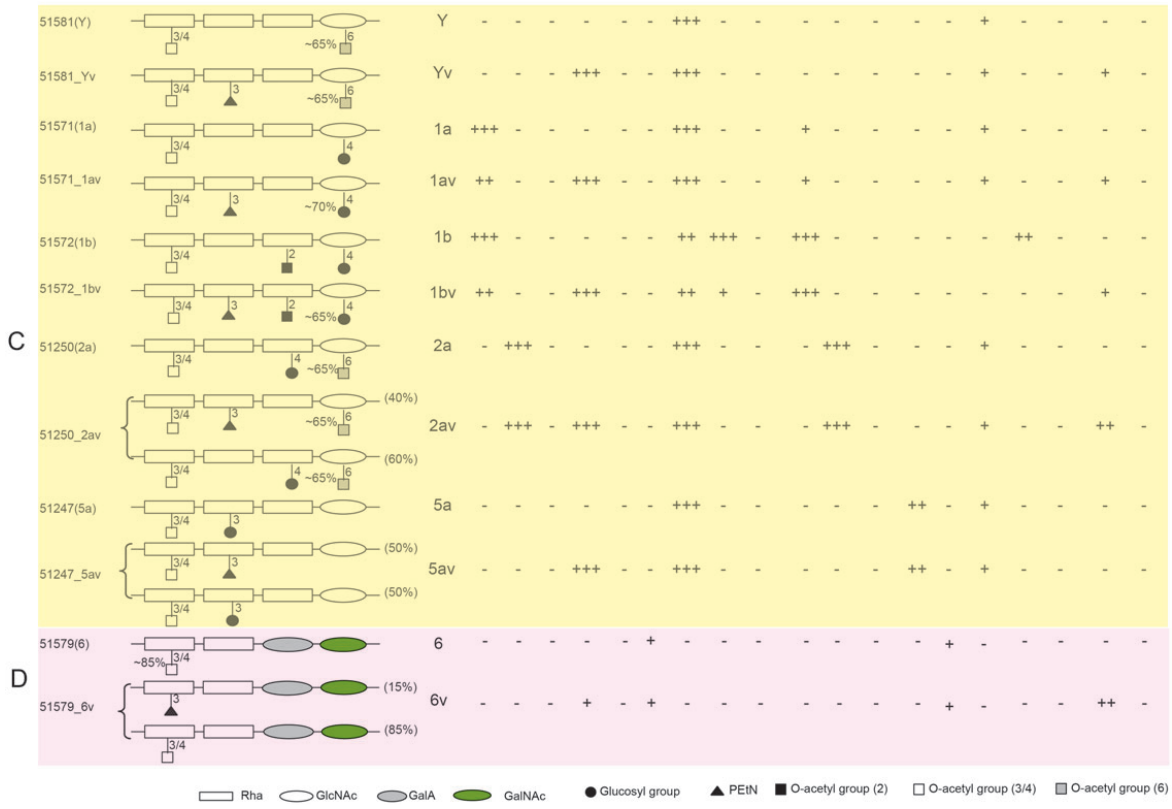

Fig. 1. *Continued*

4a (Figure 1). The Group 6 antigenicity of transformants 1bv, 3av and 4bv also decreased using both monoclonal antibody MASF 6 the monovalent antiserum 6 (Figure 1).

Interestingly, the agglutination pattern of serotype 6v was similar to that of atypical serotype 6 strains reported earlier. Three *S. flexneri* isolates collected in Bangladesh in 1985–1987 reacted with both typing antiserum VI and MASF IV-1 (Carlin et al. 1989), and six more isolates with similar serological features were identified in Bangladesh in 1997–2000 (Talukder et al. 2001). Although the molecular and structural basis of the serospecificity of these nine strains remains unknown, their serological data suggest that they can be classified into serotype 6v. Therefore, *S. flexneri* serotype 6v seems to have already emerged in nature.

