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Emended description of the genus *Trichococcus*, description of *Trichococcus collinsii* sp. nov., and reclassification of *Lactosphaera pasteurii* as *Trichococcus pasteurii* comb. nov. and of *Ruminococcus palustris* as *Trichococcus palustris* comb. nov. in the low-G+C Gram-positive bacteria

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Analyses of 16S rRNA gene sequences, restriction endonuclease digestion fingerprints of 16S–23S intergenic regions, DNA base compositions, fatty-acid profiles, cell-wall chemistry, cell physiology and fermentation end-product composition, along with other biochemical and phenotypic properties, supported the view that *Trichococcus flocculiformis* Echt^T (DSM 2094^T), *Lactosphaera pasteurii* KoTa2^T (DSM 2381^T), *Ruminococcus palustris* Z-7189^T (DSM 9172^T) and an isolate named '*Carnococcus allantoicus*' NDP were all very similar and should be merged into a single genus. Detailed characterization of strains Ben 77, Ben 200 and Ben 201 described previously as '*Nostocoida limicola*' I, a filamentous bacterium which causes bulking in activated sludge systems, revealed that these strains also belonged to the same genus as *T. flocculiformis* Echt^T, *L. pasteurii* KoTa2^T, *R. palustris* Z-7189^T and '*C. allantoicus*' NDP. In fact, their shared properties suggested that these strains all belonged to a single species. However, DNA–DNA hybridization data indicated that *T. flocculiformis* Echt^T, all of the '*N. limicola*' I isolates and '*C. allantoicus*' NDP belonged to the same species, whereas *L. pasteurii* KoTa2^T, *R. palustris* Z-7189^T and two new isolates, 37AN3*^{T} and 45AN2, represented three distinct species within the same genus. The priority of the genus name *Trichococcus* is established and since its validation predates the description of the genus *Lactosphaera* this name should take precedence. Under certain culture conditions, all of the strains mentioned above could produce chains of cocci. Furthermore, the morphology of *T. flocculiformis* Echt^T could change to a non-filamentous form on certain media. This study proposes that the above strains be reclassified as members of the genus *Trichococcus* as four species, namely *Trichococcus flocculiformis* emend. (type strain Echt^T = DSM 2094^T), *Trichococcus pasteurii* comb. nov. (type strain KoTa2^T = DSM 2381^T = ATCC 35945^T), *Trichococcus collinsii* sp. nov. (type strain 37AN3*^{T} = DSM 14526^T = ATCC BAA-296^T), and *Trichococcus palustris* comb. nov. (type strain Z-7189^T = DSM 9172^T).

Keywords: *Lactosphaera pasteurii*, *Trichococcus flocculiformis*, '*Nostocoida limicola*' I, '*Carnococcus allantoicus*', *Ruminococcus palustris*

The GenBank accession numbers for the 16S rRNA sequences reported in this paper are AF244371, AJ306611 and AJ306612 for *Trichococcus flocculiformis* Ben 200, *Trichococcus flocculiformis* Echt^T and *Trichococcus collinsii* 37AN3*^{T}, respectively.

INTRODUCTION

Scheff *et al*. (1984a) described a filamentous bacterium responsible for bulking in activated sludge plants in Germany. This filamentous bacterium, consisting of cocci in chains, was descriptively named *Trichococcus flocculiformis*, a name which was validated in 1984 (Scheff *et al*., 1984b). Surprisingly, until recently, this bacterium had not been reported as a bulking agent isolated from sludge plants in other parts of the world. This is possibly because its description was not included in the manuals (Eikelboom, 2000; Jenkins *et al*., 1993) widely used in the wastewater industry to 'identify' such filamentous bacteria. Subsequently, 16S rRNA sequence analysis has revealed that *T. flocculiformis* Echt^T is almost identical to *Lactosphaera pasteurii* KoTa2^T (Stackebrandt *et al*., 1999), a non-filamentous, aerotolerant, lactate-producing coccus belonging to the low-G+C Gram-positive bacteria, originally isolated from anoxic digester sludge (Janssen *et al*., 1995). Liu *et al*. (2000) showed that, on the basis of similarities in their 16S rRNA sequences, some isolates of a bulking filamentous bacterium they described as '*Nostocoida limicola*' I (van Veen, 1973; Jenkins *et al*., 1993), from Australia and the Czech Republic, were also very closely related to *T. flocculiformis* Echt^T, whose morphology they can resemble. This study describes an extensive characterization of these bacteria, and the data presented convincingly suggest that, despite often considerable differences in their morphologies, these bacteria should all be grouped into a single genus. This genus should also contain '*Carnococcus allantoicus*' NDP (R. S. Tanner, unpublished data), a strain isolated from duck-pond sediment as a non-filamentous organism. However, DNA–DNA hybridization data presented here suggest that *L. pasteurii* KoTa2^T should be placed into a separate species to the other strains. On the basis of their characteristics, *Ruminococcus palustris* Z-7189^T, isolated from a swamp (Zhilina *et al*., 1995), and two previously undescribed isolates, 37AN3*^{T} and 45AN2, obtained from a hydrocarbon-spill site (Gieg *et al*., 1999), are also considered to represent new species of the same genus. The genus name *Trichococcus* takes priority over *Lactosphaera* according to the *Bacteriological Code* (1990 revision) and, furthermore, is suited for circumscription of the morphology of these strains since, under some growth conditions and in some media, all can grow as chains of cocci.

METHODS

Strains and culture conditions. The isolates used in this study are listed in Table 1. Strains 37AN3*^{T} and 45AN2, isolated from a gas-condensate-contaminated soil in Colorado (Gieg *et al*., 1999), were cultured using half-strength tryptic soy broth (Difco). '*C. allantoicus*' NDP, isolated from a duck-pond sediment, was cultured in an allantoin-based medium with the mineral salts, trace metals and vitamin solution of Tanner (1997) supplemented with yeast extract (1 g l^{-1}) and allantoin (5 g l^{-1}), and it was incubated under anaerobic conditions. '*C. allantoicus*' NDP is very similar to an isolate, '*Streptococcus allantoicus*', described by Barker (1943). All cultures were maintained at −80 °C. In some experiments, where the influence of growth conditions on cell morphology was investigated, the following media were used: R2A broth and agar (Reasoner & Geldreich, 1985); SR2A broth and agar (Liu *et al*., 2001); SCY agar (van Veen, 1973); and M69 agar (Scheff *et al*., 1984a). All of the cultures were incubated at 26 °C, sometimes with shaking (180 r.p.m.) unless otherwise stated.

Phenotypic characterization. Strains grown on R2A agar were characterized using the BIOLOG system to obtain their substrate utilization patterns and the API ZYM system to obtain their enzyme profiles, following the manufacturers' instructions for both systems. The abilities of the strains to grow aerobically or anaerobically on other substrates, including allantoin, L-tartrate, citrate, DL-malate, ethanol, succinate, fumarate, L-glutamate, glycerol and crotonate, were determined by using the allantoin-based isolation medium described above in which the allantoin was replaced by the test substrate at 5 g l^{-1}, or by using the methods described by Janssen *et al*. (1995) and incorporating appropriate controls. Fatty acid methyl ester (FAME) analyses were performed by Microcheck, using cells grown on tryptic soy agar at room temperature for 3 days. The derivatization and analysis of the fatty acids was done using the methods of Miller (1982) and Sassar (1990). The fatty acid data of Zhilina *et al*. (1995) were used here for *R. palustris* Z-7189^T. The peptidoglycan type of the strains was determined according to the protocol of Schumann *et al*. (1997). Cells were prepared and viewed under a scanning electron microscope, using the methods described by Liu *et al*. (2000).

