The Epitopic and Structural Characterization of Brucella suis Biovar 2 O-Polysaccharide Demonstrates the Existence of a New M-Negative C-Negative Smooth Brucella Serovar

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Abstract

The brucellae are Gram-negative bacteria that cause an important zoonosis. Studies with the main Brucella species have shown that the O-antigens of the Brucella smooth lipopolysaccharide are 2(1→2) and 2(1→3)-linked N-formyl-perosamine polysaccharides that carry M, A and C (A = M, A>M and A<M) epitopes relevant in serodiagnosis and typing. We report that, in contrast to the B. suis biovar 1 O-antigen used as a reference or to all described Brucella O-antigens, B. suis biovar 2 O-antigen failed to bind monoclonal antibodies of C (A = M), C (M>A) and M specificities. However, the biovar 2 O-antigen bound monoclonal antibodies to the Brucella A epitope, and to the C/Y epitope shared by brucellae and Yersinia enterocolitica O:9, a bacterium that carries an N-formyl-perosamine O-antigen in exclusively 2(1→2)-linkages. By 13C NMR spectroscopy, B. suis biovar 1 but not B. suis biovar 2 or Y. enterocolitica O:9 polysaccharides showed the signal characteristic of 2(1→3)-linked N-formyl-perosamine, indicating that biovar 2 may altogether lack this linkage. Taken together, the NMR spectroscopy and monoclonal antibody analyses strongly suggest a role for 2(1→3)-linked N-formyl-perosamine in the C (A = M) and C (M>A) epitopes. Moreover, they indicate that B. suis biovar 2 O-antigen lacks some lipopolysaccharide epitopes previously thought to be present in all smooth brucellae, thus representing a new brucella serovar that is M-negative, C-negative. Serologically and structurally this new serovar is more similar to Y. enterocolitica O:9 than to other brucellae.

Introduction

Brucellosis is one of the major bacterial zoonoses in the world [1]. This disease is caused by members of the genus Brucella, a group of Gram-negative microorganisms that behave as facultative intracellular parasites of a large variety of mammals. Infected livestock is the source of human brucellosis, a grave and debilitating disease that requires prolonged antibiotic treatment. The genus Brucella includes several species, three of which (B. abortus, B. melitensis and B. suis) account for the vast majority of infections in domestic livestock and humans. These are further divided into biovars for epidemiological purposes. Regardless of the biovar, the main hosts of B. abortus and B. melitensis are cattle and small ruminants, respectively, and genetic analyses show that the strains of these two species form two distinct clusters within the genus [2]. On the other hand, the strains presently grouped under B. suis do not cluster together and are usually found in swine (biovars 1, 2 and 3), wild boars and hares (biovar 2), reindeer (biovar 4), and wild rodents (biovar 5) [2–4]. Because of their zoonotic nature and importance in ruminant husbandry and in the dairy industry, research on Brucella virulence, physiology and antigenic structure has been carried out mostly on B. abortus and B. melitensis. B. suis has received comparatively meager attention and, despite the greater internal diversity and wide host range, research has been focused on biovar 1. B. abortus, B. melitensis and B. suis cells carry a smooth (S) lipopolysaccharide (LPS), a surface molecule that is a major virulence factor and the most important serodiagnostic antigen. The O-polysaccharide (or O-antigen) section of this S-LPS is a homopolymer of N-formyl-perosamine (N-Rhap4FNO) in 2(1→2)- and 2(1→3)-linkages [5] that is bound to the lipid A through a core oligosaccharide. From extensive genetic studies in B. abortus biovar 1 and B. melitensis biovar 1, O-antigen polymerization requires at least four glycosyltransferase genes (wboA, wboB, wbkA and wbkE) [6]. All O-antigens of S Brucella studied by NMR so far...
(B. melitensis 1 and 3, B. abortus biovar 1 and B. suis biovar 4) contain at least one α-(1→3)-linked β-Rha4Nfo residue, with B. melitensis biovar 1 showing the highest proportion (four contiguous α-(1→2)-linked sugars followed by one α-(1→3)-linkage) [7]. As demonstrated with monoclonal antibodies (MAb), these linkages create three basic epitopes: A (five or more contiguous α-(1→2)-linked β-Rha4Nfo units), M (which strictly requires α-(1→3)-linked sugars), and C (which includes α-(1→2)-linked tri- or tetrascarbohydres) [7–10]. However, as expected from the polysaccharide structures and the variability in affinity intrinsic to antibody binding sites, these three basic epitopes in Brucella O-polysaccharide are not discrete entities and behave as overlapping epitopes. In fact, based on the relative MAb binding in enzyme-linked immunosorbent assays (ELISA) to A- and M-dominant Brucella strains and to Yersinia enterocolitica O:9 (which carries a β-Rha4Nfo homopolymer having only α-(1→2)-linkages [11]), the C epitope has been subdivided into five subsets: C (M>A), C (A=M), C/Y (M>A), C/Y (A=M), and C/Y (A>M), where C indicates specificity for Brucella and for C/Y Brucella-Y. enterocolitica O:9 cross-reactivity [12,13]. The A and M epitopes are not uniformly distributed in the genus: B. abortus biovars 1, 2, 3 and 6 as well as B. suis 1, 2 and 3 carry the A epitopes but not the M, while B. melitensis biovar 1, B. abortus biovars 4, 5 and 9 and B. suis biovar 5 have M but not A epitopes [14].