Plasmid pSFxv_2 can be mobilized between S. flexneri strains by conjugation

Conjugation is a common means of natural plasmid transfer (Lederberg and Tatum 1946). To test whether pSFxv_2 can be transferred by conjugation, we performed conjugation assay using strain NCTC9725_4av carrying pSFxv_2-kan which is resistant to kanamycin but sensitive to ampicillin as the donor and ampicillin-resistant isolates 01038, HN099 (both serotype 1a), 11JS12 (1b), X6 (1c), HN153 (1d), HN194 (2a), HN018 (2b), 06GS02 (3a), 2002089 and 03031(both X), 036, 03064 and 06004 (all Y) as the recipients. Double resistance to ampicillin and kanamycin was conveniently used for screen for transconjugants on LB plates containing both ampicillin and kanamycin.

After overnight culture, doubly resistant colonies were obtained from all the strains tested, except for serotype 1b strain 11JS12. Serological analysis indicated that all the transconjugants acquired additional reactivity with monoclonal antibody MASF IV-1 or monovalent typing antiserum IV. Plasmid profile analysis revealed an additional band of 7.8 kb, the expected size of plasmid pSFxv_2-kan, as well as high-molecular-weight bands (Figure 2). Southern hybridization using an *opt* gene fragment as probe confirmed that the 7.8-kb plasmid is indeed pSFxv_2-kan and also showed that the high-molecular-weight bands were positive for the probe, suggesting that they were multimers of the pSFxv_2-kan plasmid (Figure 2). The transfer frequency ranged from 4.69×10^{-10} to 5.88×10^{-7} transconjugants per donor cell.

These results indicate that plasmid pSFxv_2 can be mobilized by conjugation. Since pSFxv_2 has no conjugation elements (e.g., the *tra* cluster) (Sun et al. 2012) and is not a conjugative plasmid, unknown factors encoded elsewhere must be involved in the conjugation process. The data obtained further support that pSFxv_2 can be transferred to other *S. flexneri* serotypes.

O-antigen structure variations of the pSFxv_2 transformants and their effect on serological reactivity

To determine the effect of the pSFxv_2 transformation on the O-antigen structure, the O-antigens of the transformants and