Fermentative behaviour of strains. The sulfide-reduced, bicarbonate-buffered growth medium FM, described by Janssen *et al*. (1997), was used for these studies. Medium FH contained the salts and trace elements in FM, except that 0·5 g of $MgSO_4.7H_2O$ was used instead of $MgCl_2.6H_2O$, sulfide and bicarbonate were omitted from the medium, and the medium was buffered with 10 mM HEPES. The initial pH was 7·3, adjusted with NaOH. Growth substrates and yeast extract were added to FM and FH, as required, from filter-sterilized stock solutions. Aerobically incubated cultures were grown in 10 ml aliquots in screw-capped bottles (40 ml) with loosened lids, which were shaken at 150 r.p.m. Cultures incubated anoxically, also in 10 ml aliquots in screw-capped bottles (40 ml) with loosened lids, were grown in anaerobic jars with GasPak Plus sachets (Becton Dickinson) to remove O_2. Cultures grown under strictly anaerobic conditions in FM were in 50 ml aliquots in completely filled glass screw-capped bottles with rubber-lined metal lids. Bulk cultures for dry mass determinations and the detection of cytochromes were grown statically under air in 200 ml aliquots of FH supplemented with 10 mM glucose l^{-1} and 0·1 g yeast extract l^{-1}. Inocula were 1 % (v/v), and incubations were done at 25 °C. Yeast extract was routinely added to all media at 0·1 g l^{-1}.

Fermentation products and balances. The utilization of growth substrates and the production of organic acids and alcohols were determined by HPLC (Ehrlich *et al*., 1981) using a refractive index detector. The isomeric form of lactic acid was determined enzymically (Bergmeyer, 1974). Dry mass growth yields were calculated from culture densities measured at 600 nm in a 10 mm cuvette using a gravimetrically determined conversion factor obtained from washed (25 mM ammonium acetate) cell pellets harvested from four 200 ml cultures by centrifugation and dried to a

Table 1. Summary of main discriminatory phenotypic and chemotaxonomic characters of strains used in this study

Strains: 1, strain Ben 200; 2, strain Ben 201; 3, strain Ben 77; 4, *T. flocculiformis* Echt^T; 5, *L. pasteurii* KoTa2^T; 6, '*C. allantoicus*' NDP; 7, *R. palustris* Z-7189^T; 8, strain 37AN3*^{T}; 9, strain 45AN2. ?, Not determined.

Characteristics	1*	2*	3*	4	5	6	7§	8	9
Source	Activated sludge	Activated sludge	Activated sludge	Activated sludge	Septic pit	Duck-pond sediment	Swamp	Hydrocarbon-spill site	Spill
Growth:									
Temperature range	4 to < 37 °C	Ben 200*	?	4 to < 40 °C	0–42 °C	> 7 to < 46 °C	0–33 °C	> 7 to < 36 °C	> 7 to < 36 °C
(optimum)	(12–30 °C)	Ben 200*	?	(25–30 °C)	(25–30 °C)	(23–36 °C)	?	(23–30 °C)	(23–30 °C)
pH range	6–10 (?)	Ben 200* (?)	?	5·8–9·0 (8·0)	5·5–9·0	6–9 (8·0)	6·2–8·4	6–9 (7·5)	6–9 (7·5)
(optimum)					(?)		(7·5)		
G+C content (mol%)	48	48	48	47†	45‡	49	48	47	48
Growth on:									
Citrate	−	+	−	+	+	+	?	+	+
L-Malate	−	−	−	−	+	+	−	+	+
Allantoin	−	+	−	−	−	+	?	+	+
L-Tartrate	−	−	−	−	+	−	?	+	+
Acid from:									
Inositol	+	+	+	+	+	+	−	+	−
Mannitol	+	+	+	+	+	−	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	−
Raffinose	+	+	−	+	+	−	+	+	−
Adonitol	+	+	−	+	+	−	?	+	−
Reduction of:									
Nitrate	+	+	+	−	−	−	−	−	−
Urease	+	+	−	−	−	−	−	−	−

* Some data from Liu *et al.* (2000).

† Data from Scheff *et al.* (1984a).

‡ Some data from Janssen *et al.* (1995).

§ Data from Zhilina *et al.* (1995).

constant mass at 105 °C. D-Fructose-1,6-bisphosphate-activated lactate dehydrogenase activity was measured at 30 °C as described by Lamed & Zeikus (1980). D-Fructose-1,6-bisphosphate-independent lactate dehydrogenase activity was measured by the same method, but with the omission of D-fructose 1,6-bisphosphate. The presence of cytochromes was assayed spectrophotometrically according to Janssen (1998).

DNA analysis of strains. DNA base composition determinations and DNA–DNA hybridization experiments were carried out using the methods described by Maszenan *et al.* (2000).

Fingerprinting of the 16S–23S intergenic spacer region. DNA was extracted and purified from pure cultures using a slight modification of the method of Bond *et al.* (1995). In the absence of any sequence information on this region in these bacteria, the PCR primers selected to amplify the 16S–23S region were the primers 2 forward and 7 reverse of Gürtler & Stanisich (1996). The PCR mix used was as follows. Fifty microlitres of reaction mix containing PCR reaction buffer (Applied Biosystems), 1 μM of each primer, 1·5 mM $MgCl_2$, 200 μM of each dNTP (Roche Diagnostics), 5% (v/v) DMSO and 2·5 U Ampli*Taq* Gold (Applied Biosystems). DNA was amplified by using a GeneAmp 2400 PCR system (Applied Biosystems) with the following protocol: 10 min activation at 94 °C followed by 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 53 °C) and extension (4 min at 72 °C), followed by a further 10 min at 72 °C for the final extension. Amplified products were visualized by running them on agarose gels [1% SeaKem agarose (FMC) in Tris/borate buffer (0·045 M Tris/borate, 0·001 M EDTA, pH 8·0)] and then staining the gels with ethidium bromide. *Sau*3AI, *Msp*I and *Bst*UI (5–10 U of each; all from New England Biolabs) were used to digest the PCR products. Digests were carried out at 37 or 60 °C for 4 h in a reaction volume of 20 μl, using the recommended buffers for each enzyme. The digest products were then analysed by gel electrophoresis [3% New Sieve agarose (FMC) in Tris/borate buffer containing ethidium bromide] followed by visualization under UV light, and the gels were photographed using Polaroid 665 film.

Phylogenetic analysis of strains. Phylogenetic determination was performed by comparative 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30–1521 of the *E. coli* 16S rRNA gene) was amplified from strains Ben 200, Echt^T and 37AN3*^{T} by PCR, using conserved primers close to the 3′ and 5′ ends of the gene. The PCR products were directly sequenced using a dideoxy terminator cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of these strains were determined by performing a database search using the program FASTA of the Genetics Computer Group package (Lipman & Pearson, 1985). These sequences and those of other known related strains were retrieved from the EMBL or RDP data libraries and aligned with the newly determined sequences using the program DNATOOLS (Rasmussen, 1995). The resulting multiple-sequence alignment was corrected manually and approximately 100 bases at the 5′ end of the rRNA were omitted from further analyses because of alignment ambiguities using the program GENEDOC (Nicholas *et al.*, 1997). Pairwise evolutionary distances were then computed from a continuous stretch of 1320 bases and a distance matrix was calculated by using DNADIST (using the Kimura-2 correction parameter). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). The 16S rRNA gene sequences of Ben 200, *T. flocculiformis* Echt^T and 37AN3*^{T} have been deposited in GenBank under accession numbers AF244371, AJ306611 and AJ306612, respectively.