It has been reported that B. suis biovar 2 fails to react with MAb specific for the C (A=M) and C (M>A) epitopes [15] suggesting unknown structural peculiarities. B. suis biovar 2 represents an emerging disease in domestic swine throughout Europe and it has the striking feature of not being overtly virulent for humans [16,17,18]. Because of the importance of S-LPS in the biology of brucellae, elucidation of the fine structure of B. suis biovar 2 O-antigen should help both to understand the nature of the C and C/Y epitopes and to better characterize this atypical and increasingly important biovar. Here, we confirm a different epitopic structure of the O-antigen of B. suis biovar 2 and show that it is closer to that of Y. enterocolitica O:9 than to those of B. suis biovar 1 or other S Brucella characterized to date. We could not detect α-(1→3)-linked β-Rha4Nfo residues in the B. suis 2 O-antigen. This is in contrast to the O-antigen of other S brucellae studied thus far and strongly suggests a role for α-(1→3)-linked β-Rha4Nfo in the C (A=M) and C (M>A) epitopes.

Results and Discussion

To determine the epitopic structure of the B. suis biovar 2 O-antigen, we examined the polysaccharide (PS, i.e. the core-O-antigen polysaccharide) obtained by acid hydrolysis of the corresponding LPS using the PS from the well-characterized B. suis biovar 1 LPS as a reference [7]. These PS were subsequently analyzed by 1H,13C-H2BC and 1H,13C-HMBC experiments [23,24] in which heteronuclear 1H,13C-HMBC cross-peaks were present in the biovar 1 PS at δH 78 and δC 77 ppm, respectively. The 13C chemical shifts at the glycosylation positions per se may not be sufficient to easily distinguish between the PS of biovar 1 and 2 at the level of structure responsible for the M epitope. All these results suggest that the O-antigen of B. suis biovar 2: (i) could be serologically indistinguishable from the O-antigen of Y. enterocolitica O:9, at least with the MAb of the specificities defined so far; and (ii), that it could have levels of α-(1→3)-linkages far below those of other S brucellae described to date. To test the first hypothesis, we used MAb of C specificity, an epitope typical of S brucellae but absent in Y. enterocolitica O:9. As can be seen in Figure 1, MAb 12B12 (C (M>A)) and 07F09 (C (A=M)) reacted with the PS of B. suis biovar 1 but not with the PS of B. suis biovar 2, thus confirming that B. suis 2 PS is not distinguishable from its Y. enterocolitica counterpart with the MAb specificities defined thus far. To test the second hypothesis, we investigated the presence of α-(1→3)-linkages by NMR spectroscopy. Consistent with the serological results, the anomeric region of the 1H NMR spectra revealed differences between the B. suis biovar 1 and 2 PS. In particular, the peak at ~5.07 ppm was more intense in the B. suis biovar 1 PS spectrum than from the biovar 2 PS spectrum (Fig. 2a and b). This difference is reminiscent of 1H NMR spectral changes taking place when the PS of biovar 1 16M and biovar 1 B. abortus 11193-3 are mixed (α-(1→3)/α-(1→2) ratio for the mixture c.a. 1:2) [7]. Thus, the results from analyses of the 1H NMR spectra also suggest that the O-antigen from B. suis biovar 1 contains a higher amount of α-(1→3)-linkages than that of biovar 2.

