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***Brucella papii* sp. nov. isolated from baboons (*Papio* spp.).**

Adrian M. Whatmore[1*], Nicholas Davison[2#], Axel Cloeckaert[3,4], Sascha Al Dahouk[5], Michel S. Zygmunt[3,4], Simon D. Brew[1], Lorraine L. Perrett[1], Mark S. Koylass[1], Gilles Vergnaud[6,7,8], Christine Quance[9], Holger C. Scholz[10], Edward J. Dick Jr[11], Gene Hubbard[12], Natalia E. Schlabritz-Loutsevitch[13##]

[1] OIE/WHO/FAO Brucellosis Reference Laboratory, Department of Bacteriology, Animal Health and Veterinary Laboratories Agency (AHVLA), Woodham Lane, Addlestone, United Kingdom, KT15 3NB.

[2] Animal Health and Veterinary Laboratories Agency (AHVLA), Polwhele, Truro, United Kingdom, TR4 9AD.

[3] INRA, UMR1282 Infectiologie et Santé Publique, F-37380 Nouzilly, France.

[4] Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, F-37000 Tours, France

[5] Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, D-12277, Berlin, Germany.

[6] Univ Paris-Sud, Institut de Génétique et Microbiologie, UMR 8621, F-91405 Orsay, France.

[7] CNRS, F-91405 Orsay, France.

[8] DGA/MRIS- Mission pour la Recherche et l'Innovation Scientifique, F-92221 Bagneux, France.

[9] Mycobacteria and Brucella Section, National Veterinary Services Laboratories, USDA-APHIS, Ames, 1920 Dayton Ave., Ames, Iowa 50010, USA.

[10] Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, D-80937 Munich, Germany.

[11] Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas, USA.

[12] Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.

[13]Department of Obstetrics and Gynaecology, University of Texas Health Science Center at San Antonio (UTHSC), San Antonio, Texas, USA.

Present address: Scottish Marine Animal Stranding Scheme, SAC Veterinary Services, Drummondhill, Inverness, United Kingdom, IV2 4JZ.

Present address: Department of Obstetrics and Gynaecology, University of Tennessee Health Science Center (UTHSC), 853 Jefferson Ave, Memphis, TN 38103, USA

* Corresponding author: Department of Bacteriology, Animal Health and Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom, KT15 3NB; Phone +44 (0)1932 341111; Email: Adrian.Whatmore@ahvla.gsi.gov.uk

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Abstract

Two Gram-negative, non-motile, non-spore-forming coccoid bacteria (strains F8/08-60^T and F8/08-61) isolated from clinical specimens obtained from baboons (*Papio* spp.) during that had delivered stillborn offspring were subjected to a polyphasic taxonomic study. On the basis of 16S rRNA sequence similarities both strains, which possessed identical sequences, were assigned to the genus *Brucella*. This placement was confirmed by extended multilocus sequence analysis (MLSA), where both strains possessed identical sequences, and whole genome sequencing of a representative isolate. All the above analyses suggested that the two strains represent a novel lineage within the genus *Brucella*. The strains also possessed a unique profile when subjected to the phenotyping approach classically used to separate *Brucella* species reacting only with *Brucella* A monospecific antiserum, being sensitive to the dyes thionin and fuchsin, being lysed by bacteriophage Wb, Bk_2, and Fi phage at routine test dilution (RTD) but only partially sensitive to bacteriophage Tb and with no requirement for carbon dioxide, no production of hydrogen sulphide, but strong urease activity. Biochemical profiling revealed a pattern of enzyme activity and metabolic capabilities distinct from existing *Brucella* species. Molecular analysis of the *omp2* locus genes showed that both strains had a novel combination of two highly identical *omp2b* gene copies. Both of the strains shared a unique multiple copy IS*711* fingerprint profile of this *Brucella* specific element. Like MLSA, a multilocus variable number of tandem repeat analysis (MLVA) showed that the isolates clustered very closely together, but represent a separate cluster within the genus *Brucella*. Isolates F8/08-60^T and F8/08-61 could clearly be distinguished from all known *Brucella* species and their biovars by both phenotypic and molecular properties. Therefore, by applying the *Brucella* species concept suggested by the ICSP Subcommittee on the Taxonomy of *Brucella,* they represent a novel species within the genus *Brucella* for which the name *Brucella papii* sp. nov., with the type strain F8/08-60^T (= NCTC XXXX^T = BCCN XXXX^T), is proposed.

Body Text

The genus *Brucella* currently comprises ten species *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella neotomae*, *Brucella ovis*, *Brucella pinnipedialis*, *Brucella ceti*, *Brucella microti* and *Brucella inopinata* (Whatmore, 2009). The latter four species have been described in the last decade after a long period of stability and reflect an ongoing widening of the understanding of the host range of *Brucella* (Godfroid *et al,* 2011). Due to high DNA-DNA relatedness of the six longstanding species (relatedness >70%) it had been suggested that the genus should be monospecific and consist of only *B. melitensis* with six biovars Melitensis, Abortus, Suis, Ovis, Neotomae, and Canis (Verger *et al.*, 1985). However, in 2003, the ICSP Subcommittee on the Taxonomy of *Brucella* unanimously agreed on a return to the pre-1986 *Brucella* taxonomy of six species with recognised biovars of *B. suis*, *B. abortus* and *B. melitensis* (Osterman & Moriyon, 2006). Three of the newly described species, *B. ceti*, *B. pinnipedialis* and *B. microti* (Foster *et al*., 2007; Scholz *et al*., 2008) conform to the existing pattern of high genetic homogeneity, for example all sharing identical 16S rRNA sequences. However *B. inopinata*, although clearly much more closely related to *Brucella* than to the nearest neighbour genus *Ochrobactrum* (De *et al,* 2008), substantially extends the described genetic diversity within the group (Scholz *et al.,* 2010; Wattam *et al.*, 2012; Wattam *et al.*, 2014). This has led some authors to informally divide the genus into 'atypical' *Brucella,* including *B. inopinata* and other groups recently isolated from rodents, amphibians and humans but yet to be formally taxonomically described (Tiller *et al*., 2010a, Tiller *et al.*, 2010b; Eisenberg *et al.*, 2012; Fischer *et al*., 2012), and 'core' *Brucella,* representing the remaining nine genetically conserved species that cluster with the longstanding 'classical' *Brucella* species.

Historically classification of *Brucella* species has been based on a combination of host species preference and phenotypic biotyping that examines a range of characteristics including CO_2 requirement, H_2S production, dye-sensitivity, lysis by *Brucella* specific bacteriophage and agglutination with monospecific sera. Phenotyping is still used widely, at

least in reference laboratories, although its limitations are increasingly widely recognised and the emergence of new species is further eroding its value. Recent descriptions have relied heavily on additional data from emerging molecular approaches and a process of updating the minimal standards to describe novel *Brucella* species, written almost 40 years ago (Corbel and Brinley-Morgan, 1975), to reflect these approaches is ongoing within the current ICSP Subcommittee on the Taxonomy of *Brucella (*www.the-icsp.org/subcoms/Brucella*).*

Here we describe the classification of two strains (AHVLA F8/08-60^T = Baboon 15719 = NVSL 07-0026-1 and AHVLA F8/08-61 = Baboon 15331 = NVSL 07-0224-1) which were originally isolated in 2006 and 2007 from two cases of stillbirth and retained placenta in baboons at a primate research centre in Texas, USA (Schlabritz-Loutsevitch *et al.,* 2009). The index case was a 13 year old baboon originally captured in Tanzania with a history of three previous pregnancies including one abortion/stillbirth. The suspect *Brucella* (isolate AHVLA F8/08-60^T) was isolated from a cervical swab obtained following a stillbirth in August 2006. Banked sera from the animal was serologically positive for *Brucella* using testing validated for *B. abortus* in cattle (Brewers' Diagnostic Kits, Hynson, Westcott and Dunning Inc, Baltimore, MD, USA). A second isolate was obtained from a swab of uterine content in January 2007 one month after a stillbirth in an 8 year old colony born baboon with no previous contact with the index case. This animal was serologically negative for *Brucella*.

