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Spontaneous Excision of the O-Polysaccharide *wbkA* Glycosyltranferase Gene Is a Cause of Dissociation of Smooth to Rough *Brucella* Colonies

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The brucellae are Gram-negative pathogens that cause brucellosis, a zoonosis of worldwide importance. The genus *Brucella* includes smooth and rough species that differ in that they carry smooth and rough lipopolysaccharides, respectively. *Brucella abortus*, *B. melitensis*, and *B. suis* are typical smooth species. However, these smooth brucellae dissociate into rough mutants devoid of the lipopolysaccharide O-polysaccharide, a major antigen and a virulence determinant encoded in regions *wbo* (included in genomic island-2) and *wbk*. We demonstrate here the occurrence of spontaneous recombination events in those three *Brucella* species leading to the deletion of a 5.5-kb fragment carrying the *wbkA* glycosyltranferase gene and to the appearance of rough mutants. Analysis of the recombination intermediates suggested homologous recombination between the IS*Bm1* insertion sequences flanking *wbkA* as the mechanism generating the deletion. Excision of *wbkA* was reduced but not abrogated in a *recA*-deficient mutant, showing the existence of both RecA-dependent and -independent processes. Although the involvement of the IS*Bm1* copies flanking *wbkA* suggested a transpositional event, the predicted transpositional joint could not be detected. This absence of detectable transposition was consistent with the presence of polymorphism in the inverted repeats of one of the IS*Bm1* copies. The spontaneous excision of *wbkA* represents a novel dissociation mechanism of smooth brucellae that adds to the previously described excision of genomic island-2. This IS*Bm1*-mediated *wbkA* excision and the different %GC levels of the excised fragment and of other *wbk* genes suggest that the *Brucella wbk* locus is the result of at least two horizontal acquisition events.

The brucellae are a group of Gram-negative pathogens that cause brucellosis, one of the most important bacterial zoonosis worldwide. Based on the appearance of the colony surface and the structure of the lipopolysaccharide (LPS), these bacteria are divided into rough (R) and smooth (S) species. The R species are represented by *Brucella ovis* and *B. canis*, produce matte, granular colonies, and carry an R-type LPS lacking the O-polysaccharide (O-PS). The S species, which are zoonotically more important, include *B. melitensis*, *B. abortus*, and *B. suis*, have an LPS with an *N*-formyl-perosamine O-PS, and produce glossy colonies. The S phenotype, however, is not stable, and S brucellae dissociate to generate R mutants closely similar to the R species in colony morphology and LPS structure. Under unfavorable growth conditions or after prolonged incubation, this dissociation is very noticeable. Thus, it soon caught the attention of brucellosis researchers, who described the colonial dissociation in great detail in the first half of the past century. This early work also established that R mutants are attenuated and not useful for serological diagnostic purposes (for a review of the early literature, see reference 37), two observations accounted for by the role of the O-PS as both a key virulence factor and a major diagnostic antigen in brucellosis serological tests (21, 27). Indeed, procedures that minimize the S-R dissociation and controls to exclude batches containing R bacteria are critical in brucellosis antigen and vaccine production (2).

The genetic basis underlying the S-R dissociation of S brucellae is only partially understood. Extensive analyses (for a review, see reference 16) have shown that the O-PS biosynthetic genes are located in two major regions, *wbo* and *wbk*, with different characteristics. The *wbo* region carries two putative glycosyltransferase genes (*wboA* and *wboB*) and is included in a large *Brucella* genomic island denominated GI-2 (32). The *wbk* region, which was probably acquired by horizontal transfer (15), carries the genes putatively necessary to synthesize *N*-formyl-perosamine and to prime bactoprenol for O-PS polymerization plus those of several glycosyltransferases and of the ABC transporters that translocate the O-PS. It is noteworthy that the *wbk* genes do not form a continuous unit since the region contains a number of insertion sequence (IS) open reading frames (ORFs) (Fig. 1A). Recently, we traced one mechanism of S-R dissociation to the spontaneous excision of GI-2 mediated by the recombination activity of a phage-related integrase present in this island (23). Stabilization of GI-2 by mutation of this integrase reduces but does not eliminate the S-R dissociation, suggesting additional mechanisms. Based on the characteristics of the *wbk* region, we hypothesized that spontaneous recombination between homologous sequences adjacent to the *wbkA* glycosyltransferase gene (Fig. 1A) could lead to its excision and generate S-R dissociation. We present here evidence showing that such an excision occurs spontaneously *in vitro* by homologous recombination and report that the

Received 21 November 2011 **Accepted** 27 January 2012
Published ahead of print 10 February 2012
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doi:10.1128/JB.06561-11