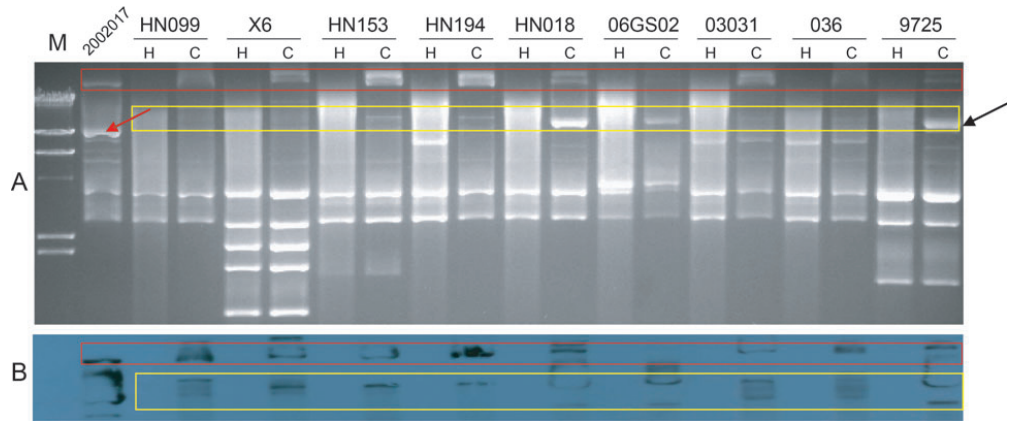

Fig. 2. Plasmid profiling and Southern hybridization analysis of transconjugatants and parental isolates. (**A**) Plasmid profiles of transconjugatants and their parental isolates. Isolates HN099 (serotype 1a), X6 (1c), 153 (1d), HN194 (2a), HN018 (2b), 06GS02 (3a), 03031(X) and 036 (Y) were selected as the recipients; serotype Xv wild isolate 2002017 was used as the control; NCTC9725_4av (NCTC9725 transformed by plasmid pSFxv_2-kan) was used as donor. Plasmid DNA was separated by electrophoresis with a Chef Mapper system (Bio-Rad Laboratories, Hercules, CA) on a 1% SeaKem Gold agarose gel and visualized by EB staining. Lambda DNA cleaved with *HindIII* (TaKaRa) was used as molecular mass markers. Yellow pane shows the location of plasmid pSFxv_2; red pane indicates the location of the high-molecular-weight plasmid (>23 kb) presented in the transconjugatants and the control wild serotype Xv strain 2002017. Black arrow indicates the band of pSFxv_2-kan (7.8 kb) of NCTC9725_4av, which is larger than that in 2002017 (red arrow, 6.8 kb). (**B**) Southern hybridization detection of pSFxv_2-kan in transconjugants. PCR product amplified from strain 2002017 using primer pair *lpt-O-2* was prepared as probe (Sun et al. 2012).

their parental strains were analyzed as descried in the Materials and methods section. The data obtained in this work and earlier (Sun et al. 2012; Knirel et al. 2013) are summarized in Figure 1. Previously, it was shown that pSFxv_2 carries the *optII* gene, which preferably mediates the addition of PEtN onto Rha^{II} (Knirel et al. 2013). In this work, we found that other O-antigen modifications affect and/or compete with PEtN modification. For the purposes of comparison and discussion, the serotypes studied were divided into four groups depending on the backbone structure and modification at Rha^{III} in the parental strains. The data are given in Table II and Figure 1.

Serotypes Y, 4a, 4b and 1c which have no modification at Rha^{III} (Group A). Group A serotypes are characterized by the lack of Glc or OAc group at Rha^{III}. In serotype Yv (strain 036), 4av and 4bv transformants, both Rha^{II} and Rha^{III} are modified. However in serotypes 4a and 4b, glucosylation at position 6 of GlcNAc interfered with PEtN modification at Rha^{II}. As a result, the content of PEtN on Rha^{II} in 4av and 4bv was lower than in Yv (~50% vs. 100%), and the degree of glucosylation at GlcNAc also reduced (~80% vs. 100%). Neither the PEtN modification nor the decrease in the content of Glc affected the Glc-associated type IV antigenicity of 4av and 4bv transformants, which showed the same agglutination level as their parental strains (Figure 1).

The PEtN modifications at Rha^{II} and Rha^{III} also interfered with O-acetylation at position 2 of Rha^{I} (group antigenic determinant 6). Only ~50% Rha^{I} in 4bv was O-acetylated and, consequently, the antiserum 6 reaction was weaker than that of parental 4b strains with a fully O-acetylated Rha^{I} (Figure 1). In contrast, 2-O-acetylation at Rha^{I} had no influence on the PEtN modification since the PEtN phosphorylation levels of Rha^{II} and Rha^{III} are the same in serotypes 4bv and 4av with the former containing OAc at Rha^{I}.

In serotype 1c, the presence of the Glc-(1→2)-Glc disaccharide on GlcNAc blocked PEtN modification of Rha^{II}. Thus, the PEtN modification in the 1cv O-antigen is restricted to Rha^{III}.

The interference among PEtN phosphorylation, glucosylation and O-acetylation is likely to produce heterogeneous repeats with different modifications forming the final O-antigen.

Serotypes X, 1d, 2b and 3a which carry Glc at position 3 of Rha^{III} (Group B). In these serotypes, Rha^{III} is occupied by Glc giving rise to group antigenic determinant 7,8, whereas Rha^{II} is not modified. However, PEtN modification was possible not only at Rha^{III} but, in a competitive manner with glucosylation, also at Rha^{III} resulting in structural heterogeneity with a reduced level of glucosylation at Rha^{III}. The decrease in the glucosylation of Rha^{III} abolished the 7,8 antigenicity of 3av and slightly diminished that of Xv when tested with MASF 7,8 but had no influence on the agglutination levels of 1dv and 2bv when tested using both MASF and Seiken typing kits (Figure 1).

