Saccharides cross-reactive with *Bacillus anthracis* spore glycoprotein as an anthrax vaccine component

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Bacillus anthracis **is a spore-forming bacterium that causes anthrax in humans and in other mammals. The glycoprotein BclA (*Bacillus* collagen-like protein of *anthracis*) is a major constituent of the exosporium, the outermost surface of *B. anthracis* spores. The glycosyl part of BclA is an oligosaccharide composed of 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucose, referred to as anthrose, and three rhamnose residues. A structure similar to anthrose, 4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucose is found in the side chain of the capsular polysaccharide (CPS) of *Shewanella* spp. MR-4. Under certain growth conditions the bacteria produce a variant CPS lacking one methyl group on the hydroxybutyrate, 4-(3-hydroxybutanamido)-4,6-dideoxy-D-glucose. Contrary to anthrose, neither of the *Shewanella* CPSs is 2-*O* methylated. Here, we report that both *Shewanella* CPS variants react with anti-*B. anthracis* spore sera. We also found that these antisera reacted with flagellae of *Pseudomonas syringae*, reported to be glycosylated with a similar terminal saccharide, 4-(3-hydroxybutanamido)-4,6-dideoxy-2-*O*-methyl-D-glucose. Sera produced by immunization with *Shewanella* or *P. syringae* cells bound to *B. anthracis* spores but not to *Bacillus cereus* spores in a fluorescent microscopy assay. These experiments show that methylation of the anthrose at the *O*-2 of the sugar ring and at the *C*-3 of 3-hydroxybutyrate are not essential for induction of cross-reactive antibodies. We report the preparation, characterization, and antibody responses to protein conjugates of the two variants of *Shewanella* CPS. Both conjugates induced antibodies that bound to both *Shewanella* CPS variants by ELISA and to *B. anthracis* spores, as detected by fluorescent microscopy. We propose the use of *Shewanella* CPS conjugates as a component of an anthrax vaccine.**

anthrose | capsule | pseudomonas syringae | shewanella | flagellae

Anthrax, a potentially lethal human infection, is a zoonotic disease contracted by humans under natural conditions directly or indirectly from domesticated animals. Disease manifestations occur according to one of three routes of encounter: (*i*) cutaneous, the most common, by contact with animals or their products; (*ii*) inhalational, the most serious; or (*iii*) gastrointestinal (1). The causative organism, *Bacillus anthracis*, exists in vegetative or in spore form. In its vegetative form, *B. anthracis* produces two virulence factors that are essential for pathogenesis: the anthrax toxin and the capsule (2). Spores are the infecting agent. They resist extreme heat, dryness, and aggressive chemical conditions and survive for decades in the soil (3).

Spores of *B. anthracis* are surrounded by a loose layer, the exosporium, composed of a number of proteins. A major protein, called BclA (*Bacillus* collagen-like protein of *anthracis*), was shown recently to be a glycoprotein containing short O-linked sugar chains. Its structure (Fig. 1*A*) was elucidated by NMR and mass spectroscopy (4). It contains three rhamnose residues substituted by an unusual nonreducing terminal sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, named anthrose. The reducing end rhamnose is most likely attached to the protein through a GalNAc moiety.

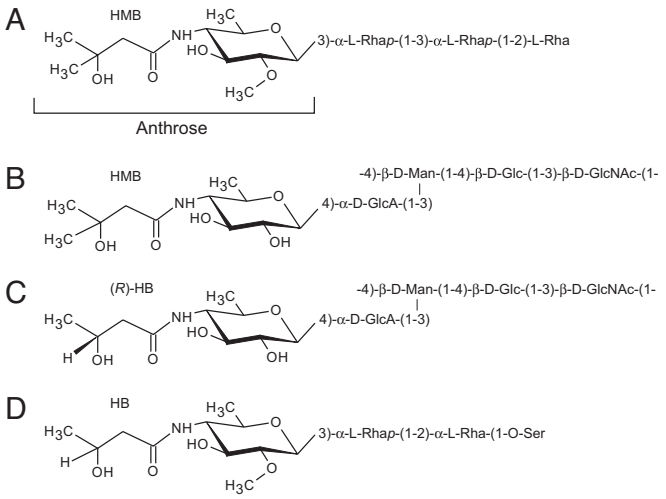

Fig. 1. Structures of saccharides of *Bacillus anthracis* BclA glycoprotein (4) (*A*); *Shewanella* spp. MR-4 CPS (both structures are present and the ratio depends on the growing conditions) (9) (*B* and *C*); and *Pseudomonas syringae* pv. *tabaci* 6605 flagellin glycan (10) (*D*).

