Biochemical and genetic evidence for the transfer of *Enterococcus solitarius* Collins *et al.* 1989 to the genus *Tetragenococcus* as *Tetragenococcus solitarius* comb. nov.

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Phylogenetic analysis of 16S rRNA gene sequences revealed that *Enterococcus solitarius* is not a member of the genus *Enterococcus*, but is related to species of the genus *Tetragenococcus*. On a phylogenetic tree, *E. solitarius* clustered with *Tetragenococcus halophilus* and *Tetragenococcus muriaticus*, with which it showed the highest 16S rRNA gene sequence similarity level (about 94 %). Phenotypic studies indicated that *E. solitarius* was also unable to produce acid from lactose, providing further evidence of its affiliation to the genus *Tetragenococcus*. DNA hybridization studies indicated that *E. solitarius* was clearly a separate species, different from *T. halophilus* and *T. muriaticus* (reassociation levels of about 23 and 54 %, respectively). As suggested in previous studies, *E. solitarius* is closely related to but clearly distinct from *T. halophilus*. Based upon properties that taxonomically distinguish it from species of the genus *Enterococcus*, it is proposed that *E. solitarius* be transferred to the genus *Tetragenococcus* and reclassified as *Tetragenococcus solitarius* comb. nov. (type strain, 885/78^T=ATCC 49428^T=CCUG 29293^T=CIP 103330^T=DSM 5634^T=JCM 8736^T=LMG 12890^T=NCTC 12193^T).

Enterococcus solitarius was first described by Collins *et al.* (1989) and isolated from a human sample. The only strain known to date – the type strain – was identified on the basis of conventional physiological tests, as described by Facklam & Collins (1989). However, it was reported that strict adherence to these tests often led to misidentification of this particular species (Buschelman *et al.*, 1993; Ruoff *et al.*, 1990; Tsakris *et al.*, 1998). With the use of 16S rRNA gene sequences in phylogenetic analyses, it was suggested that *E. solitarius* may in fact not be an enterococcal species, since its 16S rRNA gene sequence is too different from those of other *Enterococcus* species for it to be included in this genus (Collins *et al.*, 1990; Patel *et al.*, 1998; Williams *et al.*, 1991). More recently, while performing sequence analysis of the 16S rRNA genes of lactic acid bacteria inhabiting paddy rice silage, we also noticed that *E. solitarius* was only

remotely related to other enterococci (Ennahar *et al.*, 2003). Other studies based on enterococci-specific PCR assays (Ke *et al.*, 1999) or on whole-cell-protein profiles (Barros *et al.*, 2001) were consistent with 16S rRNA gene sequence data. In fact, phylogenetically, *E. solitarius* appears to be more closely related to members of the genus *Tetragenococcus* (Barros *et al.*, 2001; Collins *et al.*, 1990; Ke *et al.*, 1999; Patel *et al.*, 1998; Williams *et al.*, 1991), and it has even been suggested that *E. solitarius* and *Tetragenococcus halophilus* may constitute a single taxon (Ke *et al.*, 1999; Monstein *et al.*, 2001). However, controversy still exists, since a more recent study based on the sequence of a highly specific fragment within the enterococcal superoxide dismutase gene clearly placed *E. solitarius* among enterococcal species (Poyart *et al.*, 2000). A comparative investigation including *Enterococcus* species and *Tetragenococcus* species has yet to be carried out to establish whether *E. solitarius* is a miscategorized taxon.

In this study, the *E. solitarius* type strain JCM 8736^T was analysed with respect to its 16S rRNA gene sequence, DNA relatedness and phenotypic traits. In addition, *T. halophilus* JCM 5888^T and *Tetragenococcus muriaticus* JCM 10006^T, and the type strains of closely related *Enterococcus* species

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Tetragenococcus solitarius* DSM 5634^T is AJ301840.

Results of DNA–DNA hybridization experiments are available as supplementary material in IJSEM Online.