2-O-Acetylation at Rha^{I} and 6-O-acetylation at GlcNAc in serotype 3a did not affect the PEtN modification pattern and vice versa. However, compared with its parental strain, the OAc content on Rha^{I} in 3av decreased to ~80% (Figure 1), and correspondingly, a decrease in the reaction levels with antisera 6 and III was observed (Figure 1).

The PEtN modification led serotype 2b to lose Glc on Rha^{I} (typing antigenic determinant II) and converted serotype 2b into Xv rather than 2bv (~20% repeats remained unmodified). The Glc at position 4 of GlcNAc in 1d had no influence on the PEtN modification pattern but significantly decreased the conversion level (only ~35% repeats were modified).

Serotypes Y, 1a, 1b, 2a and 5a which are mono-O-acetylated at either position 3 or 4 of Rha^{III} (Group C). 3/4-O-Acetylation at

Table II. PEtN modification patterns of *S. flexneri* transformants

Strain	Conversion (%)	PEtN content (%) on					References
		Only Rha^{II}	Only Rha^{III}	Both Rha^{II} and Rha^{III}	Total Rha^{II}	Total Rha^{III}	
Group A (no substituent on Rha^{III})							
036_Yv	100	20	–	80	100	80	Knirel et al. (2013); Sun et al. (2012)
NCTC9725_4av	100	25	50	25	50	75	Knirel et al. (2013); Sun et al. (2012)
51577_4bv	100	25	50	25	50	75	This work
X6_1cv	100	–	100	–	0	100	This work
Group B (Glc on Rha^{III})							
51580 _Xv	100	60	–	40	100	40	Knirel et al. (2013); Sun et al. (2012)
HN153_1dv	35	70	–	30	100	30	This work
51251_2bv	80	80	–	20	100	20	This work
51575_3av	100	80	–	20	100	20	This work
Group C (100% OAc on Rha^{III})							
51581_ Yv	100	100	–	–	100	0	This work
51571_1av	100	100	–	–	100	0	This work
51572_1bv	100	100	–	–	100	0	This work
51250_2av	40	100	–	–	100	0	This work
51247_5av	50	100	–	–	100	0	This work
Group D (different backbone structure, 85% OAc on Rha^{III})							
51579_F6v	15	–	100	–	0	100	This work

position 3 or 4 of Rha^{III} in serotypes Y, 1a, 1b, 2a, 5a and 6 has been reported recently (Perepelov et al. 2009, 2010, 2012). The degree of the O-acetylation varies in the range of 30–70% and 15–30% at position 3 and 4, respectively (Perepelov et al. 2012). In serotype Y, 1a, 1b, 2a and 5a strains, 3/4-O-acetylation blocked the PEtN modification at Rha^{III} and PEtN modification occurred at Rha^{II} only. PEtN modification was not affected by O-acetylation at Rha^{I} in 1b or at GlcNAc in Yv (strain 51581) and 2av, nor by glucosylation at GlcNAc in 1a and 1b.

PEtN modification has no effect on O-acetylation at any position, but reduces glucosylation at GlcNAc to ~65% from 100% in 1av and 1bv. This also reduced agglutination with type I antiserum.

PEtN modification at Rha^{II} competed with glucosylation at Rha^{I} in 2a and Rha^{II} in 5a (type antigenic determinant II and V). As a result, the 2a and 5a repeats converted into the Yv (strain 51581) and O-deacetylated Yv repeats rather than those of 2av and 5av, respectively. In both cases, conversion was lower (40–50%) than in the other Group C serotypes that lack Glc at Rha^{I} and Rha^{II}. Therefore, two types of O-antigen repeats, PEtN-modified and nonmodified, were present in these transformants. The ability of the transformants to react with antibodies MASF II and MASF V was not affected, and they were serotyped as 2av and 5av.

