Gram-negative bacteria associated with brewery yeasts: reclassification of *Obesumbacterium proteus* biogroup 2 as *Shimwellia pseudoproteus* gen. nov., sp. nov., and transfer of *Escherichia blattae* to *Shimwellia blattae* comb. nov.

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Phylogenetic analyses of type and reference strains of *Obesumbacterium proteus* biogroups 1 and 2 plus a novel isolate of biogroup 2 were carried out based on 16S rRNA gene sequences and partial sequences of four protein-coding genes (*fusA*, *leuS*, *pyrG* and *rpoB*). Both approaches revealed that *O. proteus* biogroup 1 strains were closely related to *Hafnia alvei*. Biogroup 2 strains, however, formed a distinct monophyletic clade of generic status that included *Escherichia blattae*. Phenotypic tests were consistent with the molecular classification and provided diagnostic features. It is proposed that biogroup 2 strains be placed in a new genus, *Shimwellia* gen. nov., as *Shimwellia pseudoproteus* sp. nov., with strain 521^T (=DSM 3038^T=LMG 24835^T=NCIMB 14534^T) as the type strain, and that *Escherichia blattae* be transferred to the genus *Shimwellia* as *Shimwellia blattae* comb. nov., with strain ATCC 29907^T (=DSM 4481^T) as the type strain.

Traditional beer brewing practice involves using a portion of the yeast harvested from a current fermentation as inoculum for the next fermentation. However, this leads to the accumulation of Gram-negative bacteria with the yeast and, after about 10 fermentations, the extent of contamination is such that the yeast is generally discarded in favour of a new, pure-culture inoculum, or it is treated with acid to destroy the contaminating bacteria (Strandskov *et al.*, 1953; Van Vuuren & Priest, 2003).

Abbreviation: MLST, multilocus sequence typing.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Obesumbacterium proteus* strains NCIMB 8771^T, 511 and 540, *Shimwellia* [*Escherichia*] *blattae* DSM 4481^T, and *Shimwellia pseudoproteus* 521^T and Am-4 are FJ267521, FJ492810, FJ267522, FJ267520, FJ267523 and FJ492809, respectively. Accession numbers for protein-coding genes are as follows. *S. blattae* DSM 4481^T: *fusA*, FJ267524; *leuS*, FJ267525; *pyrG*, FJ267526; and *rpoB*, FJ267527. *O. proteus* NCIMB 8771^T: *fusA*, FJ267528; *leuS*, FJ267529; *pyrG*, FJ267530; and *rpoB*, FJ267531. *O. proteus* 511: *fusA*, FJ492813; *leuS*, FJ492816; *pyrG*, FJ492819; and *rpoB*, FJ492822. *O. proteus* 540: *fusA*, FJ492811; *leuS*, FJ492814; *pyrG*, FJ492817; and *rpoB*, FJ492820. *S. pseudoproteus* 521^T: *fusA*, FJ267532; *leuS*, FJ267533; *pyrG*, FJ267534; and *rpoB*, FJ267535. *S. pseudoproteus* Am-4: *fusA*, FJ492812; *leuS*, FJ492815; *pyrG*, FJ492818; and *rpoB*, FJ492821.

Details of strains used in the construction of MLST and 16S rRNA gene trees are available as supplementary material with the online version of this paper.

These bacteria were first isolated in pure culture in 1936 and classified as *Flavobacterium proteus* (Shimwell, 1936; Shimwell & Grimes, 1936). Later, the genus *Obesumbacterium* was created for these bacteria, with *Obesumbacterium proteus* as the sole species (Shimwell, 1963, 1964). More detailed taxonomic investigation assigned *Obesumbacterium* to the *Enterobacteriaceae* and revealed two distinct taxa within *O. proteus*, referred to as biogroups 1 and 2 (Priest *et al.*, 1973). These two biogroups have been substantiated by various phenotypic (Prest *et al.*, 1994; van Vuuren *et al.*, 1981) and molecular methods such as ribotyping (Koivula *et al.*, 2006; Prest *et al.*, 1994) and PCR procedures (Maugueret & Walker, 2002). DNA–DNA hybridization studies indicated that biogroup 1 strains share 75–78% renaturation with *Hafnia alvei* and that biogroup 2 strains have an affinity with *Escherichia blattae*, a bacterium isolated from the hindgut of cockroaches (Burgess *et al.*, 1973; Brenner, 1981). In this study, the almost complete sequences of the 16S rRNA genes and partial sequences of four protein-coding genes (*fusA*, *leuS*, *pyrG* and *rpoB*) of reference strains of *O. proteus*, a novel isolate of biogroup 2 from brewery yeast, and the type strain of *Escherichia blattae* have been determined. Phylogenetic analyses using these and reference sequences confirmed that *O. proteus* biogroup 1 is closely related to *Hafnia alvei*, but revealed that *O. proteus* biogroup 2 strains and *Escherichia blattae* are phylogeneti-