RESULTS

Morphology of the isolates

All of the strains examined in this study were pleiomorphic, often with cell forms different to those expected from their original descriptions. These morphological variations are illustrated in Figs 1 and 2. For example, although *T. flocculiformis* Echt^T grew as regular cocci in chains, as described by Scheff *et al.* (1984a), on most of the media (e.g. SR2A agar) (Fig. 1a), it grew on R2A agar as much larger tapered rod-shaped cells, which often occurred in pairs (Fig. 1b). A similar morphology was seen for *L. pasteurii* KoTa2^T when it was (Fig. 1d) grown on R2A agar, but it grew as regular cocci on SR2A agar (Fig. 1c), in accordance with the description of Janssen *et al.* (1995). '*C. allantoicus*' NDP had a similar appearance when grown on these two media, displaying tapered cells in pairs (Fig. 1e, f), whereas strains 37AN3*^{T} and 45AN2 grew quite differently on R2A and SR2A agar (Fig. 1g–j). Strain 37AN3*^{T} appeared as swollen, often tapered, paired, irregular cells on both SR2A and R2A agar, but its cells were much larger on the latter medium (Fig. 1g, h). Strain 45AN2 grew as more regular single or paired cocci on SR2A agar, whereas on R2A agar irregular, much larger coccoid or tapered cells, often arranged in pairs were seen (Fig. 1i, j). Ben 200 changed from regular cocci in chains on SR2A agar (Fig. 1k) to highly irregular pleiomorphic cells in chains on R2A agar (Fig. 1l).

Similar changes were seen in the above cultures grown in R2A broth when compared to SR2A broth, whether these media were shaken or not. Examples of these differences, which were often substantial, are shown in Figs 2(a–l). Again, the Ben strains were especially pleiomorphic. For example, in R2A broth, Ben 200 produced irregular swollen cells under static conditions (Fig. 2a), but paired cocci were seen when the cultures were shaken (Fig. 2b). Equally striking were the change in appearance of *L. pasteurii* KoTa2^T, which in R2A broth produced short chains of coccoid and irregular cells (Fig. 2e, f); this change was also seen with 37AN3*^{T} (Fig. 2i, j) and 45AN2 when they were grown in R2A broth (Fig. 2k, l). Highly irregular cells also were produced by '*C. allantoicus*' NDP in R2A broth (Fig. 2g, h), especially when the culture was shaken (Fig. 2h). *T. flocculiformis* Echt^T grew in a regular filamentous form in both R2A and SR2A broths under shaken and static conditions (Fig. 2c, d).

The possible pleiomorphic nature of *R. palustris* Z-7189^T was not studied in such detail but, in agreement with the observations of Zhilina *et al.* (1995), its cells

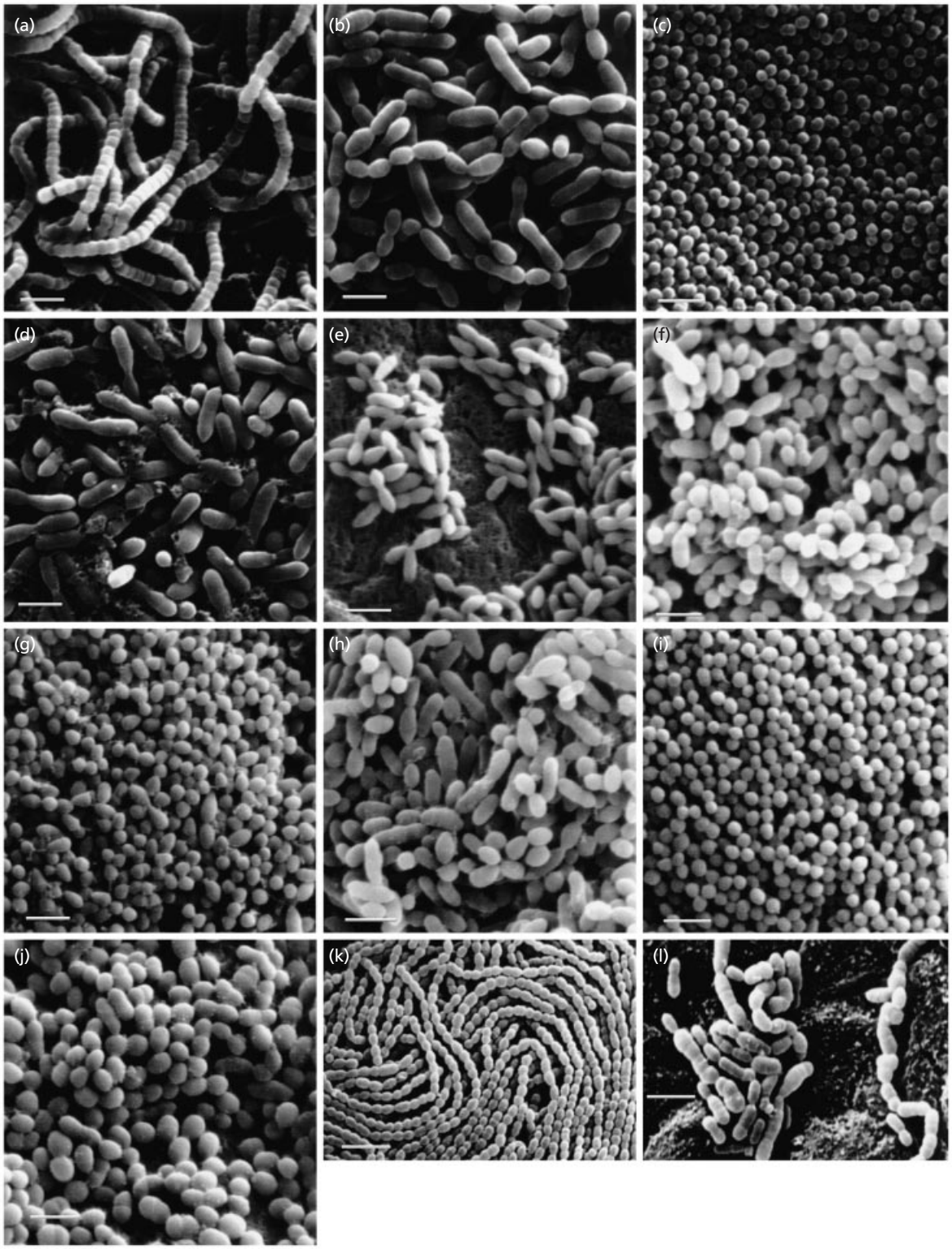

Fig. 1. Scanning electron micrographs of *T. flocculiformis* Echt^T (a, b), *L. pasteurii* KoTa2^T (c, d), '*C. allantoicus*' NDP (e, f), strain 37AN3*^{T} (g, h), strain 45AN2 (i, j) and strain Ben 200 (k, l) growing on SR2A agar (a, c, e, g, i, k) and on R2A agar (b, d, f, h, j, l), showing the morphological variations in cell shape and arrangement of these organisms. Further details are given in the text. Bar, 2 μm for all, except for (k) in which the bar is 4 μm.