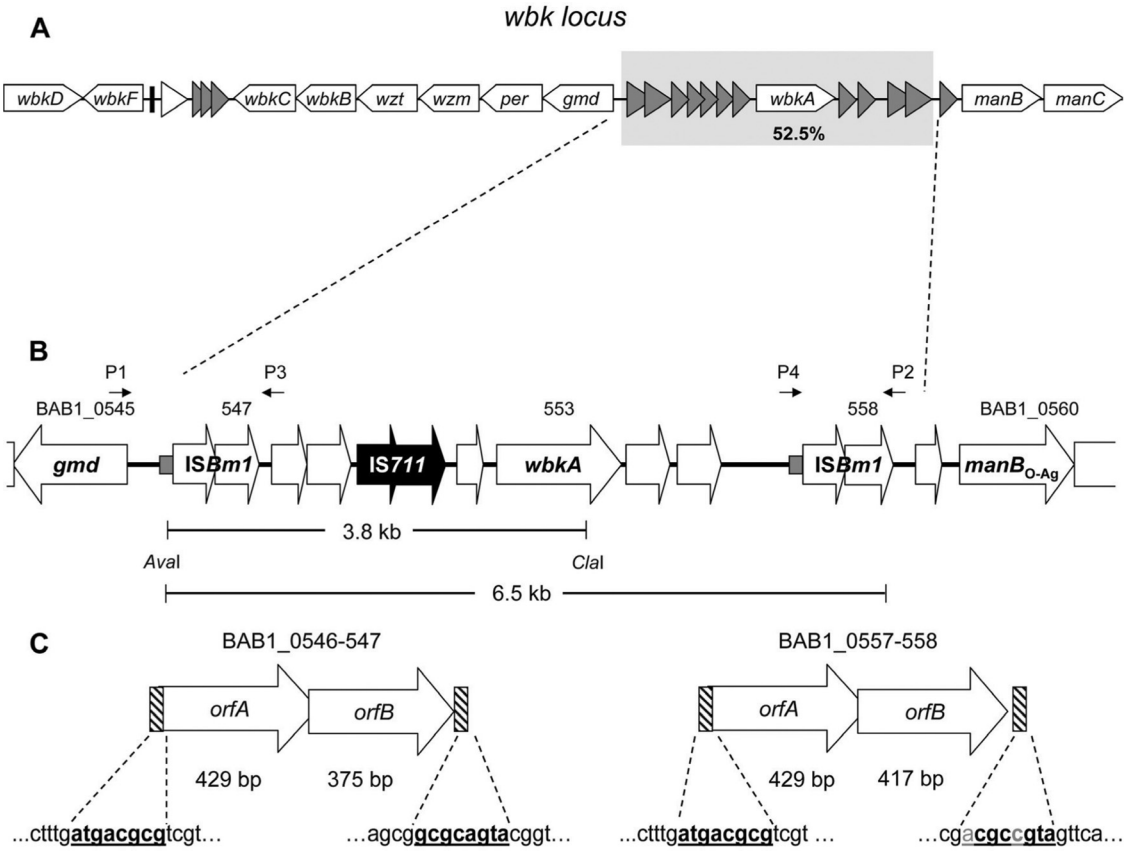

FIG 1 Genetic organization of the *wbk* locus of *B. abortus* 2308. (A) *wbk* locus (white arrows represent ORFs encoding LPS biosynthesis genes, gray triangles show transposase ORFs, a white triangle represents a gene encoding a putative regulatory protein, and the black bar indicates a gene encoding a $tRNA^{Gln}$). (B) *wbkA* region. Small gray boxes indicate the 60-nt repeat sequences located upstream of each IS*Bm1* (the sequences are AAAATTTTTTGGCATTTATCGGCCGCTA TGATTCAAGGCTTCGAAATAGGGGAGCCTTTG and AAAATTTCTTGGCATTTATCGGCCGCTATGTTCAAGGCTTCGAAATAGGGAAGTCTTTG for left and right IS*Bm1* copies, respectively). The primer positions are depicted by small black arrows (P1, BAB1_0545Fb; P2, BAB1_0558R; P3, BAB1_0548R; P4, BAB1_0557F). The AvaI-ClaI segment indicates the location of the corresponding IS*711*-carrying 3.8-kb restriction fragment. The IS*711* element is indicated in black. (C) Flanking IS*Bm1* with their corresponding inverted repeats (in boldface; polymorphism is indicated in gray).

deletion was found in a spontaneous R mutant directly isolated in a pure culture from goat milk.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the laboratory bacterial strains used in the present study. *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330 are strains commonly used in brucellosis studies that have been maintained as master stocks in either skimmed milk or Trypticase soy broth (Becton Dickinson, Sparks, MD)–5% sterile decomplemented calf serum–7% dimethyl sulfoxide at −85°C. Bacteria taken from these stocks were not passed more than once *in vitro*, and repeated controls showed the absence of R mutants detectable by the crystal violet dye exclusion test. In

TABLE 1 Bacterial strains used in this study

Strain	Characteristics/relevant phenotype[a]	Source or reference[b]
2308 Nal^r	*B. abortus* biovar 1 reference strain; S, Nal^r	UNAV
R6	Spontaneous *B. abortus* 2308 Nal^r Δ*wbkA* mutant; R	This study
B10	Chilean *B. abortus* field strain; S	24
R7	Spontaneous Δ*wbkA* mutant, *B. abortus* B10 derivative; R	This study
R6/c-*wbkA*	R6 complemented with plasmid pMM14; S	This study
M939	*B. melitensis* Δ*wbkA* field strain isolated from goat milk; R	CITA
BabΔIS*Bm*-L	*B. abortus* 2308 Nal^r mutant carrying a deletion of 150 nt in BAB1_0546; S	This study
BabΔIS*Bm*-R	*B. abortus* 2308 Nal^r mutant carrying a deletion of 150 nt in BAB1_0557; S	This study
BabΔIS*Bm*-LR	Double mutant in the *wbkA*-flanking IS*Bm1* copies; S	This study
BabΔ*recA*	*B. abortus* 2308 Nal^r mutant with a *recA*(Δ*80-283*) gene deleted; S	This study
BabΔIS*Bm*LRΔ*recA*	*recA* mutant constructed on BabΔIS*Bm*-LR; S	This study

[a] Nal^r, nalidixic acid resistance.
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TABLE 2 Primers used in this study