cally distinct and together merit separate generic status within the family *Enterobacteriaceae* as two species.

Bacteria were grown routinely on nutrient agar at 30 °C. They included *O. proteus* biotype 1 strain NCIMB 8771^T and two biotype 1 isolates from ale brewery yeasts from the UK, strains 511 and 540 (=LMG 3048) (Priest *et al.*, 1973). *O. proteus* biotype 2 strain 521 (=DSM 3038=LMG 24835) has been described previously (Priest *et al.*, 1973). *Escherichia blattae* DSM 4481^T was obtained from the DSMZ, Braunschweig, Germany. *O. proteus* Am-4 (available as LMG 24836) was isolated from ale brewery yeast from Edinburgh; the yeast sample was plated directly on MacConkey agar supplemented with 25 µg cycloheximide ml⁻¹ and incubated at 30 °C for 2 days. Small, lactose-negative colonies were purified by plating on the same medium. DNA was prepared from strains grown in nutrient broth for 18 h at 30 °C. The culture (2 ml) was centrifuged and DNA was isolated using a PureGene isolation kit as described by the manufacturer. 16S rRNA genes were amplified and sequenced using standard primers at an annealing temperature of 56 °C: 27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 926F, 5'-AAACTCA-AAGGAATTGACGG-3'; 685R3, 5'-TCTACGCATTTCACCGCTAC-3'; 1100R, 5'-GGGTTGCGCTCGTTG-3'; and 1492R, 5'-TACGGYTACCTTGTACGACTT-3'. Genes for *fusA*, *leuS*, *pyrG* and *rpoB* were amplified and sequenced using the primers described by Salerno *et al.* (2007) (annealing temperatures in parentheses): *fusA3-fusA4* (58 °C), *leuS3-leuS4* (58 °C), *pyrG3-pyrG4* (55 °C) and *VIC4-VIC6* (55 °C). PCR mixtures (50 µl) contained 0.2 mM of each nucleotide in 10 × NH₄ buffer (Bioline), 0.25 µM primers, 2.5 mM MgCl₂ for protein-coding genes (2.0 mM MgCl₂ for 16S rRNA genes), and ~100 ng DNA template, made up to 49.7 µl with sterile deionized water. The PCR comprised denaturation at 95 °C for 5 min, followed by the addition of 0.3 µl Biotaq enzyme (5 U µl⁻¹; Bioline). There were 30 cycles of denaturation at 95 °C for 1 min, annealing for 2 min and elongation at 72 °C for 2 min. Final elongation was at 72 °C for 10 min and samples were stored at 4 °C. Amplification products were checked using agarose gel electrophoresis, purified by polyethylene glycol precipitation (Embley, 1991) and sequenced by MWG Biotech. Forward and reverse sequences were assembled using STADEN software.

16S rRNA gene sequences (1349–1352 bases) for representative *Enterobacteriaceae* species were obtained from GenBank. Wherever possible, sequence data from the type strains were chosen and strains were selected to match the multilocus sequence typing (MLST) data of Salerno *et al.* (2007). Details of the strains used and accession numbers of the gene sequences are given in Supplementary Table S1 (available in IJSEM Online). Sequences were aligned using CLUSTAL_X and neighbour-joining trees were constructed. The trees were visualized using TREEVIEW 1.6.6 (Page, 1996). Bootstrap values (%) were determined for 1000 replications using CLUSTAL_X. Maximum-parsimony trees were constructed using MEGA version 4 (Tamura *et al.*, 2007).