Phenotype was examined by characterising growth on different media, CO_2 requirement, H_2S production, growth in presence of dyes (thionin and basic fuchsin), reaction with *Brucella* unabsorbed and monospecific A and M antisera and microscopy. Metabolic activity in comparison with other *Brucella* was assessed using API20E, API20NE & APIZYM (BioMerieux, France) with some confirmatory standalone biochemical testing and application of the Micronaut® BfR *Brucella* assay (Al Dahouk *et al.,* 2010). Molecular analysis comprised multiplex PCR, DNA-DNA hybridisation studies (Ziemke *et al*., 1988), 16S rRNA sequence analysis, *omp2a* and *omp2b* sequencing, multilocus sequence analysis (MLSA), multilocus variable number of tandem repeat analysis (MLVA with 16 loci), and IS*711* fingerprinting, all

of which have been used in recent descriptions of new *Brucella* species, with recently completed whole genome sequencing supporting findings.

Phenotypic Analysis

Both isolates grow slowly on sheep blood agar (SBA) after incubation both aerobically and in CO_2 with small, raised, circular, convex, non-haemolytic, greyish colonies, ~0.5mm in diameter appearing after 3 days incubation at 37^{o}C increasing to ~1mm in diameter after 6 days. Growth is poor at 28^{o}C. On Farrell's agar growth also occurs without additional CO_2 with both isolates producing pinpoint colonies after 3 days incubation at 37^{o}C becoming cream in colour, circular, entire, convex and ~ 0.5-1mm in diameter after 6 days incubation. On Serum Dextrose agar (SDA) growth is apparent after 24 hours at 37^{o}C without additional CO_2 with colonies of around 1 mm increasing to 3-4 mm after 72 hours incubation. Colonies are smooth, entire and circular and show characteristic blue iridescence using obliquely transmitted light (Henry, 1933). Growth is poor at 28^{o}C. On nutrient agar there is poor growth at 37^{o}C and no growth at 28^{o}C. Both isolates are sensitive to doxycycline (MIC <0.016 µg/ml), rifampicin (MIC 0.032 µg/ml), ciprofloxacin (MIC 0.094 µg/ml F8/08-60; 0.064 µg/ml F8/08-61) and streptomycin (MIC 0.125 µg/ml F8/08-60; 0.094 µg/ml F8/08-61) in SDA and fail to grow in nutrient both with 6% NaCl.

Both isolates are Gram-negative cocci, coccobacilli or short rods approximately 0.5-0.7µm in diameter and 0.5-1.5 µm in length arranged singly or, occasionally, in pairs, small groups and chains. Both isolates are consistent with *Brucella* using the modified Ziehl-Neelsen stain (Brinley-Morgan *et al.*, 1978) being resistant to decolourisation with 0.5% acetic acid. Flagellation was determined by transmission electron microscopy. Both isolates were dried onto formvar/carbon coated support grids, which had been subjected to plasma glow discharge, negatively stained with 2% phosphotungstic acid (pH6.6) and immediately

examined in a Phillips CM10 transmission electron microscope at 80 kV. Cells are unflagellated coccobacilli approximately 0.5-1.0µm in length arranged as individual cells or irregular clusters (Figure 1). Both isolates were examined by slide agglutination test using unabsorbed *Brucella* antiserum (Weybridge Working Standard - WWS), negative control serum and sterile normal saline and agglutinate with WWS8 but not with either the negative control or saline.

Using classical phenotyping approaches that are traditionally used to divide *Brucella* into species and biovars (Alton *et al*., 1988) the organism does not conform to the characteristics of any recognized species of *Brucella* (Table 1). The unusual profile includes no requirement for carbon dioxide, no production of hydrogen sulphide, sensitivity to the dyes thionin and basic fuchsin at 1:50,000, agglutination only with mono-specific anti-A serum, and lysis with bacteriophage Wb, Bk_2 and Fi.

Both isolates were run through API20E, API20NE incubated aerobically at 37^{o}C and 30^{o}C respectively and APIZYM (BioMerieux, France). As observed for other classical *Brucella* species, and in contrast to the recently described *B. inopinata* and *B. microti*, the isolates show limited metabolic reactivity. After 24 hrs incubation at 37^{o}C API 20E strips for both strains show positive reactions only for urea, VP, and fermentation of D-glucose and L-arabinose. Both strips were re-incubated for a further 24 hours (not normally done under manufacturer instructions) and this did produce a slight colour change in the sorbitol cupule perhaps indicating slow fermentation in isolate F8/08-61. After 24 hrs incubation at 30^{o}C the API 20NE strips for both isolates were read - reactions only occurred in urea, there was no reduction of nitrates or indole production. Reincubation of the API 20NE strips for a further 24 hours as per manufacturer instructions did not produce any changes to the profile of either isolate. APIZYM strips were inoculated and incubated for 4.5 hours as per manufacturer instructions. Both isolates produced identical results: alkaline phosphatase –; esterase (C 4) ++; esterase lipase (C 8) +; lipase (C 14) –; leucine arylamidase ++; valine

arylamidase +; cystine arylamidase +; trypsin ++; α-chymotrypsin –; acid phosphatase ++; napthol-AS-bi-phosphohydrolase ++; α-galactosidase –; β-galactosidase –; β-glucuronidase –; α-glucosidase –; B-glucosidase –; N-acetyl-β-glucosaminidase –; α-mannosidase – and α-fucosidase – where + = positive (produced) ++ = positive (produced strong reaction) and – = negative (not produced).

Oxidase and catalase production were examined. Both isolates produce catalase but are negative for oxidase production by both pyotest strips (Medical Wire and Equipment, Bath, UK) and freshly made oxidase reagent. Urea slopes were also inoculated and both isolates rapidly hydrolyse urea within 30 minutes consistent with the positive urea results recorded on API20E and API20NE. Nitrate broths were inoculated with both isolates and incubated for 24 hrs and 3 days at 37^{o}C along with a positive control (*Serratia marcescens*), negative control (*Acinetobacter lwoffii*) and an uninoculated broth. Both isolates do not reduce nitrate.

In addition to classical biochemical reactions extended metabolic activity was examined using the Micronaut® BfR *Brucella* assay (Merlin Diagnostika, Bornheim-Hersel, Germany) which assesses reaction with 93 different metabolites (Al Dahouk *et al.,* 2010). Once again, and in contrast to *B. inopinata* and *B. microti* the isolates display limited metabolic reactivity, especially in sugar metabolism, consistent with the 'classical' *Brucella* species group. The metabolic profile is distinct from any extant *Brucella* species (Table 2) and hierarchical cluster analysis (BioNumerics version 6.6, Applied Maths, Belgium), performed using the Ward's linkage algorithm (simple matching), confirmed this with the closet metabolic relationship appearing to be with *B. suis* bv 5 (Figure 2). Within the same cluster but in a different branch *B. canis*, *B. ovis*, *B. neotomae*, *B. ceti* and *B. pinnipedialis* are found. Interestingly, the enzymatic reaction using H-hydroxyproline-βNA (hitherto defining the genus *Brucella* together with a positive urease as well as negative Glu(pNA)-OH and Pyr-pNA (Al Dahouk *et al.*, 2010; Al Dahouk *et al.*, 2012) for the first time revealed a negative result. Hence metabolically *B. papii* sp. nov. differs from existing *Brucella* species.

Molecular analysis

The virtually complete 16S rRNA sequence was determined from both strains using an approach described previously (Hunt *et al.,* 2013). Sequences of 1407bp obtained from both isolates are identical to each other and confirm the identity of isolates as members of the genus *Brucella*. Sequences show 2 bp differences from the nine 'core' *Brucella* species previously shown to share identical 16S rRNA sequences (Gee *et al.*., 2004; Al Dahouk *et al.*, 2012) and 7bp differences from *Brucella inopinata*, the only *Brucella* species previously shown to have a divergent 16S rRNA sequence (Scholz *et al.,* 2010).

Results from DNA–DNA hybridizations using labelled DNA of *B. melitensis* 16M^{T} showed 79.1% ± 0.7 DNA relatedness with strain F8/08-60^{T}. These results are in agreement with previous reports (Verger *et al*., 1985) which showed that, if the 70% DNA-relatedness threshold value is applied, species of this genus cannot be separated based on results from DNA–DNA hybridizations. The DNA relatedness to the phylogenetically closest neighbour *Ochrobactrum.intermedium* LMG 3301^{T} was 45.7%.