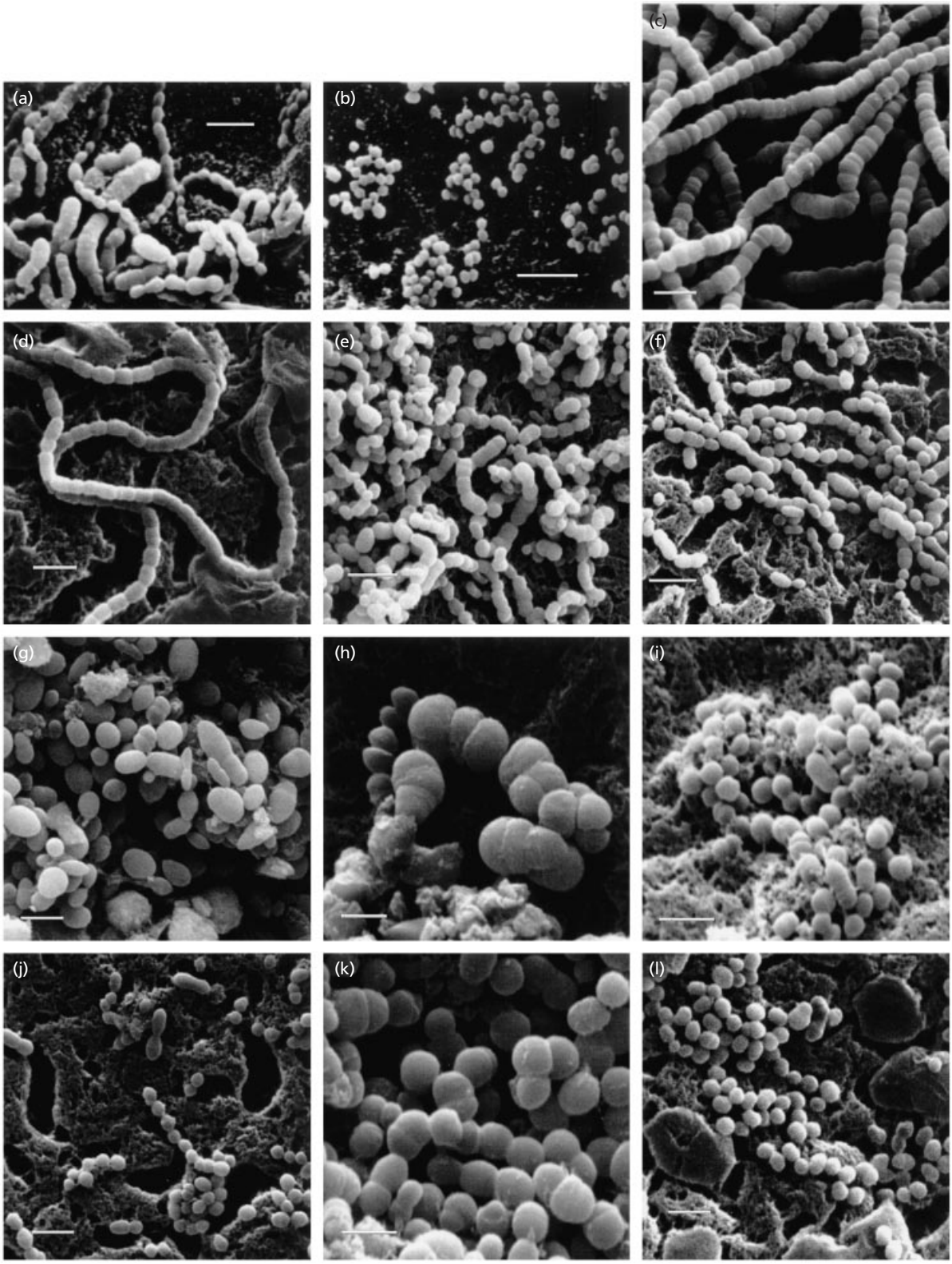

Fig. 2. Scanning electron micrographs of strain Ben 200 (a, b), *T. flocculiformis* Echt^T (c, d), *L. pasteurii* KoTa2^T (e, f), '*C. allantoicus*' NDP (g, h), strain 37AN3*^{T} (I, j) and strain 45AN2 (k, l) growing in R2A broth without shaking (Fig. 1a, c, e, g, l, k) and with shaking (Fig. 1b, d, f, h, j, l), showing the variations in the cell shape and arrangements of these strains. Further details are given in text. Bar, 2 μm for all, except for (g) and (j) in which the bars are 3 μm.

Table 2. Differences in substrate utilization patterns between isolates determined using the BIOLOG system

Strains: 1, strain Ben 77; 2, strain Ben 200; 3, strain Ben 201; 4, *T. flocculiformis* Echt^T; 5, *L. pasteurii* KoTa2^T; 6, *R. palustris* Z-7189^T; 7, '*C. allantoicus*' NDP; 8, strain 45AN2; 9, strain 37AN3*^{T}. All other substrates examined gave the same results for all strains. +, Positive; −, negative.

Substrate	1	2	3	4	5	6	7	8	9
Cellobiose	−	+	+	+	+	+	+	+	+
D-Galactose	−	+	+	+	+	−	+	+	+
Gentiobiose	−	+	+	+	+	+	+	+	+
α-D-Lactose	−	+	−	+	+	−	+	+	−
Lactulose	−	+	−	+	+	−	+	−	−
Maltose	+	+	+	+	+	−	+	+	−
Maltotriose	+	+	+	+	+	−	+	+	+
D-Mannitol	−	−	−	−	−	−	+	+	−
D-Melezitose	−	−	−	−	−	−	−	+	−
Methyl α-D-galactoside	−	−	−	−	−	−	+	−	−
Methyl β-D-galactoside	−	−	−	−	−	+	+	−	−
3-Methyl glucose	−	+	+	−	−	−	−	−	−
Methyl β-D-glucoside	+	+	+	+	+	−	+	+	+
Palatinose	−	+	+	+	+	−	+	+	+
D-Psicose	−	+	+	+	+	+	+	+	+
D-Raffinose	−	+	+	−	−	−	+	+	+
D-Ribose	−	−	−	−	−	−	+	+	−
Stachyose	−	−	−	−	−	−	+	−	−
D-Trehalose	+	+	+	+	+	−	+	+	+
Turanose	−	+	+	+	+	−	−	−	−
L-Lactic acid	−	−	−	−	−	+	−	−	−
Methyl pyruvate	−	+	+	+	+	+	+	+	+
Pyruvate	−	+	+	+	+	+	+	+	+
Adenosine	−	+	−	+	+	−	−	+	−
2′-Deoxyadenosine	−	+	−	+	+	−	−	−	−
Uridine	−	+	−	+	+	−	−	+	−
Thymidine	−	+	−	+	+	−	−	−	−

were oval with tapered ends and showed a tendency to form pairs or short chains on R2A agar.