To confirm the above spectral analysis, the PS from B. suis biovar 1 and 2 were further analyzed by two-dimensional NMR spectroscopy. In the anomeric region of the 1H,13C-HSQC spectra from the two PS, six cross-peaks in common were observed at δH 5.26/δC 101.6, 5.20/101.3, 5.17/101.3, 5.10/103.0 5.06/102.7 and 4.60/101.8. Furthermore, four additional cross-peaks were present in the biovar 1 PS at δH 5.14/101.5, 5.07/102.4, 5.07/101.4 and 5.05/101.5 (Fig. 2c) but not in biovar 2 PS. The distinction between 2- or 3-linked residues may be performed by analysis of 13C NMR chemical shifts [20,21] or the glycosylation shifts, i.e., chemical shift displacements as a result of substitution by a sugar residue [22] at either O2 or O3 of the β-Rha4Nfo residues. For a non-subsituted α-linked β-Rha4Nfo, the 13C chemical shifts of C2 and C3 are ~70 and ~69 ppm, respectively, whereas glycosylation displace these chemical shifts to ~78 and ~77 ppm, respectively. The 13C chemical shifts at the glycosylation positions per se may not be sufficient to easily distinguish between the different substitutions but the large glycosylation shifts of ~8 ppm facilitate their differentiation. The two PS were subsequently analyzed by 1H,13C-H2BC and 1H,13C-HMBC experiments [23,24] in which heteronuclear 1H,13C-correlations over two bonds are observed in the first experiment and correlations over two and/or three bonds are present in the second one. Correlations over three bonds in 1H,13C-HMBC
Brucella suis Biovar 2 O-Polysaccharide

- biovar 1
- biovar 2

MAb reactivity (O.D.)

A68/03F03/D05 (R)
04F03 (M)
04F9 (C/Y (A>M))
05D4 (C/Y (A>M))
18H08 (C/Y (A=M))
16C10 (C/Y (M>A))
12B12 (C (M>A))
07F09 (C (A=M))

Log MAb dilution
spectra may be due to intraresidue as well as interresidue $J$-couplings and are thus not necessarily conclusive with respect to the substitution position in a $\beta$-Rha$\beta$4NfO residue that is part of a polysaccharide. In the $^1H,^13C$-H2BC spectrum of the $B. suis$ biovar 1 PS, but not of biovar 2, correlations from $\delta_H$ 5.07 were observed to 69.6 and 78.3 ppm. The former correlation supports the presence of an $\alpha$-linked $\beta$-Rha$\beta$4NfO residue in the PS of biovar 1 that is not 2-substituted; consequently, it should be 3-substituted (unless it is the terminal residue of the polysaccharide).

In the $^1H,^13C$-HSQC spectra the $^13C$ spectral region between 51 and 58 ppm also showed marked differences. Major cross-peaks were observed at $\delta_H/\delta_C$ 3.99/52.7 and 3.42/57.7. In the PS from both strains, the H2/C2 cross-peak from N-acetyl-quinovosamine (QuiNAc) was observed at $\delta_H/\delta_C$ 3.84/55.2 [6]. QuiNAc is a component of the LPS core oligosaccharide of Brucella [6] and its detection is consistent with the reactivity of these PS with the MAb of rough LPS specificity (see above). However, at the level where the H2/C2 cross-peak from QuiNAc was clearly visible, additional cross-peaks at $\delta_H/\delta_C$ 4.04/51.7 and 3.50/56.2 were present in the $B. suis$ biovar 1 PS but absent in the biovar 2 PS. The former correlation is fully consistent with the chemical shifts for H4/C4 of a 3-substituted $\alpha$-linked $\beta$-Rha$\beta$4NfO residue in the major $\alpha$-isomer also referred to as the $\alpha$-cis conformation of the formyl group [20] and the latter with the minor $\beta$-isomer ($\beta$-trans conformation) of the amide substituent [21]. From $^1H,^13C$-HSQC-$^1H$-H2BC experiments [25,26] with mixing times up to 200 ms, correlations from C4 atoms to H1 atoms were observed in the 2D NMR spectra. In particular, a correlation between $\delta_C$ 51.7 and $\delta_H$ 5.07 was present, i.e., between atoms from a structural element present only in the $B. suis$ biovar 1 PS. Again, these results show that the extent of 3-substituted $\alpha$-linked $\beta$-Rha$\beta$4NfO residues in the $B. suis$ biovar 2 PS is lower than in the biovar 1 PS.

