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DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers

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Abstract

**Background:** The lipopolysaccharide is a major antigen and virulence factor of *Brucella*, an important bacterial pathogen. In smooth brucellae, lipopolysaccharide is made of lipid A-core oligosaccharide and N-formylperosamine O-polysaccharide. *B. ovis* and *B. canis* (rough species) lack the O-polysaccharide.

**Results:** The polymorphism of O-polysaccharide genes *wbkE, manAO-Ag, manBO-Ag, manCO-Ag, wbkF* and *wbkD* and *wbo* (*wboA* and *wboB*), and core genes *manBcore* and *wa**, was analyzed. Although most genes were highly conserved, species- and biovar-specific restriction patterns were found. There were no significant differences in putative N-formylperosamyl transferase genes, suggesting that *Brucella* A and M serotypes are not related to specific genes. In *B. pinnipedialis* and *B. ceti* (both smooth), *manB0-Ag* carried an IS711, confirming its dispensability for perosamine synthesis. Significant differences between smooth and rough species were found in *wbkF* and *wbkD*, two adjacent genes putatively related to bactoprenol priming for O-polysaccharide polymerization. *B. ovis* *wbkF* carried a frame-shift and *B. canis* had a long deletion partially encompassing both genes. In smooth brucellae, this region contains two direct repeats suggesting the deletion mechanism.

**Conclusion:** The results define species and biovar markers, confirm the dispensability of *manB0-Ag* for O-polysaccharide synthesis and contribute to explain the lipopolysaccharide structure of rough and smooth *Brucella* species.

**Background**

The members of the genus *Brucella* are gram-negative bacteria that cause brucellosis, a zoonotic disease of great importance worldwide. Currently, several *Brucella* species are recognized [1]. *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ovis*, and *B. canis* have been known for a long
time and are traditionally distinguished according to their preferential host, biochemical tests and cell surface characteristics [2]. In addition, Brucella strains isolated from cetaceans and pinnipeds during the last fifteen years have been grouped into B. ceti and B. pinnipediais, [3]. Very recently, some Brucella strains have been isolated from the common vole and a new species, B. microti, proposed [4]. B. abortus, B. melitensis and B. suis have been classically subdivided into biovars according to H$_3$S production, CO$_2$-dependence, dye sensitivity and distribution of the A and M epitopes (see below) [2]. However, because these tests are difficult to standardize, molecular markers have been investigated [5-9].

Wild type B. melitensis, B. abortus, B. suis, B. neotomae, B. ceti, B. pinnipediais and B. microti express a smooth (S)-type lipopolysaccharide (LPS) formed by an O-polysaccharide connected to a core oligosaccharide which, in turn, is linked to lipid A, the section embedded into the outer membrane. However, both B. ovis and B. canis lack the O-polysaccharide and, accordingly, their LPS is termed rough (R) (R-LPS). Brucella LPS is of great interest not only because of these species differences but also because it is the foremost diagnostic antigen and a major virulence factor [10]. Despite this, the structure and genetics of Brucella LPS is only partially understood. The O-polysaccharide is a homopolymer of N-formyl-perosamine (α1-3) linkages [11], and these variations relate to the main serovars in Brucella S species (A dominant, related to the α1-2 linkage; M dominant [α1-2] plus α1-3 in a 4:1 proportion); or A = M [α1-2] plus α1-3 in a > 4:1 proportion). Previous studies in B. melitensis 16 M and H38 (both biovar 1) have identified two genetic regions involved in O-polysaccharide synthesis and translocation ([Figure 1](page number not for citation purposes))(reviewed in [12]). Region who encodes two putative glycosyltransferases (wboA and wboB) and region wbk contains the genes putatively involved in perosamine synthesis ([gmd](GDP-mannose 4, 6 dehydratase) and [per](perosamine synthetase)), its formylation ([wbkC]) and polymerization (glycosyltransferases) ([wbkA] and [wbkE]), as well as those for bactoprenol priming ([wbkD] and [wbkF]) and O-PS translocation ([wzm] and [wzt]). In addition, wbk contains genes ([manAO$_{Ag}$], [manBO$_{Ag}$] and [manCO$_{Ag}$]) which may code for the enzymes that furnish mannose, the perosamine precursor. Intriguingly, wbbK and manB$_{O,Ag}$ do not generate R phenotypes upon disruption [12,13], and B. ovis and B. canis carry wbk genes despite the absence of the O-polysaccharide [14]. Much less is known on the Brucella core oligosaccharide. Reportedly, it contains 2-keto, 3-deoxyoctulosonic acid, mannose, glucose, glucosamine and quinovosamine [12,15] but the structure is unknown. Thus far, only three genes have been proved to be involved in core synthesis: [pgm](phosphoglucomutase, a general biosynthetic function), [manB$_{core}$] (mannose-synthesis) and [wa$^{**}$] (putative glycosyltransferase) [12]. Obviously, genetic analysis encompassing a variety of strains could shed light on the differences behind the phenotypes of S and R species, confirm or rule out a role for known genes, and identify differences that could serve as serovar or biovar markers. With these aims, wbkE, manAO$_{Ag}$, manBO$_{Ag}$, manCO$_{Ag}$, wbkF, wkdD, wboA, wboB, wa$^{**}$ and manB$_{core}$ were analyzed for polymorphism in the classical Brucella spp., B. ceti, and B. pinnipediais.