Phenotypic properties

All of the strains in this study were very similar phenotypically. They were all oxidase and catalase negative, H_2S negative and ornithine decarboxylase negative. All grew with glucose (both oxically and anoxically – see below), produced acid from sucrose and lactose metabolism and all, except for *R. palustris* Z-7189^T, could grow with xylose, rhamnose and D-arabinose. None of the strains grew in the presence of ethanol and all of the strains, except for Ben 77, *R. palustris* Z-7189^T and Ben 200, grew with citrate. Only Ben 201, '*C. allantoicus*' NDP and strains 37AN3*^{T} and 45AN2 grew in the presence of allantoin, whereas *L. pasteurii* KoTa2^T and strains 37AN3*^{T} and 45AN2 alone grew with L-tartrate (Table 1). The results obtained with the BIOLOG system showed that all of the strains assimilated arbutin, cellobiose, D-fructose, α-D-glucose, D-mannose, salicin, sucrose and pyruvate. None of the following substrates were utilized according to the results of BIOLOG screening: β-cyclodextrin, inulin, Tween 40, Tween 80, L-arabinose, D-arabitol, L-fucose, D-galacturonic acid, D-gluconic acid, *myo*-inositol, methyl α-D-glucoside, methyl α-D-mannoside, sedoheptulosan, D-sorbitol, D-tagatose, xylitol, D-xylose, acetic acid, α-hydroxybutyrate, β-hydroxybutyrate, γ-hydroxybutyrate, β-hydroxyphenylacetate, 2-oxoglutarate, 2-oxovalerate, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, monomethylsuccinic acid, propionic acid, succinamic acid, *N*-acetylglutamic acid, alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, inosine, thymidine, adenosine 5′-monophosphate, thymidine 5′-monophosphate, uridine 5′-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, DL-α-glycerol phosphate. For some substrates (e.g. L-rhamnose, D-xylose, L-malate, citrate and salicin) different results were obtained with the BIOLOG system from those obtained from the growth experiments using conventional methods. The BIOLOG substrate utilization patterns that were

Table 3. API ZYM profiles showing differences in detectable enzymes in strains

Strains: 1, strain Ben 77; 2, strain Ben 200; 3, strain Ben 201; 4, *T. flocculiformis* Echt^T; 5, *L. pasteurii* KoTa2^T; 6, '*C. allantoicus*' NDP; 7, *R. palustris* Z-7189^T; 8, strain 37AN3*^{T}; 9, strain 45AN2. 0, No activity; 1–5 indicate relative levels of activity, with 1 being the least active and 5 being the most active.

Enzyme	1	2	3	4	5	6	7	8	9
Alkaline phosphatase	0	1	0	0	0	0	0	1	0
Esterase (C4)	0	3	2	1	1	1	1	1	1
Leucine arylamidase	0	1	2	0	0	1	1	1	1
Trypsin	0	0	0	1	0	0	0	0	0
Chemotrypsin	0	1	2	1	0	0	0	0	0
β-Glucuronidase	0	1	0	0	0	0	0	0	1
α-Glucosidase	1	0	1	5	2	2	1	0	1
β-Glucosidase	0	0	0	3	1	0	1	0	3

Table 4. Balances of glucose metabolism by *T. flocculiformis* Echt^T under oxic and anoxic conditions

Cultures were grown with 4 mM glucose in sulfide-reduced FM, anoxically in FH in anaerobic jars or under air in FH.

Conditions	Products formed [mol (mol glucose)$^{-1}$]				Balances (%)*		Yield [g dry mass cells (mol glucose)$^{-1}$]	Final pH
	Lactate	Formate	Acetate	Ethanol	Carbon	Available H		
Sulfide reduced	1·13	1·02	0·35	0·39	98	96	23·3	5·7
Anaerobic jar	1·55	0·46	0·27	0·15	99	98	20·5	4·9
Under air	0·91	0·00	0·90	0·00	75	90	21·3	5·0

* Balances were calculated excluding cell matter, which was assumed to have been produced largely from the yeast extract in the medium.

Table 5. Cellular fatty acid compositions of strains expressed as a per cent of the total fatty acids

Strains: 1, strain 45AN2; 2, strain 37AN3*^{T}; 3, *T. flocculiformis* Echt^T; 4, *L. pasteurii* KoTa2^T; 5, '*C. allantoicus*' NDP; 6, *R. palustris* Z-7189^T; 7, strain Ben 200; 8, strain Ben 201. ND, Not detected.

Fatty acid	1	2	3	4	5	6†	7	8
$C_{12:0}$	12	6	ND	1	ND	ND	ND	ND
$C_{14:0}$	57	46	14	28	8	21	14	11
$C_{iso\text{-}15:1}$	ND	ND	ND	ND	ND	ND	ND	1
$C_{16:0}$	14	18	15	16	14	15	24	12
$C_{16:1}$	18	27	46	42	44	20	39	50
$C_{iso\text{-}17:1}$	ND	ND	1	ND	1	ND	1	4
$C_{18:0}$	ND	2	2	2	2	4	3	1
$C_{18:1(\omega 9c)}$	ND	2	18	6	23	22	16	14
$C_{18:1(\omega 7c)}$	ND	ND	ND	ND	ND	2	ND	ND

† Values for *R. palustris* are taken from Zhilani *et al.* (1995).

different for each of the different strains are given in Table 2.

All isolates produced detectable levels of esterase lipase (C8), phosphohydrolase and naphthol-AS-B1 phosphohydrolase with the API ZYM system. None expressed α-fucosidase, α-mannosidase, *N*-acetyl β-glucosaminidase, β-galactosidase, α-galactosidase, cystine arylamidase, valine arylamidase or lipase (C4) activities. The differences between their enzyme profiles are shown in Table 3. Where tested, some strains were able to grow at 0–4 °C and at above 40 °C (Table 1). Growth was also recorded at pH values up to 9 and above this value in a few of the strains (Table 1).

Fermentation of sugars

All of the strains examined were able to grow with and to ferment glucose (added at 5 mM) under anoxic conditions, whether grown in FH in anaerobic jars or in sulfide-reduced FM. All produced lactate, formate, acetate and ethanol as their organic end products of glucose fermentation, and the pH dropped from an initial value of about 7·3 to values of 5·7–5·9 in FM and 4·8–5·3 in FH. All strains, except for Ben 77, were able to grow with glucose in FH incubated under oxic conditions. Lactate and acetate were produced by these cultures, and there was a drop in pH, from 7·3 to between 4·8 and 5·2.

Glucose fermentation by *T. flocculiformis* Echt^T

The fermentative behaviour of *T. flocculiformis* Echt^T was analysed in detail here. It exhibited a mixed-acid fermentation of glucose, producing lactate, formate, acetate and ethanol as its organic end products. At more acidic pH values, Echt^T produced less formate, acetate and ethanol, and more lactate (data not shown). Only the L-isomer of lactate was formed. The activity of D-fructose-1,6-bisphosphate-activated lactate dehydrogenase was detected, whereas the activity of D-fructose-1,6-bisphosphate-independent lactate dehydrogenase was not.

Under anoxic conditions *T. flocculiformis* Echt^T fermented glucose to lactate, formate, acetate and ethanol, both in the FH medium in anaerobic jars and in the sulfide-reduced FM medium. The balances of carbon and available substrate suggest that these fermentation end products, together with biomass production, account for the glucose utilized (Table 4). In contrast, Echt^T produced only acetate and lactate when grown in the presence of O_2, and formate and ethanol were not detected under these growth conditions. The carbon balances of oxically incubated cultures suggested that there was a significant production of CO_2, whereas the balance of available H suggests that O_2 was reduced (Table 4). Cells grown with glucose under air did not contain cytochromes. Biomass yields from glucose in oxically and anoxically grown cultures were almost identical (Table 4). The slightly higher yields in sulfide-reduced FM may, in fact, be a consequence of the better buffering capacity of that medium (see above and Table 4).