Both strains produce a strong band of identical size to the *B. melitensis* control in PCR performed to determine the presence of the *Brucella* genus specific *bscp31* gene (Bailey *et al.*, 1992). Fingerprinting using probes against the *Brucella* specific IS*711* (Halling *et al,* 1993) has been shown to be a useful tool for differentiating *Brucella* at species and/or sub-species level (Ouahrani *et al.,* 1993; Bricker *et al.,* 2000; Dawson *et al.*, 2008). By Southern blot the two baboon isolates share an identical high copy number IS*711* profile that is distinct from any profile seen previously (data not shown). Some of these IS*711* copies have been located in the genome sequence of one of the baboon isolates (Audic *et al*., 2011). While some IS*711* chromosomal locations are common to other *Brucella* species but at least 7 may be specific to the baboon isolates.

193 Two frequently applied multiplex PCRs were applied to the isolates. AMOS (*B. abortus*, *B.*
194 *melitensis*, *B. ovis*, and *B. suis*) PCR (Bricker and Halling, 1994; Ewalt and Bricker, 2000;
195 Bricker *et al*., 2003) with additional species-specific primers confirms that the isolates are
196 *Brucella* species because of the generation of the specific 180 bp amplicon. However, both
197 isolates fail to produce any of the expected PCR products that would identify them as a
198 known species (Schlabritz-Loutsevitch *et al.,* 2010). The strains were examined by
199 Bruceladder PCR, a multiplex PCR that can differentiate all known *Brucella* species (López-
200 Goñi *et al*., 2008). The profile of bands obtained is identical to the *B. melitensis* profile (data
201 not shown) and thus the existing Bruceladder protocol would need to be adapted to
202 distinguish *B. papii* sp. nov. The novel IS*711* localities described above give a means to do
203 this.
204
205 Studies of DNA polymorphism at the *omp2* locus have also been used extensively to
206 characterise *Brucella*. The baboon strains were shown uniquely to possess two almost
207 identical *omp2b* copies, as previously observed for *B. ceti*, but distinct from these latter by
208 being more *omp2b*-like at the 3' end of both *omp2* gene copies (Figure 3).
209
210 Both isolates were examined using an MLSA scheme that characterises the sequences of
211 21 independent genomic fragments equating to >10.2 Kbp (Al Dahouk *et al.,* 2012). Both
212 baboon isolates share identical sequences at all loci but possess unique alleles at 11/21 loci
213 not seen in characterisation of over 700 *Brucella* isolates representing all known species and
214 biovars. Phylogenetic analysis comparing the baboon isolates with type strains representing
215 all extant *Brucella* species and their biovars confirms that they represent a well separated
216 lineage most closely related to *B. ovis* (Figure 4).
217
218 Both isolates were typed using the MLVA assay described by Le Flèche *et al.* (2006) and Al
219 Dahouk *et al.* (2007) comprising 16 loci (Scholz & Vergnaud, 2013) with allele coding
220 convention following version 3.6 of the table for allele assignment at http://mlva.u-

221 psud.fr/brucella/spip.php?rubrique29. All loci could be amplified with the exception of
222 Bruce11. The MLVA genotype is 2-5-0-14-2-2-5-3 for the 8 panel 1 loci (0 reflects lack of
223 amplification at locus Bruce11), 6-36-9 for panel 2A and 2-2-3-4-10 or 2-2-3-5-11 for the
224 most variable panel 2B. The two strains differ only at loci Bruce16 and Bruce30 typical of the
225 minor variation seen in closely related strains (Garcia-Yoldi *et al.*, 2006: Maquart et al.
226 2009). Figure 5 shows the relative position of the two strains compared to approximately
227 1900 different MLVA16 genotypes. In this minimum spanning tree (MST) connecting lines
228 longer than 6 are masked. Seven unconnected groups are identified. The largest one
229 contains *B. melitensis*, *B. abortus*, *B. pinnipedialis, B. ceti, B. suis* biovar 5*, B. microti*. The
230 second largest is made by *B. suis* biovar 2. The third group aggregates *B. canis*, and *B. suis*
231 biovars 1, 3, 4. *B. inopinata*, *B. ovis*, *B. neotomae* and *B. papii* (shown in red) constitute the
232 last four groups.
233
234 In addition one of the isolates (NVSL 07-0026-1) was subjected to whole genome
235 sequencing. Two independent comparative genomic analyses have been published based
236 on this sequence (Audic *et al*., 2011; Wattam *et al.*, 2014) comparing this strain with all other
237 *Brucella* species type strains. Both confirm the phylogenetic position suggested by MLSA
238 showing the baboon isolate and *B. ovis* united by an extremely shallow branch indicating a
239 shared common ancestor but with the long branch length indicative of significant divergence
240 since that time (Figure 6). Further, the *in silico* MLVA genotyping tool accessible at
241 http://mlva.u-psud.fr was applied to the whole genome sequence (WGS). The deduced code
242 was exactly as determined by PCR and electrophoresis size measurement, illustrating that
243 *Brucella* tandem repeats have been correctly assembled from the initial 454 WGS sequence
244 data. Investigation of the WGS data shows that one of the two flanking sequences of the
245 Bruce11 VNTR contains an approximately 141 bp deletion (101 bp of flanking sequence,
246 40bp out of 63 bp of the first repeat unit, plus potentially an unknown number of full repeat
247 units). This explains the lack of amplification of Bruce11 in *B. papii* since one of the usual
248 Bruce11 PCR primers is within this deletion. If primer GTAGCGATTGACACCTTGCCTG is

used instead of CTGTTGATCTGACCTTGCAACC, an identical 396 bp PCR product is obtained in both strains whereas the Bruce11 PCR product is 93 bp longer in the other *Brucella* (data not shown).

Conclusions

In summary, extensive phenotypic and genotypic analyses demonstrate that isolates F8/08-60^T and F8/08-61 are members of the genus *Brucella*, but can be clearly differentiated from all established species of this genus, including their biovars. Hence, by applying the *Brucella* species concept suggested by the ICSP Subcommittee on the Taxonomy of *Brucella* (Osterman & Moriyon, 2006), the two strains represent a novel species of the genus *Brucella*, for which we propose the name *Brucella papii* sp. nov.

Description of *Brucella papii* sp. nov.

Brucella papii (L. gen. n. papii, of the baboon, from which the first strains of this species have been isolated).

Cocci, coccobacilli or short rods, ~0.5-0.7µm in diameter, ~0.5-1.5 µm in length, arranged singly and, occasionally in pairs, small groups and chains. Gram negative and resistant to decolourisation with 0.5% acetic acid. Non-motile and non-spore forming. Aerobic. Does not require supplementary CO_2 for growth. Growth occurs at 30^{o}C to 37^{o}C. Colonies on sheep blood agar and Farrell's are visible at 3-4 days and are small, raised, circular, entire and convex of ~0.5-1mm in diameter. Non-haemolytic and greyish in colour (on blood agar) and honey coloured (on Farrell's). No growth occurs on MacConkey agar. No growth in broth with 6.5% NaCl. Isolates do not grow in the presence of thionin or basic fuchsin at 1/50 000. Both strains agglutinate with monospecific anti-A serum but not anti-M or anti-R serum. Cells are lysed by Wb, Bk_2, Tb and Fi phage at routine test dilution (RTD). Cells are sensitive to

doxycycline, rifampicin, ciprofloxacin and streptomycin. Catalase positive. Oxidase negative. Strongly urease positive. Indole negative. No production of H_2S. Acetoin (Vogues Proskauer) is produced. Nitrates are not reduced. No production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase, β-glucosidase or gelatinase. Positive (API20E) for L-arabinose, D-glucose (weak at 37^{o}C but not 30^{o}C) and variable, but weak, fermentation of D-sorbitol. No fermentation or oxidation of D-mannitol, inositol, L-rhamnose, D-sucrose, D-melibiose or amygdalin at 37^{o}C. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid at 30^{o}C. Esterase (C 4), esterase lipase (C 8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, and napthol-AS-Bi-phosphohydrolase are all produced. Alkaline phosphatase, lipase (C 14), α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and tryptophane deaminase are not produced. Differentiating physiological reactions (Micronaut® BfR *Brucella* assay) of strains F8/08-60^{T} and F8/08-61 with respect to other *Brucella* species are given in Table 2.