**Results**

**LPS genes in Brucella spp. and biovars**

Figure 1 shows the organization of LPS genes in B. melitensis 16 M [12]. PCR amplification of wbkE, manBO$_{Ag}$, manAO$_{Ag}$, manCO$_{Ag}$, wbkF, wkdD, wboA, wboB, wa$^{**}$ and manB$_{core}$ was conducted on representative strains of each of the Brucella species included in this study and their biovars with attention to the LPS characteristics (i.e. S versus R; and A dominant, M dominant, or A = M for the S-LPS). Except for wboA and wboB in B. ovis, all genes were successfully amplified in the strains of all Brucella species and biovars tested. These results confirm the absence of the who region in B. ovis [16,17]. They also suggest that conservation of wbk extends beyond those genes (wbkA to wbkC) examined in a previous work [14] and that wa$^{**}$ and manB$_{core}$ were also conserved in the genus. Further analyses were then conducted to examine these possibilities.

**Gene polymorphism in wbk**

**wbkE**

For all strains, the wbkE PCR-amplified product displayed the same EcoRV, HindI, PstI and PvuII RFLP patterns. Although B. melitensis 63/3 biovar 2 showed a different StyI pattern, only one of eight additional B. melitensis biovar 2 strains tested showed this StyI pattern (data not shown).

**manAO$_{Ag}$**

B. neotomae had a distinct manAO$_{Ag}$ restriction pattern consisting of an additional AvaII site (Figures 2 and 3, Table 1). Moreover, in silico analysis showed a specific profile for B. ovis consisting of a nucleotide substitution (GAA to GGA) at position 497 which modified the ManA C-terminal sequence at amino acid 165 (not shown). Also, a single nucleotide deletion (CAAT to C-A-T) was detected at position 738; this frame shift leads to a change in amino acid sequence after position 246. Nucleotide sequence of PCR products from several strains confirmed the deletion in manAO$_{Ag}$ as characteristic of B. ovis (not shown).

**manCO$_{Ag}$**

Despite the use of several endonucleases (BamHI, AvaI, AvaII, BglII, ClaI, PstI), manCO$_{Ag}$ restriction patterns were
wbk region

Figure 1
Regions and genes encoding LPS biosynthetic enzymes in *B. melitensis* 16M Region *wbk* contains genes coding for: (i), enzymes necessary for N-formylperosamine synthesis (*gmd, per, wbkC*); (ii), two O-PS glycosyltransferase (*wbkE, wbkA*); (iii), the ABC transporter (*wzm, wzt*); (iv) the epimerase/dehydratase necessary for the synthesis of an N-acetylaminosugar (*wbkD*); and (v), the polyisoprenyl-phosphate N-acetylhexosamine-1-phosphate transferase enzyme that primes bactoprenol (*wbkF*). Genes *manAO-Ag, manBO-Ag, manCO-Ag* could be involved in the synthesis of mannose, the perosamine precursor. Restriction sites: *A, AluI; AvI, AvoI; Av, Avall; B, BglII; Bg, BglII; C, Clal; E, EcoRI; EV, EcoRV; H, HindIII; Ha, HaeII; Hf, HinfI; P, PstI; Pv, PvuII; S, Sau3A; Sa, Sall; St, StyI.
| Species   | Biovar | Serotype | Strain     | Host/source | Geographic origin | wbkE | manA O-Ag | manC O-Ag | manB O-Ag | wbkF | wbkD | wboA | wboB | manB core | wa*** |
|-----------|--------|----------|------------|-------------|-------------------|------|-----------|-----------|-----------|------|------|------|------|-----------|-------|
| Terrestrial mammal: |
| **B. melitensis** | 1      | M        | 16 M (ATCC 23456; BCCN R1) | Goat | United States | A | A | A | A | A | A | A | A | A | A | A |
| | 2      | A        | 63/9 (ATCC 23457; BCCN R2) | Goat | Turkey | A | A | A | B | B | A | A | A | A | A | A |
| | 3      | AM       | Ether (ATCC 23458; BCCN R3) | Goat | Italy | A | A | A | A | B | A | A | A | A | A |
| **B. abortus** | 1      | A        | 544 (ATCC 23448; BCCN R4) | Cattle | England | A | A | A | C | A | B | B | A | A | A |
| | 2      | A        | 86/8/59 (ATCC 23449; BCCN R5) | Cattle | England | A | A | A | C | C | B | B | A | A | A |
| | 3      | A        | Tulya (ATCC 23450; BCCN R6) | Human | Uganda | A | A | A | A | B | A | B | A | A |
| | 4      | M        | 292 (ATCC 23451; BCCN R7) | Cattle | England | A | A | A | C | A | B | B | A | A | A |
| | 5      | M        | 83196 (ATCC 23452; BCCN R8) | Cattle | England | A | A | A | C | A | B | B | A | A | A |
| | 6      | A        | 870 (ATCC 23453; BCCN R9) | Cattle | Africa | A | A | A | C | A | B | B | A | A | A |
| | 9      | M        | C68 (ATCC 23455; BCCN R11) | Cattle | England | A | A | A | C | A | B | B | A | A | A |
| | R      |          | 45/20 (BCCN V2) | Cattle | England | A | A | A | C | C | B | B | A | A | A |
| **B. suis** | 1      | A        | 1330 (ATCC 23444; BCCN R12) | Swine | United States | A | A | A | D | A | B | A | A | A | A |
| | 2      | A        | Thomsen (ATCC 23445; BCCN R13) | Swine | Denmark | A | A | A | E | A | B | A | A | A | B |
Table 1: *Brucella* strains used in this study. (Continued)

