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Research article

Genetic comparison of *Brucella* spp. and *Ochrobactrum* spp. erroneously included into the genus *Brucella* confirms separate genera

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Abstract

The facultative intracellular pathogen *Brucella* and the free-living bacteria *Ochrobactrum* are both α-proteobacteria and very close to each other. A group of researchers recently clustered *Ochrobactrum* strains into the genus *Brucella* according to a BLAST distance approach. Thus, we performed a deeper comparative genetic analysis for eleven *Ochrobactrum* strains and twelve different *Brucella* isolates to demonstrate important differences between these bacteria. In addition to the clear differences between *Brucella* and *Ochrobactrum*, like the differences in genes contents, and different genome sizes, the *Brucella*-specific gene *bsc*p31 was not found in *Ochrobactrum*, as well as other important *Brucella*-specific proteins and virulence factors. Differences in antimicrobial resistance genes content and the presence or absence of plasmids were obvious between *Brucella* and *Ochrobactrum* spp. Genome alignment of *Brucella* spp. and *Ochrobactrum* spp. revealed a genome similarity of 85.7% maximum, whereas all analyzed *Brucella* spp. in this study had a similarity of 97.6-99.9%, and all compared *Ochrobactrum* spp. 82.6-98.0%. Because of these facts mentioned in this work, *Brucella* and *Ochrobactrum* should be considered separate genera.

Keywords: *Brucella*, *Ochrobactrum*, Genome analysis, WGS

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Introduction

Brucella spp. are zoonotic bacteria causing brucellosis. These microorganisms are risk class three pathogens, which can cause serious illness in humans with high undulant fever, liver and spleen inflammation, joints and bursa infestation as well as spinal and testicular infections, or placental retention (Young, 1995). Infected animals usually suffer from abortions. *Ochrobactrum* is a risk class one pathogen, an emerging pathogen in immunodeficient and immunocompetent patients with possible clinical symptoms like fever, headache, and disorder of consciousness (Zhu et al., 2018). *Brucella* and *Ochrobactrum* spp. belong to the class 2 *alphaproteobacterial* (Dorsch et al., 1989; Moreno et al., 1990; Velasco et al., 1998). Recently, *Ochrobactrum* spp. have been falsely included in the genus of *Brucella* and, therefore, renamed *Brucella* (Hördt et al., 2020). However, there are many arguments against classifying *Ochrobactrum* as *Brucella*. The doubt about including *Ochrobactrum* and *Brucella* in the same genus was based on several differences such as the genome size, existence of plasmids, cell envelope permeability, metabolic redundancy, and therapy regimes and responses for treatment in cases of illness. The genome size of both pathogens differs noticeably (*Brucella*: 3.1-3.4 Mb vs. *Ochrobactrum*: 4.7-8.3 Mb). *Ochrobactrum* possesses up to six plasmids, whereas in *Brucella*, no plasmid could be found (Teyssier et al., 2005).

Regarding the cell envelope permeability, *Brucella* is permeable to hydrophobic probes and resistant to destabilization by polycationic peptides, while *Ochrobactrum* is impermeable to hydrophobic probes but sensitive to polycationic peptides (Velasco et al., 2000; Barquero-Calvo et al., 2009). The next point is that *Brucella* has a low metabolic redundancy in contrast to *Ochrobactrum*, which shows a high metabolic redundancy (Diaz et al., 2018; Gohil et al., 2020). In infections cases, *Ochrobactrum* have to be threatened based on a short monotherapy, while infections with *Brucella* are complicated and need a long bi-therapy (Corbel, 2006; Ryan and Pembroke, 2020; Yagel et al., 2020). Another aspect is that *Ochrobactrum anthropic* or *Ochrobactrum intermedium*, representing the closest *Brucella* relatives, shows 900-3000 gene differences to

Brucella spp. (Moreno et al., 2022).

Furthermore, there are about 170 *Brucella* proteins whose genes could not be found in *Ochrobactrum* genomes (Wattam et al., 2014; Gohil et al., 2020). Based on the previous information, the current comparative genomic analysis between eight different *Ochrobactrum* spp. erroneously referred to as *Brucella* spp. and 11 known *Brucella* spp. was carried out to emphasize that both bacteria are not belonging to the same genus.