Table 1. Acid production from carbohydrates by *E. solitarius*, *Tetragenococcus* species and closely related species

Taxa: 1, *Enterococcus casseliflavus* JCM 8723^T; 2, *E. faecalis* JCM 5803^T; 3, *E. gallinarum* JCM 8728^T; 4, *E. cecorum* JCM 8724^T; 5, *E. saccharolyticus* JCM 8734^T; 6, *E. solitarius* JCM 8736^T; 7, *T. halophilus* JCM 5888^T; 8, *T. muriaticus* JCM 10006^T. All of the strains were positive for hydrolysis of aesculin and acid production from *N*-acetylglucosamine, D-fructose, D-glucose, D-mannose and salicin. None of the strains tested produced catalase or acid from adonitol, L-arabitol, erythritol, D-fucose, L-fucose, glycogen, 5-ketogluconate, D-lyxose, methyl xyloside, sorbose, L-xylose and xylitol. +, Positive; –, negative; (+), weak or delayed reaction.

Character	1	2	3	4	5	6	7	8
Amygdalin	+	+	+	+	+	+	+	–
L-Arabinose	+	–	+	+	–	–	+	–
D-Arabitol	–	–	–	–	–	–	+	–
Arbutin	+	–	+	+	+	+	+	–
Cellobiose	+	+	+	+	+	+	+	–
Dulcitol	–	–	–	–	–	+	–	–
Galactose	+	+	+	+	+	+	+	–
Gluconate	+	(+)	+	(+)	–	+	+	–
Glycerol	+	+	–	–	–	–	–	–
Inulin	(+)	–	(+)	+	+	–	–	–
2-Ketogluconate	–	(+)	–	–	+	–	(+)	–
Lactose	+	+	+	+	+	–	–	–
Maltose	+	+	+	+	+	+	+	–
Mannitol	+	+	+	(+)	+	+	–	+
Melibiose	+	–	+	+	+	–	–	–
Melezitose	(+)	(+)	–	+	+	+	–	–
Methyl α -D-glucopyranoside	(+)	(+)	(+)	–	+	–	–	–
Methyl α -D-mannopyranoside	–	–	+	–	–	–	–	–
D-Raffinose	(+)	–	+	+	+	–	–	–
Rhamnose	(+)	–	+	–	–	–	–	–
Ribose	+	+	+	+	–	–	+	+
Sorbitol	–	+	–	–	+	–	–	–
Starch	–	(+)	(+)	(+)	+	–	–	–
Sucrose	+	+	+	+	+	+	+	–
D-Tagatose	–	+	+	–	–	+	–	–
Trehalose	+	+	+	+	+	+	–	–
D-Turanose	+	+	+	+	+	+	+	–
D-Xylose	+	–	+	–	–	–	+	–

were included as references (Table 1). Maintenance and propagation of cultures have been described previously (Ennahar *et al.*, 2003).

E. solitarius JCM 8736^T was compared with the type strains of *Tetragenococcus* species and closely related *Enterococcus* species using carbohydrate assimilation/fermentation on API 50 CH strips (bioMérieux), as described previously (Ennahar *et al.*, 2003). Analyses of phenotypic features other than those analysed by the API system were performed as described by Cai *et al.* (1999). Our results indicate that, unlike other *Enterococcus* species, *E. solitarius* JCM 8736^T did not ferment lactose, which is a common trait of both members of the genus *Tetragenococcus* (Table 1). In addition, *T. halophilus* displayed the closest fermentation pattern to *E. solitarius*, with 18 identical reactions among the 28 differential carbohydrates tested. Both species produced

acids from amygdalin, arbutin, cellobiose, galactose, gluconate, maltose, sucrose and trehalose, but not from glycerol, inulin, lactose, melibiose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, D-raffinose, rhamnose, sorbitol or starch. The remaining carbohydrates yielded the same pattern of fermentation for all *Enterococcus* and *Tetragenococcus* species tested (Table 1).

Phylogenetic analysis based on 16S rRNA gene sequences was carried out. Amplification, purification and sequencing were performed as described previously (Ennahar *et al.*, 2003). The almost-complete 16S rRNA gene sequences were determined for *E. solitarius* JCM 8736^T, *T. halophilus* JCM 5888^T and *T. muriaticus* JCM 10006^T. These were identical to sequences determined by other investigators (see GenBank/EMBL/DDJB accession numbers in Fig. 1). The highest levels of sequence similarity for *E. solitarius* were

Fig. 1. Phylogenetic tree showing the relative positions of *Enterococcus* species and *Tetragenococcus* species as inferred by the neighbour-joining method with 16S rRNA gene sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the GenBank/EMBL/DBJ accession numbers for all 16S rRNA gene sequences (in parentheses). *Bacillus subtilis* was used as an outgroup. Bar, 1 % sequence divergence.

found with *T. halophilus* (93.8%) and *T. muriaticus* (93.7%), with values similar to that obtained between the two *Tetragenococcus* species (93.8%). Similarity values between *E. solitarius* and other *Enterococcus* species were lower, generally ranging from about 88 to 90 %, while values between enterococci (except *E. solitarius*) ranged from about 95.0 to 99.1 %.