|   |   | 686 (ATCC 23446; BCCN R14) | Swine | United States | A | A | A | D | A | B | A | A | A | A |
|---|---|-----------------------------|-------|---------------|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | AM | 40 (ATCC 23447; BCCN R15)  | Reindeer | Russia       | A | A | A | D | A | B | A | A | A | A |
| 5 | M  | 513 (BCCN R21)              | Wild rodent | Russia      | A | A | A | D | A | B | A | A | A | A |

*B. ovis*

|   |   | 63/290 (ATCC 25840; BCCN R17) | Sheep | Africa       | A | A | A | F | A | B | NA | NA | A | C |
|---|---|-----------------------------|-------|---------------|---|---|---|---|---|---|-----|-----|---|---|
|   |   | Reo 198 (BCCN R22)           | Sheep | United States | A | A | A | F | A | B | NA | NA | A | C |
|   |   | BCCN 76–250                  | Sheep | France       | A | A | A | F | A | B | NA | NA | A | C |

*B. canis*

|   |   | RM6/66 (ATCC 23365; BCCN R18) | Dog | United States | A | A | A | D | D | C | A | A | A | A |
|---|---|------------------------------|-----|---------------|---|---|---|---|---|---|---|---|---|---|---|---|
|   |   | D519 (BCCN C1)               | Dog | Madagascar   | A | A | A | D | D | C | A | A | A | A |
|   |   | BCCN 87.65                   | Dog | Canada       | A | A | A | D | D | C | A | A | A | A |

*B. neotomae*

|   |   | SK33 (ATCC 23459; BCCN R16) | Desert rat | United States | A | B | A | D | A | A | A | A | A | A |

Marine mammal:

*B. pinnipedialis*

|   |   | B2/94                        | Common seal | Scotland     | A | A | A | G | A | A | A | A | A | A |

*B. ceti*

|   |   | B1/94                        | Porpoise    | Scotland     | A | A | A | G | A | A | A | A | A | A |

*ATCC, American Type Culture Collection; BCCN, Brucella Culture Collection, Nouzilly, France.*

NA: Not Amplified
Figure 2
Restriction maps of the core- and O-polysaccharide genes with the restriction enzymes used. For each gene, restriction map A corresponds to that deduced from the nucleotide sequence of B. melitensis 16 M. Only differences compared to the nucleotide sequences of B. melitensis 16 M are indicated in restriction maps B, C, D, E, F and G. The restriction patterns A, B, C, D, E, F and G are further indicated in Table 1 for each gene and for each Brucella strain studied. Additional sites and their most probable location according to restriction patterns are indicated by the restriction name (e.g. Hf) and by the position name and an asterisk.
identical in all Brucella strains (Figure 2, Table 1). Therefore, no polymorphism was observed by this method.

**manB**

*B. melitensis* 16 M (biovar 1) and *B. abortus* Tulya (biovar 3) presented a similar *manB* restriction pattern (pattern A), and *B. melitensis* biovars 2 and 3 showed a Sau3A site absent in other strains (pattern B). All *B. abortus* (except *B. abortus* Tulya (biovar 3)) strains tested showed a specific pattern characterized by the absence of the EcoRV site at position 1238 (pattern C). *B. suis* biovars 1, 3, 4 and 5, *B. canis* and *B. neotomae* formed a separate group (pattern C) on the basis of the Sau3A restriction patterns of this gene. *B. ovis* shared this pattern only partially because it lacked one more Sau3A site (pattern F). *B. suis* biovar 2 strains lacked the *manB* Sau3A site and showed an additional HinfI site in this gene (pattern E). When this gene was amplified (primers *manB*-A and *manB*-B; (Table 2) from *B. ovis* 63/290, sequenced, and aligned with the homologous genes of *B. melitensis* biovar 1, *B. abortus* biovar 1, and *B. suis* biovar 1, polymorphism in both sequence and length was detected. As compared to *B. melitensis* biovar 1 and *B. abortus* biovar 1, two more nucleotides were found at position 1265–1266 in *B. suis* biovar 1 and *B. ovis* which should lead to a modification of C-terminal sequence of the protein (not shown). All strains isolated from marine mammals yielded restriction *manB* patterns very different from those of the six classical species (pattern G, Table 1) as well as a larger PCR product (2,933 bp and 2,091 bp, respectively) (Figure 3). Sequencing of the PCR product of three strains (B2/94, B1/94 and B14/94) revealed an IS711 element (842 bp) inserted into the gene (from position 780 to 1622) (Figure 2), and this insertion was confirmed by PCR in 82 additional marine mammal strains (not shown).