The four protein-coding alleles were concatenated to provide sequences of 2082 bases (*fusA*, 633; *leuS*, 642; *pyrG*, 306; and *rpoB*, 501), with the exception of *Haemophilus influenzae* Rd KW20, which was 2085 bases due to a 3 bp insertion in *rpoB*. Nucleotide sequences for MLST were obtained from GenBank (EU010012–EU010119). Sequences were aligned and neighbour-joining trees were constructed as described above.

Yeast from three breweries in the south of Scotland was examined for the presence of Gram-negative bacteria by culturing on MacConkey agar containing cycloheximide to inhibit yeast growth. Only one sample contained lactose-negative bacteria typical of *O. proteus*, which were recovered in pure culture as strain Am-4. The bacterium was isolated from the only brewery that did not practice acid-washing of its yeast to remove contaminating organisms. Almost complete 16S rRNA gene sequences (1412–1496 bases) were prepared from: *O. proteus* biogroup 1 strains NCIMB 8771^T, 511 and 540; *O. proteus* biogroup 2 strains 521 and Am-4; and *Escherichia blattae* DSM 4481^T. The sequences were aligned with 16S rRNA gene sequences of representatives of the *Enterobacteriaceae* and a phylogenetic tree was constructed (Fig. 1). This revealed that the two biogroups of *O. proteus* were distantly related. The three biogroup 1 strains had identical 16S rRNA genes that showed 99% similarity with that of *Hafnia alvei* ATCC 13337^T. In the *O. proteus* NCIMB 8771^T sequence of 1436 bases, there were seven ambiguous bases at positions 434 (A or G), 971 (A or G), 972 (G or C), 973 (C or T), 980 (A or G), 981 (G or C) and 982 (C or T). These probably represent intragenomic variation in the rRNA genes since they lie in regions (H17 and H33) noted for such heterogeneity (Case *et al.*, 2007). The two 16S rRNA gene sequences from *O. proteus* biogroup 2 strains 521 and Am-4 were identical and shared 99% similarity with that of *Escherichia blattae* DSM 4481^T. Moreover, the *Escherichia blattae* 16S rRNA gene sequence shared little similarity with those of the type strains of *Escherichia coli* and *Escherichia fergusonii* (95 and 96%, respectively) showing that the bacterium is incorrectly placed in the genus *Escherichia*. Trees constructed using maximum-parsimony were similar topologically to the neighbour-joining tree and, in particular, confirmed that *O. proteus* biogroup 1 strains were closely related to *Hafnia alvei* with *Serratia liquefaciens* as a close relative, whereas the biogroup 2 strains included *Escherichia blattae* and formed a cluster with *Erwinia amylovora* and representatives of the genus *Pantoea* (data not shown).

To understand the phylogenetic positions of the *O. proteus* biogroups in more detail, the four protein-coding genes as used for the MLST of *Plesiomonas shigelloides* (Salerno *et al.*, 2007) were analysed. The fifth gene from the MLST study, *recG*, was not amplified in our strains using the recommended primers. Trees derived from the individual gene sequences, together with reference sequences from GenBank, showed limited topological variation suggesting limited horizontal gene transfer (data not shown). A

Fig. 1. Neighbour-joining tree of representative strains from the *Enterobacteriaceae* using almost complete 16S rRNA gene sequences. Numbers at nodes are bootstrap values (%; 1000 replicates). Bar, 0.01 nt substitutions per site.

consensus tree derived from the concatenated gene sequences is shown in Fig. 2. The three *O. proteus* biogroup 1 strains had identical sequences for all alleles, as did the two biogroup 2 strains. The MLST tree supported the 16S rRNA gene tree and again showed that the two biogroups of *O. proteus* are phylogenetically distinct: biogroup 1 strains were closely related to *Hafnia alvei*, whereas the biogroup 2 strains formed a monophyletic group with *Escherichia blattae*. As with the rRNA tree, *Escherichia blattae* was distinct phylogenetically from other species of the genus *Escherichia*.