Integration of the methyl group resonance of the N-acetyl group of QuiNAc at $\delta_H$ 2.07 in the $^1H$ NMR spectrum from the $B. suis$ biovar 1 PS compared to protons in the anomeric region between $\delta_C$ 4.97 and 5.39 showed that the average number of sugars in the O-antigen polysaccharide was 50 to 60. The result for the $B. suis$ biovar 2 PS was similar, which rules out the possibility that the above-described differences could be caused by a different degree of polymerization of the O-antigens. It was also noted that the anomeric protons at $\delta_H$ 5.26 and 5.10 showed correlations in the $^1H,^13C$-HMBC spectrum to resonances at $\delta_C$ $\sim$ 74.3 which could be confirmed by intraresidue correlations since these connectivities were also observed in the HSQC-TOCSY spectra; notably these sugar residues did not give any correlations to methyl groups at $\delta_H$ $\sim$ 1.21 and should originate from sugar residues other than $\beta$-Rha$\beta$4NfO in the polymer, such as sugars in the core region. We did not find any support for a significant amount of consecutively $\alpha$-(1$\rightarrow$3)-linked $\beta$-Rha$\beta$4NfO residues since the $^1H$ chemical shifts of the anomeric protons should then reside at slightly lower chemical shifts than observed herein, like in the O-antigen polysaccharide from Citrobacter gillenii O9a,9b lipopolysaccharide [27].

The one-dimensional $^{13}C$ NMR spectra of the $B. suis$ biovar 1 and 2 PS were further analyzed in comparison with the spectra of $F. enterolitica$ O:9 PS, and a few additional conspicuous differences were observed. In particular, the following resonances were present in the $B. suis$ biovar 1 PS but absent in the biovar 2 PS: 18.0, 51.7, 102.4 and in particular 165.2 (Fig. 2d and 2e) and, noteworthy, they were also absent in the $^{13}C$ NMR spectrum of $Y. enterocolitica$ O:9 PS. However, in the latter PS, resonances of low intensity were observed at, inter alia, 102.5, 165.5 and 165.8 ppm (Fig. 2f). The $^{13}C$ NMR chemical shift at 165.2 ppm in $B. suis$ biovar 1 PS is fully consistent with a previous interpretation showing that an $\alpha$-(1$\rightarrow$3)-linked N-formylated perosamine residue is present in this polysaccharide [7] and confirms that the degree of $\alpha$-(1$\rightarrow$3)-linked $\beta$-Rha$\beta$4NfO residues is higher in the $B. suis$ biovar 1 than in the biovar 2 O-antigen polysaccharides. Thus, the latter biovar may altogether lack the $\alpha$-(1$\rightarrow$3)-linkage in the O-antigen part of the PS.

To explore possible gene differences that could account for the O-antigen structural variation, the four known $Brucella$ O-antigen glycosyltransferases were examined. To this end, the amino acid sequences of the $B. melitensis$ 16M (biovar 1; highest proportion [21%] of $\alpha$-(1$\rightarrow$3)-linked $\beta$-Rha$\beta$4N); $B. abortus$ (BMEI0997); $B. melitensis$ (BMEI0998); WhkE (BMEI1194) and WhkA (BMEI1404) were compared with the homologous genes in $B. suis$ biovar 1 and 2 (no detectable $\alpha$-(1$\rightarrow$3)-linked $\beta$-Rha$\beta$4N). These analyses did not reveal any significant differences in any of these proteins.

### Table 1. Characteristics, origin and reactivity with $B. suis$ biovar 1 and 2 PS of the MAb used.1

| MAb          | Specificity | Isotype | $B. abortus$ biovar 1 | $B. melitensis$ biovar 1 | $Y. enterocolitica$ O:9 | References | $B. suis$ biovar 1 | $B. suis$ biovar 2 |
|--------------|-------------|---------|----------------------|--------------------------|-------------------------|------------|-------------------|-------------------|
| A68/03F03/005 | R (LPS core) | IgG2b   | N.D.2                | N.D.                     | N.D.                    | [9,12,13]  | ++++              | ++++              |
| 04F03        | M           | IgM     | ++/−                 | +++                      | –                       | [12,13,35,36] | ++++              | –                 |
| 04F9         | C/Y (A$>$M) | IgG2a   | +++                  | −/−                      | +++                     | [12,13,35,36] | ++++              | ++++              |
| 05D4         | C/Y (A$>$M) | IgG1    | This work            |                          |                          |            | ++++              | ++++              |
| 18H08        | C/Y (A=M)   | IgA     | [12,13]              |                          |                          |            | ++++              | ++++              |
| 16C10        | C/Y (M$>$A) | IgG3    | ++                    | +++                      | +                       | [12,13,36]  | ++                | –                 |
| 12B12        | C (M$>$A)   | IgG3    | ++                    | +++                      | –                       | [9,12,13]  | ++                | –                 |
| 07F09        | C (A=M)     | IgG1    | +++                  | +++                      | –                       | [9,12,13]  | ++++              | –                 |

1 Reactivity is reported as no reactivity (−) to strong (++++) as judged from the titers reported in the corresponding references.
2 N.D. = not determined.