Materials and methods

Downloaded sequences from GenBank and species identification

The sequences (assemblies) of eleven different *Ochrobactrum* (*O.*) spp, namely *O. haematophila*, *O.cytisi*, *O. lupini*, *O. pseudogrignonense* strain SHIN, *O. anthropi*, *O. cicero*, *O. daejeonense*, *O. endophytica*, *O. intermedia*, *O. pituitosa*, and *O. rhizospaerae* were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). These mentioned strains were falsely renamed into *Brucella* (*B.*) *pseudogrignonensis*, *B. anthropic*, *B. cicero*, *B. daejeonesis*, *B. endophytica*, *B. intermedia*, *B. pituitosa*, *B. rhizospaerae*. Additionally, 12 *Brucella* spp, namely *B. abortus* 2308, *B. melitensis* 16M, *B. suis* 1330, *B. ovis*, *B. microti*, *B. canis*, *B. pinnipedialis*, *B. ceti*, *B. inopinata*, *B. vulpis*, *B. neotomae*, and *B. papionis* were used in the comparison. The reference sequences or its BioProject or BioSample are listed in Table 1. The B4 and B5 markers were used to determine the *Brucella*-specific gene *bsc*p31 (Baily et al., 1992). The PCR was carried out *in-silico* based on contigs using the program Geneious v.11.1.5. The sequences of the primers are B4 (5'-TGG CTC GGT TGC CAA TAT CAA-3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG-3').

Whole-genome sequencing and bioinformatic procedure

The sequences of the *Ochrobactrum* strains were compared to *Brucella* strains. For analyzing the downloaded 23 genome assemblies in a standardized and automated manner, the Linux-based bioinformatic WGSBAC (v.2.1) pipeline (https://gitlab.com/FLI_Bioinfo/WGSBAC/-/tree/version2, accessed on 02 September 2022) was used for running certain software. The pipeline input consisted of a metadata file and genome assemblies fastq files.

Comparison with entries in public databases

The tool pyANI v. 0.2.10 (https://github.com/widdowquinn/pyani#conda), accessed on 19 June 2021) is a module for whole-genome classification of microbes using average nucleotide identity. This module was used to compute a pairwise ANI and other metrics between *Brucella* assemblies and *Ochrobactrum* contigs.

Antimicrobial resistance and plasmid determination

In-silico detection of AMR genes and virulence-associated determinants was performed using different databases, i.e., the Resistance Gene Identifier (RGI) based on the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), the ResFinder database (Zankari et al., 2012), and the NCBI AMR Finder Plus (https://github.com/ncbi/amr/wiki/Running-AMRFinderPlus, accessed on 19 June 2022) (Feldgarden et al., 2019) for the identification of resistance genes and chromosomal mutations mediating antimicrobial resistance. Identifying the potential virulence-associated determinants was retrieved from the virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/) using the core dataset (Liu et al., 2019). Plasmid identification was performed with the PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/ (accessed on 19 June 2022), and Platon (https://github.com/oschwengers/platon, accessed on 10 January 2023).

In-silico MLST and MLVA-16 analysis

Multilocus Sequence Typing (MLST) (Maiden et al., 1998) was carried out *in-silico* (*https://github.com/tseemann/mlst*, accessed on 02 November 2022). A scheme using the nine distinct genes *gap*, *aro*A, *glk*, *dna*K, *gyr*B, *trp*E, *cob*Q, *int_hyp* and *omp25* was used, of which seven represent housekeeping genes, whereas the two other genes, omp25 and *int_hyp*, represent an outer membrane protein and a hypothetical protein (Whatmore et al., 2007). Multiple-Locus Variable-Number Tandem Repeat Analysis using 16 markers (MLVA-16) genotyping system was carried out *in-silico* using MISTReSS (https://github.com/Papos92/MISTReSS, accessed on 22 February 2022) with primers adapted for *Brucella* (Sacchini et al., 2019). To avoid multiple primer binding sites, the forward primer sequence of Bruce21 was extended to (5'-GGCAGTGGGGCAGTGAAGAATATGGTCGCTG-CGCTCATGCGCAACCAAAACA-3'). The number of repeats at each locus was determined by the fragment size according to the published *Brucella* allele assignment table (Al Dahouk et al., 2007).

Results and discussion

Species identification with specific Brucella primer and comparative genomics

Comparing *Ochrobactrum* to *Brucella* showed a clear difference. The binding of the *Brucella*-specific primers B4 and B5, identifying the *Brucella*-specific *bsc*p31 gene, was not detectable in the *Ochrobactrum* spp. The MLST and MLVA-16 analysis based on the whole genome revealed that no MLST or MLVA results exist for all *Ochrobactrum* strains. Measurement of the genome lengths of both genera showed that the genome size of the *Ochrobactrum* strains varies between 4,393,164 bp and 5,937428 bp and has a GC content of 53.0-60.7%, whereas *Brucella* spp. have a genome size of up to 3.4 Mb (Jumas-Bilak et al., 1998; Moreno et al., 2022) and a GC content of 57.2-57.3% (Table 1).