Serotype 6 with a different O-antigen backbone structure (Group D). The serotype 6 O-antigen has a Rha^{III}-(1 → 2)-Rha^{II} disaccharide rather than a Rha trisaccharide in other *S. flexneri* serotypes. Upon pSFxv_2 transformation, PEtN modification of serotype 6 occurred at Rha^{III} in ~15% O-antigen repeats giving rise to IV-1 positive serotype 6v (Figure 1). The extent of modification is evidently restricted to the proportion of non-O-acetylated repeats since about ~85% Rha^{III} was O-acetylated in both parent and transformant. No PEtN modification of Rha^{II} was observed, most likely, owing to a different neighboring monosaccharide: GalA in serotype 6 vs. Rha^{I} in all other *S. flexneri* serotypes.

Conclusions

This study demonstrated that *optII*-carrying plasmid pSFxv_2 isolated from a serotype Xv strain can be transferred into, and stably maintained in, all other *S. flexneri* serotypes (1–6) by transformation. The transformants expressed the MASF IV-1 epitope and possessed modified O-antigens with PEtN group(s) at position 3 of Rha^{III} or Rha^{II} or both in the tetrasaccharide repeat. In some serotypes, PEtN modification competed or/and interfered with glucosylation and O-acetylation at the same or a neighboring sugar residue, which can account for changes in serological characteristics of the transformants. pSFxv_2 plasmid has been demonstrated to be mobilizable by conjugation. The data obtained showed that the *opt*-carrying pSFxv_2 and pSFyv_2 plasmids potentially can disseminate to other serotypes of *S. flexneri* giving rise to novel serotypes, which may confer a significant advantage to the bacteria. Further studies of effect of PEtN modification on virulence would be warranted.

Materials and methods

Biosafety statements

All procedures involving the recombinant DNA work were performed in a biosafety level-2 facility according to standard operating procedures approved by the institutional biosafety committee. The recombinant plasmids and strains will be held in biosafety level-2 facility for 5 years, and disposed of using autoclave methods, following the Regulation of Pathogenic Microorganism Laboratory Biosafety Management of China. The containment will ensure the safety and security of these plasmids and strains.

Strains, plasmids and culture conditions

Serotype Xv strain 2002017 (Ye et al. 2010; Sun et al. 2012) was used as the source of plasmid pSFxv_2. *Shigella flexneri* isolates 51571 (1a), 51572 (1b), X6 (1c), HN153 (1d) (Luo et al. 2012), 51250 (2a), 51251 (2b), 51575 (3a), NCTC 9725 (4a) (Sun et al. 2012; Knirel et al. 2013), 51577 (4b), 51247 (5a), 51580 (X) (Sun et al. 2012; Knirel et al. 2013), 51581 (Y) (Sun et al. 2012; Knirel et al. 2013) and 51579 (6) were used as reference strains for transformation analysis. Thirty-two more *S. flexneri* isolates belonging to 10 serotypes [1a (5), 1b (3), 2a (4), 2b (2), 3a (1), 4a (3), 4b (2), 5a (3), X (4) and Y (5)] were selected for transformation analysis to further confirm the observation. For conjugation assay, strain NCTC9725 (4a) was transformed with plasmid pSFxv_2-kan, and the transformant NCTC9725_4av (carrying pSFxv_2-kan, kan^r, amp^s) was used as the donor. Kanamycin-sensitive but ampicillin-resistant isolates 01038 and HN099 (both serotype 1a), 11JS12 (1b), X6 (1c), HN153 (1d), HN194 (2a), HN018 (2b), 06GS02 (3a), 2002089 and 03031(both X), 036, 03064 and 06004 (all serotype Y) were selected as the recipients. All strains were isolated from diarrheal patients in China or purchased from NCTC (National Center for Typical Collection). Plasmid pRS551 (kindly donated by Dr Long-Fei Wu) and pMD20T (TaKaRa, Japan) were used as template for kanamycin and ampicillin resistance gene amplification. *Shigella flexneri* strains were routinely grown in a 37°C incubator or orbital shaker in LB supplemented with ampicillin (100 μg mL^{-1}) and kanamycin (40 μg mL^{-1}) when appropriate.