Chemotaxonomic properties

All of the strains studied possessed the cell wall type A4α with an L-Lys-D-Asp interpeptide bridge, and their DNA-base compositions fell between 45 and 49 mol% G+C (Table 1). Fatty-acid analyses revealed some differences between them (Table 5). Thus, while *T. flocculiformis* Echt^T, '*C. allantoicus*' NDP and Ben 200 and Ben 201 had very similar profiles to each other, *L. pasteurii* KoTa2^T differed to these strains in its $C_{14:0}$ and $C_{18:1(\omega 9c)}$ content. Strains 37AN3*^{T} and 45AN2 both had much higher proportions of $C_{12:0}$ and $C_{14:0}$ than the other strains, and they shared, with *R. palustris* Z-7189^T, a lower $C_{16:1}$ content than the rest of the strains.

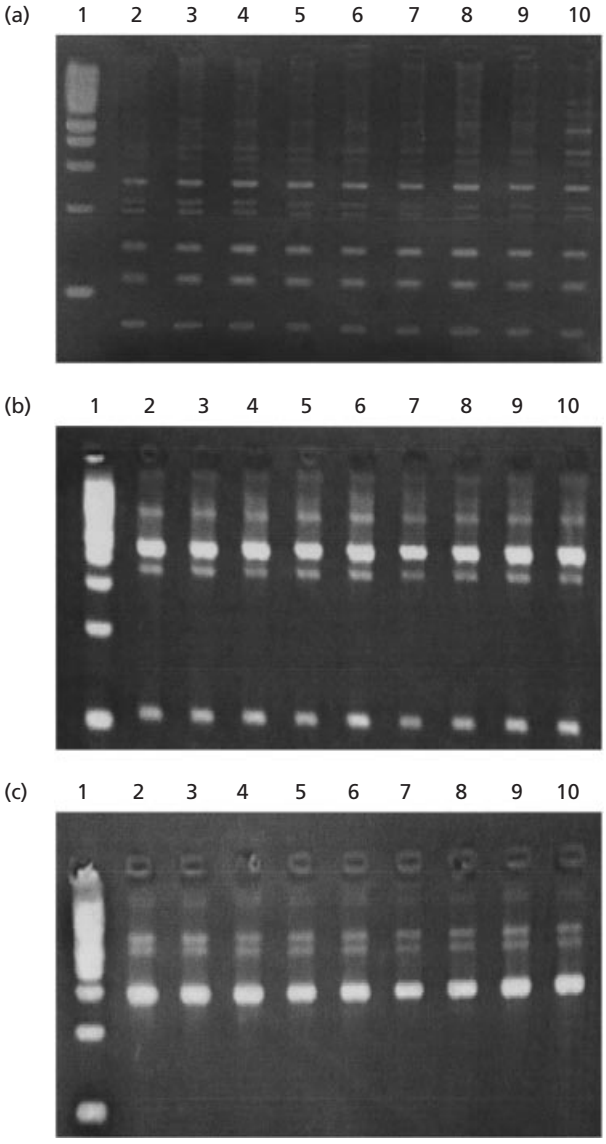

Fig. 3. Fingerprints of fragments of the 16S–23S intergenic regions of strains after digestion with restriction endonuclease *Sau*3AI (a), *Msp*I (b) and *Bst*UI (c) and separated on agarose gels. Lanes: 1, 100 bp marker; 2, strain 37AN3*^{T}; 3, strain 45AN2; 4, strain Ben 77; 5, strain Ben 200; 6, strain Ben 201; 7, strain Ben 206; 8, *T. flocculiformis* Echt^T; 9, *L. pasteurii* KoTa2^T; and 10, '*C. allantoicus*' NDP. See text for details.

16S–23S rDNA intergenic fingerprinting

Although different numbers of bands were seen with the different enzymes used, fingerprints obtained after digestion of the 16S–23S PCR products with the endonucleases *Sau*3AI, *Msp*I and *Bst*UI (Fig. 3) showed that all of the strains gave identical patterns to each other with each of the three enzymes. Only slight quantitative differences in band intensities between the

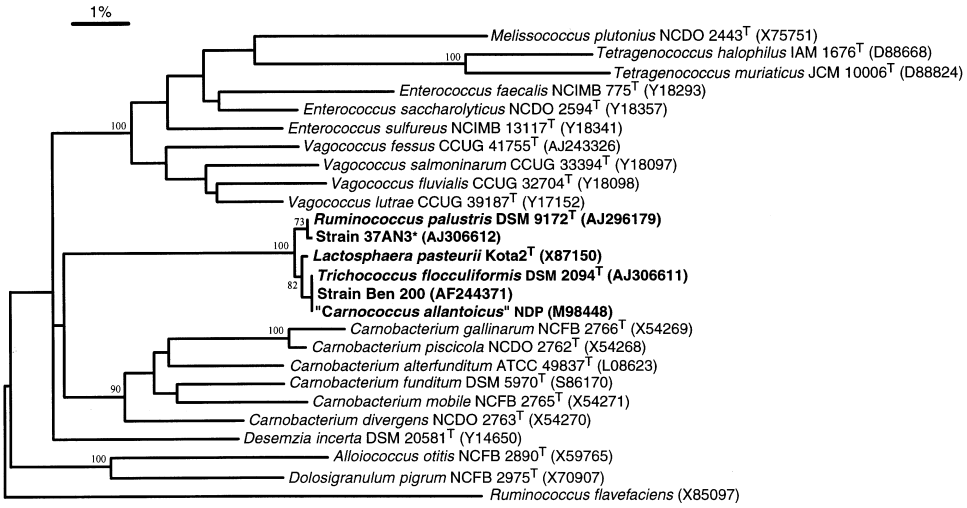

Fig. 4. Tree showing the phylogenetic relationships of strains of the genus *Trichococcus* in the catalase-negative group of the low-G+C Gram-positive bacteria. The tree, constructed using the neighbour-joining method, was based on approximately 1320 nt. Bootstrap values (500 replications) are given at branch points. Bar, 1 substitution per 100 nt positions.

strains were noticed for patterns derived with each enzyme. *R. palustris* Z-7189^T was not analysed.

Phylogenetic relationships between strains

The almost complete 16S rRNA gene sequences (> 1400 nt) of these strains were determined. Pairwise analysis revealed that strains 37AN3*^{T} and 45AN2 were 100 % identical, and that strains Ben 77, Ben 200 and Ben 201 were almost identical to each other (99·9–100 % sequence similarity). Sequence searches of the GenBank and RDP libraries also showed that these strains were all members of the low-G+C Gram-positive, catalase-negative cocci within the lactic acid group of bacteria. A tree constructed by the neighbour-joining method is shown in Fig. 4. It is evident that strains 37AN3*^{T} and 45AN2 (represented by 37AN3*^{T}) form a cluster with *L. pasteurii* KoTa2^T and *R. palustris* Z-7189^T. More specifically, 37AN3*^{T} shares a branching node with *L. pasteurii*, but this relationship was not statistically significant (recovered in 52 % of the re-samplings). Strains Ben 77, Ben 200 and Ben 201, as exemplified by Ben 200, were most closely related to *T. flocculiformis* Echt^T and '*Carnococcus allantoicus*' NDP. However, all of the strains demonstrated a 16S rRNA sequence similarity of greater than 99 %, forming a tight robust cluster that was supported by a significant bootstrap re-sampling value of 100 %. All of the major groupings were confirmed using maximum-parsimony analysis (data not shown).