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biovar 1 PS with a resonance of low intensity at 165.2 ppm (d), and biovar 2 PS (e); selected carbonyl region of the $^{13}$C NMR spectrum from $Y$. enterocolitica O:9 PS with resonance of low intensity at 165.5 and 165.8 ppm (f). The major $^{13}$C resonances from N-formyl groups are observed at 165.7 and 168.6 ppm.

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Similarly, no differences were found in the regions situated 300 bp upstream (putative promoter region) for any of the corresponding genes. A comparison of the amino acid sequences of other glycosyltransferases of $B$. melitensis 16M, $B$. suis biovar 1 and $B$. suis biovar 2 identified in data bases as presumably involved in LPS synthesis did not reveal significant differences but for $B$. melitensis BMEII 0728 and BMEII 1129 and their orthologues, BMEII 0728 carries a frame shift that results in the loss of 140 amino acids with regard to the orthologues (614 amino acids) in biovars 1 of $B$. abortus and $B$. suis. Since $B$. melitensis biovar 1 O-antigen shows about 21% of these linkages and $B$. abortus and $B$. suis show a similarly low proportion (about 2%) of $\alpha$-(1→3)-linkages [7], these ORF are unlikely to play a role in the structural differences. BMEII 1129 is conserved in the genomes of biovars 1 of $B$. melitensis, $B$. abortus and $B$. suis but not in $B$. suis biovar 2, where it carries a frame shift. However, since BMEII 1129 mutant has been shown to maintain the O-antigen [6], either this glycosyltransferase is not related to the $\alpha$-(1→3)-linkage or this linkage is not necessary for O-antigen polymerization in $B$. melitensis. Research is in progress to test these possibilities. Nonetheless, it seems that alternative and/or additional mechanisms of control are necessary to account for the different levels of $\alpha$-(1→3)-linkages observed in the biovar 1 strains of $B$. melitensis, $B$. abortus and $B$. suis.

The proportions of $\alpha$-(1→3)-linkages reported previously for the O-antigens of brucellae vary from 21% for $B$. melitensis biovar 1 strain 16M to 2% in $B$. abortus biovar 1 strain 1119-3, with intermediate values for $B$. melitensis biovar 3 (8%) and $B$. suis biovar 4 (13%) [7]. Therefore, the present work extends this range to include brucellae where this linkage is not detectable and could even be absent. Furthermore, during the last decade, Brucella has been identified as a pathogen of several marine mammals, and MAb reactivity patterns almost identical to that described here for $B$. suis biovar 2 have been reported for $B$. ceti strains isolated from some dolphin species [28]. Also, like $B$. suis biovar 2, several Brucella strains isolated from wild rodents in Australia do not react with MAb of C and M specificities [29]. Thus, it seems that the A-positive, C and M-negative epitopic structure is not infrequent outside the $B$. abortus and $B$. melitensis clusters and that it represents a new and extended Brucella serovar. The zoonotic nature of those dolphin and wild rodent strains is not known but considerable epidemiological evidence shows that $B$. suis biovar 2 displays little virulence for human beings. S-LPS is a major virulence factor of brucellae and it has been shown [30] that the O-antigen of virulent $B$. suis biovar 1 plays a critical role in selecting lipid rafts to enter into murine macrophages indicating a critical role at the port of entry. Since the O-antigen is the major surface structure, it is tempting to speculate that an absence of $\alpha$-(1→3)-linkages may alter the interaction with human cells. Although this possibility cannot be ruled out, the fact is that the structural difference is small.