Table 1: Genome length of the examined *Brucella* and *Ochrobactrum* spp. and their GC content.

Genus and species	Total length [bp]	GC content [%]	RefSeq
B. melitensis	3294931	57.22	NC_003317.1, NC_003318.1
B. abortus	3278307	57.22	NC_007618.1, NC_007624.1
B. suis	3315175	57.25	NC_004310.3, NC_004311.2
B. neotomae	3329628	57.23	BioSample SAMEA104210778
B. inopinata	3442381	57.15	NZ_LT605585.1, NZ_LT605586.1
B. vulpis	3238137	57.13	LN997863.1, LN997864.1
B. ovis	3275590	57.19	NC_009505.1, NC_009504.1
B. canis	3312769	57.24	NC_010103.1, NC_010104.1
B. ceti	3278034	57.23	NC_022905.1, NC_022906.1
B. microti	3329628	57.23	NC_013119.1, NC_013118.1
B. pinnipedialis	3331029	57.24	NZ_CP007743.1, NZ_CP007742.1
B. papionis	3255082	57.26	BioProject PRJNA251693
O. Rhizosphaerae	4903046	53.01	SAMN07258022
O. endophytica	4932019	60.73	PRJDB10509
O. pituitosa	4885407	53.44	SAMN08100214
O. intermedia	4727886	57.74	SAMEA3146534
O. anthropi	4858647	56.11	SAMN16619790
O. ciceri	4741587	57.63	SAMN25207262
O. daejeonensis	4642379	58.49	SAMN1202516
O. pseudogrignonensis	5622438	54.11	SAMN08166471
O. lupini	5582483	56.35	SAMN07259926
O. cytisi	5937428	55.46	SAMN05941866
O. haematophilum	5503262	56.67	SAMN11855631

Furthermore, using pyANI alignment to all mentioned *Brucella* isolates compared to the *Ochrobactrum* spp. showed that there was a genome similarity of 83.2%-85.7%, whereas all compared *Brucella* spp. had a similarity of 97.6-99.9% to each other (Table 2 and Supplementary Table S1). Recently, the genus *Ochrobactrum* was included in the genus *Brucella* (Hördt et al., 2020). The reason for renaming *Ochrobactrum* as *Brucella* was a phylogenic BLAST distance approach and a supposed equivalence with some genera of pathogenic bacteria. In this study, eleven known *Ochrobactrum* spp. were taken for deeper genome analysis and compared with the genomes of twelve known taxonomy-accepted *Brucella* spp.

The absence of the *Brucella*-specific *bsc*p31 genes, as well as the absent MLST and MLVA profiles from the genome of all tested *Ochrobactrum*, in addition to the differences in the genome length, point out that both bacteria are completely different and including them in one genus is doubtful. Despite *Brucella* being phylogenetically close to *Ochrobactrum*, the alignment that has been carried out to include *Brucella* and *Ochrobactrum* together in one genus is not enough for the classification of the genus (Moreno et al., 2022).

Antimicrobial resistance and plasmid contents

In this section, clear differences could be demonstrated. The *in-silico* detection of AMR genes in *Ochrobactrum* and *Brucella* strains successfully identified only the *Brucella suis mpr*F gene and *bep*C, *bep*D, *bep*E, *bep*F, and *bep*G genes in all tested brucellae except for *B. vulpis*, where *bep*G and *bep*F are missing for whatever reason. However, none of those genes were found in the genomes of all tested *Ochrobactrum* spp. (except *bep*E for *O. anthropi*, *O. cicero*, *O. intermedia* and *O. lupini*). In contrast, resistance genes mediating resistance to β-lactamase (*bla*OXA and *bla*OXA-919), carbapenems (IMP-8), phenicol (*flo*R), tetracycline (*tet*G), and aminoglycoside such as gentamicin (*aac*(6')-Ib) were found only in the genomes of *Ochrobactrum* and no classical AMR genes were found in all tested brucellae (Table 3).