**wbkD** and **wbkF**

The *wbkD* PCR product was tested with HinfI, Avall, Sau3A, BglI, Clal and Styl, but a very low degree of DNA polymorphism was observed (Figures 2 and 3, and Table 1). For *B. melitensis*, *B. neotomae* and all marine mammal strains, all strains showed the same Sau3A pattern. An additional Sau3A site was observed for all *B. abortus*, *B. suis* and *B. ovis* strains (pattern B). Interestingly, the *B. canis* product showed a reduced size of around 400 bp and, therefore, yielded species specific restriction patterns (Figures 2 and 3). This result indicated the existence of a deletion in *B. canis* *wbkD* (see below). The *wbkF* PCR product showed also a low degree of polymorphism when tested with EcoRV, Haell, HinfI, Alul, Sau3A and Styl (Figures 2 and 3, and Table 1). One pattern, however, was specific for *B. melitensis* biovar 2 which lacked an Alul site, and a distinct pattern for two *B. abortus* biovar 2 and 45/20, was also observed with Alul site. Remarkably, no amplification was obtained for *B. canis*, suggesting that...
the sequence of the \( \text{wbkF}-\text{B} \) primer corresponded to a deletion extending from the adjacent \( \text{wbkD} \) gene (see above). In fact, when the appropriate primer was used, the \( \text{wbkF} \) PCR product showed a reduced size of about 400 bp. To examine this point further, the \( \text{wbkF}-\text{wbkD} \) locus was amplified and sequenced in \( \text{B. melitensis} \), \( \text{B. ovis} \) and \( \text{B. canis} \). The sequences showed a 351 bp deletion in \( \text{B. canis} \) extending from \( \text{wbkD} \) nucleotide 1594 (in BMEI 1425) to \( \text{wbkF} \) nucleotide 918 (in BMEI 1427) (Figure 3 and 4) as confirmed by the genome sequence of \( \text{B. canis} \) RM 6/66 (ATCC 23365) (Genbank accession # CP000872 and CP000873). Moreover, as compared to their homologs in \( \text{B. melitensis} \), \( \text{B. abortus} \) and \( \text{B. suis} \), gene \( \text{wbkF} \) of \( \text{B. ovis} \) showed a single nucleotide deletion at position 35. This frame shift mutation necessarily leads to an extensive modification of cognate protein (Figure 5).

### Table 2: DNA Primers used

| Target DNA | Primer name | Sequence (5'-3') | Amplicon size |
|------------|-------------|-----------------|---------------|
| manA0-Ag   | manA-A      | CATCACCATCGTTTCAAGCA | 1804 bp       |
| manA0-B    | manA-B      | GCCAGGGGAATGATAATGA |               |
| manB0-Ag   | manB-A      | GGGTGGCAAGTTGCGATCG | 2091 bp       |
| manB0-B    | manB-B      | CTAATGCTGTCCGGACC |               |
| manC0-Ag   | manC-A      | TGAAAGACTGTTTATTCG | 2210 bp       |
| manC0-B    | manC-B      | GCAAGACTGGATAGAAACC |               |
| \( \text{wbkE} \) | \( \text{wbkE-A} \) | CGCAAAACTGAATGGGAA | 2452 bp       |
| \( \text{wbkF} \) | \( \text{wbkF-A} \) | GCCAGGGAATGGCAAGGAC | 1234 bp       |
| \( \text{wbkD} \) | \( \text{wbkD-A} \) | TGGCTGGAGTGTCGGAAGG | 2165 bp       |
| \( \text{wa}^{**} \) | \( \text{wa}^{**}-A \) | CATTCGTCATGGCCAGT | 2342 bp       |
| \( \text{manBcore} \) | \( \text{manBcore-A} \) | CCAGCCGACGATTGGAACCTG | 1589 bp       |
| \( \text{manBcore} \) | \( \text{manBcore-B} \) | AAGCGTGGCAACCCGACTGCC |               |
| \( \text{wboA} \) | \( \text{wboA-A} \) | TGTGCAATACGGTCCTTCG | 1334 bp       |
| \( \text{wboA} \) | \( \text{wboA-B} \) | GCTTTTACCGCAACAGTTC |               |
| \( \text{wboB} \) | \( \text{wboB-A} \) | CTGCGGATGCGAAACTACCG | 1656 bp       |
| \( \text{wboB} \) | \( \text{wboB-B} \) | GCTCAGCTTCCGGAAATC |               |

