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Reclassification of *Enterococcus flavescens* Pompei *et al.* 1992 as a later synonym of *Enterococcus casseliflavus* (*ex* Vaughan *et al.* 1979) Collins *et al.* 1984 and *Enterococcus saccharominimus* Vancanneyt *et al.* 2004 as a later synonym of *Enterococcus italicus* Fortina *et al.* 2004

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The taxonomic relatedness between the species *Enterococcus casseliflavus* and *Enterococcus flavescens* and between *Enterococcus italicus* and *Enterococcus saccharominimus* was investigated. Literature data had already indicated the synonymy between *E. casseliflavus* and *E. flavescens*, but this observation had not been formally published. Additional evidence that the two taxa represent a single species was provided by comparison of the partial sequences for three housekeeping genes, phenylalanyl-tRNA synthase alpha subunit (*pheS*), RNA polymerase alpha subunit (*rpoA*) and the alpha subunit of ATP synthase (*atpA*). Additional genomic data derived from DNA–DNA hybridization demonstrated that the two species are synonymous. For *E. italicus* and *E. saccharominimus*, two recently described taxa, a high 16S rRNA gene sequence similarity of $>99\,\%$ and analogous phenotypic features indicated a close taxonomic relatedness. The same multilocus sequence analysis scheme for the three housekeeping genes was also applied for *E. italicus* and *E. saccharominimus* and indicated possible conspecificity, an observation that was also confirmed by a high DNA–DNA hybridization value ($\geqslant 78\,\%$). Data from the present study led to the proposal that *E. flavescens* should be reclassified as a later synonym of *E. casseliflavus* and that *E. saccharominimus* should be reclassified as a later synonym of *E. italicus*.

The description of *Enterococcus casseliflavus* can be traced after the revival of the genus *Enterococcus* by Schleifer & Kilpper-Bälz (1984) to include the Lancefield group D faecal streptococci *Streptococcus faecalis* and *Streptococcus faecium*, as *Enterococcus faecalis* and *Enterococcus faecium* (Farrow *et al.*, 1983; Leclerc *et al.*, 1996; Ludwig *et al.*, 1985). *E. casseliflavus* was originally described as *Streptococcus faecium* var. *casseliflavus* (Mundt & Graham, 1968), later elevated to species rank as *Streptococcus casseliflavus* (Vaughan *et al.*, 1979) and finally transferred to the genus *Enterococcus* (Schleifer & Kilpper-Bälz, 1984). The species has been isolated from plants, silage and soil (Schleifer & Kilpper-Bälz, 1984), but it is also clinically significant and has been incriminated in blood infections (Nauschuetz *et al.*, 1993). *Enterococcus flavescens* was described based on four strains of enterococci isolated from humans with severe infections (Pompei *et al.*, 1991). These strains were further investigated and designated *E. flavescens* (Pompei *et al.*, 1992). Phenotypically, *E. casseliflavus* and *E. flavescens* are yellow-pigmented, motile and possess intrinsic low-level resistance to vancomycin; *E. flavescens* can be differentiated from *E. casseliflavus* through its inability to produce acid from the fermentation of ribose (Pompei *et al.*, 1992) and its failure to produce α-haemolysis on sheep blood (Descheemaeker *et al.*, 1997). Since its initial identification, some doubt has

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Abbreviations: MLSA, multilocus sequence analysis; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the *pheS*, *rpoA* and *atpA* gene sequences determined in this study are given in Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online.

Neighbour-joining trees based on *rpoA* and *atpA* gene sequences of enterococcal strains are available as supplementary material in IJSEM Online.

remained over the validity of describing *E. flavescens* as a distinct species. The 16S rRNA gene sequences of the type strains of *E. casseliflavus* and *E. flavescens* show about 100 % similarity (Patel *et al.*, 1998). Furthermore, DNA–DNA hybridization experiments confirmed that *E. casseliflavus* and *E. flavescens* constitute a single species (Teixeira *et al.*, 1997), in contrast to the data reported by Pompei *et al.* (1992). Other literature data were in complete accordance with Teixeira *et al.* (1997), supporting the synonymy between the two taxa. Descheemaeker *et al.* (1997) were unable to discriminate between the two taxa using PFGE or oligonucleotide D11344-primed PCR. Clark *et al.* (1998) and Dutta & Reynolds (2003) reported an extensive similarity between the sequences of the vancomycin resistance genes *vanC-2* of *E. casseliflavus* and *vanC-3* of *E. flavescens*. Other techniques, such as $(GTG)_5$-PCR (Švec *et al.*, 2005), randomly amplified polymorphic DNA (RAPD) analysis (Quednau *et al.*, 1998), tRNA intergenic spacer PCR (Baele *et al.*, 2000) and sequence comparison of genes encoding manganese-dependent superoxide dismutase ($sodA_{int}$) (Poyart *et al.*, 2000) and D-alanine–D-alanine ligase-related enzymes (*ddl*) (Navarro & Courvalin, 1994) and *vanC* genes (Dutka-Malen *et al.*, 1995) were also unable to differentiate between *E. casseliflavus* and *E. flavescens*. Despite these data supporting the synonymy between *E. casseliflavus* and *E. flavescens*, no formal reclassification has been proposed to date.