This oligosaccharide (OS) was chemically synthesized in several laboratories and, when covalently attached to a protein, shown to be immunogenic in animals (5–8).

The spore OS was reported to be unique for bacilli spores (4, 9). However, carbohydrates of similar structures are present on two other bacteria. The capsular polysaccharide (CPS) of the marine organism *Shewanella* spp. MR-4 (10) contains side chains with terminal residues of 4-amino-4,6-dideoxy-D-glucopyranose (Qui4N) substituted with 3-hydroxy-3-methylbutyrate or 3-hydroxybutyrate, in different ratios depending on the growth medium (Fig. 1 *B* and *C*). Another structure similar to anthrose was found on flagella of *Pseudomonas syringae*, a plant pathogen (Fig. 1*D*). It contains two rhamnose residues and a terminal 4-(3-hydroxybutanamido)-4,6-dideoxy-2-*O*-methyl-D-glucopyranose, thus differing from the anthrax OS by the replacement of 3-hydroxy-3-methylbutyrate with 3-hydroxybutyrate at the amino group of Qui4N (11). In this study we report the serological cross-reactivity of *Shewanella* and *P. syringae* saccharides with anthrax spores and the preparation of *Shewanella* CPS–protein conjugates as a potential component of improved anthrax vaccines.

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Table 1. Binding of rabbit anti-spore and rabbit anti-anthrose sera to *Shewanella* spp. MR-4 CPS

	IgG titers*	
Coating antigen	Anti-spore	Anti-anthrose
CPS_{TSB}	1600	12,800
CPS_{CDM}	3200	500

*IgG titer was defined as the serum dilution that gave OD_{405} of 1 by ELISA. The OD_{405} of preimmune rabbit serum was <0.08 at the dilution of 1:20.

Results

Characterization of *Shewanella* spp. MR-4 CPS. Two types of CPS were purified from *Shewanella* spp. MR-4: CPS_{TSB} (CPS purified from bacteria grown in Tryptic Soy Broth) and CPS_{CDM} (CPS purified from bacteria grown in chemically defined media). NMR analyses confirmed that the terminal Qui4N was substituted with 3-hydroxy-3-methylbutyrate and 3-hydroxy-butyrate at an approximately 1:1 ratio in CPS_{TSB} and almost entirely (>95%) with 3-hydroxy-butyrate in CPS_{CDM}, in agreement with the reported structures (10). Both CPSs formed viscous solutions and eluted as a single broad peak starting at the void volume of the Sepharose CL-6B column.

Anti-*B. anthracis* spore and anti-anthrose sera precipitated with both CPS_{TSB} and CPS_{CDM} by immunodiffusion. Table 1 shows that both antisera bound to both forms of *Shewanella* CSP by ELISA, but the anti-spore serum had a higher titer against CPS_{CDM} than against CPS_{TSB}; the reverse was observed with the anti-anthrose serum.

Fluorescence microscopy showed that antiserum induced by *Shewanella* spp. MR-4 bound to *B. anthracis* spores (Fig. 2). There was no binding to *Bacillus cereus* spores (data not shown).

Characterization of *P. syringae* Flagellae and LPS. Anti-*B. anthracis* spore and anti-*Shewanella* spp. MR-4 sera precipitated with *P. syringae* flagellae by immunodiffusion, confirming that the common sugar, present on *B. anthracis* spores, the *Shewanella* capsule, and the *P. syringae* flagellae, is cross-reactive. Because *P. syringae* flagellae could not be isolated free of LPS, the O-specific polysaccharide (O-SP) of *P. syringae* LPS was isolated, and its structure was analyzed. The results are presented in Table 2: the structure of the O-SP was found to be identical to that of the described *P. syringae* pv. tabaci 225 serogroup VIII O-SP (12). It did not contain any anthrose-like sugar, and thus the cross-reactivity was ascribed to the glycosylated flagellae. This result was confirmed by Western blot analyses, in which flagellae but not LPS reacted with both anti-spore and anti-anthrose sera (data not shown).