Figure 4

The \( \text{B. melitensis} \) 16 M chromosome I region absent in \( \text{B. canis} \) and the adjacent DNA. The two 7 bp direct repeats located in \( \text{B. melitensis} \) 16 M at both sides of the fragment absent in \( \text{B. canis} \) are in bold.
Comparison of the \textit{B. suis} ManB\textsuperscript{core} and WbkF with the corresponding \textit{B. ovis} proteins

\textbf{Figure 5}

Comparison of the \textit{B. suis} ManB\textsuperscript{core} and WbkF with the corresponding \textit{B. ovis} proteins. Conserved amino acids are indicated by stars. The alignment was performed using the Clustal W program.
Gene polymorphism in wboA

A low degree of DNA polymorphism was observed in wboA. However, one pattern was specific of B. abortus since all strain tested lacked an AluI site. As described above, no amplification was observed for any B. ovis strain. This confirms [16,17] that absence of wboA (and wboB) is a B. ovis species-specific marker.

Polymorphism in core LPS genes

Despite using six restriction enzymes, all brucellae displayed the same RFLP pattern for the manBcore amplicon. In silico, the four genomes available showed low polymorphism. A single nucleotide deletion at position 812 was detected in B. ovis, which should modify the C-terminal sequence of the protein (Figure 5). Similarly, a low degree of polymorphism was observed in wa* *. With the exception of B. suis biovar 2, one PsI pattern was specific of B. suis. Biovar 2 also lacked an Avall site, which could be considered as a biovar marker. With Hinfl, a pattern was specific of B. ovis (Figure 2, Table 1).

Discussion

Despite the high DNA homology of brucellae, gene polymorphism and biovar-specific markers have been consistently found. Concerning outer membrane molecules, both have been found in genes of proteins [16,18,19] but not in the LPS genes examined, all of the wbk region (wbkA, gnd, per, wzm, wzt, wkbB, and wkbC). Interestingly, these O-polysaccharide genes were found to be highly conserved not only in the classical S Brucella species and biovars but also in B. ovis and B. canis, the two species that lack the O-polysaccharide [14]. Therefore, an implication of these observations is that the R phenotype of B. ovis and B. canis cannot be explained by the absence of any of those seven wbk genes. More recently, the wbk region has been extended to include wkbE, manAOAg, manBOAg, manCOAg, wkbF, and wkdD [12]. The present study includes an analysis of some of these genes and the results not only show the existence of specific markers but, more importantly, they also improve our understanding of the genetics-structure relationship in Brucella LPS. Concerning the O-polysaccharide, the results are relevant to interpret the variations in O-polysaccharide linkages of S Brucella and add further weight to our previous finding [12] that the putative mannose genes in wbk are not essential for perosamine synthesis. Furthermore, they help to explain the differences existing between S and R Brucella species.

Despite extensive transposon mutagenesis searches, only four putative glycosyltransferase genes have been implicated in N-formylperosamine polymerization in Brucella: wbkA, wkbE, wboA and wboB. As mentioned above, wbkA is conserved in classical Brucella species [14], and the results reported here show that wboA, wboB and wkbE are similarly present in S B. melitensis, B. abortus, B. suis, B. pinnipedialis and B. ceti. Moreover, these genes displayed low polymorphism, no matter the A or M serotype. It has to be noted that the consensus sequences of glycosyltransferases are conspicuous enough to make unlikely the existence of O-polysaccharide transferases other than wboA, wboB, wkbA and wkbE, and that, although the α (1–3) linkage relates to the M serotype, there is evidence showing that at least some A dominant strains generate a very small proportion (i.e. 2%) of α (1–3) linkages [20]. In keeping with this, it has been observed that strain RB51 (a wboA mutant of the A-dominant B. abortus 2308 S strain [21]) generates small amounts of atypical M-type polysaccharides [22]. All this evidence suggests that, rather than the presence of a α (1–3)-specific transferases in the M serotype, there are subtle variations in the expression of wboB, wkbA or wkbE, or in the activity of the corresponding glycosyltransferases that lead to the increase in α (1–3) linkages typical of the M and A = M serotypes.