The results of this study show that the O-antigen of $B$. suis biovar 2 differs in a subtle but epitopically significant feature related to $\alpha$-(1→3)-linkages in Brucella O-antigens. In this context, our results support the early interpretation that the $\alpha$-(1→3)-linkage is partially responsible for the reactivity of those antibodies that recognize A and M dominant brucellae but not $Y$. enterocolitica O:9 [7]. Originally described as part of A [7], they are better defined as overlapping C (A = M and M>A) epitope(s) recognized
by antibodies whose reactivity strictly requires the χ-(1→3)-linkage in addition of a variable number of χ-(1→2)-linked sugar residues. They are, therefore, different from those (the overlapping A and C/Y epitopes) attributable to χ-(1→2)-linkages only. From a practical point of view, the C epitope(s) marks the only known serological difference between S. brucellae and \( B. \) \textit{enterolitica} O:9 but is undetectable in \( B. \) \textit{suis} biovar 2. Swine are infected by \( B. \) \textit{suis} biovar 2 in areas where infections by \( B. \) \textit{enterolitica} O:9 also occur making necessary a differential diagnosis. The absence of antigenically detectable differences in the O-antigen of these two bacteria poses a very difficult challenge to the differentiation of these infections in standard LPS tests.

**Methods**

**Strains, Growth Conditions and Polysaccharide Preparations**

\( B. \) \textit{suis} 1330 (biovar 1) and \( B. \) \textit{suis} Thomsen (biovar 2; ATCC23445) are both defined serologically as A-dominant in biovar typing schemes [14]. \( B. \) \textit{enterolitica} O:9 MY79 is an avirulent (plasmid-less) strain showing the expected cross-reactivity with \( S. \) \textit{brucellae} [31]. The bacteria were grown and harvested as described previously [19] and S-LPS was extracted by the modified phenol-water method and purified by nuclease and protease K digestion [32]. The lipid A-free polysaccharide (PS) was obtained by hydrolysis of S-LPS with dilute aqueous acetic acid followed by ultracentrifugation and the purity of these fractions was demonstrated by quantitative immunoprecipitation [19] and with anti-core MAb (see Results).

**Enzyme-linked Immunosorbent Assay (ELISA)**

ELISA was performed on polystyrene plates coated by incubation with the lipid A-free PS (see above) at 2.5 μg per ml in 10 mM phosphate buffered saline (pH 7.2) at 4°C overnight [15]. The relevant characteristics of the MAb used and appropriate references on their origin and production are summarized in Table 1.

**NMR Spectroscopy**

1D and 2D NMR spectra of \( B. \) \textit{suis} biovar 1 PS (10 mg in 0.5 mL \( \text{D}_2\text{O} \)), biovar 2 PS (5 mg in 0.5 mL \( \text{D}_2\text{O} \)) and \( B. \) \textit{enterolitica} O:9-PS (9 mg in 0.5 mL \( \text{D}_2\text{O} \)) were recorded at 47°C on a Bruker AVANCE III 700 MHz spectrometer equipped with a 5 mm TCI Z-Gradient CryoProbe. \( ^1\text{H} \) chemical shifts were referenced to internal sodium 3-trimethylsilyl-(2,2,3,3,-\( ^2\text{H}_4 \))-propanoate (TSP, \( \delta_{\text{TSP}} \) 0.00) and \( ^1\text{C} \) chemical shifts were referenced to external dioxane in \( \text{D}_2\text{O} \) (\( \delta_{\text{D}_2\text{O}} \) 67.40). Data processing was performed using vendor-supplied software. The assignments of the \( ^1\text{H} \) and \( ^1\text{C} \) resonances of the PS were obtained by analysis of 1D \( ^1\text{H} \) and \( ^1\text{C} \) NMR spectra together with 2D NMR spectra from multiplicity-edited \( ^1\text{H} \),\( ^1\text{C} \)-HSQC experiments [33]. \( ^1\text{H} \),\( ^1\text{C} \)-HMBC experiments [24] with a 45 ms delay for evolution of long-range couplings and \( ^1\text{H} \),\( ^1\text{C} \)-HSQC-\( ^1\text{H} \)-TOCSY experiments [25,26] with mixing times of 50, 100, 150 and 200 ms. A \( ^1\text{H} \),\( ^1\text{H} \)-NOESY experiment [34] with a mixing time of 100 ms was also performed for the \( B. \) \textit{suis} biovar 1 PS.