The two identified AMR genes, the *Brucella suis mpr*F, and *bep*C-G, were also found in genomes of almost all *B. abortus* and *B. melitensis* previously investigated (Wareth et al., 2021). The multiple peptide resistance factor *mpr*F (*Brucella suis mpr*F gene) is an integral membrane protein encoding a peptide, which modifies anionic phosphatidylglycerol for repulsion of cationic antimicrobial peptides (CAMPs), leading to resistance to CAMPs (Ernst et al., 2009) as well as resistance to methicillin, oxacillin, bacitracin, gentamycin, β-lactams, and other cationic peptides (Andrä et al., 2011) which were found in *Brucellaceae*.

The *mpr*F also promises resistance to moenomycin, vancomycin, human defensins (HNP1-3), and oxygen-

Table 2: pyANI results classification of microbes using average nucleotide identity (%) showing the homogeneity between *Ochrobactrum pseudogrignonensis* strain SHIN as an example and the different *Brucella* spp. The entire detailed table, including all *Ochrobactrum* spp, is shown in Table S1.

Genus and species	Percent identity												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. *O. pseudogrignonensis* SHIN	100.00	83.88	83.81	83.81	83.81	83.78	84.05	83.83	83.81	83.82	83.82	83.82	83.80
2. *B. vulpis*		100.00	97.70	97.77	97.78	97.78	97.58	97.76	97.78	97.83	97.65	97.79	97.73
3. *B. melitensis* 16M			100.00	99.64	99.65	99.64	97.71	99.64	99.65	99.72	99.53	99.65	99.70
4. *B. canis*				100.00	99.73	99.70	97.78	99.70	99.73	99.78	99.59	99.90	99.66
5. *B. microti*					100.00	99.73	97.78	99.72	100.00	99.80	99.62	99.75	99.68
6. *B. papionis*						100.00	97.78	99.71	99.72	99.78	99.60	99.73	99.67
7. *B. inopinata*							100.00	97.76	97.79	97.84	97.66	97.80	97.73
8. *B. ceti*								100.00	99.71	99.83	99.59	99.71	99.67
9. *B. neotomae*									100.00	99.80	99.62	99.75	99.68
10. *B. pinipedialis*										100.00	99.68	99.81	99.75
11. *B. ovis*											100.00	99.62	99.56
12. *B. suis*												100.00	99.69
13. *B. abortus*													100.00

Table 3: Results of antimicrobial resistance genes analysis in *Brucella* and *Ochrobactrum*.

AMR genes found	**Antimicrobial group and mechanism of action**	***Brucella* spp.**	***Ochrobactrum* spp.**
*bla*OXA-919	β-Lactam	Absent	Present
*flo*R_2	Phenicol (Chloramphenicol/florfenicol)	Absent	Present
tet(G)	Tetracycline	Absent	Present
aac(6')-Ib	Aminoglycoside (Gentamicin)	Absent	Present
*bla*IMP-8	β-Lactam (Carbapenem)	Absent	Present
*B.suis_mpr*F cationic antimicrobial peptides	Integral membrane protein modifying the negatively-charged phosphatidylglycerol on the membrane	Present	Absent
Bep-C, D, E, F, G	Efflux pump	Present	Absent (except *bep*E was found in *O. anthropi*, *O. ciceri*, *O. lupini O. intermedia*)

independent neutrophil killing (Andrä et al., 2011). It is worth mentioning that the *mpr*F plays a crucial role in the virulence and pathogenesis of *Staphylococcus aureus* (*S. aureus*) and is involved in resistance to daptomycin (Ernst and Peschel, 2019), which is used for the treatment of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). However, it is ineffective in Gram-negative bacteria, and the presence of this resistance is of no clinical relevance for treating brucellosis. The *mpr*F1 and *mpr*F2 also play a role in the pathogenicity of *Enterococcus faecalis* (Bao et al., 2012). The outer membrane efflux protein *bep*C-G of *B. suis* (strain 1330) is involved in the efflux of toxic and relatively hydrophobic compounds.

The presence of the *bep*C confers resistance to drugs such as chloramphenicol and acriflavine. An insertional mutation in the *bep*C gene in *B. suis* decreased the resistance to antibiotics such as tetracycline, erythromycin, ampicillin, and norfloxacin; consequently, *bep*C-dependent efflux processes of toxic compounds contribute to *B. suis* survival inside the host (Posadas et al., 2007). Furthermore, the presence of *bep*D-E increased resistance to tetracycline, doxycycline, ampicillin, norfloxacin, and ciprofloxacin in *B. suis* (Martin et al., 2009). No resistances genes were present for the efflux pump system *bep*C, *bep*D, *bep*F, and *bep*G, in the genomes of *Ochrobactrum*, which are indeed present in *Brucella*, contributing to resistance to some drugs like quinolones (Ravanel et al., 2009). However, *O. anthropi*, *O. ciceri*, *O. lupini*, and *O. intermedia* have bepE, a part of the efflux system. But because it occurs singly, it is not able to lead to the resistance of quinolones.