A surprising feature of the wbk is the presence of genes that are not essential for O-polysaccharide synthesis. Godfroid et al. [13] analyzed the functions of the ORFs between BME11404 (wbkA), encoding a putative mannosyltransferase [perosaminyltransferase since mannose and perosamine are related] and BME11418 (wbkC, encoding a putative formyltransferase) and found that disruption of ORF BME11417 (wbkB) generated no R phenotype. Later, it was found that the genome of B. melitensis contains three putative mannosynthese genes (ORFs BME11394 to BME11396) adjacent to wkbA. Because mannosynthe is the direct precursor of perosamine and O-polysaccharide genes usually cluster together, Monreal et al. [23] proposed the names of manAOAg, manBOAg, manCOAg, for BME11394 to BME11396, and their assignment to wkb is supported by the finding by González et al. [12] that disruption of ORF BME1393 (wkbE) blocks O-polysaccharide synthesis. The latter authors provided proof that at least manBOAg is dispensable for perosamine synthesis but also pointed out that the existence of manBOcore (ORFs BME110900 and BME110899) preclude to rule out any role for the wkb putative mannosynthese genes since there could be internal complementation [12]. All these results are fully consistent with the observation that, although manBOAg is disrupted by IS711 in B. pinnipedialis and B. ceti, these two species keep the S phenotype. The wbk region has features suggestive of horizontal acquisition [14] whereas manBOcore (and manCOcore) are Brucella older genes necessary for the synthesis of the LPS core oligosaccharide [23,24]. Accordingly, a drift to dysfunction of the wbk man genes may have been made possible by the redundancy created after horizontal acquisition of wbk, and the similarity in this regard between B. ceti and B. pinnipedialis suggests a common ancestor.
The results of this research also shed additional light on the genetic basis behind the R phenotype of B. ovis and B. canis. Previous work has shown a large deletion in B. ovis that encompasses wboA and wboB [16,17]. The present work confirms the absence of these two putative perosaminyltranferase genes in B. ovis, an absence that can account by itself for the lack of O-polysaccharide in this species [12,25]. To this evidence, the present work adds the nucleotide deletion detected in B. ovis wbbK [26]. Indeed, the frame-shift thus created predicts a very modified protein. Presumably, WbbK is involved in catalyzing the transfer of an acetylated aminosugar to undecaprenylphosphate, thus priming this carrier for O-chain polymerization. The N-terminal region of the E. coli WbbK homologue was found to be necessary for this function [26] and, therefore, it seems likely that the frame-shift in B. ovis wbbK introduces a non-functional protein, thus explaining in part the R phenotype of this species. Other changes detected in several B. ovis LPS genes do not have this dramatic effect. As discussed above, the man wbbk genes are dispensable and, therefore, the nucleotide substitution and frame shift detected in B. ovis manA \textsubscript{O-Ag} do not contribute to the R phenotype. Since disruption of manB \textsubscript{core} generates a deep R-LPS [24,24], the presence of two more nucleotides in the sequence of B. ovis manB \textsubscript{core} was interesting. However, this deletion modified only the C-terminal sequence (5 last amino-acids) of the protein making unlikely a change severe enough to contribute to the R phenotype. In support of this interpretation, B. ovis R-LPS is not deeply truncated like that of manB \textsubscript{core} mutants. Moreover, the same two nucleotide addition was detected in B. suis, and it is known that a functional manB \textsubscript{core} is required for the synthesis of S-LPS in this species [27].

A DNA deletion of 351 bp, including 3' end of \textit{wbbK} and 3' end of \textit{wbbD} was detected in B. canis, which might have occurred by a slipped mispairing mechanism (a direct repeat sequence of 7 bp «GGATCAT» is present at both sides of the deleted sequence in the other \textit{Brucella} species (Figure 5). It is clear that this deletion has profound consequences in the synthesis of LPS. We have discussed above the essential role of \textit{wbbK} in O-polysaccharide synthesis, and \textit{wbbD} seems involved in the synthesis of quinovosamine, a sugar that is possibly linking the \textit{Brucella} O-polysaccharide to the R-LPS [12]. This double mutation clearly explains the R phenotype of B. canis and is consistent with the absence of quinovosamine in this species [28].

**Conclusion**

The analyses carried out suggest new hypothesis to study the genetics of \textit{Brucella} O-polysaccharide serotypes and provide evidence on both the dispensability of some \textit{wbb} genes which is consistent with their horizontal acquisition. They also confirm the essential role of \textit{wbbD} and \textit{wbbK} in O-polysaccharide synthesis and, at the same time, contribute to understand the R phenotype of B. ovis and B. canis. Finally, they provide several biovar and species specific markers that can be used to design the corresponding molecular typing tools.

**Methods**

**Brucella strains**

The strains (Table 1) were maintained freeze-dried in the INRA \textit{Brucella} Culture Collection, Nouzilly (BCCN), France. For routine use, they were grown on tryptic soy agar (Difco)-0.1% (w/v) yeast extract (Difco). Fastidious strains (\textit{B. abortus} biovar 2 and B. ovis) were grown on the same medium supplemented with 5% sterile horse serum (Gibco BRL). All strains were checked for purity, and species and biovar confirmed by standard procedures [2].

**DNA preparation**

Bacteria were cultured at 37°C for 24 h, suspended in 3 ml sterile distilled water, harvested (2000 × g, 10 minutes) and resuspended in 567 μl of 50 mM Tris, 50 mM EDTA, 100 mM NaCl (pH 8.0). Then, 30 μl of 10% (w/v) SDS and 3 μl of 2% (w/v) proteinase K were added, the mixture was held at 37°C for 1 h and extracted twice with phenol-chloroform. Nucleic acids in the aqueous phase were precipitated with two volumes of cold ethanol, dissolved in 100 μl of 10 mM Tris, 1 mM EDTA (pH 8.0) and the amount of DNA estimated by electrophoresis on 0.8% agarose gels using appropriate DNA solutions as the standards.

**Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)**

The 20-mer primers were selected to amplify \textit{manB}_{O-Ag}, \textit{manA}_{O-Ag}, \textit{manC}_{O-Ag}, \textit{wbbF}, \textit{wkdD}, \textit{wbbE}, \textit{wboA} and \textit{wboB}, \textit{wua} and \textit{manB}_{core} according to the \textit{B. melitensis} 16 M genome sequence (Genbank accession numbers AE008917 and AE008918) (Table 2). Amplification mixtures were prepared in 100 μl volumes containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.1% Triton X-100, 0.2 mg ml\textsuperscript{-1} gelatin (1 × PCR buffer; Appligene), 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 100 ng of genomic DNA, and 2.5 U of Taq DNA polymerase (Appligene). Amplification was performed in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer) as follows: cycle 1, 94°C for 5 minutes (denaturation); the next 30 cycles, 58°C for 30 s (annealing), 70°C for 30 s (extension) and 94°C for 30 s (denaturation); the last cycle, 58°C for 30 s (annealing) and 70°C for 10 minutes (extension). For PCR-RFLP, \textit{AluI}, \textit{AvaiI}, \textit{Aval}, \textit{BamHI}, \textit{BglII}, \textit{BglII}, \textit{ClaI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII}, \textit{HaeII}, \textit{HinfI}, \textit{PstI}, \textit{PvuII}, \textit{SalI}, \textit{SshI}, \textit{StyI} were used. The restriction enzymes were chosen according to the \textit{B. melitensis} 16 M genomeic sequences of the above-listed genes.
2.4. Nucleotide sequence and data analysis

PCR products of the expected sizes were purified from 1% agarose gels (Invitrogen) with a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), cloned into pGEM-T Easy vector (Promega, Madison, Wis.), and transformed into competent JM109 Escherichia coli cells (Promega). The transformants were selected with ampicillin, and recombinants were selected by blue-white differentiation. Plasmids were isolated from several clones with a Qiagen Plasmid Mini kit. To check for the presence of the correct insert, plasmids were digested with EcoRI and the restriction products were separated on 1% agarose gels. Nucleotide sequencing of clone was performed by automated cycle sequencing with Big Dye terminators (ABI 377XL; PE Applied Biosystems, Foster City, Calif.) and primers RP (reverse primer) and UP (universal primer M13-20). Multiple DNA and amino acid sequence alignments were performed with CLUSTAL W http://www2.ebi.ac.uk/clustalw/.

Nucleotide sequence accession numbers

The Brucella nucleotide sequences determined in this work have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: FJ376556, FJ 376557 for the manB0_Ag gene of B. pinnipedialis B2/94 and B. ceti B1/94.

Authors’ contributions

MSZ, IM and AC conceived the study. MSZ designed and performed the experimental work. All authors analyzed the data. MSZ wrote the manuscript. IM, and AC helped to draft the manuscript. All authors read, corrected and approved the final manuscript.