In the present study, we provide additional evidence that the two taxa represent a single species. Multilocus sequence analysis (MLSA) is a polygenic approach applied for accurate identification of all enterococcal species (Naser *et al.*, 2005a, b). Partial sequences for the genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*), RNA

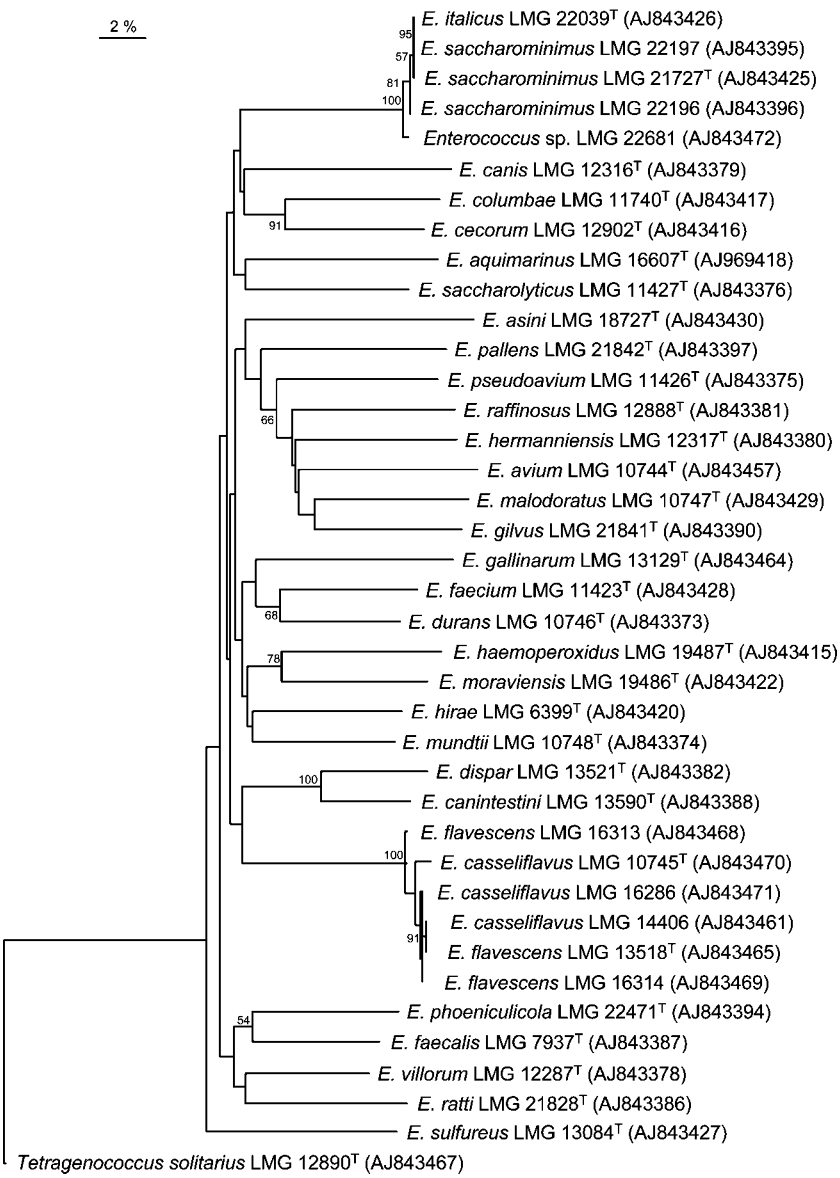

Fig. 1. Neighbour-joining tree based on *pheS* gene sequences of enterococcal strains. *Tetragenococcus solitarius* LMG 12890^T was included as an outgroup. Bootstrap percentages (⩾50) after 500 simulations are shown. Bar, 2 % sequence divergence.