**Sequence Analysis**

Homologies of \textit{Brucella} O-antigen glycosyltransferases \textit{wboA}, \textit{wboB}, \textit{wbkA} and \textit{wbkE} and upstream sequences and of the predicted proteins were studied using the NCBI (National Center for Biotechnology Information) and TIGR (The Institute for Genomic Research) sequences accessible through the \textit{Brucella} Bioinformatics Portal (http://www.phidias.us/bbip/data/index.php) and BLASTN and BLASTP tools. For other \textit{Brucella} LPS possible glycosyltransferases, the data available at the Carbohydrate Active Enzymes database (http://www.cazy.org/GlycosylTransferases.html) were used.

**Author Contributions**

Conceived and designed the experiments: IM GW. Performed the experiments: MVZ TA MI MSZ AC AW IM GW. Contributed reagents/materials/analysis tools: IM. Wrote the paper: MVZ IM GW.

**References**

1. Boschirolli ML, Foulongne V, O’Callaghan D (2001) Brucellosis: a worldwide zoonosis. Current opinion in microbiology 4: 58–64.
2. Whatmore AM (2009) Current understanding of the genetic diversity of \textit{Brucella}, an expanding genus of zoonotic pathogens. Infection, genetics and evolution: Journal of molecular epidemiology and evolutionary genetics in infectious diseases 9: 1168–1184.
3. Moreno E, Cloeckaert A, Moriyón I (2002) \textit{Brucella} evolution and taxonomy. Veterinary microbiology 90: 209–227.
4. Zheludkov MM, Truelson LE (2010) Reservoirs of \textit{Brucella} infection in nature. Biol Bull 37: 709–715.
5. Perry MB, Bundle DR (1990) Lipopolysaccharide antigens and carbohydrates of \textit{Brucella}. Advances in brucellosis research. College Station: Texas A&M University Press. 76–78.
6. González D, Grillo MJ, De Miguel MJ, Ali T, Arce-Groev L, et al. (2008) Brucellosis vaccines: assessment of \textit{Brucella melitensis} lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLOS ONE 3: e2760.
7. Mölle PJ, Perry MB, Cherwonogrodzky JW, Bundle DR (1989) Fine structure of A and M antigens from \textit{Brucella} biovars. Infect Immun 57: 2820–2828.
8. Bundle DR, Cherwonogrodzky JW, Méndez MA, Meikle PJ, Perry MB, et al. (1989) Definition of \textit{Brucella} A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. Infect Immun 57: 2829–2836.
9. Cloeckaert A, Zygmunz MS, Dubay G, Linet JP (1993) Characterization of O-polysaccharide specific monoclonal antibodies derived from mice infected with the rough \textit{Brucella melitensis} strain B115. Microbiology 139: 1531–1536.
10. Douglas JT, Palmer DA (1980) Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth \textit{Brucella} species. J Clin Microbiol 26: 1353–1356.
11. Carof M, Bundle DR, Perry MB, Cherwonogrodzky JW, Duncan JR (1984) Antigenic S-type lipopolysaccharide of \textit{Brucella abortus} 1119–3. Infect Immun 46: 384–389.
12. Weynants V, Gilson D, Cloeckaert A, Tibor A, Denoeil PA, et al. (1997) Characterization of smooth lipopolysaccharides and O-polysaccharides of \textit{Brucella} species by competition binding assays with monoclonal antibodies. Infect Immun 65: 1939–1943.
13. Cloeckaert A, Weynants V, Godfroid J, Verger J-M, Grayon M, et al. (1998) O-polysaccharide epitope heterogeneity at the surface of \textit{Brucella} spp. studied by enzyme-linked immunosorbent assay and flow cytometry. Clin Diagn Lab Immunol 5: 862–870.
14. Allot GG, Jones LM, Angus RD, Verger J-M (1989) Techniques for the Brucellosis laboratory INRA, Paris, France.
15. Weynants V, Gilson D, Cloeckaert A, Denoeil PA, Tibor A, et al. (1996) Characterization of a monoclonal antibody specific for \textit{Brucella} smooth lipopolysaccharide and development of a competitive enzyme-linked immunosorbent assay to improve the serological diagnosis of brucellosis. Clin Diagn Lab Immunol 3: 309–314.
16. Al Dahouk S, Nöckler K, Tomasø H, Splettsstoesser WD, Jungersen G, et al. (2005) Serorecurrence of brucellosis, tularemia, and yersiniosis in wild boars (\textit{Sus scrofa}) from North-Eastern Germany. J Vet Med B 52: 444–455.