Fluorescence microscopy showed that sera induced by *P. syringae* bound to *B. anthracis* (Fig. 2) but not to *B. cereus* spores. The *P. syringae* anthrose-like sugar contained only 3-hydroxy-butyrate groups, but the antibodies induced by it bound to spores that were reported to carry only 3-hydroxy-3-methylbutyrate groups, indicating that this methyl group is not essential for cross-reactivity.

Characterization of *Shewanella* spp. MR-4 CSP Conjugates. Both conjugates, BSA/CPS_{TSB} and BSA/CPS_{CDM}, formed a line of identity with anti-BSA and anti-spore sera by immunodiffusion. Both conjugates had high molecular masses, as shown by their elution at the void volume of the Sepharose CL-6B column. The protein/sugar ratios are shown in Table 3. BSA/CPS_{TSB} had lower sugar content than BSA/CPS_{CDM}, probably because the BSA used for its preparation had a lower hydrazide content (5.2%) than the one used for BSA/CPS_{CDM} (9.8%). Both conjugates had <5 endotoxin units/μg as determined by the limulus amoebocyte lysate assay.

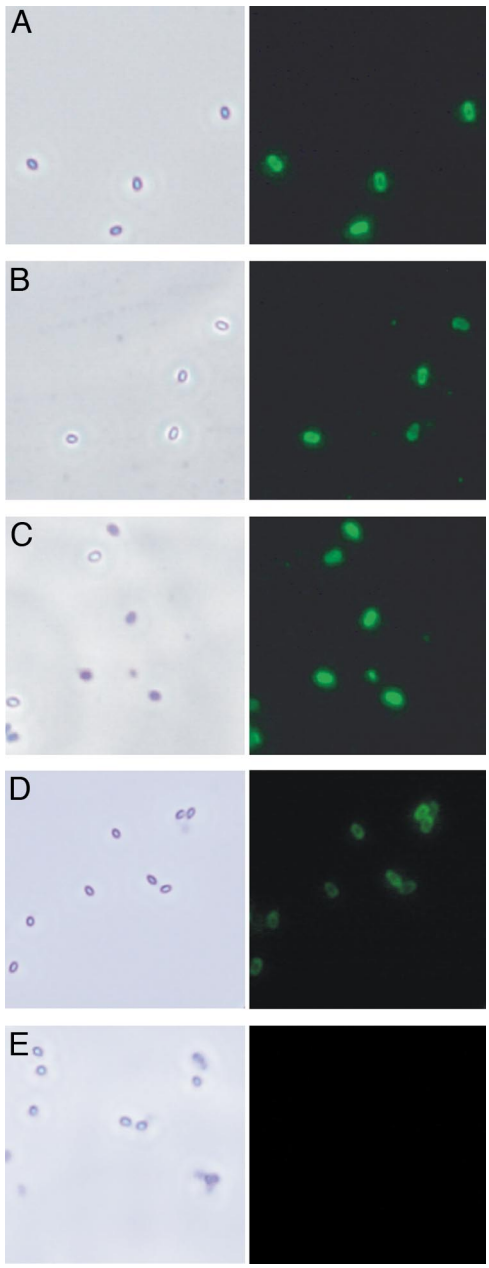


Fig. 2. Immunofluorescent staining of *B. anthracis* spores. (*Left*) Phase-contrast microscopy showing the spores. (*Right*) Spores treated with hyperimmune anti-*Shewanella* spp. MR-4 (*A*); anti-Conjugate no. 1: BSA/CPS_{TSB} (*B*); anti-Conjugate no. 2: BSA/CPS_{CDM} (*C*); hyperimmune anti-*P. syringae* (*D*); and preimmune control serum (*E*).

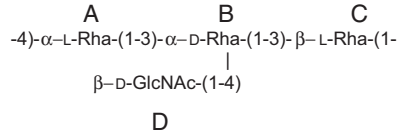
BSA/CPS_{TSB} induced higher antibody levels to CPS_{TSB} than to CPS_{CDM} [54 vs. 43 ELISA units (EU)] but the difference was not statistically significant. There was no difference between antibody levels induced by BSA/CPS_{CDM} and the two CPS (31 vs. 32 EU; Table 2).