The type strain is F8/08-60^{T} (BCCN XXXX^{T} NCTC XXXX^{T}) isolated in 2006 from a cervical swab taken from a *Papio* spp. following stillbirth in an animal handling facility in Texas, USA.

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Nucleotide Accession Numbers

The EMBL accession numbers for the gene sequences of 16S rRNA and *omp2a*/*omp2b* are HG932316/HG932317 and KJ493822, KJ493823, KJ510540 and KJ510541 respectively.

Figure and Table Legends

Table 1. Differentiating characteristics of *B. papii* from other *Brucella* species and biovars based on classical biotyping approaches.

Table 2. Metabolic activity of *Brucella papii* sp. nov. in comparison with classical and newly described species tested with the Micronaut® BfR *Brucella* assay. (-, no metabolic activity; +, metabolic activity; v, variable metabolic activity. *Brucella*-specific traits are shown in boldface, and potentially distinctive features of *Brucella papii* sp. nov. are shaded).

Figure 1. Electron micrographs showing non-flagellated F8/08-61 cells. The appearance of F8/08-60^T is identical.

Figure 2. Hierarchical cluster analysis of *Brucella* species including *Brucella papii* sp. nov. performed by the Ward's linkage algorithm (simple matching) on the basis of 93 biochemical reactions tested with the Micronaut® BfR *Brucella* assay.

Figure 3. Schematic multiple nucleotide sequence alignment of the *omp2a* and *omp2b* genes of *Brucella* strains: Nucleotide sequences are represented by large rectangles divided into boxes of 30 nucleotides. *B. microti omp2b* gene is taken as the reference sequence. The yellow colour is typical of *B. microti omp2a*-specific nucleotides. The numbers in the corresponding boxes indicate the number of *omp2a*-specific nucleotides present in the sequence considered. The numbers in parentheses show insertions and deletions. The numbers in red indicate nucleotide differences that are not due to gene conversion.

Figure 4. Phylogenetic analysis of the baboon isolates in comparison with type strains (see http://www.the-icsp.org/taxa/Brucellalist.htm for details of strains) of all *Brucella* species and biovars inferred by MLSA. Bar = 0.002 substitutions per nucleotide position. Analysis was performed with the MEGA5 package using the neighbour-joining approach.

Figure 5. Minimum spanning tree from the MLVA16 data. Published MLVA16 data which can be accessed from the cooperative *Brucella* MLVA database at http://mlva.u-psud.fr was compared to the *B. papii* MLVA16 data. The colour code reflects species and sometimes biovar or significant sub-species clustering. The two *B. papii* strains are shown in red. Categorical distance was used. The creation of hypothetical intermediate links was allowed. Only connecting branches up to a length of 6 are shown.

Figuure 6. Phylogenetic representation of species in the genus *Brucella* based on a concatenated alignment of orthologous genes of complete or nearly complete genome sequences of 34 *Brucella* strains, including *B. papii* sp. nov.

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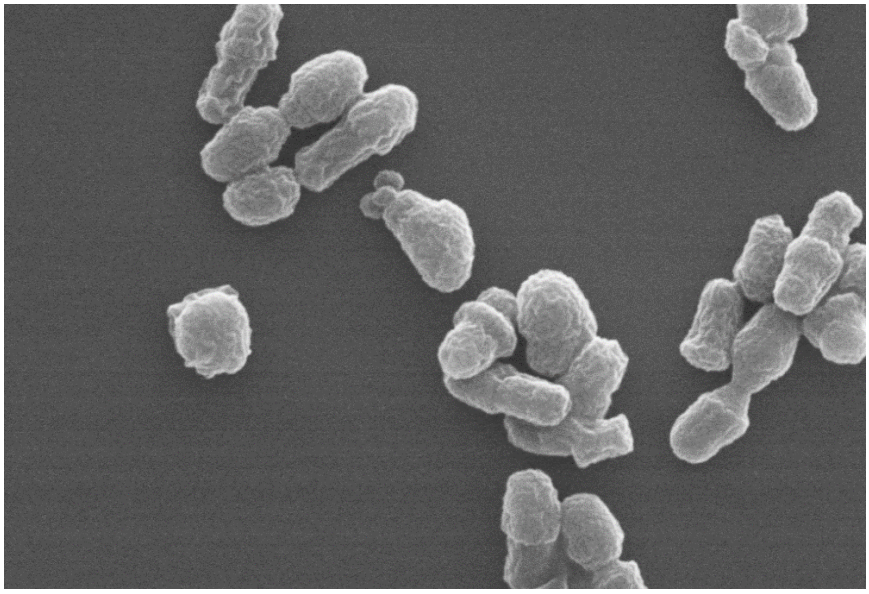
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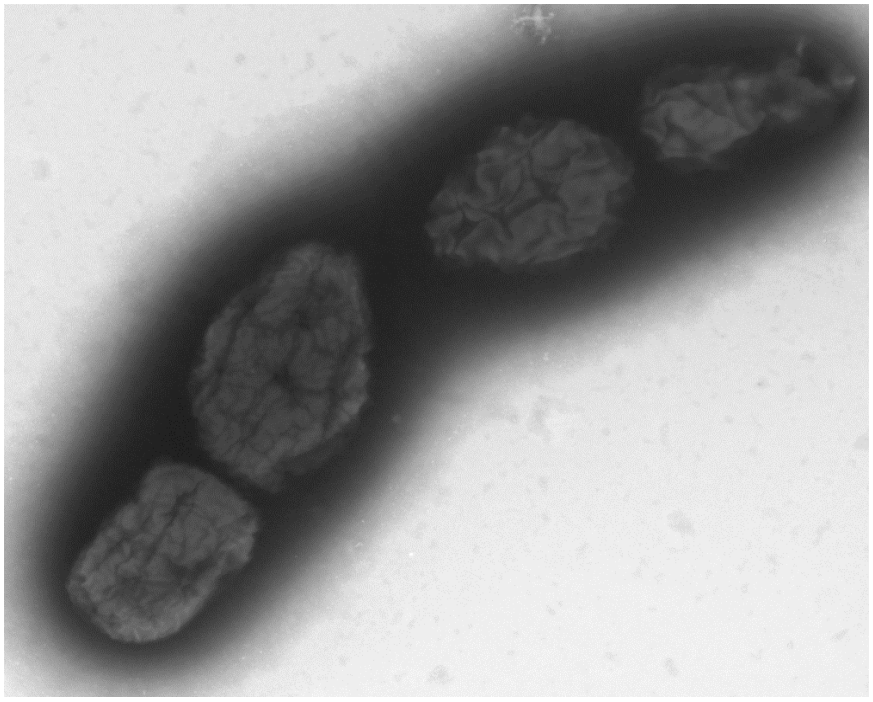
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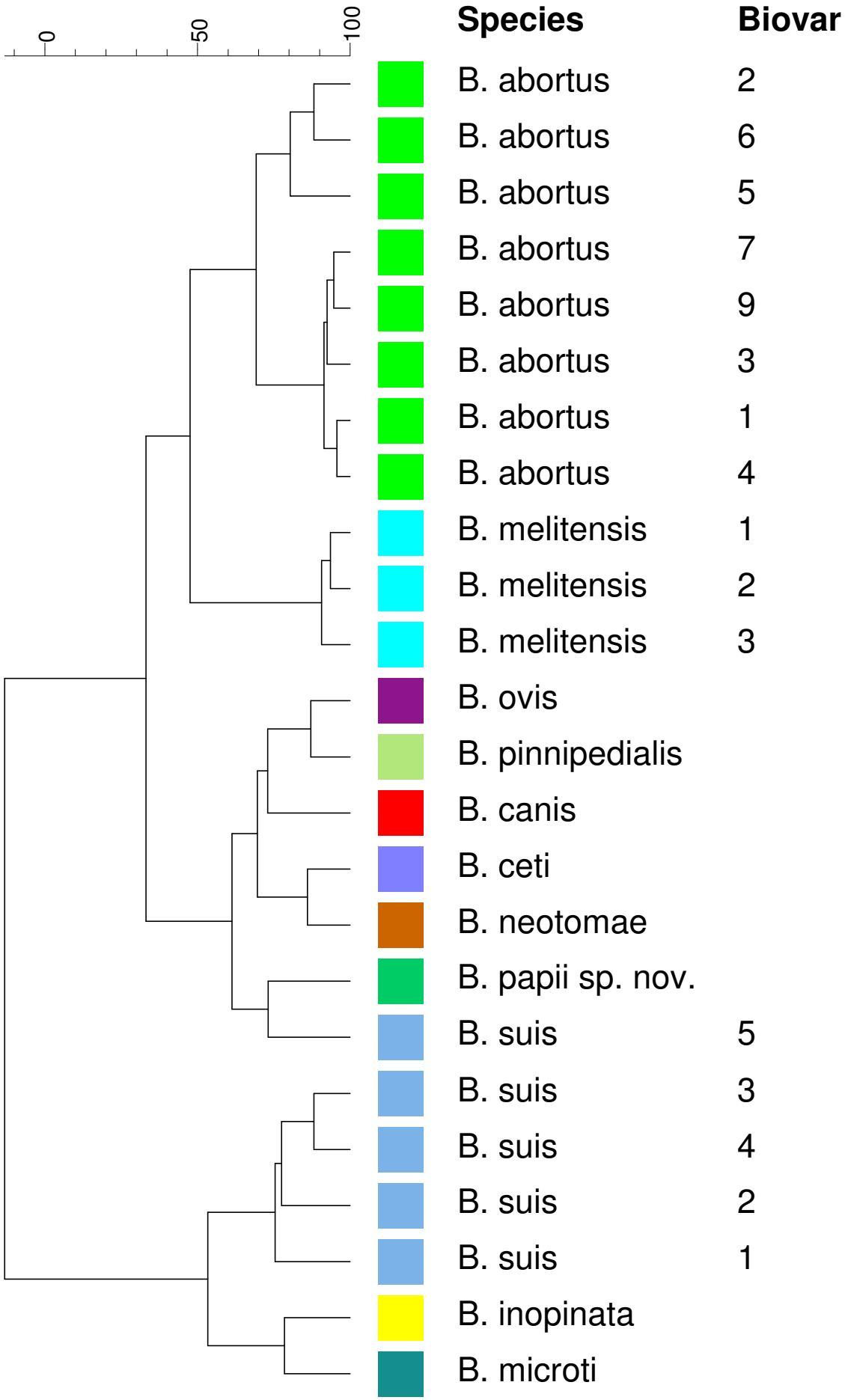
Figure 1