DNA–DNA hybridization between strains

DNA hybridizations between the different strains revealed that *T. flocculiformis* Echt^T, '*C. allantoicus*' NDP, Ben 77, Ben 200 and Ben 201 had hybridization values displaying more than 70 % similarity with each other. Conversely, *L. pasteurii* KoTa2^T, *R. palustris* Z-7189^T, and strains 37AN3*^{T} and 45AN2 showed DNA hybridization values of less than 70 % with any of the other strains for which data were obtained. Thus, the values for *L. pasteurii* KoTa2^T/*R. palustris* Z-7189^T, *L. pasteurii* KoTa2^T/*C. allantoicus* NDP, *L. pasteurii* KoTa2^T/Ben 201 and *L. pasteurii* KoTa2^T/37AN3*^{T} were 40·4, 56, 59·5 and 57·9 %, respectively. Those for *R. palustris* Z-7189^T/Ben 201, *R. palustris* Z-7189^T/37AN3*^{T}, 37AN3*^{T}/Ben 201 and 45AN2/Ben 201 were 35·8, 34·4, 53·3 and 51·7 %, respectively, whereas that for 37AN3*^{T}/45AN2 was 97·8 %.

DISCUSSION

T. flocculiformis Echt^T, *L. pasteurii* KoTa2^T, '*C. allantoicus*' NDP, *R. palustris* Z-7189^T, and strains 37AN3*^{T} and 45AN2, together with the '*N. limicola*' I strains Ben 77, Ben 200 and Ben 201, are all aerotolerant, fermentative bacteria with similar fermentation end products, and which probably have similar biochemical pathways. Two different fermentation pathways seem to be operating under anaerobic conditions in *T. flocculiformis* Echt^T, chosen as representative of the strains studied here and for comparison with *L. pasteurii* KoTa2^T, whose fermentation behaviour has been examined previously by Janssen *et al.* (1995). Both species are very similar in their fermentation behaviour. In the first pathway in *T. flocculiformis* Echt^T, a homolactic fermentation results in the formation of (theoretically) 2 mol lactate (mol glucose metabolized)$^{-1}$. In this pathway, electrons arising from the oxidation of 1 mol of glucose to 2 mol of pyruvate are used to reduce 2 mol of pyruvate to 2 mol of lactate, via the lactate dehydrogenase. The second pathway, which is less active at more acidic pH

values, leads to the production of formate, acetate and ethanol. In this pathway, electrons arising from the oxidation of 1 mol of glucose to 2 mol of pyruvate are used to reduce 1 mol of pyruvate to 1 mol of ethanol (via acetyl-CoA), leaving 1 mol of pyruvate to be metabolized to acetate (also via acetyl-CoA). In this pathway, the oxidation of pyruvate to acetyl-CoA leads to the production of 2 mol of formate, and the pathway is balanced by the absence of lactate formation. These two pathways appear to operate simultaneously in *T. flocculiformis* Echt^T, as they do in *L. pasteurii* KoTa2^T (Janssen *et al.*, 1995). In oxically incubated cultures, no ethanol was produced and an increase in acetate production was observed. This indicates that electrons normally disposed of in the reductive pathway from acetyl-CoA to ethanol may instead flow to O_2, allowing more acetyl-CoA flow to acetate. In addition, the lack of formate production in aerobically incubated cultures suggests that electrons also flow to O_2 at the steps normally resulting in formate production. The production of lactate did not appear to be influenced by the presence of O_2, nor was there evidence that acetate (or acetyl-CoA) is oxidized to CO_2.

If it is assumed that formate is oxidized to CO_2 (or that CO_2 is formed directly from pyruvate), and that the carbon flux through this pathway is the same as in the formate-producing pathway under anoxic conditions, the estimated CO_2 production would account for 17–21 % of the carbon balance, and would therefore increase the carbon balance under oxic conditions to 92–96 %. Since there was no yield increase in the presence of O_2, and no cytochromes were detected in oxically grown cells, ATP production linked to O_2 respiration does not occur. It therefore seems that *T. flocculiformis* Echt^T uses O_2 as a terminal electron acceptor as a means of disposing of electrons, without any ATP formation linked to electron transport. Such a transfer of electrons to O_2 by lactic acid bacteria has been described previously (van Beelen *et al.*, 1986; Fukui *et al.*, 1988). Some small increase in ATP formation would result as a consequence of increased flow through the acetate-producing pathway with concomitant phosphotransacetylase-dependent ATP formation in the absence of the need for ethanol formation as a route for disposing of electrons arising from glucose oxidation to pyruvate, but this may be offset by a higher maintenance energy requirement to repair oxidative damage under air.

The data presented here support the view that all of the isolates examined in this study are sufficiently similar in their phenotypic, chemotaxonomic and phylogenetic attributes to be considered members of the same genus in the low-G+C Gram-positive bacteria (Table 1, Fig. 4). As well as being aerotolerant, all of the strains are oxidase- and catalase-negative, possess the same cell-wall composition and have very similar mol % G+C values. They also have almost identical 16S rRNA sequences to each other (> 99 % sequence similarity), and all (except for *R. palustris* Z-7189^T, which was not included in the analysis) give identical fingerprints after digestion with three different restriction endonucleases of their 16S–23S intergenic spacer regions. This is despite the differences in their cell morphologies and arrangements suggested from their original descriptions, which have been revealed here as inadequate, since all of the strains appear to be able to grow either in chains or as single or paired cells, often highly irregular in shape and size, depending on the culture conditions used (Figs 1 and 2).

Apart from these considerable shared phenotypic properties, some small differences were recorded among the strains. For example, only *L. pasteurii* KoTa2^T and strains 37AN3*^{T} and 45AN2 grew with L-tartrate, an unusual ability considered by Janssen *et al.* (1995) to be distinctive of *L. pasteurii* KoTa2^T. Other differences among the strains were apparent in their abilities to grow on allantoin and L-malate (Table 1), and their fatty-acid profiles also showed distinctive features (Table 5). It was not possible to distinguish between the different strains using phenotypic characters, and the phenotypic and phylogenetic data together suggested that the strains all belonged to a single species. However, the DNA–DNA hybridization data do not support this view. Similarity values would suggest that *T. flocculiformis* Echt^T, '*C. allantoicus*' NDP and strains Ben 200, Ben 201 and Ben 77 should all be placed in the same genomic species (similarity values of > 70 %), whereas strains 37AN3*^{T} and 45AN2 (similarity value of 98 %) should be placed in a separate genomic species. On this evidence, both *L. pasteurii* KoTa2^T and *R. palustris* Z-7189^T, despite being 99 % similar to all of the other strains on the basis of their 16S rRNA sequences, each belong to separate genomic species.

Other bacteria have been described with very high or identical 16S rRNA sequences, which after DNA–DNA hybridization determinations emerged as separate species (Fox *et al.*, 1992; Yassin *et al.*, 1997; Maszenan *et al.*, 2000). For example, *Sporosarcina psychrophila* W16A^T and W5 and *Sporosarcina globispora* W25^T exhibited more than 99·5 % similarity in their 16S rRNA sequence, yet DNA–DNA hybridization values between them were too low (23 and 50 %) for them to be considered as the same species, even though they exhibited very similar phenotypic properties (Fox *et al.*, 1992; Yoon *et al.*, 2001). Yassin *et al.* (1997) also reported that the high levels of 16S rDNA sequence similarity (> 99 %) between *Tsukamurella* strains were not supported by DNA–DNA hybridization data. These experiences emphasize that 16S rDNA genes may be conserved between members of closely related bacterial species and that they cannot be used to differentiate between them at the species level (Fox *et al.*, 1992; Goodfellow, 2000).