Fluorescence microscopy showed that serum induced by both conjugates reacted with *B. anthracis* spores (Fig. 2). In CPS_{CDM} and in *P. syringae* flagella, a 3-hydroxy-butyrate group was found in place of the 3-hydroxy-3-methylbutyrate present in *B. anthracis* spores. Also, both *Shewanella* CPSs lacked the methyl group at *O*-2, which was described in anthrose. This suggests that none of these methyl groups is essential for cross-reactivity.

Table 2. NMR data for *P. syringae* pv. tabaci 6605 O-SP (δ, ppm; 60°C)

Residue	Nucleus	1	2	3	4	5	6
α-Rha A	H	4.93	4.04	4.14	3.59	4.43	1.32
	C	97.1	71.1	70.1	84.0	68.2	17.9
α-Rha B	H	4.96	4.15	3.96	3.73	4.02	1.29
	C	97.2	67.9	73.5	77.3	69.5	18.0
α-Rha C	H	4.67	4.29	3.64	3.47	3.49	1.36
	C	101.8	68.2	78.1	71.5	73.4	18.1
β-GlcNAc D	H	4.55	3.77	3.52	3.38	3.41	3.76/3.94
	C	102.0	57.2	75.1	71.9	77.3	63.0

Structure



Pseudomonas syringae pv. tabaci 6605 O-SP repeating unit structure is as shown. NAc at E2: C-1 175.9 ppm, H-2/C-2 2.01/23.3 ppm.

Discussion

The anthrose-containing oligosaccharide present on the exosporium protein BclA was reported to be unique to *B. anthracis* spores (4, 9); however, the anthrose biosynthesis genes were recently also identified in other bacilli (13). Rabbit antibodies to *B. anthracis* spores protected mice against *B. anthracis* infection (14), as did immunization of mice and guinea pigs with live or attenuated spores, improving the protection afforded by the Protective Antigen alone (15, 16). We have shown that saccharides of similar structures in other bacteria cross-react with *B. anthracis* spores. We describe binding of *Shewanella* spp. MR-4 CPS and *P. syringae* flagellae to antibodies induced by live *B. anthracis* spores or by synthetic anthrose-containing trisaccharide conjugates. Moreover, antibodies induced by whole cells of either of these two bacterial strains or by conjugates prepared with two variants of *Shewanella* CPS bound to *B. anthracis* spores but not to *B. cereus* spores. Our results also show that the presence of methyl groups in anthrose, either on the *O*-2 of the monosaccharide or the *C*-3 of butyrate, are not essential for the cross-reactivity.

Antibodies induced by these and possibly other cross-reactive moieties, as well as low levels of anti-toxin and anti-capsule found in adults, may explain the relative resistance of humans to anthrax (1, 17). Similarly, immunity to *Haemophilus influenzae* type b (Hib) in adults is induced by cross-reactive saccharides containing ribitol-phosphate, including those in *Escherichia coli* K100 CPS, *Staphylococcus aureus* teichoic acid, and other bacteria (18). It is therefore possible that the group most susceptible to anthrax is infants and children who have not yet developed such antibodies. This group may be the one most in need of active vaccination were protection against bioterroristic attack deemed essential.

Table 3. Composition and geometric means (GM) of mouse IgG anti-*Shewanella* MR-4 CPS induced by BSA conjugates of CPS_{TSB} and CPS_{CDM}

		GM of IgG after 3rd injection, EU	
Conjugate	Protein/saccharide ratio, wt/wt	Coating antigen CPS_{TSB}	Coating antigen CPS_{CDM}
BSA/CPS_{TSB}	2:1	54	43
BSA/CPS_{CDM}	0.7:1	31	32

Mice (10 per group) were injected s.c. with 2.5 μg saccharide as a conjugate/mouse 3 times (2 wk apart) and killed 1 wk after the last injection. Antibody levels were calculated relative to a hyperimmune anti-*Shewanella* spp. MR-4 mouse serum that was assigned a value of 100 EU.

The conjugates of *Shewanella* CPS described here were prepared and injected in accordance with a clinical protocol and in a similar manner to the preparation of Hib conjugates. We propose to add a *Shewanella* conjugate to future improved anthrax vaccines. Furthermore, whole-cell vaccines prepared with either *Shewanella* or *P. syringae* may be useful in veterinary practice.