1 µm

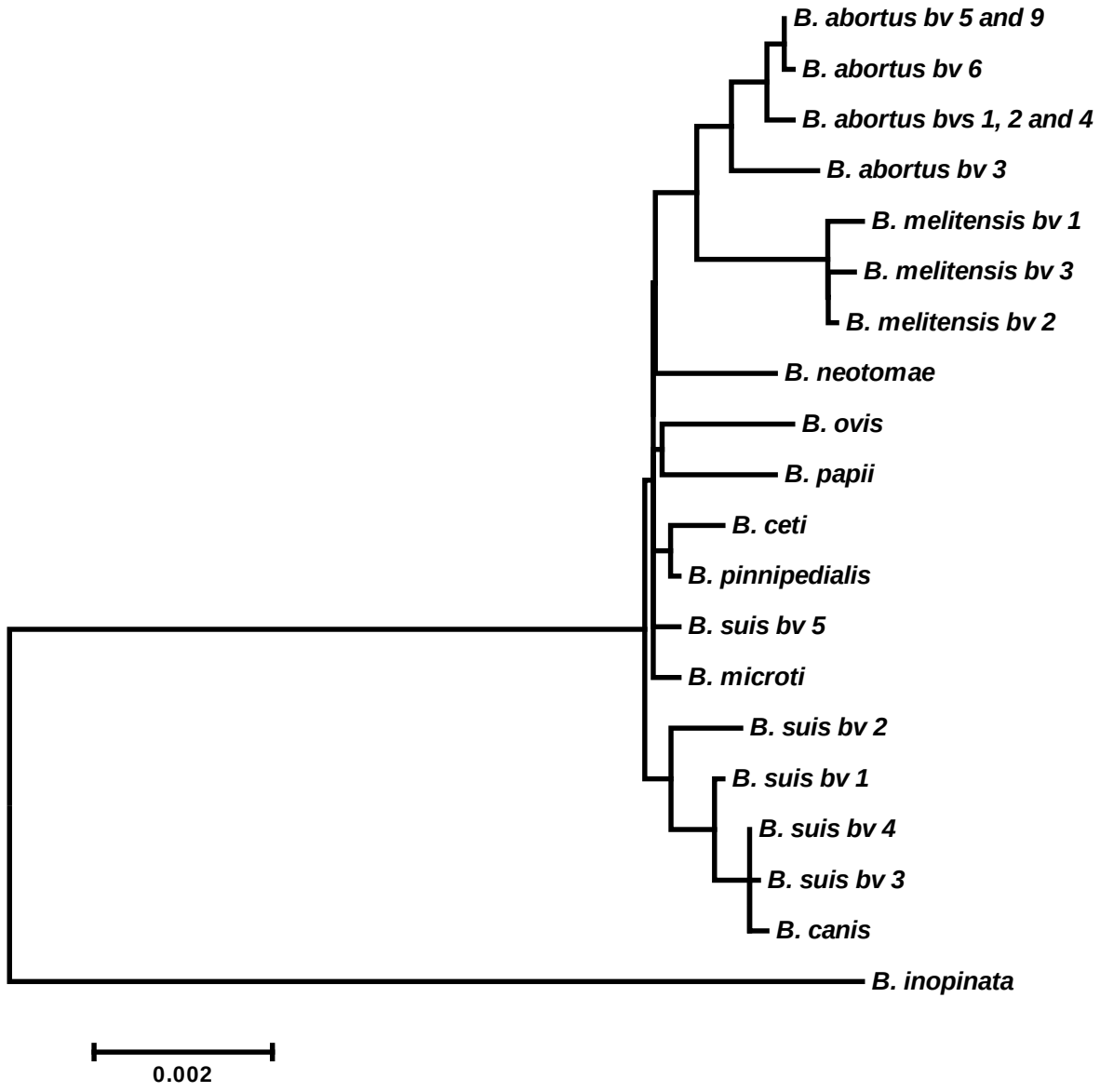


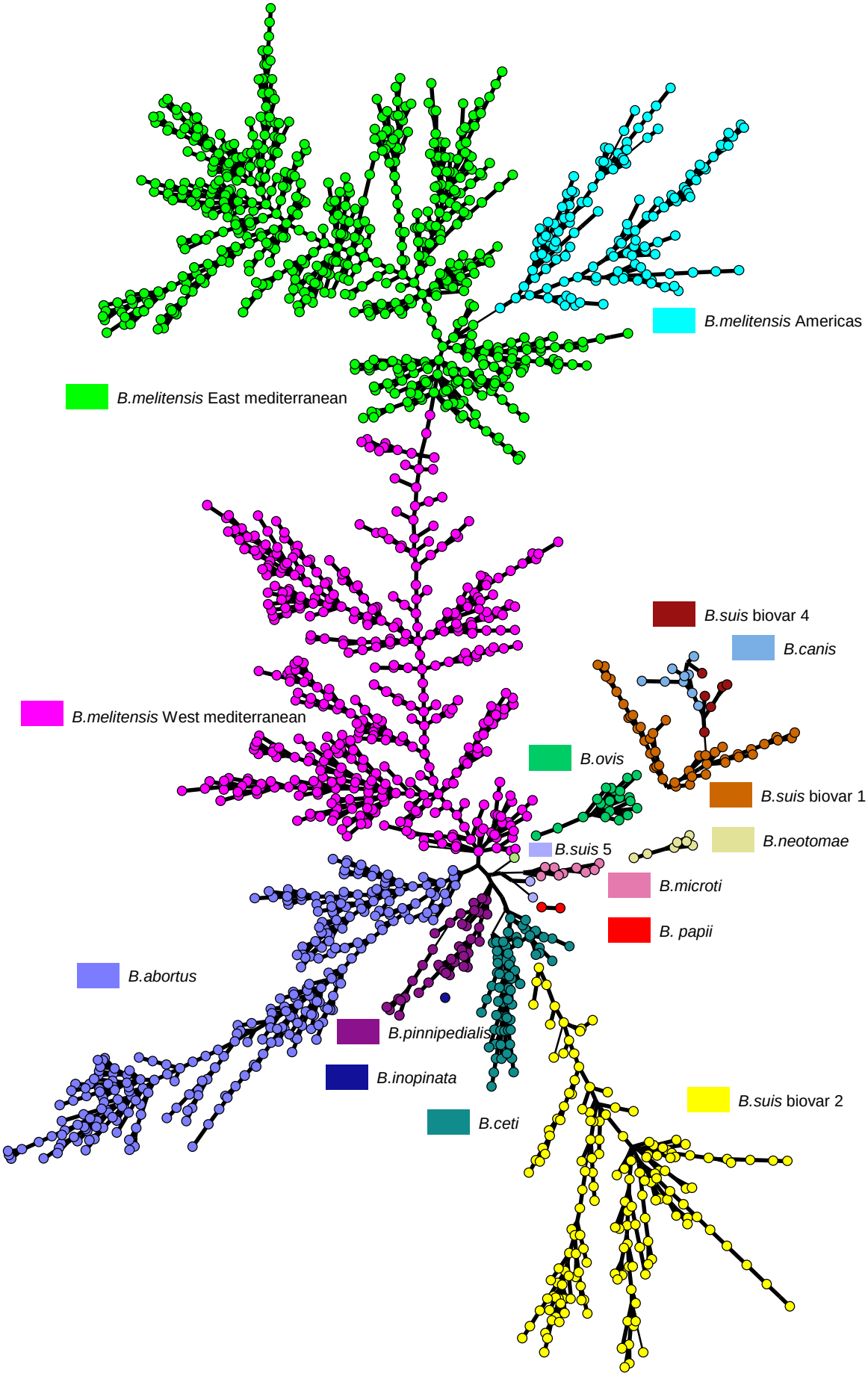
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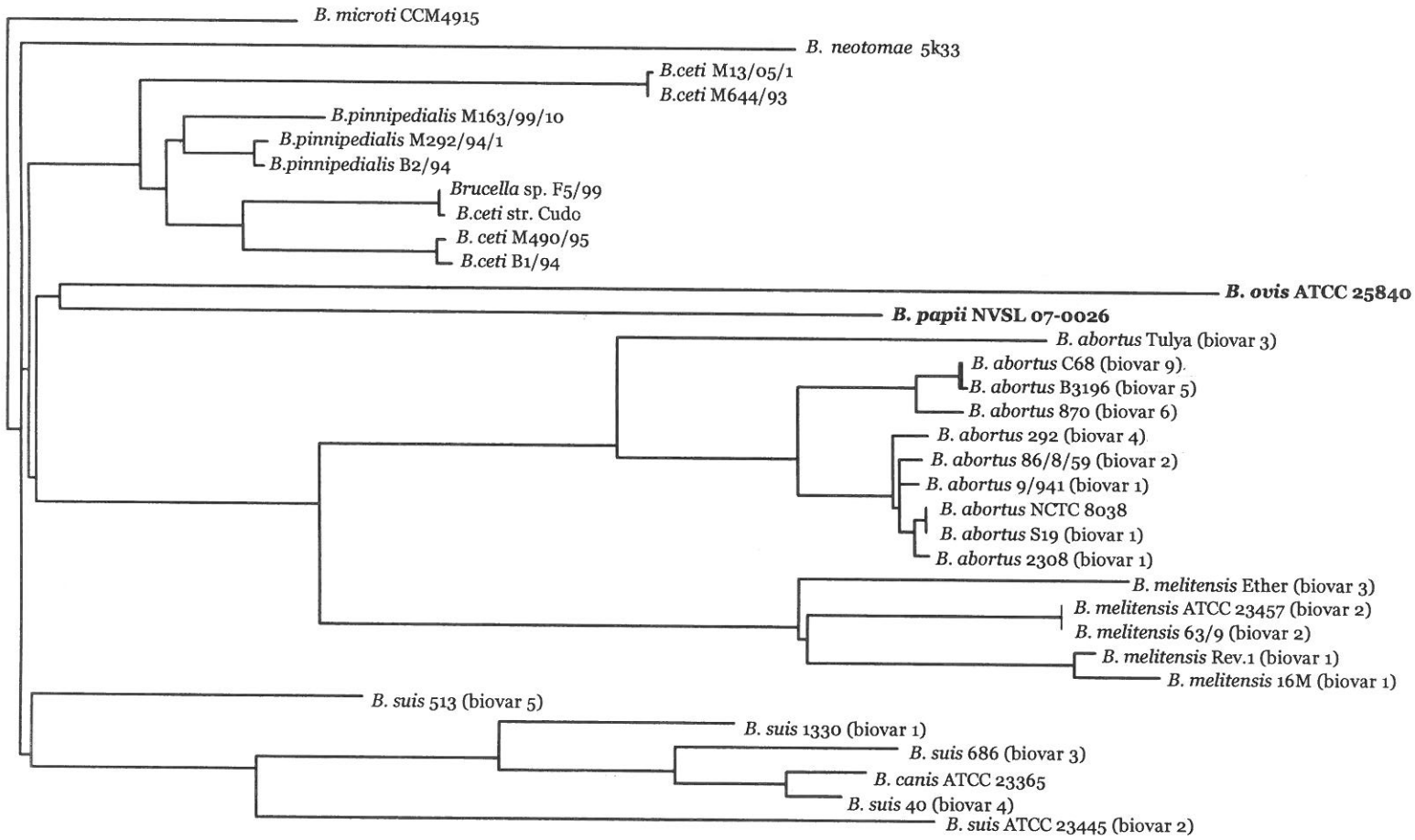

0
50
100
Species
Biovar
B. abortus 2
B. abortus 6
B. abortus 5
B. abortus 7
B. abortus 9
B. abortus 3
B. abortus 1
B. abortus 4
B. melitensis 1
B. melitensis 2
B. melitensis 3
B. ovis
B. pinnipedialis
B. canis
B. ceti
B. neotomae
B. papii sp. nov.
B. suis 5
B. suis 3
B. suis 4
B. suis 2
B. suis 1
B. inopinata
B. microti

GenBank Accession N°	Species	Strain	Host or source	*omp2*gene	90	180	270	360	450	540	630	720	810	900	990	1080	1140
AM943813	*B. microti*	BMS 20	Soil	*omp2b*													
AM943807				*omp2a*	1	4 1 9	1 10 9 12	5 (12)	6 (18)	8	7	1 6	2	1 1	8 (8)	3 (7)	1	2	2 5	14 1 4 7	1	2	2
U26440	*B. melitensis*	16M	Goat	*omp2b*	1		1										
U26440				*omp2a*	1		1	10 9 12	5 (12)	6 (18)	8	7	1 6	2	1 1	8 (8)	3 (7)	1	2	2 5	14 1 4 7	1	2	2
U26443	*B. suis*	1330	Swine	*omp2b*	1												