Serotyping

Shigella flexneri strains were serotyped by slide agglutination test using two commercial serotyping kits: monovalent antisera of Denka Seiken and monoclonal antibody reagents of Reagensia AB according to the manufacturer's instructions. The agglutination was sensitively scored according to the following scale: +++, 100% agglutination of the cells; ++, >50% agglutination; +, <50% agglutination; −, no agglutination detected.

Isolation of plasmid pSFxv_2 and labeling with antibiotic resistance genes

For most of the reference *S. flexneri* isolates which were sensitive to ampicillin, ampicillin was used as a selection marker for pSFxv_2. For ampicillin-resistant isolates (X6 and HN153), kanamycin was used. Plasmid DNA mix containing pSFxv_2 was extracted from serotype Xv strain 2002017 using the plasmid purification kit (Qiagen, Germany). To further isolate and purify pSFxv_2, the plasmid DNA mix was separated by electrophoresis, and the target band of 6.8 Kb was cut and purified using DNA purification Kit (Qiagen). Primer pairs amp-U: GCGAATTCATCTCAGTTCGGTGTAGGTC and amp-L: GCGAATTCACGTCAGGTGGCACTTTT and kan-U: GCGAATTCCACGT TGTGTCTCAAAATCT and kan-L: GCGAATTCCGTCCCGTCAAGTCAGCGTA, were used to amplify the functional ampicillin and kanamycin resistance fragments, respectively from plasmid pMD20T and pRS551. Each fragment contains the ampicillin resistance gene (*amp*) or kanamycin adenyl transferase gene (*kan*), as well as the promoter and terminator sequences up- and downstream of it. After digestion with endonuclease *EcoR*I, the fragments were inserted into the *EcoR*I site (base 5180) of pSFxv_2 to form recombinant plasmids pSFxv_2-amp and pSFxv_2-kan, respectively. Figure 3 shows the genetic maps of plasmid pSFxv_2-amp and pSFxv_2-kan. Importantly, as the *EcoR*I site of pSFxv_2 is located in the none-coding region, the integration of antibiotic resistance fragments should not affect the function of pSFxv_2. The recombinant plasmids were transformed into commercial *E. coli* DH5α competent cells (TaKaRa) and transformants were grown on LB plates supplemented with ampicillin (100 μg mL^{-1}) or kanamycin (40 μg mL^{-1}). The recombinant plasmids were further confirmed by PCR amplification and restriction digestion.

Transformation of S. flexneri using pSFxv_2-amp and pSFxv_2-kan

Plasmid transformation of *S. flexneri* was performed using a standard chemical protocol (Sambrook et al. 1989). To prepare the competent cells, overnight *S. flexneri* cultures were transferred to 50 mL of LB liquid media and allowed to grow until the cell density reached to 0.6 (OD_{600}). One milliliter of cells were collected by centrifugation, washed twice with cold calcium chloride (0.1 M) and resuspended in 200 μL of the same solution. For transformation, the appropriate quantity of plasmid DNA (1.5 mg in a volume of 10 μL) was mixed with the competent cells and incubated on ice for 30 min. After heat-

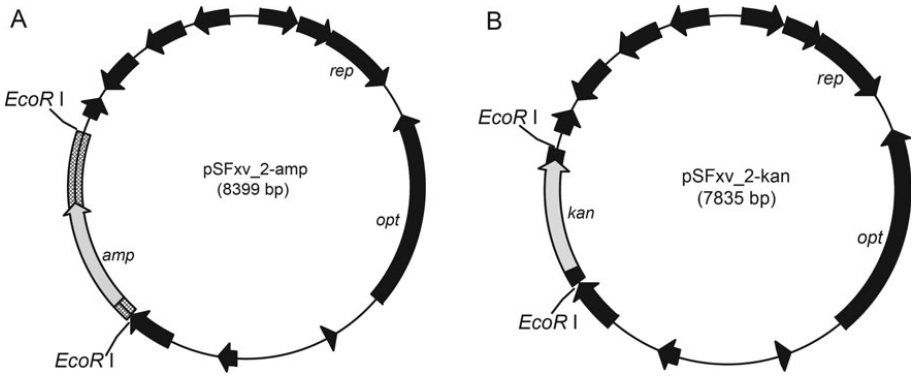

Fig. 3. Genetic maps of recombinant plasmids pSFxv_2-amp and pSFxv_2-kan. Thick black arrows represent the annotated *orfs* of pSFxv_2. Black strip indicates the functional antibiotic fragments, and gray arrow indicates the ampicillin resistance gene (*amp*) or kanamycin adenyl transferase gene (*kan*). The restriction site of *EcoR*I is shown.