Materials and Methods

Growth of Bacteria. The following bacterial strains were used in the study: *B. anthracis* Ames 35, an avirulent, Sterne-type strain lacking plasmid pXO2 (19); *B. cereus* strain 569 (20); *Shewanella* spp. strain MR-4, a gift from J. A. Gralnick (Department of Microbiology, BioTechnology Institute, University of Minnesota, St. Paul, MN); and *P. syringae* pv. tabaci 6605, a gift from Yuki Ichinose (Meiji University, Kawasaki, Japan). *Shewanella* spp. MR-4 was grown in either TSB (Difco Laboratories) or in CDM as described (10). *P. syringae* were kept on King's medium plates and cultured in LB broth (Difco Laboratories) for 48 h at 25°C as described (11).

Isolation and Purification of *Shewanella* CPS. CPS was isolated from the cell surface by vigorous stirring and purified as described (21), followed by ultracentrifugation (Sorvall Discovery 100SE, rotor type 55.2TI, Hitachi, Ltd., Tokyo) at 35,000 rpm for 5 h at 5°C to remove LPS. The supernatant was freeze-dried and passed through a Sepharose CL-6B column (1 × 100 cm) in 0.2 M NaCl. A single high-molecular-mass polysaccharide was obtained for each CPS and designated CPS_{TSB} and CPS_{CMP} according to its culture media. CPS structures were confirmed by NMR (10).

Isolation of *P. syringae* Flagella, LPS, and O-SP. Flagella were separated from *P. syringae* cells by vigorous stirring and purified by ultracentrifugation as described (22). LPS was extracted by hot phenol (23). To obtain the O-SP, LPS was treated with 1% acetic acid at 100°C for 1 h, Lipid A was removed by centrifugation, and the soluble products separated by a Sephadex G-50 column (1 × 100 cm) in pyridine/acetic acid/water buffer (4/8/988). Void volume fractions were freeze-dried and analyzed by NMR.

NMR Spectroscopy. NMR spectra were recorded at 60°C in D_2O on a Varian UNITY INOVA 500 instrument, using acetone as reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, ROESY (mixing time of 400 ms), TOCSY (spinlock time of 120 ms), HSQC, and HMBC (long-range transfer delay of 100 ms) were used. Spectra were assigned by using the Topspin program (Bruker).

Analytical Methods. Sugar concentration was measured by the phenol/H_2SO_4 assay (24), protein concentration was measured by the method of Lowry (25), and hydrazide was measured by the trinitrobenzenesulphonic acid assay (26). SDS/PAGE was done using 14% gels, and separated material was electroblotted onto PVDF membranes according to the manufacturer's protocol (Bio-Rad) and reacted with anti-spore and anti-anthrose sera. Double immunodiffusion was performed by using 1% agarose in PBS. Endotoxic activity was

measured by the limulus amoebocyte lysate assay as described by the manufacturer (Cambrex).

Antisera. Hyperimmune sera against *Shewanella* sp. MR-4 and *P. syringae* pv. tabaci 6605 were prepared with heat-killed whole bacteria as described (27). Rabbit sera against whole anthrax spores (anti-spore) and against synthetic anthrax spore trisaccharide-keyhole limpet hemocyanin conjugate (anti-anthrose) (5) were supplied by Conrad Quinn (Centers for Disease Control, Atlanta, GA).

Preparation of Conjugates. ***Conjugate No. 1.*** BSA was derivatized with adipic acid dihydrazide (ADH) as described (26). The product [BSA-adipic acid hydrazide (BSA-AH)] contained 5.2% AH groups (wt %). CPS_{TSB} (10 mg) was dissolved in 1 ml 0.2 M NaCl and the pH was adjusted to 5.5. Next, 10 mg of BSA-AH in 0.5 ml 0.2 M NaCl was added, followed by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) to a final concentration of 0.1 M EDC. The pH was maintained with an automatic titrator (MeterLab) at pH 5.5 for 4 h at room temperature. Next, the product was dialyzed against 0.2 M NaCl, overnight at 4°C and purified by gel filtration by using a Sepharose CL-6B column (1×100 cm) in 0.2 M NaCl. The obtained conjugate was designated BSA/CPS_{TSB}.