Analyzing the sequence of *Ochrobactrum* spp. revealed the presence of one plasmid. This may explain the presence of some classical AMR genes in the genome of *Ochrobactrum*. In comparison, analyzing the sequences of *Brucella* spp. could not detect any plasmids, as it is known that *Brucellaceae* have no plasmids.

Important proteins and virulence factors contents

Forty-three virulence-related genes corresponding to five virulence factors were identified in the genomes of

all tested brucellae, i.e., lipopolysaccharide (LPS) associated genes, type IV secretion system (*vir*B1-B12), TIR domain-containing effectors BtpA and BtpB, the Rab2 interacting conserved protein A (*ric*A), and *cgs* gene which is belonging to cyclic β-1,2 glucans (Supplementary Table S2). Among them, the LPS corresponding genes (*lpx*A-*lps*E, *lps*A, *lps*B, *lpc*C, *wbd*A, *wbk*A-C, *wbo*A, *wbp*L, *wbp*Z, *wzm* and *wzt*) could not be detected in the genomes of all tested *Ochrobactrum* spp. except *O. intermedia* and *O. daejeonensis*.

In the genome of *O. intermedia*, only the *wbp*Z gene was found, and the genome of *O. daejeonensis* only has the *lpx*A gene. The same applies to the genes of *Btp*A and *Btp*B, the Vir System (*vir*B1-*vir*B12), which were only found in the *Brucella* isolates. *Brucella wbo*A, coding for a glycosyltransferase is playing a role in the establishment of the O-antigen in the lipopolysaccharide (LPS) biosynthesis. In general, the LPS of *Brucella* is different from other *Enterobacteriaceae*, like *Escherichia coli* (Christopher et al., 2010), and acts as a virulence factor (Cardoso et al., 2006). The genes *lps*A, *lps*B/*lpc*C, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*E, *gmd*, *per*, *wbk*A, *wbk*B, *wbk*C, *wbp*L, *wbd*A, *wzm* and *wzt* regulating the LPS synthesis and its functions could only be found in the *Brucella* isolates. In *Brucellaceae*, the important *vir*B type IV secretion system (T4SS) genes coding proteins for cell entry, intracellular trafficking, and survival genes (Christopher et al., 2010; Ke et al., 2015). The major outer membrane protein omp25, as well as *Btp*A, and *Btp*B proteins, act as virulence factors and interfere with toll-like receptors by interrupting the signaling pathway (Felix et al., 2014).

The major mechanisms known to contribute to virulence in the intracellular pathogens of the genus *Brucella* are intracellular survival via LPS, genes *csg* (encoding for a glycoprotein), and the protein *ric*A, which interacts with the human Rab2 (de Barsy et al., 2011). Rab2 is a small GTPase required for protein transport from the endoplasmatic reticulum to the Golgi apparatus. Furthermore, immune avoidance in brucellae occurs via *Btp*A/*Btp*B/*Btp*1/*Tcp*B, regulating the expression of the two-component BvrR/BvrS regulatory system and the T4SS secretion system (Głowacka et al., 2018). The cyclic β-1,2 glucan is a key virulence factor for the pathogenesis of brucellae and is described as a potent immune stimulator facilitating intracellular survival of *Brucella* (Roset et al., 2014; Degos et al., 2015). Rab2a is also required for a *Brucella*-containing vacuole (BCV) biogenesis and intracellular replication of brucellae (Smith et al., 2020). The absence of those genes and other important *Brucella* virulence proteins in the genome of *Ochrobactrum*, according to the results of the current study, supports that both genera must be maintained separately, and including *Ochrobactrum* in the genus *Brucella* is not correct.

Conclusion

According to the results mentioned above, like the complete difference in antimicrobial resistant gene content between brucellae and *Ochrobactrum*, the absence of important *Brucella* virulent factors and the *bsc*p31 gene in *Ochrobactrum*, support the idea of not clustering *Ochrobactrum* to the genus *Brucella*. This statement is supported by the alignment between both bacteria (*Brucella* and *Ochrobactrum* strains: up to 85.7%, whereas *Brucella* spp. among themselves: at least 97.6%). Therefore, we recommend separating both genera again and keeping them separated.

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