Primer[a]	Sequence (5′-3′)	Purpose	Source or reference
BAB1_0553F[a]	TGGTATATCCGGGTGTCTCG	*wbkA* detection	This study
BAB1_0553R	AAGCGTCCGTGCATTTCTAT		This study
BAB1_0545F	CCGAATTCTTGGAATGGAGA	*gmd* detection	This study
BAB1_0545R	CAGTCGCGTAATGAGTCCAA		This study
BAB1_0560F	CTTATGCAATGGCTCCCAAT	$manB_{O\text{-}Ag}$ detection	This study
BAB1_0560R	CATGTTCGGCGATAAATGTG		This study
BAB1_0545F[b]	TGCGACTTTCTTCACGATTG	Detection of *wbkA* deletion	This study
BAB1_0558R	GATCTTGGTATCGGCCTGTC		This study
BAB1_0557F	CGCTTTAATATCTCGCGTTCC	Detection of excised circle	This study
BAB1_0548R	GGTCCCATCGGCATATCTT		This study
recA_F1	TGTTATCCCTGTCGCCAAAT	To construct *recA* deletion mutant	This study
recA_R2	CTTTCCGGCCCATAGATTTC		This study
recA_F3	GAAATCTATGGGCCGGAAAGGCTGGTCGATCTTGGTGTC		This study
recA_R4	ACCGCTTTCCTGTCCAGATA		This study
BAB1_0546_F1	AGTCCACCTTGACATGCACA	To construct a 150-bp deletion IS*Bm1*-left	This study
BAB1_0546_R2	CCATAGCGTTCGGGAACTT		This study
BAB1_0547_F3	AAGTTCCCGAACGCTATGGAAAAAGGGGATGGAGACGAT		This study
BAB1_0547_R4	ACGATTGCAGCCTCTTCCAC		This study
BAB1_0557_F1	CGACGAAGGCGTTTTACAG	To construct a 150-bp deletion IS*Bm1*-right[b]	This study
BAB1_0558_R4	GATCTTGGTATCGGCCTGTC		This study
BAB1_0553F-gw[c]	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCGAAT TGGTGTCGAC	To complement the spontaneous Δ*wbkA* mutant	This study
BAB1_0553R-gw	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CTTAATAGGT CATGAGCTTAGATT		This study
711u	CACAAGACTGCGTTGCCGACAGA	IS*711* fingerprinting	28
711d	CATATGATGGGACCAAACACCTAGGG		28
scar_Fq	TTTGAAACGGTGCCCAAG	qPCR for chromosomal scar	This study
scar_Rq	ATAGACCCAGCGCGAAAAC		This study
wbkA_Fq	TGCCGTCTCTCTACGAAGGT	qPCR for *wbkA* gene	This study
wbkA_Rq	TTCGGCTACGTTCAGAGGAT		This study

[a] F, forward; R, reverse.
[b] These primers were used along with the primers BAB1_0546_R2 and BAB1_0547_F3 to construct the same deletion into IS*Bm1*-right.
[c] Sequences including *att* sites for BP clonase reaction are indicated in boldface.

addition, a *B. melitensis* R mutant obtained in pure culture during routine bacteriological examination of goat milk was studied. The brucellae were grown on *Brucella* Trypticase soy agar (Becton Dickinson) plates (prepared at least 24 h before use and dried to remove syneresis water to avoid conditions known to favor S-R dissociation) at 37°C for 48 h. For cloning, *E. coli* was grown in Luria-Bertani broth supplemented with kanamycin (50 μg/ml) or chloramphenicol (20 μg/ml).

Dissociation. To promote dissociation, a protocol described previously was used (23). Bacteria taken directly from the frozen master stock (see above) were inoculated onto Trypticase soy agar plates, and a loopful of bacteria was transferred to a flask with 10 ml of brucella broth (Becton Dickinson) and grown at 37°C until reaching the stationary phase (typically within 48 h). An aliquot of this culture was adjusted to an A_{750} of 0.109 ± 0.005 using sterile broth, and 100 μl was inoculated in 10 ml of tryptic soy broth or the same broth supplemented with McIlvaine's buffer (pH 6.6) (3). After incubation for 10 days at 37°C, the CFU were counted, and the extent of S-R dissociation was determined by the crystal violet dye exclusion test (40). Two plates with approximately 1,500 colonies per plate (the limit that allows obtaining isolated colonies in 36 to 48 h) were examined, and three independent experiments performed.

Sequence analyses. The complete sequence of *B. abortus* 2308 chromosome I was downloaded from GenBank (accession no. AM040264) (8), and similarity searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). *In silico* restriction analysis of the *wbk* locus was performed with Vector NTI software (Invitrogen, Carlsbad, CA). The primers used for conventional PCR and real-time quantitative PCR (qPCR) assays (Table 2) were designed using the Primer3 web tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). DNA repeat sequences were predicted by use of the REPuter web tool (http://bibiserv.techfak.uni-bielefeld.de/reputer). DNA sequencing by the dideoxy method was performed using the Sequencing Unit of Centro de Investigación Médica Aplicada (CIMA; Universidad de Navarra). The sequence of the PCR product derived from the R6 strain was deposited in GenBank under accession no. JN982244.