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References

1. Euzéby JP: List of prokaryotic names with standing in nomenclature. 2008 [http://www.bacterio.cict.fr/index.html].
2. Alton GG, Jones LM, Angus RD, Verger JM: Techniques for the brucellosis laboratory Paris, France: INRA; 1988.
3. Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A: Brucella ceti sp. nov. and Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol 2007, 57:2688-2693.
4. Scholz HC, Hubalek Z, Sedlacek I, Verenaug G, Tomascho H, Al DS, Melzer F, Kamber P, Neubauer H, Cloeckaert A, Maquart M, Zygmun MTS, Whatmore AM, Falsen E, Bahn P, Gollner C, Pfeffer M, Huber B, Busse H, Nockler K: Brucella microti sp. nov., isolated from the common vole Microtus arvalis. Int J Syst Evol Microbiol 2008, 58:375-382.
5. Le Fléche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denœud F, Nockler K, Neubauer H, Guillotoue LA, Verenaug G: Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. BMC Microbiol 2006, 6:9.
6. Marianelli C, Ciuchi M, Tarantino M, Pasquale P, Adone R: Molecular characterization of the rpoB gene in Brucella species: new potential molecular markers for genotyping. Microbes Infect 2006, 8:860-5.
7. Garcia-Yoldi D, Marín CM, de Miguel MJ, Muñoz PM, Vizmanos JL, López-Goñi I: Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1. Clin Chem 2006, 52:779-781.
8. Foster JT, Okinaka RT, Shaw K, De BK, Robison RA, Probert WS, Kerenf LJ, Brown WD, Keim P: Real-time PCR assays of Single-Nucleotide Polymorphisms defining the major Brucella clades. J Clin Microbiol 2008, 46:296-301.
9. Whatmore AM, Perrett LL, MacMillan AP: Characterisation of the genetic diversity of Brucella by multilocus sequencing. BMC Microbiol 2007, 7:34.
10. Lapaque N, Moriyón I, Moreno E, Gorvel JP: Brucella lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol 2005, 8:560-66.
11. Perry MB, Bundle DR: Advances in brucellosis research Edited by: Adams LG. Texas A&M University Press, College Station: 1990:76-88.
12. González D, Grillo MJ, De Miguel M, Ali T, Arce-Gorvel V, Delruie R-M, Cande-Alvarez R, López-Goñi I, Moriyón I, Dobín A, Widalm M, Gygumt MS, Letesson JI, Gorvel JP, Blasco JM, Moriyón I: Brucella vaccines: assessment of Brucella melitensis lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS ONE 2008, 3:e2760.
13. Godfroid F, Cloeckaert A, Tamainiaux B, Danese I, Tabor A, de Bolle X, Mertens P, Letesson JJ: Genetic organisation of the lipopolysaccharide O-antigen biosynthesis region of Brucella melitensis 16 M (wkb). Res Microb 2000, 151:653-683.
14. Cloeckaert A, Verger JM, Grayon M, Paquet Y, Garin-Bastuji B, Foster G, Godfroid J: Conservation of seven genes involved in the biosynthesis of the lipopolysaccharide O-side chain in Brucella spp. Res Microb 2000, 151:209-216.
15. Iriarte M, González D, Delruie R-M, Monreal D, Conde R, Lópe-Goñi I, Letesson JJ, Moriyón I: Brucella. Molecular and Cellular Biology Edited by: López-Goñi I, Moriyón I. Horizon Bioscience, Wymondham, UK; 2004:159-192.
16. Vizcaíno N, Caro-Hernández P, Cloeckaert A, Fernández-Lago L: DNA polymorphism in the omp23/omp31 family of Brucella spp.: Identification of a 1.7-kb inversion in Brucella cetaceae and of a 11.1-kb genomic island, absent from Brucella ovis, related to the synthesis of smooth lipopolysaccharide. Microbes Infect 2004, 6:821-834.
17. Garcia-Yoldi D, Marín CM, López-Goñi I: Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of Brucella spp. FEMS Microbiol Lett 2005, 245:79-84.
18. Cloeckaert A, Verger JM, Grayon M, Paquet Y, Garin-Bastuji B, Foster G, Godfroid J: Classification of Brucella spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. Microbes Infect 2001, 3:729-738.
19. Ficht TA, Hussein HS, Derr J, Bearden SW: Species-specific sequences at the omp2 locus of Brucella type strains. Int J Syst Bacterial 1996, 46:329-331.
20. Mekle PJ, Perry MB, Chiewongongrodzky JW, Bundle DR: Fine structure of A and M antigens from Brucella biovars. Infect Immun 1989, 57:2820-2828.
21. Vemulapalli R, McQuiston JR, Schuring GG, Sirirangsaleam NA, Halling SM, Boyle SM: Identification of and IS151 element interrupting the polA gene of Brucella abortus vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other Brucella species and strains. Clin Diagn Lab Immunol 1999, 6:760-764.
22. Cloeckaert A, Gygumt MS, Guillotoue LA: Brucella abortus vaccine strain RB51 processes low levels of M-like O-antigen. Vaccine 2002, 20:1820-1822.
23. Monreal D, Griló MJ, González D, Marín CM, de Miguel MJ, López-Goñi I, Blasco JM, Cloeckaert A, Moriyón I: Characterization of Brucella abortus O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against Brucella abortus and Brucella ovis in the mouse model. Infect Immun 2003, 71:3261-3271.
24. Allen CA, Adams LG, Ficht TA: **Transposon-derived Brucella abortus rough mutants are attenuated and exhibit reduced intracellular survival.** Infect Immun 1998, **66**:1008-1016.

25. McQuiston JR, Vemulapalli R, Inzana TJ, Schuring GG, Sriranganathan NM, Fritzinger D, Hadfield TL, Warren RA, Snellings N, Hoover DL, Halling SM, Boyle SM: **Genetic characterization of a Tn5-disrupted glycosyltransferase gene homologue in Brucella abortus and its effect on lipopolysaccharide composition and virulence.** Infect Immun 1999, **67**:3830-3835.

26. Amer AO, Valvano MA: **The N-terminal region of the Escherichia coli WecA (Rfe) protein, containing three predicted transmembrane helices, is required for function but not for membrane insertion.** J Bacteriol 2000, **182**:498-503.

27. Foulongne V, Bourg G, Cazevieille C, Michaux-Charachon S, O'Callaghan D: **Identification of Brucella suis genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis.** Infect Immun 2000, **68**:1297-1303.

28. Bowser DV, Wheat RW, Foster JW, Leong D: **Occurrence of quinovosamine in lipopolysaccharides of Brucella species.** Infect Immun 1974, **9**:772-774.