shock at 42°C for 90 s, cells were mixed with 0.8 mL super optimal broth with catabolite repression (SOC) medium and incubated at 37°C for 45 min. 100 µL of the recovered culture was spread on LB plates with appreciated antibiotics and incubated at 37°C overnight. An aliquot of this suspension was incubated on LB agar plates without antibiotics to determine the recipient cell number. Transformation frequency is represented as the number of CFU grown per mg of DNA in 2.0×10^9 competent cells. The serological features of transformants were identified using monoclonal antibody of the MASF scheme, or monovalent antisera of the Seiken scheme.

Plasmid stability assay

Plasmid stability assays were done essentially as described (Sota et al. 2010). Briefly, transformants containing pSFxv_2-amp or pSFxv_2-kan were first grown overnight in LB plates supplemented with ampicillin or kanamycin to obtain single colonies. One hundred single colonies of each transformant were selected randomly and replicated on LB plates with and without ampicillin or kanamycin. The ratio of ampicillin or kanamycin-resistant colonies to total colonies was considered the plasmid-containing fraction. This procedure was repeated every 24 h for 3 days.

Determination of the plasmid copy number in transformants

The copy number of pSFxv_2 in *S. flexneri* 2002017 and transformants was measured by real-time quantitative PCR (Lee et al. 2006). The pSFxv_2-specific gene *opt* and the chromosomal gene *arcA* were selected as molecular markers of plasmid and chromosome, respectively. The *arcA* and *opt* gene PCR amplified from strain 2002017 were cloned into pMD20-T at a ratio of 1:1 to generate the reference plasmid pMD20-T-*arcA*-*opt*. Primer pairs *arcA*-U: GTTGCGTTGATGTTCCTGAC and *arcA*-L: CTACGACGTTCTTCGCTGAC, and *opt*-U: TCTTCTGATGGG TAACTTGCA and *opt*-L: CTTGGCTTAGATAGCCCTGAT were used to detect the *arcA* and *opt* genes in the respective samples. Total DNA prepared by boil water methods was used for PCR template. The plasmid copy number in the transformants can be determined as the copy ratio of *opt* to *arcA*.

Conjugation experiments

Conjugation experiments were carried out essentially as described (Munshi et al. 1987). Briefly, donor and recipient strains were grown separately in LB broth for 4 h (OD_{600}, 0.8), and then 0.4 mL of donor culture was mixed with 4.6 mL of recipient culture. Cells were collected by centrifugation, bacteria were resuspended in 200 µL LB and spread onto a nitrocellulose membrane overlaid on a LB agar plate and incubated overnight. The filter was placed in 3 mL sterile saline and 100 µL of the solution was spread onto LB plate containing ampicillin (100 µg mL^{-1}) and kanamycin (40 µg mL^{-1}). Strains resistant to both ampicillin and kanamycin were further characterized by serotyping and plasmid profiling. The transfer frequency of the resistance plasmid was calculated by a method described (Munshi et al. 1987).

Isolation and structure analysis of O-polysaccharides

Lipopolysaccharides (LPSs) were isolated by the phenol-water extraction of dried bacterial cells (Westphal and Jann 1965). 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 and the supernatant was dialyzed against distilled water and freeze-dried. The purified LPSs were degraded with aqueous 2% acetic acid at 100°C, and the O-polysaccharides were isolated in yields of 27–44% by gel-permeation chromatography on Sephadex G-50 Superfine in 0.05 M pyridinium acetate buffer, pH 4.5, monitored with a differential refractometer (Knauer, Germany).