Based on the 16S rRNA gene sequences, a phylogenetic tree was constructed by using the neighbour-joining method, as described previously (Ennahar *et al.*, 2003). This analysis showed that *E. solitarius* formed a very well defined cluster with *T. halophilus* and *T. muriaticus*, with a bootstrap value of 100 % supporting monophyly (Fig. 1). Among the two *Tetragenococcus* species, *T. halophilus* was the closest phylogenetic relative of *E. solitarius* in 94 % of bootstrap analyses, which reflects a higher 16S rRNA gene sequence similarity. The cluster making up the remaining enterococcal species was clearly distinct from the first one and was recovered in 98 % of bootstrap analyses. This finding, along with the phenotypic data, is in agreement with previous reports suggesting a close phylogenetic relationship between *E. solitarius* and *Tetragenococcus* species (Barros *et al.*, 2001; Ke *et al.*, 1999; Patel *et al.*, 1998; Williams *et al.*, 1991).

The relatedness of the genomic DNAs of *E. solitarius* JCM 8736^T, *T. halophilus* JCM 5888^T and *T. muriaticus* JCM 10006^T was tested. Chromosomal DNA was purified by

standard methods (Sambrook *et al.*, 1989) as modified by Satomi *et al.* (1997). DNA–DNA hybridization experiments were performed by the method of Ezaki *et al.* (1989) using photobiotin labelling and colorimetric detection, as described previously (Ennahar *et al.*, 2003). The results are presented in Table A, available as supplementary material in IJSEM Online. *E. solitarius* showed reassociation levels of 25.7 to 28.6 % with *T. halophilus* and 25.4 to 27.2 % with *T. muriaticus*. This is far lower than the DNA reassociation threshold value recommended for species delineation (70 %; Wayne *et al.*, 1987). There were no great differences between the levels of homology measured when the genomic DNAs were used as targets or as probes. Therefore, as opposed to previous suggestions (Barros *et al.*, 2001; Collins *et al.*, 1990), *E. solitarius* constitutes a separate species, distinct from known *Tetragenococcus* species. On the other hand, DNA base composition, determined by HPLC as described previously (Ennahar & Cai, 2004), showed the G+C content for *E. solitarius* (38.3 mol%) to be only slightly different from those of *T. halophilus* (37.0 mol%) and *T. muriaticus* (36.5 mol%).

As described above, *E. solitarius* resembles *Tetragenococcus* species in its fermentative metabolism and 16S rRNA gene sequence. The low DNA–DNA reassociation values obtained for *Tetragenococcus* species clearly support the validity of *E. solitarius* as a separate *Tetragenococcus* species, with *T. halophilus* and *T. muriaticus* as its nearest

phylogenetic relatives. Therefore, we conclude that *E. solitarius* (Collins *et al.*, 1989) is a misclassified taxon that should be included in the genus *Tetragenococcus* as a separate species. The name *Tetragenococcus solitarius* comb. nov. is proposed.

Description of *Tetragenococcus solitarius* comb. nov.

Tetragenococcus solitarius (sol.i.tar.i'us. L. adj. *solitarius* alone, lonely).

Basonym: *Enterococcus solitarius* Collins *et al.* 1989.

Characteristics of the species are as described previously by Collins *et al.* (1989) with the following additions and corrections. Carbohydrate utilization pattern is as reported in Table 1 of this study. The DNA base ratio is about 38 mol% G + C (as determined by HPLC). The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence is AJ301840.

The type strain is 885/78^T (=ATCC 49428^T=CCUG 29293^T=CIP 103330^T=DSM 5634^T=JCM 8736^T=LMG 12890^T=NCTC 12193^T).

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