DNA purification and PCR assays. Genomic DNA (gDNA) was isolated from liquid cultures according to standard protocols (41), and PCR products were purified from agarose gels with a QIAEXII kit (Qiagen, Hilden, Germany). To determine the boundaries of spontaneous *wbkA* deletion, conventional PCR with primer pairs (Table 2) targeting the adjacent genes *gmd* (BAB1_0545) and $manB_{O\text{-}Ag}$ (BAB1_0560) and *wbkA* (BAB1_0553) was performed. The mixture included 10 ng of gDNA, 10 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, and 1 U of Immolase DNA polymerase (Bioline, London, United Kingdom) in a final volume of 25 μl. The amplification conditions were as follows: an initial step at 95°C for 5 min, followed by 30 cycles at 95°C for 20 s, annealing at 60°C for 25 s, and extension at 72°C for 30 s, with a final extension step of 5 min at 72°C. Recombination intermediates were amplified from 0.2-μg portions of gDNA samples using the primer pairs BAB1_0545Fb/BAB1_0558R (chromosomal scar) and BAB1_0557F/BAB1_0548R (excised circle) under the same conditions but for the extension steps that were 1 min. PCR assays were carried out in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). Amplicons were resolved by electrophoresis in 1.0 to 2.0% TBE (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) agarose gels.

qPCR assessment of *wbkA* excision. To assess the extent of *wbkA* excision in each mutant, a qPCR assay was developed based on the relative

TABLE 3 Plasmids and *E. coli* strains used in this study

Plasmid or *E. coli* strain	Characteristics[a]	Source or reference
Plasmids		
pCR2.1 TOPO	Cloning plasmid; Km^r	Invitrogen
pJQK	Suicide vector for mutagenesis by gene replacement; Km^r	31
pDONR221	Cloning plasmid (Gateway Technology); Km^r	This study
pMR10	Destination vector (Gateway Technology); Km^r Cm^r	19
pMM13	Donor vector, pDONR221 derivative containing a copy of the *B. abortus wbkA* gene (BAB1_0553); Km^r	This study
wbkA-pMM14	Complementation vector, pMR10 derivative carrying the *att* fragment from pMM13, Km^r Cm^r	This study
pMM62	pCR2.1 TOPO derivative containing a mutant allele of *recA*(Δ*80-283*) (BAB1_1224)	This study
pMM63	Mutator plasmid, pJQK derivative containing the BamHI-XbaI fragment of pMM62	This study
pMM72	pCR2.1 derivative containing F1R4ΔBAB1_0557 fragment generated by PCR overlap	This study
pMM73	Mutator plasmid, pJQK derivative containing the BamHI-XbaI fragment of pMM72	This study
pMM77	pCR2.1 derivative containing F1R4ΔBAB1_0546 fragment generated by PCR overlap	This study
pMM78	Mutator plasmid, pJQK derivative containing the BamHI-XbaI fragment of pMM77	This study
pMM99	pCR2.1 derivative containing a fragment of 853 bp from a *wbkA* deletion intermediate	This study
E. coli strains		
TOP10F	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80*lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
OmniMAX 2-t1	F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(TetR) Δ(*ccdAB*)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80(*lacZ*)ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*	Invitrogen
S17-1λ*pir*	Mating strain with plasmid RP4 inserted into the chromosome	35

[a] Cm^r, chloramphenicol resistance; Str^r, streptomycin resistance; Km^r, kanamycin resistance.

quantification protocol described previously (5). The absolute quantification of chromosomal scars determined with the primers scar_Fq and scar_Rq was normalized to the amount of gDNA in each sample obtained by the absolute quantification of *wbkA* using the primers wbkA_Fq and wbkA_Rq (Table 2). Serial double dilutions of gDNA from *B. abortus* 2308 and R6 strains were used to generate calibration curves for the corresponding assay by plotting copy number versus $\log(C_T)$ value. PCR fragments of 389 bp (scar) and 143 bp (*wbkA* gene) were obtained from a 20-μl reaction mixture containing 10 μl of 2× SYBR green PCR master mix (Applied Biosystems), the appropriate primers at a concentration of 0.5 μM, and 0.1 ng of gDNA for *wbkA* quantification or 100 ng for scar quantification. Amplification was carried out in a 7500 Real-Time PCR system (Applied Biosystems). The cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and then 45 cycles of 15 s at 95°C and 1 min at 60°C. The amplification efficiency was obtained from the equation $E = 10^{(-1/\text{slope})}$, using slope values determined from the standard curves. The results were analyzed with Applied Biosystems 7500 Fast System SDS software v1.3. For simplicity, the ratio R_{scar} obtained from three independent experiments was expressed as a percentage (i.e., the number of scars in 100 genomes). A fragment of 853 bp from circular intermediates was quantified using 100 ng of gDNA and the same amplification conditions with the primers BAB1_0557F and BAB1_0548R (Table 2) and dilutions of plasmid pMM99 as a standard. The efficiencies of the amplifications of the scar, *wbkA*, and the circular intermediate were 1.82, 1.98, and 1.60, respectively.