Conjugate No. 2. BSA was derivatized with ADH by using the conditions listed above. The obtained product, BSA-AH, contained 9.8% AH groups. CPS_{CMP} (10 mg) was dissolved in 1 ml 0.2 M NaCl and the pH was adjusted to 5.5, 10 mg of BSA-AH was added, and the reaction proceeded as above. The obtained conjugate was designated BSA/CPS_{CMP}.

Immunization. Five- to 6-week-old female National Institutes of Health Swiss–Webster mice were injected s.c. three times at 2-wk intervals with 2.5 μg CPS as a conjugate in 0.1 ml PBS. Groups of 10 mice were killed 7 d after the third injections (27). Controls received PBS.

Antibody. Antibody levels to *Shewanella* CPS were measured by ELISA by using CovaLink plates (Nalge Nunc Intern) (28). The plates were coated with 100 μl per well CPS_{TSB} or CPS_{CMP} (5 μg/ml, determined to be optimal by checker board titration) dissolved in 10 mM 1-methylimidazole buffer (pH 7.0), and EDC was added to a final concentration of 50 mM and incubated at 37°C overnight. The plates were washed six times with 0.1% Brij 35-saline and blocked with 1% human serum albumin (HSA) in PBS for 1 h at room temperature. Twofold dilutions of the sera were made in 1% HSA-0.1% Brij 35-saline, incubated at 37°C for 4 h, and then washed six times. Goat anti-mouse IgG conjugated to alkaline phosphatase was added, and the mixture was incubated at 37°C for 3 h. Then, 4-nitrophenylphosphate [1 mg/ml in 1 M Tris·HCl buffer (pH 9.8), containing 0.3 mM $MgSO_4$] was added. The OD_{405} was read after 30 min in an MR600 microplate reader (Dynatech).

Fluorescence Microscopy. *B. anthracis* Ames 35 and *B. cereus* 569 were grown on nutrient sporulation agar at 37°C for 2 d (29). Spores were purified by washing the plates with deionized water, and the spore suspension incubated at 65°C for 30 min. The spore suspension was then washed twice with deionized water and subjected to immunofluorescent staining as described (30) with the following modification. The spore suspensions were applied to coverslips treated with 1% polylysine (Sigma), blocked with 3% milk in PBS for 30 min, and rabbit anti-spore or anti-anthrose, or mouse anti-*Shewanella* or anti-*P. syringae* sera added. After three washes in PBS, the coverslips were treated with a secondary antibody, either goat anti-mouse or goat anti-rabbit IgG conjugated to fluorescent dye AF488 (Molecular Probes). After staining, the coverslips were mounted onto slides and examined with a Nikon Eclipse TE2000-U fluorescence microscope.