polymerase alpha subunit (*rpoA*) and the alpha subunit of ATP synthase (*atpA*) were determined and compared for *E. casseliflavus* LMG 10745^T, LMG 16286 and LMG 14406 and *E. flavescens* LMG 13518^T, LMG 16313 and LMG 16314. Primer sequences, amplification conditions and sequencing reactions were as described by Naser *et al.* (2005a, b). In general, the interspecies-level gene sequence similarities of *pheS*, *rpoA* and *atpA* for all enterococcal species tested were at most 86, 97 and 92 %, respectively. Strains of the same species showed at least 97 % *pheS*, 99 % *rpoA* and 96·3 % *atpA* gene sequence similarity. The neighbour-joining trees of *pheS*, *rpoA* and *atpA* gene sequences revealed high relatedness between the investigated strains of *E. casseliflavus* and *E. flavescens*, with at least 99 % *pheS*, 100 % *rpoA* and 99 % *atpA* gene sequence similarity, confirming that *E. flavescens* and *E. casseliflavus* represent the same species (Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online).

As a next step, DNA–DNA hybridizations were performed between *E. casseliflavus* LMG 10745^T and LMG 14406 and between *E. flavescens* LMG 13518^T and LMG 16314. Genomic DNA was prepared according to the protocol of Pitcher *et al.* (1989) with the following modifications: the washed cell pellet was resuspended and lysed in buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8·0) containing RNase (200 μg ml^{-1}; Sigma), mutanolysin (100 U ml^{-1}; Sigma) and lysozyme (25 mg ml^{-1}; SERVA) for 1 h at 37 °C. The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to the microplate wells. Hybridizations were performed at 36 °C in hybridization mixture (2× SSC, 5× Denhardt's solution, 2·5 % dextran sulfate, 50 % formamide, 100 μg denatured salmon sperm DNA ml^{-1} and 1250 ng biotinylated probe DNA ml^{-1}). Reciprocal reactions (e.g. A × B and B × A) were performed. The DNA–DNA binding values reported are the mean values of a minimum of four hybridization experiments, the reciprocal reactions included. *E. casseliflavus* LMG 10745^T and LMG 14406 and *E. flavescens* LMG 13518^T and LMG 16314 showed high DNA–DNA hybridization values in the range 78–94 %, confirming that the two taxa belong to the same species.

On the basis of the evidence presented, it is proposed that the two species *E. casseliflavus* and *E. flavescens* be united under the same name; as a rule of priority (Rules 38 and 42 of the *Bacteriological Code*; Lapage *et al.*, 1992), the name *E. casseliflavus* should be retained and strains of *E. flavescens* should be reclassified as such. The type strain of *E. casseliflavus* is LMG 10745^T (=ATCC 25788^T=NCDO 2372^T= MUTK 20^T). The description of *E. casseliflavus* remains essentially the same.

In the present study, we also investigated the taxonomic relatedness between *Enterococcus italicus* and *Enterococcus saccharominimus*, as the two taxa have a 16S rRNA gene sequence similarity of >99 % and are phenotypically highly similar. The two species were described almost simultaneously in 2004. *E. italicus* was described by Fortina *et al.* (2004), who isolated the organism from cows' raw milk used in the production of artisanal Italian cheeses. *E. saccharominimus* was described by Vancanneyt *et al.* (2004) and was isolated from Belgian, Morrocan and Romanian dairy products.

MLSA of three housekeeping genes (see above) was used as an initial screening test to investigate the relatedness of the two species. Gene sequences were determined and compared for *E. saccharominimus* LMG 21727^T, LMG 22196 and LMG 22197, *E. italicus* LMG 22039^T and *Enterococcus* sp. CDC PNS-E1 (=LMG 22681), which was designated as a strain of *E. italicus* (R. R. Facklam, personal communication). The results confirmed that *E. saccharominimus* and *E. italicus* are very highly related, with 100 % *pheS*, *rpoA* and *atpA* gene sequence similarities (Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online).

Finally, DNA–DNA hybridizations were performed as described above between *E. italicus* LMG 22039^T and LMG 22681 and beween *E. saccharominimus* LMG 21727^T and LMG 22196. The DNA–DNA hybridization level between the four strains was in the range 78–87 %, clearly indicating that the two species constitute a single species.

On the basis of the evidence presented, it is proposed that the two species *E. saccharominimus* and *E. italicus* be united under the same name; as a rule of priority (Rules 38 and 42 of the *Bacteriological Code*; Lapage *et al.*, 1992), the name *E. italicus* should be retained and strains of *E. saccharominimus* should be reclassified as such. The type strain of *E. italicus* is DSM 15952^T (=LMG 22039^T). The description of *E. italicus* remains essentially the same.

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