U26443				*omp2a*	1		9	1 10 9 12	5 (12)	6 (18)	8	7	1 6	2	1 1	8 (8)	3 (7)	1	2	2 5	14 1 4 7	1	2	2
AF268035	*B. ovis*	76-250	Sheep	*omp2b*	1	4 1 9	1 10 8, 1	11, 1 5 (12)	6 (18)	7, 1	7	1 1					1
AY008720				*omp2a*	1	4 1 9	1 10 9 12	5 (12)	6 (18)	8	7	1 1	2	1 1	8 (8)	3 (7)	1	2	2 5	14 1 4 7	1	2	2
U26442	*B. ovis*	63/290	Sheep	*omp2b*	1	4 1 9	1 10 8, 1	12 5 (12)	6 (18)	8	7	1 1	2	1 1	8 (8)	3 (7)	1	2	2 5				
U26442				*omp2a*	1	4 1 9	1 10 9 12	5 (12)	6 (18)	8	7	1 1	2	1 1	8 (8)	3 (7)	1	2	2 5	14 1 4 7	1	*	*
AF300816	*B. ceti*	B1/94	Porpoise	*omp2b*	1	4 1 9												2	2 5	14 1 4 4			
AF300817				*omp2a*	1	4 1 9												2	2 5	14 1 4 4		2	2
AF300814	*B. ceti*	B14/94	Common dolphin	*omp2b*	1															14 1 4 7	1	2	2
AF300815				*omp2a*	1															14 1 4 7	1	2	2
KJ510540	*B. papii* sp. nov.	F8/08/60	Baboon	*omp2b*	1																1	2	2
KJ493822				*omp2a*	1																1	2	2
KJ510541	*B. papii* sp. nov.	F8/08/61	Baboon	*omp2b*	1																1	2	2
KJ493823				*omp2a*	1																1	2	2