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1. Spencer RC (2003) *Bacillus anthracis. J Clin Pathol* 56:182–187.
2. Scorpio A, Blank TE, Day WA, Chabot DJ (2006) Anthrax vaccines: Pasteur to the present. *Cell Mol Life Sci* 63:2237–2248.
3. Williams RP (1986) Bacillus anthracis and other spore forming bacilli. *Infectious Disease and Medical Microbiology*, eds Braude AI, Davis LE, Fierer J (Saunders, Philadelphia), pp 270–278.
4. Daubenspeck JM, *et al.* (2004) Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. *J Biol Chem* 279:30945–30953.
5. Mehta AS, *et al.* (2006) Synthesis and antigenic analysis of the BclA glycoprotein oligosaccharide from the *Bacillus anthracis* exosporium. *Chemistry* 12:9136–9149.
6. Saksena R, Adamo R, Kovác P (2005) Studies toward a conjugate vaccine for anthrax. Synthesis and characterization of anthrose [4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl-D-glucopyranose] and its methyl glycosides. *Carbohydr Res* 340:1591–1600.
7. Werz DB, Seeberger PH (2005) Total synthesis of antigen *Bacillus anthracis* tetrasaccharide—creation of an anthrax vaccine candidate. *Angew Chem Int Ed Engl* 44:6315–6318.
8. Crich D, Vinigradova O (2007) Synthesis pf the antigenic tetrasaccharide side chain from the major glycoprotein of *Bacillus anthracis* exosporium. *J Org Chem* 72:6513–6520.
9. Tamborrini M, Werz DB, Frey J, Pluschke G, Seeberger PH (2006) Anti-carbohydrate antibodies for the detection of anthrax spores. *Angew Chem Int Ed Engl* 45:6581–6582.
10. Vinogradov E, Nossova L, Korenevsky A, Beveridge TJ (2005) The structure of the capsular polysaccharide of *Shewanella oneidensis* strain MR-4. *Carbohydr Res* 340:1750–1753.
11. Takeuchi K, *et al.* (2007) Flagellin glycans from two pathovars of *Pseudomonas syringae* contain rhamnose in D and L configurations in different ratios and modified 4-amino-4,6-dideoxyglucose. *J Bacteriol* 189:6945–6956.
12. Vinogradov E, *et al.* (1991) Somatic antigens of pseudomonads: Structure of the O-specific polysaccharide chain of *Pseudomonas syringae* pv. tabaci 225 (serogroup VIII) lipopolysaccharide. *Carbohydr Res* 212:307–311.
13. Dong S, *et al.* (2008) Anthrose biosynthetic operon of *Bacillus anthracis. J Bacteriol* 190:2350–2359.
14. Enkhtuya J, *et al.* (2006) Significant passive protective effect against anthrax by antibody to *Bacillus anthracis* inactivated spores that lack two virulence plasmids. *Microbiology* 152:3103–3110.
15. Brossier F, Levy M, Mock M (2002) Anthrax spores make an essential contribution to vaccine efficacy. *Infect Immun* 70:661–664.
16. Cohen S, *et al.* (2000) Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. *Infect Immun* 68:4549–4558.
17. Singer DE, *et al.* (2008) Serum IgG antibody response to the protective antigen (PA) of *Bacillus anthracis* induced by anthrax vaccine adsorbed (AVA) among U.S. military personnel. *Vaccine* 26:869–873.
18. Bradshaw MW, Schneerson R, Parke JC, Jr, Robbins JB (1971) Bacterial antigens cross-reactive with the capsular polysaccharide of *Haemophilus influenzae* type b. *Lancet* 1:1095–1096.
19. Pomerantsev A, Sitaraman R, Galloway CR, Kivovich V, Leppla SH (2006) Genome engineering in *Bacillus anthracis* using cre recombinase. *Infect Immun* 74:682–693.
20. Ruhfel RE, Robillard NJ, Thorne CB (1984) Interspecies transduction of plasmids among *Bacillus anthracis*, *B cereus*, and *B thuringiensis*. *J Bacteriol* 157:708–711.
21. Rodrigues LP, Schneerson R, Robbins JB (1971) Immunity to *Hemophilus influenzae* type b. I. The isolation, and some physicochemical, serologic and biologic properties of the capsular polysaccharide of *Hemophilus influenzae* type b. *J Immunol* 107:1071–1080.
22. Taguchi F, *et al.* (2006) Identification of glycosylation genes and glycosylated amino acids of flagellin in *Pseudomonas syringae* pv. tabaci. *Cell Microbiol* 8:923–938.
23. Westphal O, Jann K (1965) Extraction with phenol-water and further application of the procedure. *Methods Carbohydr Chem* 5:83–91.
24. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356.
25. Lowry JO, Rosenbrough NJ, Farr AL, Randall R (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
26. Schneerson R, Barrera O, Sutton A, Robbins JB (1980) Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. *J Exp Med* 152:361–376.
27. Chu CY, *et al.* (1991) Preparation, characterization, and immunogenicity of conjugates composed of the *O*-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. *Infect Immun* 59:4450–4458.
28. Zielen S, *et al.* (1996) Simple determination of polysaccharide specific antibodies by means of chemically modified ELISA plates. *J Immunol Methods* 193:1–7.
29. Schaeffer P, Ionesco H, Ryter A, Balassa G (1963) Sporulation of Bacillus subtilis: Genetic and physiological study. *Colloq Int CNRS* 124:553–563.
30. Hu H, Sa Q, Koehler TM, Aronson AI, Zhou D (2006) Inactivation of *Bacillus anthracis* spores in murine primary macrophages. *Cell Microbiol* 8:1634–1642.