Although the genus name *Trichococcus* predates the genus name *Lactosphaera*, it is not really a descriptive name for this group of bacteria (e.g. Figs 1 and 2). The reasons why cell morphologies in this group of bacteria

vary so much are not known, but they have particular relevance when using microscopic identification methods to detect these bacteria in activated sludge.

This study suggests that this genus should include four species, namely *Trichococcus flocculiformis*, to include *T. flocculiformis* Echt^T (Scheff *et al.*, 1984a), '*C. allantoicus*' NDP, Ben 77, Ben 200 and Ben 201, *Trichococcus pasteurii*, to include *L. pasteurii* KoTa2^T, *Trichococcus palustris*, to include *R. palustris* Z-7189^T, and *Trichococcus collinsii*, to include strains 37AN3*^{T} and 45AN2. The descriptions of these four species are given below.

Emended description of the genus *Trichococcus* (Scheff *et al.* 1984b)

Spherical to ovoid, sometimes olive-shaped cells with a mean cell diameter of 1·0–1·5 μm and a mean cell length of 1·0–2·5 μm, usually arranged in chains but sometimes appearing as single or paired cocci.

Aerotolerant, fermentative organisms in the low-G+C Gram-positive bacteria. Cells grow with glucose to produce lactate and acetate oxically, and to produce lactate, acetate, formate and ethanol anoxically. Cells also grow with sucrose and lactose, with acid production. All members of the genus utilize arbutin, cellobiose, D-fructose, α-D-glucose, D-mannose, salicin, sucrose and pyruvate with the BIOLOG system. None of the following substrates are utilized: β-cyclodextrin, inulin, Tween 40, Tween 80, L-arabinose, D-arabitol, L-fucose, D-galacturonic acid, D-gluconic acid, *myo*-inositol, methyl α-D-glucoside, methyl α-D-mannoside, sedoheptulosan, D-sorbitol, D-tagatose, xylitol, D-xylose, acetic acid, α-hydroxybutyrate, β-hydroxybutyrate, γ-hydroxybutyrate, β-hydroxyphenylacetate, 2-oxoglutarate, 2-oxovalerate, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, monomethylsuccinic acid, propionic acid, succinamic acid, *N*-acetylglutamic acid, alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, inosine, thymidine, adenosine 5′-monophosphate, thymidine 5′-monophosphate, uridine 5′-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, DL-α-glycerol phosphate. No indole is produced. The DNA base compositions range from 45 to 49 mol % G+C. The peptidoglycan type is type A4α, L-Lys-D-Asp (A 11.31). The natural habitats of members of the genus are activated sludge, soil and sediment. The type species of the genus is *Trichococcus flocculiformis* Echt^T.

Emended description of *Trichococcus flocculiformis* (Scheff *et al.* 1984b)

T. flocculiformis is a previously described organism, originally isolated from bulking sludge in Germany (Scheff *et al.*, 1984a). Results in this study suggest that '*N. limicola*' I (strains Ben 200, Ben 201 and Ben 77) and '*C. allantoicus*' NDP should be merged into this species, even though some of these strains exhibit morphologies which may differ from the original descriptions of cocci in chains. Under certain culture conditions, *T. flocculiformis* Echt^T grows as single cells or as paired, rod-shaped cells. In addition to the properties given in the genus description above and the original species description, members of this species have the following characteristics. Cells are spherical to ovoid; some strains grow as olive-shaped cells. Colonies on SR2A agar are white, opaque, circular and convex. All strains show no growth with L-tartrate. Only some strains grow on allantoin and L-malate. Only some produce acid from inositol, sorbitol, mannitol, raffinose and adonitol; only some reduce nitrate and are urease positive. Fatty acid composition is $C_{14:0}$ (8–14 %), $C_{16:0}$ (12–24 %), $C_{16:1}$ (39–50 %), $C_{iso\text{-}17:1}$ (1–4 %), $C_{18:0}$ (1–3 %) and $C_{18:1(\omega 9c)}$ (14–23 %). The G+C content is 47–49 mol %. Isolated from activated sludge in different parts of the world or from duck-pond sediment in the USA. The type strain of *Trichococcus flocculiformis* is Echt^T (= DSM 2094^T).

Description of *Trichococcus collinsii* sp. nov.

Trichococcus collinsii (coll.ins′i.i. N.L. gen. n. *collinsii* referring to M. D. Collins, a contemporary English microbiologist who has contributed significantly to our understanding of the systematics of the lactic acid bacteria).

This species is proposed to include strains 37AN3*^{T} and 45AN2, isolated from a hydrocarbon-spill site (Geig *et al.*, 1999). In addition to the properties given in the genus description above, members of this species have the following characteristics. They can grow with citrate, L-malate, allantoin and L-tartrate, and produce acid from mannitol. Only strain 37AN3*^{T} produces acid from inositol, sorbitol, raffinose and adonitol. Fatty acid compositions show this species has a distinctively high $C_{14:0}$ fatty acid content, which ranges between 46 and 57 %. Other major fatty acids are $C_{12:0}$ (6–12 %) and $C_{16:0}$ (14–18 %). The type strain of *Trichococcus collinsii* is 37AN3*^{T} (= DSM 14526^T = ATCC BAA-296^T).

Emended description of *Trichococcus pasteurii* (Schink 1985; emend. Janssen *et al.* 1995) comb. nov.

T. pasteurii was originally transferred from the genus *Ruminococcus* (*R. pasteurii*; Schink, 1984) by Janssen *et al.* (1995) into the genus *Lactosphaera*, as *L. pasteurii*, as this organism has several distinctive properties, including the ability to produce significant amounts of lactic acid and being aerotolerant. Its description is the same as that for *L. pasteurii* KoTa2^T, as given by Janssen *et al.* (1995), except that cells usually appear as single regularly shaped and sized cocci, except when growing in R2A broth, when they become irregular in shape and size and are arranged in short chains. The strain grows with L-tartrate but not

with allantoin, and produces acid from inositol, mannitol, sorbitol, raffinose and adonitol. Cellular fatty acid composition is $C_{12:0}$ (1%), $C_{14:0}$ (28%), $C_{16:0}$ (16%), $C_{16:1}$ (42%), $C_{18:0}$ (2%) and $C_{18:1(\omega 9c)}$ (6%). The type strain of *Trichococcus pasteurii* is KoTa2^T (= DSM 2381^T = ATCC 35945^T).

Emended description of *Trichococcus palustris* (Zhilina *et al.* 1997) comb. nov.

T. palustris was originally described as *R. palustris* Z-7189^T (Zhilina *et al.*, 1995). 16S rRNA sequence data suggest that this organism should be merged into the genus *Trichococcus*, but DNA–DNA hybridization data suggest it is a separate species, namely *T. palustris*. The features of this organism are those given by Zhilina *et al.* (1995), except that it is an aerotolerant fermentative organism and is not anaerobic (Zhilina *et al.*, 1995). The type strain of *Trichococcus palustris* is Z-7189^T (= DSM 9172^T).

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