Figure 4:


B. abortus bv 5 and 9
B. abortus bv 6
B. abortus bvs 1, 2 and 4
B. abortus bv 3
B. melitensis bv 1
B. melitensis bv 3
B. melitensis bv 2
B. neotomae
B. ovis
B. papii
B. ceti
B. pinnipedialis
B. suis bv 5
B. microti
B. suis bv 2
B. suis bv 1
B. suis bv 4
B. suis bv 3
B. canis
B. inopinata
0.002


B.melitensis Americas
B.melitensis East mediterranean
B.suis biovar 4
B.canis
B.melitensis West mediterranean
B.ovis
B.suis biovar 1
B.suis 5
B.neotomae
B.microti
B. papii
B.abortus
B.pinnipedialis
B.inopinata
B.ceti
B.suis biovar 2


B. microti CCM4915
B. neotomae 5k33
B.ceti M13/05/1
B.ceti M644/93
B.pinnipedialis M163/99/10
B.pinnipedialis M292/94/1
B.pinnipedialis B2/94
Brucella sp. F5/99
B.ceti str. Cudo
B. ceti M490/95
B.ceti B1/94
B. ovis ATCC 25840
B. papii NVSL 07-0026
B. abortus Tulya (biovar 3)
B. abortus C68 (biovar 9)
B. abortus B3196 (biovar 5)
B. abortus 870 (biovar 6)
B. abortus 292 (biovar 4)
B. abortus 86/8/59 (biovar 2)
B. abortus 9/941 (biovar 1)
B. abortus NCTC 8038
B. abortus S19 (biovar 1)
B. abortus 2308 (biovar 1)
B. melitensis Ether (biovar 3)
B. melitensis ATCC 23457 (biovar 2)
B. melitensis 63/9 (biovar 2)
B. melitensis Rev.1 (biovar 1)
B. melitensis 16M (biovar 1)
B. suis 513 (biovar 5)
B. suis 1330 (biovar 1)
B. suis 686 (biovar 3)
B. canis ATCC 23365
B. suis 40 (biovar 4)
B. suis ATCC 23445 (biovar 2)
0.001

Species	Biotype	Urease	CO_2 rq't	H_2S prod'n	Growth on media containing: Thionin[1]	Fuchsin[1]	Agglutination with monospecific antisera A	M	R	Lysis by phage at RTD[2] Tb	Wb	Bk_2	Fi	R/C	Tb10[4]
***B. papii* sp. nov.**		+	-	-	-	-	+	-	-	PL or NL	L	L	L	NL	PL
B. abortus	1	(+)*	(+)	+	-	+	+	-	-	L	L	L	L	NL	L
	2	+	(+)	+	-	-	+	-	-	L	L	L	L	NL	L
	3**	+	(+)	+	+	+****	+	-	-	L	L	L	L	NL	L
	4	+	(+)	+	-	+	-	+	-	L	L	L	L	NL	L
	5	+	-	-	+	+	-	+	-	L	L	L	L	NL	L
	6**	+	-	(+)	+	+	+	-	-	L	L	L	L	NL	L
	7***	+	-	(+)	+	+	+	+	-	L	L	L	L	NL	L
	9	+	-	+	+	+	-	+	-	L	L	L	L	NL	L
B. suis	1	+	-	+	+	-*****	+	-	-	NL	L	L	PL	NL	L
	2	+	-	-	+	-	+	-	-	NL	L	L	PL	NL	L
	3	+	-	-	+	+	+	-	-	NL	L	L	PL	NL	L
	4	+	-	-	+	(-)	+	+	-	NL	L	L	PL or L	NL	L
	5	+	-	-	+	-	-	+	-	NL	L	L	PL	NL	L
B. melitensis	1	+	-	-	+	+	-	+	-	NL	NL	L	NL	NL	NL
	2	+	-	-	+	+	+	-	-	NL	NL	L	NL	NL	NL
	3	+	-	-	+	+	+	+	-	NL	NL	L	NL	NL	NL
B. ovis		-	+	-	+	(+)	-	-	+	NL	NL	NL	NL	L	NL
B. canis		+	-	-	+	-	-	-	+	NL	NL	NL	NL	L	NL
B. neotomae		+	-	+	-	-	+	-	-	NL or PL	L	L	L	NL	L
B. ceti		+	(-)	-	(+)	(+)	+	(-)	-	NL^{α}	L^{β}	L^{β}	NL or PL	NL	
B. pinnipedialis		+	(+)	-	+	+	(+)	(-)	-	NL^{α}	L^{β}	L^{β}	NL or PL	NL	
B. microti		+	-	-	+	+	-	+		NL	L		NL		L
B. inopinata		+	-	+	+	+	-	+******		NL			NL		PL

[1]Concentration = 1/50 000 w/v.

[2]Phage lysis: RTD = Routine Test Dilution Tb = Tbilisi Wb = Weybridge Bk_2 = Berkeley Fi = Firenze R/C = Rough strains $Tbx10^4$ = RTD x 10^4.

L = Confluent Lysis, PL = Partial Lysis, NL = No Lysis, α Most strains no lysis, β Most strains lysis.

(+) = Most strains positive, (-) = most strains negative.

*Reference strain is -ve but most field strains are positive.

**For more certain differentiation of biotype 3 and 6, thionin at 1/25 000 (w/v) is used in addition. Biovar 3 = +, Biovar 6 = -.

***The status of biovar 7 is under investigation (Garin-Bastuji *et al.*, 2014).

****Some strains of this biotype are inhibited by basic fuchsin, *****Some isolates may be resistant to fuchsin, ****** Weak agglutination.

Table 2.

Substrate designation		Substrate class	*Brucella* species										
			B. abortus	*B. melitensis*	*B. suis*	*B. ovis*	*B. canis*	*B. neotomae*	*B. ceti*	*B. pinnipedialis*	*B. microti*	*B. inopinata*	*B. papii sp. nov.*
d-Ala-pNA	DANA	aminopeptidases with pNA	+	+	+	+	+	-	+	+	+	+	-
Gly-pNA	GNA	aminopeptidases with pNA	+	+	+	+	+	-	+	+	+	+	-
Leu-pNA	LNA	aminopeptidases with pNA	-	v	v	-	-	-	v	-	+	+	+
Lys-pNA	KNA	aminopeptidases with pNA	v	+	v	-	v	-	+	-	+	+	+
Gly-Pro-pNA	GPNA	aminopeptidases with pNA	v	+	+	-	+	+	+	-	+	+	-
Ala-Phe-Pro-pNA	AFPNA	aminopeptidases with pNA	-	+	+	-	-	-	-	-	-	+	-
Glu(pNA)-OH	**ENAOH**	**aminopeptidases with pNA**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Pyr-pNA	**PYRNA**	**aminopeptidases with pNA**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
H-hydroxyproline-ßNA	**HP**	**aminopeptidases with βNA**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**
Leu-ßNA	lL	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-
Lys-ßNA	K	aminopeptidases with βNA	+	+	+	+	+	-	+	+	+	+	+
Arg-Arg-ßNA	RR	aminopeptidases with βNA	v	-	v	+	+	-	+	-	v	+	+
Gly-Gly-ßNA	GG	aminopeptidases with βNA	+	+	+	+	+	-	+	+	+	+	+
Pro-ßNA	P	aminopeptidases with βNA	+	+	+	+	+	+	+	+	+	+	-
Ac-Gly-Lys-βNA	AcGK	aminopeptidases with βNA	-	-	-	+	+	-	v	-	-	+	+
Ala-Phe-Pro-Ala-ßNA	AFPA	aminopeptidases with βNA	v	+	+	-	+	+	+	v	-	+	v
Asn-ßNA	N	aminopeptidases with βNA	v	v	+	+	+	-	-	v	-	+	-
Trp-βNA	W	aminopeptidases with βNA	+	+	v	-	-	+	+	+	+	+	-
Glu-His-ßNA	EH	aminopeptidases with βNA	+	+	+	-	-	+	-	-	+	+	-
Ac-Lys-Ala-ßNA	AcKA	aminopeptidases with βNA	-	v	+	+	+	+	+	-	-	+	+
D-Ala-D-Ala-ßNA	dAdA	aminopeptidases with βNA	+	-	v	-	-	-	+	v	+	v	-
Val-ßNA	V	aminopeptidases with βNA	-	-	v	-	-	-	-	-	-	-	-
Ile-ßNA	I	aminopeptidases with βNA	-	v	v	-	-	-	-	v	-	-	-
Val-Tyr-Ser-ßNA	VTS	aminopeptidases with βNA	-	v	+	+	-	-	-	-	-	+	-
Asp-ßNA	D	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-
Glu-ßNA	E	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-
Pyr-ßNA	Pyr	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-