The NMR spectra were run for solutions in 99.95% D_2O at 30° C using an Avance II 600 instrument (Bruker, Germany). Internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0.00) and acetone (δ_C 31.45) were used as references for calibration of the ^1H and ^{13}C NMR spectra, respectively. Prior to the measurements, O-polysaccharide samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O. Two-dimensional NMR spectra were obtained using the standard Bruker software. Mixing time of 100 ms was used in total correlation spectroscopy (TOCSY) and ROESY experiments, respectively.

Structures of the O-polysaccharides were elucidated using two-dimensionally NMR spectroscopy, including $^1H,^1H$ correlation spectroscopy (COSY), TOCSY, $^1H,^{13}C$ heteronuclear single-quantum coherence (HSQC) and $^1H,^{31}P$ heteronuclear multiple-bond correlation (HMBC) experiments. To establish the carbohydrate backbone structure, including the pattern of lateral glucosylation, the ^1H and ^{13}C NMR chemical shifts were assigned using COSY, TOCSY and HSQC spectra and compared with published data of phosphorylated (Perepelov et al. 2012; Sun et al. 2012; Knirel et al. 2013) and nonphosphorylated (Perepelov et al. 2009, 2010, 2012) *S. flexneri* O-polysaccharides. The degree of glucosylation was determined by a ratio of intensities of ^1H NMR signals of the lateral Glc and main-chain Rha or/and GlcNAc residues.

In case of the presence of OAc groups, the O-polysaccharides were O-deacetylated with aqueous 12% ammonia at 37°C for 16 h. After structure elucidation of the O-deacetylated polysaccharide, the positions of the O-acetyl groups were determined by a comparison of the two-dimensional NMR spectra of the initial and O-deacetylated polysaccharides taking into account known effects of O-acetylation on ^1H and ^{13}C NMR chemical shifts (Jansson et al. 1987). Particularly, the signals of the protons at the O-acetylation sites (position 2 in Rha^I, positions 3 and 4 in Rha^{III} and position 6 in GlcNAc) were shifted downfield by 1.08–1.35 ppm for Rha and 0.5–0.7 ppm for GlcNAc due to a deshielding effect of the O-acetyl group. In addition, diagnostic downfield displacements by 2.2–3.0 ppm were observed for signals of the carbons at the O-acetylation sites and upfield displacements by 1.5–2.5 ppm for the neighboring carbon signals. The degree of O-acetylation was estimated as a ratio of the signal intensities of the corresponding O-acetylated and nonacetylated sugar residues as well as those of the O-acetyl group(s) and the N-acetyl group of GlcNAc.

The position(s) of PEtN was established by a correlation of phosphorus with proton H3 of Rha at the phosphorylation site revealed by $^1H,^{31}P$ HMBC spectra. Cross-peaks at δ_H ~4.25 or/and ~4.35 ppm indicated PEtN at Rha^{III} or/and Rha^{II}, respectively. In the $^1H,^{13}C$ HSQC spectrum, both H3/C3 and H2/C2 cross-peaks of the phosphorylated Rha residues shifted

significantly to a characteristic region of δ_H/δ_C 4.23–4.35/76–79 ppm and were easily identified. The degree of PEtN modification was determined by the ratio of integral intensities of the signals belonging to PEtN group(s) at δ_H 3.28–3.29 (CH_2N) and 4.13–4.17 (CH_2O) and those of phosphorylated and nonphosphorylated Rha residues.

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Conflict of interest

None declared.

Abbreviations

COSY, correlation spectroscopy; GalA, galacturonic acid; GalA, galacturonic acid; Glc, glucosyl; GlcNAc, *N*-acetylglucosamine; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; LB, Luria-Bertani broth; LPS, lipopolysaccharide; OAc, O-acetyl; PEtN, phosphoethanolamine; TOCSY, total correlation spectroscopy; MASF, monoclonal antibody of *Shigella flexneri*; PCR, polymerase chain reaction; NMR, nuclear magnetic resonance; ROESY, rotating frame Overhauser effect spectroscopy.

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