Mutagenesis. To construct a Δ*recA* mutant of *B. abortus* 2308, PCR overlap was used to generate an in-frame deleted allele that was then introduced into the genome by allelic exchange (7). Briefly, the two fragments obtained with the primer pairs recA-F1/recA-R2 and recA-F3/recA-R4 (Table 2) were ligated by overlap PCR, and the resulting fragment (containing the Δ*recA* allele lacking the nucleotides corresponding to amino acids 80 to 283) was cloned into pCR2.1 TOPO (Invitrogen) to produce plasmid pMM62 (Table 3). A BamHI-XbaI fragment from this plasmid was subcloned into pJQK (31), and the resulting plasmid (pMM63) was introduced into *B. abortus* by mating using the *E. coli* S17-1λ*pir* strain (35). Single-crossover transconjugants were selected on medium containing nalidixic acid (25 μg/ml) and kanamycin (50 μg/ml). After 24 h in broth without antibiotics, double-crossover mutants were selected on nalidixic acid–5% sucrose plates and confirmed by PCR. Mutants were characterized for sensitivity to methyl methanesulfonate and H_2O_2 by using the Kirby-Bauer disk diffusion assay (34). The same mutational strategy was utilized to produce IS*Bm1* mutants by making 150-nucleotide (nt) deletions of each *orfA*, which introduced several stop codons affecting the transposase catalytic domain. The BAB1_0557_F1, BAB1_0546_R2, BAB1_0547_F3, and BAB1_0558_R4 primers were used to construct the mutator plasmid pMM73, which was then introduced into *B. abortus* 2308 to obtain the BabΔIS*Bm*-R mutant. Similarly, the primers pairs BAB1_0546_F1/BAB1_0546_R2 and BAB1_0547_F3/BAB1_0547_R4 were used to generate the mutator plasmid pMM78 for the construction of a BabΔIS*Bm*-L mutant. Next, a double BabΔIS*Bm*-LR mutant was generated by the introduction of plasmid pMM73 in BabΔIS*Bm*-R. For the complementation of Δ*wbkA* (BabΔ*wbkA*) spontaneous mutants, a copy of *wbkA* generated by PCR with the primers BAB1_0553F-gw and BAB1_0553R-gw (Table 2) was introduced into the pDONR221 donor vector using clonase BP (Gateway Technology; Invitrogen). The complementation plasmid pMM14 was obtained by clonase LR reaction between the pMR10 destination vector (19), and the donor plasmid pMM13 was cloned into the *E. coli* OmniMAX 2-t1 strain. After this, plasmid pMM14 was transferred to the *E. coli* S17-1λ*pir* strain, which was then used to introduce pMM14 into *B. abortus* Δ*wbkA* mutants. Restoration of the S phenotype was assessed by using the crystal violet dye exclusion test, the agglutination of whole bacteria, and Western blotting of sodium dodecyl sulfate-proteinase K bacterial extracts with the serum of a rabbit hyperimmunized with S *B. abortus* and purified S and R-LPS as controls (16).

IS*711*-fingerprinting. The IS*711* sequence was detected by Southern blotting with 1 to 2 μg of AvaI-ClaI double-digested *B. abortus* gDNA resolved in 1% agarose in TBE buffer at 25 mA for 10 h. The IS*711* probe was generated by PCR with the primers 711u and 711d (Table 2) (28) and biotinylated by direct labeling (GE Healthcare, Buckinghamshire, United Kingdom). Chemiluminescent detection of the hybridized DNA was done using a commercial kit (Amersham/GE Healthcare), and films were developed by conventional photographic methods.

RESULTS

Excision of *wbkA* occurs spontaneously without affecting other *wbk* genes. Previous works have shown that all *wbk* O-PS genes

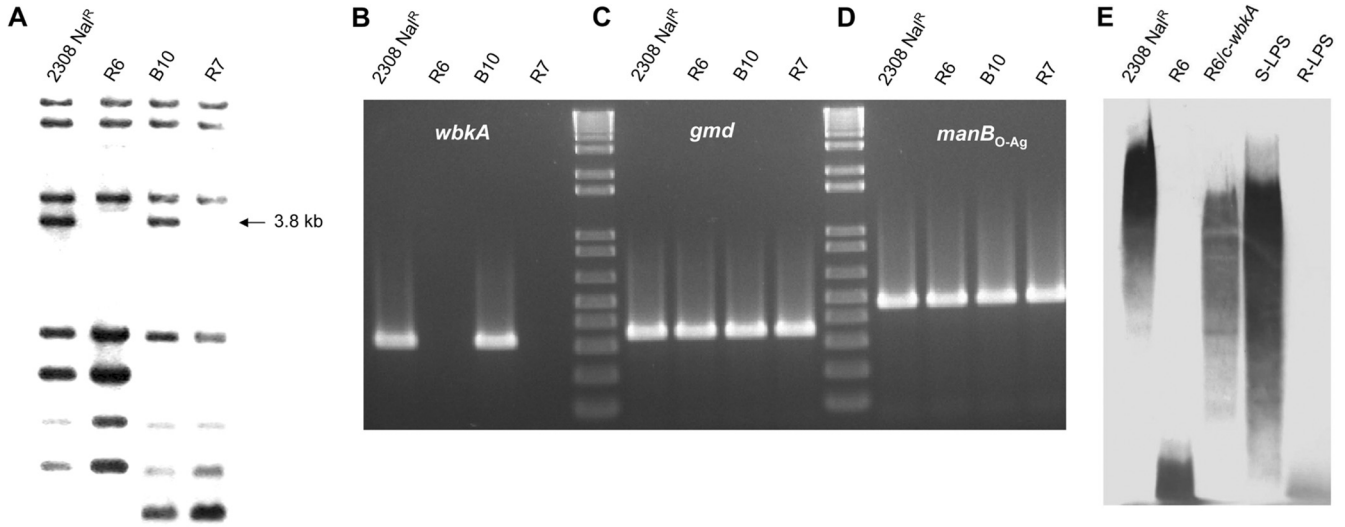

FIG 2 The spontaneous deletion of *wbkA* is linked to the emergence of R mutants. (A) IS*711* fingerprinting characterization of R mutants (the arrow indicates the 3.8-kb fragment absent in R mutants). (B) PCR detection of *wbkA* gene (BAB1_0553). (C) PCR detection of *gmd* gene (BAB1_0545). (D) PCR detection of $manB_{O\text{-}Ag}$ gene (BAB1_0560). (E) Western blot analysis with LPS antibodies. Strains 2308 Nal^r and B10 are the *B. abortus* parental S strains of R mutants R6 and R7, respectively, and strain R6/c-*wbkA* is plasmid *wbkA*-pMM14 complemented R6 mutant (Table 1). S-LPS and R-LPS are the purified products used as controls.