Protein-coding genes tend to accumulate more mutations than rRNA genes and can provide insight into species relationships at a higher resolution than rRNA genes. Alignments of the MLST fragments (2082 bases) were compared for all cases in which there was more than one species representing a genus. The two most closely related species within a genus were *Escherichia coli* and *Escherichia fergusonii* (98 %) and the two most distant species within a genus were *Pantoea agglomerans* and *Pantoea stewartii* subsp. *stewartii* (86 %). For the 12 comparisons covering *Citrobacter*, *Escherichia*, *Enterobacter*, *Pantoea* and

Fig. 2. Neighbour-joining tree of representative strains from the *Enterobacteriaceae* using the concatenated sequences of four loci (*fusA*, *leuS*, *pyrG* and *rpoB*). With the exception of *Morganella morganii* subsp. *morganii*, strains used are the same as those for Fig. 1, but in some cases type strains were from different culture collections (see Supplementary Table S1 available in IJSEM Online). Numbers at nodes are bootstrap values (%; 1000 replicates). Bar, 0.05 nt substitutions per site.

Salmonella species, the mean similarity over the 2082 bases was 92.2% (3.25 standard deviation). In this context, *O. proteus* biogroup 2 strains and *Escherichia blattae* shared 93% similarity, providing convincing evidence that they represent distinct species.

The molecular classification of these bacteria is generally supported by phenotype. The original numerical classification adequately separated *O. proteus* biotypes 1 and 2 and key diagnostic tests are shown in Table 1. Phenotypic descriptions of these bacteria based on larger numbers of

strains have been published by Priest *et al.* (1973), van Vuuren *et al.* (1981) and Farmer & Brenner (2005).

The genus *Obesumbacterium* has been associated with brewery yeasts for more than 40 years and we have shown that biogroup 2 strains, at least, still occupy this environmental niche. Biogroup 1 strains are less common in breweries than those of biogroup 2 (Priest *et al.*, 1973) and have also been associated with the intestines of fish (Skrodenyte-Arbaciauskiene *et al.*, 2006) and edible snails (Charrier *et al.*, 2006), whereas biogroup 2 strains have not

Table 1. Differential phenotypic characteristics of *Obesumbacterium proteus* biogroup 1, *Shimwellia pseudoproteus* sp. nov., *Shimwellia blattae* comb. nov. and *Hafnia alvei*

Taxa: 1, *O. proteus* biogroup 1; 2, *Shimwellia pseudoproteus* sp. nov. (previously *O. proteus* biogroup 2); 3, *Shimwellia blattae* comb. nov. (previously *Escherichia blattae*); 4, *Hafnia alvei*. +, 90 % or more positive; –, 0–9.9 % positive; v, 10.0–89.9 % positive. Data from Brenner (1981) and Farmer & Brenner (2005).

Characteristic	1	2	3	4
Catalase	+	Delayed*	+	+
Aesculin hydrolysis	+	–	–	v
Malonate utilization	v	–	+	v
Motility (24 h)	–	–	–	+
Acid production from:				
L-Arabinose	v	–	+	+
Mannitol	+	–	–	+
Salicin	+	–	–	v
D-Xylose	–	v	+	+
Gas from glucose	–	–	+	+
Voges–Proskauer test	+	–	–	+

*The catalase reaction is weak and delayed by about 30 s.