17. Godfroid J, Cloeckaert A, Liétard J-P, Kohler S, Frelin D, et al. (2005) From the discovery of the Malta fever’s agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res 36: 315–326.
18. Garin-Bastuji B, Vaillant V, Albert D, Tourrand B, Danjean MP, et al. (2006) Is \textit{Brucella} culture confirmatory? Two case reports in wild boar and hare hunters. Proceedings of the International Society of Chemotherapy Disease Management Meeting, 1st International Meeting on Treatment of Human Brucellosis. Ioannina, Greece.
19. Aragón V, Díaz R, Moreno E, Moriyón I (1996) Characterization of \textit{Brucella abortus} and \textit{Brucella melitensis} native haptens as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide. J Bacteriol 176: 1670–1679.
20. Kenne L, Unger P, Wehler T (1988) Synthesis and nuclear magnetic resonance studies of some N-acylated methyl 4-amino-4,6-dideoxy-α-D-mannopyranosides. J Chem Soc, Perkin Trans 1: 1183–1186.
21. Peters T, Brazon J-R, Bundle DR (1990) Conformational analysis of key disaccharide components of Brucella A and M antigens. Can J Chem 68: 979–988.
22. Söderman P, Jansson P-E, Widmalm G (1996) Synthesis, NMR spectroscopy and conformational studies of the four anomic methyl glycosides of the triosaccharide α-Glc(1→3)-β-Glc(1→4)-α-Glc. J Chem Soc, Perkin Trans 2: 639–648.
23. Nyberg NT, Duaa JO, Sorensen OW (2003) Editing of H2BC NMR spectra. Magn Reson Chem 41: 971–974.
24. Bax A, Summers MF (1986) 1H and 13C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J Am Chem Soc 108: 2093–2094.
25. Domke T (1991) A new method to distinguish between direct and remote signals in proton-relayed X,H correlations. J Magn Reson 95: 174–177.
26. de Beer T, Van Zuylen CWEM, Hard K, Boelens R, Kaptein R, et al. (1994) Rapid and simple approach for the NMR resonance assignment of the carbohydrate chains of an intact glycoprotein. Application of gradient-enhanced natural abundance 1H-13C HSQC and HSQC-TOCSY to the α-subunit of human chorionic gonadotropin. FEBS Lett 348: 1–6.
27. Lipinski T, Zatonksy GV, Kocharova NA, Jachinid M, Forest E, et al. (2002) Structures of two O-chain polysaccharides of Citrobacter gilleni O9a, O9b lipopolysaccharide: A new homopolymer of 4-amino-4,6-dideoxy-α-mannose (perosamine). Eur J Biochem 269: 93–99.
28. Baucheron S, Grayon M, Zygmunt MS, Cloeckaert A (2002) Lipopolysaccharide heterogeneity in Brucella strains isolated from marine mammals. Res Microbiol 153: 277–280.
29. Zygmunt MS, Jacques I, Bernardet J, Cloeckaert A (2012) Lipopolysaccharide heterogeneity in the Atypical Group of Novel Emerging Brucella Species. Clin Vaccine Immunol 19: 1370–1373.
30. Porte F, Naroomi A, Ouahram-Bettache S, Lantard J-P (2003) Role of the Brucella suis lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. Infect Immun 71: 1481–1490.
31. Fernandez-Lago L, Moriyon I, Toyos J, Diaz R (1982) Immunological identity of Brucella native hapten, polysaccharide B, and Treponema pallidum serotype 9 native hapten. Infect Immun 36: 776–783.
32. Velasco J, Bengoechea JA, Brandenburg K, Lindner B, Seydel U, et al. (2000) Brucella abortus and its closest phylogenetic relative, Ochrobactrum spp., differ in outer membrane permeability and cationic peptide resistance. Infect Immun 68: 3210–3218.
33. Parella T, Sánchez-Ferrando F, Virgili A (1997) Improved Sensitivity in Gradient-Based 1D and 2D Multiplicity-Edited HSQC Experiments. J Magn Reson 126: 274–277.
34. Kumar A, Ernst RR, Wuthrich K (1980) A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. Biochem Biophys Res Commun 95: 1–6.
35. Lamet J, Pismmet A-M, Dubray G, Pismmet M (1987) Immunity conferred upon mice by anti-LPS monoclonal antibodies in murine Brucellosis. Annales de l’Institut Pasteur/Immunologie 138: 417–424.
36. Lamet JN, Bosserez N, Dubray G, Pismmet M (1989) Humoral immunity in mice mediated by monoclonal antibodies against the A and M antigens of Brucella. Journal of Medical Microbiology 30: 37–43.