except *wbkA* are concatenated in sections of 2 to 6 ORFs and that *wbkA* is flanked by a high number of transposase ORFs (Fig. 1A) (6, 15, 16). Although the presence of transposases and inactivated remnants results in various sets of repeated sequences that make uncertain an accurate prediction of boundaries, the size of the fragment bearing *wbkA* could be estimated in 6.5 kb. This fragment (referred to here as the *wbkA* region) contains one IS*711* plus two nearly identical IS*Bm1* copies at each end with a 60-nt imperfect repeat sequence upstream of each *orfA* (Fig. 1B). A closer analysis of the IS*Bm1* copies revealed the existence of inverted repeats susceptible to transpositional recombination (Fig. 1C). These features are consistent with the hypothesis that *wbkA* could be excised through events involving the flanking sequences but no adjacent *wbk* genes. To address this possibility, 12 spontaneous R mutants obtained *in vitro* from different *B. abortus* cultures were screened for *wbkA* deletions by IS*711* fingerprinting, taking advantage of the existence (predicted by *in silico* restriction analysis) of a 3.8-kb AvaI-ClaI fragment carrying IS*711* (Fig. 1B). Two mutants (named R6 and R7) lacked the 3.8-kb fragment (Fig. 2A). Additional analyses by PCR with primers targeting *wbkA*, *gmd*, and $manB_{O\text{-}Ag}$ (Table 2) showed that mutants R6 and R7 carried a deletion encompassing *wbkA* but not the two adjacent LPS genes (Fig. 2B, C, and D), suggesting that the deletion affected only *wbkA*. This was confirmed by complementation of R6, R7, and four additional *B. abortus* 2308 spontaneous R Δ*wbkA* mutants with the plasmid *wbkA*-pMM14, which restored the S phenotype as judged by the crystal violet exclusion test, agglutination, and Western blotting with antibodies to S *B. abortus* (Fig. 2E). The rate of spontaneous Δ*wbkA* mutation was calculated by probing R colonies (i.e., crystal violet positive) obtained in dissociation experiments. Under the conditions used (prolonged incubation in static, oxygen-limiting cultures), BaΔ*wbkA* mutants represented approximately 8 to 10% of the R mutants, a value lower than that reported for GI-2 deletion (23).

The excision of *wbkA* is generated by a recombination event involving the flanking IS*Bm1* copies. Two possible *wbkA* excision mechanisms are deletion by homologous recombination and IS-mediated transposition. The former involves RecA activity, and the latter involves transposition or cooperatively acting IS-encoded proteins (13, 17, 18, 22). Concerning the first possibility, RecA can recognize nearly identical IS pairs as substrates of excisive recombination if they are oriented as direct repeats (13, 22). Accordingly, an intrachromosomal exchange between IS*Bm1* copies should result in the products illustrated in Fig. 3A and B. PCR assays with the primers pairs BAB1_0545Fb/BAB1_0558R and BAB1_0557F/BAB1_0548R showed that *B. abortus* 2308 (Fig. 3C and D, lane 1), as well as *B. melitensis* 16M and *B. suis* 1330 (data not shown), yielded both predicted products. Moreover, the circular intermediate that should result from active recombination was also demonstrated by qPCR, a method that also revealed a progressive increase in this product as the bacteria entered the stationary phase of growth (Fig. 3E). A spontaneous *B. melitensis* R mutant isolated in a pure culture from goat milk also showed the chromosomal scar corresponding to the *wbkA* deletion (not shown). Consistent with the homologous recombination mechanism, sequencing revealed a crossover between IS*Bm1* ORFs. In order to study the involvement of RecA, a *recA*-deficient mutant (BabΔ*recA*) was constructed by allelic exchange. The BabΔ*recA* mutant failed to grow in the presence of methyl methanesulfonate, a potent DNA damage inductor (38), and was refractory to the integration of plasmids successfully used in several allelic exchange experiments in our laboratory (not shown), confirming that it was defective in RecA-dependent homologous recombination. Although with less intensity, BabΔ*recA* yielded the same products as the wild-type strain (Fig. 3C and D, lane 2), strongly suggesting some degree of RecA-independent homologous recombination.

To test the existence of IS-mediated transposition, IS*Bm1* mutants were constructed by removing 150 nt from identical