been isolated outside the brewery. The original *O. proteus* isolate of Shimwell has been lost (Farmer & Brenner, 2005) and the current type strain was provided by F. B. Strandkov and J. B. Bockelmann, who isolated the bacterium from yeast in their North American brewery, although its similarity to the original isolate was substantiated by J. L. Shimwell. However, the descriptions at that time were limited and, as discussed by Farmer and Brenner, were not definitive (Farmer & Brenner, 2005). So we do not know whether Shimwell's original isolates were of biogroup 1 or 2. Farmer & Brenner (2005) refer to *O. proteus* biogroup 1 strains as *Hafnia alvei* biogroup 1 because of their close relationship with *Hafnia alvei* on the basis of DNA–DNA reassociation (75 % renaturation). However, our comparison of the MLST sequences showed only 95 % similarity between these taxa which, while greater than the mean similarity of 92 % between species, is less than that which separates the two *Escherichia* species included in this study (98 %) or *Citrobacter freundii* from *Citrobacter braakii* (96 %). It is therefore recommended that *O. proteus* biogroup 1 strains retain the name *O. proteus* until the systematics of the genus *Hafnia* have been clarified (Janda & Abbott, 2006) and the relationships between these bacteria and *Hafnia alvei* are understood more clearly. This leaves the *O. proteus* biogroup 2 strains, which represent a distinct taxonomic entity within the *Enterobacteriaceae* and for which the name *Shimwellia pseudoproteus* gen. nov., sp. nov. is proposed. Strain 521, isolated from yeast from Davenports Brewery, Birmingham, UK, in 1972 is the earliest extant strain and, as such, it is proposed that this strain be designated the type strain of the novel species. It is also recommended that

Escherichia blattae is transferred to the genus *Shimwellia* as *Shimwellia blattae* comb. nov. Phenotypic features that distinguish these taxa are shown in Table 1.

Description of *Shimwellia* gen. nov.

Shimwellia (Shim.wel'li.a. N.L. fem. n. *Shimwellia* named after J. L. Shimwell who first isolated the bacterium).

Cells are non-motile, straight Gram-stain-negative rods that occur singly. Members conform to the general description of the family *Enterobacteriaceae*. Aerobic and facultatively anaerobic. Oxidase-negative and catalase-positive, although this reaction is delayed and weak in the type species. Cells reduce nitrate to nitrite, do not grow in KCN and do not produce H₂S. The type species is *Shimwellia pseudoproteus*.

Description of *Shimwellia pseudoproteus* sp. nov.

Shimwellia pseudoproteus (pseu.do.pro'te.us. Gr. adj. *pseudes* false; L. n. *proteus* the ancient sea-god, noted for being able to change his form at will, and also a bacterial epithet; N.L. masc. n. *pseudoproteus*, the false [*Obesumbacterium*] *proteus*).

This description is taken from Priest *et al.* (1973), Brenner (1981) and Farmer & Brenner (2005). Displays the following properties in addition to those given in the genus description. There is a tendency for shapes to be pleomorphic on initial isolation. Mesophilic with optimum growth near 30 °C and between pH 4.5 and 8.0. After growth on nutrient agar for 48 h, cells form small, circular, entire colonies, 1.0–1.5 mm in diameter with a convex elevation and smooth glassy surface. Acid, but no gas, is produced from glucose, rhamnose and trehalose. No acid is produced from lactose, mannitol, salicin or numerous other sugars. Does not utilize malonate. Arginine dihydrolase-negative, lysine decarboxylase-positive. Voges–Proskauer-negative. The DNA G+C content is 48 ± 1 mol %.

The type strain is 521^T (=DSM 3038^T=LMG 24835^T=NCIMB 14534^T), isolated from ale brewery yeast in Birmingham, UK. Strain Am-4 is an additional strain of the species.

Emended description of *Shimwellia blattae* comb. nov.

Shimwellia blattae (blat'tae. L. fem. n. *blatta* cockroach; L. gen. n. *blattae* of the cockroach).

Basonym: *Escherichia blattae* Burgess *et al.* 1973.

The description is the same as that given by Burgess *et al.* (1973) and Scheutz & Strockbine (2005) with the following additional properties, including those given in the genus description. After growth on nutrient agar for 48 h, colonies are 1–5 mm diameter, circular, smooth, glossy, creamy in colour with a convex elevation and an entire margin. Acid and gas are produced from glucose; acid is

produced from L-arabinose, glycerol, D-mannose, L-rhamnose and D-xylose. Methyl-red-positive, indole-negative. Arginine dihydrolase-negative; positive for lysine and ornithine decarboxylases. Voges-Proskauer-negative. The DNA G+C content has not been determined.

The type strain is ATCC 29907^T (=CDC 9005-74^T=CIP 103175^T=CIP 104942^T=DSM 4481^T=HAMBI 1692^T=JCM 1650^T=LMG 3030^T=NCTC 12127^T).

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