ß-Ala-ßNA	βA	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-
H-Val-4-MßNA	V4M	aminopeptidases with βNA	-	-	-	-	-	-	-	-	-	-	-
bis-p-nitrophenyl phosphate	BISPH7	phosphatases	-	-	v	-	-	-	-	-	+	+	-
p-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol)	PHOS7	phosphatases	-	-	-	-	-	-	-	-	+	+	-
p-nitrophenyl-a-d-glucopyranoside	aGLU7	glucosidases	-	-	+	-	v	+	-	-	+	+	-
p-nitrophenyl-a-d-maltoside	aMAL7	glucosidases	-	-	-	-	-	-	-	-	+	+	-
p-nitrophenyl-a-d-glucopyranoside	aGLU5	glucosidases	-	-	v	-	-	-	-	-	+	+	-
p-nitrophenyl-a-d-xylopyranoside	aXYL7	glucosidases	-	-	+	-	-	-	-	-	+	+	-
p-nitrophenyl-n-acetyl-ß-D-glucosaminide	CHIT7	esterases	-	-	v	-	-	-	-	-	+	+	-
i-erythritol	EROL	sugar derivates	+	+	v	-	-	+	v	-	+	v	-
L(-)-fucose	L-FUC	monosaccharides	-	v	-	-	v	-	v	-	+	+	-
D-glucose-L-cysteine	GLUCY	sugar derivates	v	v	+	-	-	+	v	v	+	+	v
D(-)-ribose	D-RIB	monosaccharides	v	-	v	-	+	-	-	-	+	v	-
D(-)-arabinose	D-ARA	monosaccharides	-	v	-	-	-	-	-	-	v	v	-
D(+)-glucose	D-GLU	monosaccharides	-	v	v	-	-	+	-	-	+	v	-
L(+)-arabinose	L-ARA	monosaccharides	v	-	v	-	-	+	v	-	+	+	-
2-deoxy-D-galactose	DOGAL	monosaccharides	-	-	-	-	-	-	-	-	-	-	-
D(+)-galactose	D-GAL	monosaccharides	v	-	v	-	-	-	-	-	+	+	-
D(+)-xylose	D-XYL	monosaccharides	-	-	v	-	-	-	-	-	+	-	-
a-D-talose	D-TAL	monosaccharides	+	+	+	-	-	+	+	-	+	-	-
D-threitol	D-TOL	sugar derivates	+	-	v	v	+	-	+	-	+	+	-
adonite	ADON	monosaccharides	+	+	v	v	-	+	+	+	+	-	+
sucrose	SUCR	disaccharides	-	-	+	-	+	-	-	-	+	+	-
iso-maltose	MALTO	disaccharides	-	-	+	-	+	-	-	v	+	+	-
D(-)-fructose	D-FRU	monosaccharides	v	+	+	-	+	+	+	-	+	+	+
1-deoxy-1-nitro-D-sorbitol	DNSOL	sugar derivates	-	-	-	-	-	-	-	-	-	-	-
D(-)-lyxosylamine	LYXA	sugar derivates	-	-	+	-	+	+	+	-	+	+	+
palatinose	PAL	disaccharides	-	-	+	-	+	-	-	-	+	+	-
myo-inositol	INOL	sugar derivates	-	-	-	-	-	-	-	-	-	+	-
inosine	INON	sugar derivates	-	-	v	-	+	-	+	-	+	-	-
gallic acid	Galli	organic acids	-	+	+	-	+	+	v	-	+	v	+
DL-lactic acid	dlLac	organic acids	-	-	+	-	-	-	-	-	+	v	-
acetate	Acet	amino acid derivates	-	-	+	+	+	+	-	-	+	+	+

L-asparagine	L-Asn	amino acids	+	+	v	-	-	-	-	-	+	+	-
L-glutamic acid	L-Glu	amino acids	v	+	v	-	v	+	-	-	+	+	-
ala-gln	AlaGln	amino acids	v	-	-	-	-	-	-	-	+	v	-
guanidinosuccinic acid	GuaSu	organic acids	v	-	v	-	v	-	-	-	+	v	-
L-cystine	L-Cyss	amino acids	+	+	v	+	-	-	v	+	+	-	v
D-alanine	D-Ala	amino acids	v	+	v	-	-	+	-	-	+	+	-
propionic acid	Propn	organic acids	-	+	v	-	-	+	v	-	-	+	-
L-alanine	L-Ala	amino acids	+	+	v	-	v	-	-	-	+	+	-
DL-ß-hydroxybutyric acid	ßHBut	organic acids	-	-	+	-	+	+	+	-	+	+	+
D-asparagine	D-Asn	amino acids	v	+	-	-	-	+	-	-	-	v	-
L-arginine	L-Arg	amino acids	-	-	+	-	v	-	-	-	+	+	-
Na-acetyl-l-arginine	AcArg	amino acid derivates	-	-	+	-	+	-	-	-	v	+	-
glyoxylic acid	Glyx	organic acids	-	-	-	-	-	-	-	-	-	+	-
L-serine	L-Ser	amino acids	-	v	-	-	-	-	-	-	+	+	-
hippuryl-arg	HipArg	peptides	-	-	v	-	v	-	-	-	v	+	-
L-carnosine	L-Caro	amino acids	-	-	v	-	-	-	-	-	+	+	-
glycine	Gly	amino acids	-	-	-	-	-	-	-	-	+	+	-
adipic acid	Adipa	organic acids	-	-	v	-	+	-	+	-	+	+	-
L-proline	L-Pro	amino acids	-	+	-	-	-	-	-	-	+	+	-
D-proline	D-Pro	amino acids	-	+	-	-	-	-	-	-	+	+	-
fumaric acid	Fuma	organic acids	-	-	-	-	-	-	-	-	+	+	-
D-serine	D-Ser	amino acids	-	+	-	-	-	-	-	-	-	-	-
glycolic acid	Glyc	organic acids	-	-	-	-	-	-	-	-	-	+	-
Adenine	Adeni	amino acid derivates	+	+	v	-	+	-	+	v	-	-	+
glutaric acid	Gluta	organic acids	-	-	-	-	-	-	-	-	+	+	v
mesaconic acid	Mesac	organic acids	-	-	-	-	-	-	-	-	+	-	-
D-histidine	D-His	amino acids	-	-	-	-	-	-	-	-	-	+	-
nitrite	NTI	classical reactions	-	-	-	-	-	-	-	-	+	+	-
nitrate	NTA	classical reactions	+	v	+	-	v	-	+	+	+	v	-
pyrazinamidase	PCA	classical reactions	v	+	v	-	-	+	+	+	+	v	+
Voges Proskauer	VP	classical reactions	+	+	v	-	+	+	+	+	+	+	+
Urease	**Urease**	**classical reactions**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
hydrogen sulfide	H2S	classical reactions	-	-	v	-	-	-	-	-	+	+	-