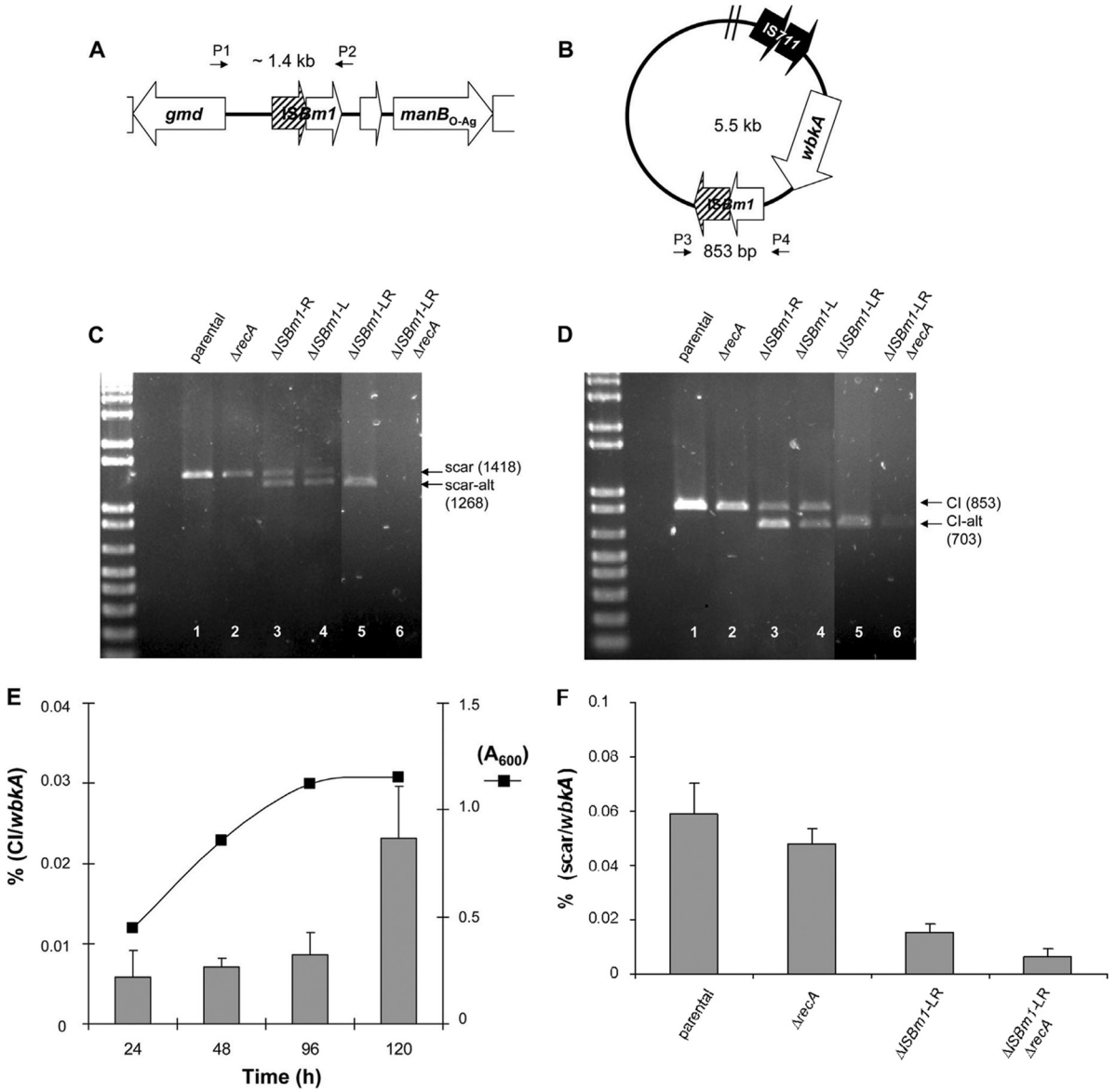

FIG 3 IS-mediated recombination is responsible for the loss of *wbkA*. (A) Genetic organization of the chromosomal scar left behind the excision. (B) Genetic organization of the closed-circular intermediate or CI carrying the copy of IS*711* (in both panels A and B, hatched arrows indicate the crossover between the IS*Bm1* ORFs, and the primer [see the legend for Fig. 1] positions are depicted by small black arrows). (C and D) PCR detection of recombination intermediates in *B. abortus recA* and IS*Bm1*-related mutants (note that scar-alt and CI-alt correspond to alternative recombination products). (E) PCR quantification of circular intermediates during growth (results are the average ± the standard error of triplicate measurements). (F) PCR quantification of chromosomal scars (results are the average ± the standard error of duplicate measurement from three independent experiments).

sites of one (mutants BabΔIS*Bm*-L and BabΔIS*Bm*-R) or both (BabΔIS*Bm*-LR) flanking transposases (Table 1). PCR analyses demonstrated products of the same size as those produced by the *wbkA* in the parental strain. In addition, these analyses showed the alternative 150-bp-lacking recombination species (Fig. 3C and D, lanes 3, 4, and 5) that result from the resolution of the Holliday junction produced by the strand exchange between asymmetric repeats. Since a chromosomal joint with no traces of IS*Bm1* sequences is expected from a transpositional deletion (10), these results were consistent with homologous recombination but not with transposition. To test whether a RecA-independent recombination takes place in the IS*Bm1* mutants, the mutation used to construct BabΔ*recA* was introduced in BabΔIS*Bm*-LR by allelic exchange to generate BabΔIS*Bm*-LRΔ*recA*. The presence of the scar was then investigated by conventional PCR. The results showed that the scar was still detectable, thus demonstrating that the recombination was not only RecA dependent. Moreover, although conventional PCR did not show the products resulting from the scar (1,268 bp; Fig. 3C, lane 6), traces of the expected circular intermediate (703 bp; Fig. 3D, lane 6) were detected in the triple mutant. To confirm the existence of a residual RecA-independent recombination, quantitative measurements of chromosomal scars were performed by qPCR. This method revealed

that, although with a nearly 10-fold reduction with respect to the parental strain, the triple mutant displayed the *wbkA* excision scar (Fig. 3F). These same experiments also showed that the deletions created in the IS repeats impaired the excision rate, as expected from homologous recombination. In summary, *wbkA* is undergoing a spontaneous excision by both RecA-dependent and RecA-independent pathways.

DISCUSSION

S-R dissociation has a deep impact on the antigenic structure and virulence of the major zoonotic brucellae and on the stability of S vaccines (2, 37). Early work showed that unfavorable growth conditions, oxygen limitation in particular, facilitate the establishment of R variants (20) and that the effect of low oxygen tension can be abrogated by supplying alternative proton/electron acceptors such as nitrate, resazurine, or methylene blue (14). Two complementary hypotheses that could explain these observations are (i) that mutants not synthesizing the costly O-PS are more competitive when energy shortage and no selective pressure to maintain the O-PS concur and (ii) that S brucellae have genetic features introducing instability in O-PS genes. Concerning the second hypothesis, we have shown previously the role of the phage-related integrase in GI-2 excision and S-R dissociation (23), and we present here evidence for an additional mechanism involving the deletion of *wbkA* by homologous recombination. Clearly, these two excision pathways belong to a set of mechanisms affecting LPS biosynthesis genes in Gram-negative bacteria, including site-specific recombination (23), complex genome rearrangements not dependent on homology at boundaries (39), insertion-deletions, and point mutations (1, 39). Moreover, in the case of the S-R dissociation of brucellae, the instability may be connected to the origin of the O-PS genes. GI-2 bears traits that indicate horizontal acquisition, and this may be also true for *wbk* and, within this region, for *wbkA*. The %GC of the 6.5-kb fragment is 52.5, higher than the 50% of the entire *wbk* region, and both are different from the average %GC of the *Brucella* genome (56 to 58%). These percentages and the peculiarities of *wbkA* and its flanking sections are compatible with the acquisition of *wbkA* by a preexisting region comprising the remaining *wbk* genes. Horizontal acquisition of the complete *wbk* has been proposed before (6, 15), an event that may have happened before the brucellae became intracellular parasites. Noteworthy, the *wbkA* region is absent from the O-PS encoding region of *Yersinia enterocolitica* O:9, a bacterium that also makes an *N*-formylperosamine homopolymers, albeit with some structural differences with that of S brucellae (30, 36). The analysis of the structure of the IS*Bm1* copies flanking *wbkA* showed that, in spite of the absence of any evident duplicated insertion site, they carry canonical terminal inverted repeat of 8 bp which might be involved in the transpositional release of the 6.5-kb fragment. In addition, the organization of this region resembles that of composite transposons (22), and there is experimental evidence for the expression of IS*Bm1*-related ORFs at the beginning of the host-pathogen interaction (33). Thus, an excision mechanism mediated by transposition seemed possible, which would be in accordance with a foreign origin of this locus in *wbk*. Nevertheless, the predicted transpositional joint was not detected, suggesting that the ability to transpose has been lost. In fact, the single nucleotide polymorphisms (SNPs) present in inverted repeats in one of the IS*Bm1* copies (Fig. 1C) could prevent transposition, so that only excision by homologous recombination remains.

Homologous recombination is one of the mechanisms responsible for large rearrangements in bacterial genomes. These changes are part of the plasticity showed by chromosomes and are often involved in intra- or interspecies polymorphism (9, 22, 29). There are examples in the literature in which ISs appear as rearrangement-promoting elements, playing a role in the deletion of fragments encoding virulence factors in pathogens such as *Y. pestis* and uropathogenic *E. coli* (12, 26). In bacteria belonging to the *Mycobacterium tuberculosis* complex, comparative analyses suggest that IS*6110*-mediated deletions have shaped extensively their genomes (4, 11). In a way similar to the *wbkA* region, the homologous recombination between IS*1610* copies participates in the formation of R variants in *M. avium* (10). In all of these cases, RecA occupies a central role prompting these genome deletions. Our results also show that, after inactivation of *recA*, homologous recombination still operates at an extent that is low but enough to produce *wbkA* deletions in *Brucella*. This observation strongly suggests that recombination-promoting genes other than *recA* participate in the excision of the *wbkA* region. The mechanism of homologous recombination and the genes involved have not been studied in detail in brucellae. In the closely related *Rhizobiaceae*, the products of the genes *ruvB* and *radA* migrate to the Holliday intermediary and, although modestly, seem to contribute to homologous recombination (25, 29). In *Brucella*, a previous work has shown the presence of a putative *recA* homolog (*radA*, BAB1_0474), which does not perform a protective DNA repair function and is devoid of physiological importance (34). Preliminary results indicate that the residual homologous recombination observed in the *wbkA* excision is not *radA* dependent.

In regard to the frequency of dissociation, the data from this and previous works (23, 39) show that deletion of *wbkA* and of GI-2, plus mutations in the mannose genes involved in LPS core oligosaccharide synthesis, is responsible for the majority of R mutants obtained in the laboratory. In this context, it is worth commenting the stochasticity of mutations involved in S-R dissociation. Mutations affecting mannose synthesis genes outside *wbk* correspond to point mutations, indels, and extensive deletions and, indeed, it seems possible that stochastic mutations affect other LPS genes. In contrast, *wbkA* and GI-2 deletions are generated through well-defined paths and, therefore, the corresponding mutants are not randomly generated. Accordingly, the identification of S-R dissociation mechanisms such as those dependent on GI-2 and *wbkA* entail the possibility of controlling these excisions to obtain more stable strains for antigen and vaccine production. Moreover, it has been recently hypothesized that dissociation may be part of the natural course of brucellosis infection (39). In this context, the isolation of a Δ*wbkA B. melitensis* R mutant strain from goat milk that lacked detectable S colonies is interesting. Isolation of R mutants in pure culture from goat milk but not from other infected samples has been occasionally observed in the laboratory of one of the authors, suggesting that these animals carry R mutants in the mammary glands. The biological significance of the loss of O-PS expression *in vivo* is currently being investigated.

ACKNOWLEDGMENTS

We thank A. Delgado for skilful technical assistance.

This study was funded by the Ministerio de Ciencia y Tecnología of

Spain (AGL2008-04514) and the Universidad Austral de Chile (DID S-2009-33).

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