Comparison between *Streptococcus macedonicus* and *Streptococcus waius* strains and reclassification of *Streptococcus waius* (Flint *et al.* 1999) as *Streptococcus macedonicus* (Tsakalidou *et al.* 1998)

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Two species of dairy streptococci, *Streptococcus waius* and *Streptococcus macedonicus*, were originally characterized by 16S–23S intergenic spacer sequence analysis, random amplified polymorphic DNA fingerprinting, PFGE analysis and DNA–DNA reassociation experiments. All genetic data suggested that *S. waius* strains belong to the previously described species *S. macedonicus*. Likewise, the phenotypic characterization showed that strains of *S. macedonicus* and *S. waius* were highly related and easily differentiated from the closest phylogenetic neighbour, *Streptococcus bovis*, principally by their failure to produce a blackening reaction in medium containing aesculin. The utilization of maltose and cellobiose by *S. macedonicus*/*S. waius* strains allowed their differentiation from the most studied dairy species, *Streptococcus thermophilus*. On the basis of genetic and phenotypic data *S. macedonicus* and *S. waius* species should be considered synonyms and *S. macedonicus* has the priority.

Keywords: dairy streptococci, *Streptococcus macedonicus*, *Streptococcus waius*

INTRODUCTION

Thermophilic dairy streptococci have usually been identified as *Streptococcus thermophilus* or as '*S. thermophilus*-like' (Moschetti *et al.*, 1998; Flint *et al.*, 1999) micro-organisms. The identification of dairy streptococci as *S. thermophilus*-like micro-organisms is principally due to their unusual carbohydrate fermentation pattern and/or their different genotype, compared with those of *S. thermophilus* strains. Furthermore, these unusual strains fail to produce a blackening reaction in medium containing aesculin, a characteristic which has allowed the discrimination of these dairy streptococci from the other dairy and non-dairy *Streptococcus* species, such as *Streptococcus salivarius*, *Streptococcus bovis*, *Streptococcus alactolyticus* and *Streptococcus equinus*. Two species, *Streptococcus macedonicus* (Tsakalidou *et al.*, 1998) and

Streptococcus waius (Flint *et al.*, 1999) have been described which have allowed some clarification of the taxonomically undefined group of '*S. thermophilus*-like' organisms. The dairy-associated *S. macedonicus* strains were isolated from the naturally fermented Greek Kasser cheese and from sour mash, while *S. waius* strains were isolated from biofilms on stainless steel samples exposed to pasteurized skimmed milk and from dairy products from dairy manufacturing plants. On the basis of phenotypic characterization, *S. macedonicus* and *S. waius* are easily differentiated from *S. thermophilus* by their ability to produce acid from cellobiose, maltose, melibiose and raffinose. *S. macedonicus* and *S. waius* do not produce acid from aesculin, β -gentibiose and glycogen, and are bile-intolerant, hence they are more closely related to *S. thermophilus* than to the other dairy streptococci. However they are phylogenetically located within the heterogeneous *S. bovis* rRNA group, whereas *S. thermophilus* is closely related to *S. salivarius* (Schleifer *et al.*, 1991).

Abbreviations: ITS, intergenic spacer; RAPD, random amplified polymorphic DNA.

The phenotypic descriptions of *S. macedonicus* and *S. waius* allow a clear discrimination of the two species from the other dairy streptococci, but in the same taxonomic descriptions it is hard to find significant phenotypic traits that allow the differentiation between these two species. The only exception is the β -galactosidase activity that was reported to be negative in *S. macedonicus* strains and positive in *S. waius* strains. Likewise, there are no comparable and available genetic data that allow discrimination between *S. macedonicus* and *S. waius*, with the exception of the G + C content that is reported to be 38 and 34.6 mol %, respectively.

In this study we provide genotypic and phenotypic evidence that *S. macedonicus* and *S. waius* should be considered synonyms and that *S. macedonicus* has priority.

METHODS

Strains and laboratory media. Strains were routinely maintained at 4 °C after growth at 37 °C for 18 h in M17 broth (Difco). For longer term maintenance, stock cultures were stored in 20% (v/v) glycerol, 80% (v/v) M17 at –20 and –80 °C. The strains used in this work and their origin are shown in Table 1. Carbohydrate fermentation was carried out using BSM medium containing chlorophenol red at a final concentration of 4% (w/v) and the desired filter-sterilized carbohydrate at a final concentration of 0.2% (w/v), as described by De Man *et al.* (1960). Acetoin production was evaluated as described by Lanyi (1987). Exopolysaccharide production was evaluated in ruthenium

red milk plates consisting of 0.5% yeast extract, 10% skim milk powder, 1% sucrose, 1.5% agar and 0.08 g ruthenium red l⁻¹. Ruthenium red stains the bacterial cell wall producing red colonies for non-ropy strains and white colonies for ropy strains (Stingle *et al.*, 1996).

DNA preparation. For the PCR reaction total DNA was extracted from 100 µl of an overnight culture in M17 broth as described previously (Mora *et al.*, 2000). For larger amounts of DNA, cells from an overnight culture in 100 ml M17 broth were processed as described previously (Manachini *et al.*, 1985). All the DNA solutions obtained were stored at –20 °C.

PCR experiments. Amplification of the 16S–23S intergenic spacer (ITS) region was performed in a volume of 25 µl containing 1 µl bacterial genomic DNA solution obtained as described above and about 100 ng DNA, 2.5 µl 10 × PCR reaction buffer, 200 µM each dNTP, 2.5 mM MgCl₂, 0.3 µM each primer (ITSF, 5'-GTCGTAACAAGGTAGCCGTA-3', and ITSr, 5'-CAAGGCATCCACCGT-3') (Mora *et al.*, 1998) and 0.5 U *Taq* polymerase (Amersham Pharmacia). The temperature profile was as follows: 5 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and 30 cycles of 92 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min. Random amplified polymorphic DNA (RAPD) experiments with primers OPI02-mod (5'-GCTCGGAGGAGAGG-3') and AP2 (5'-AGTCAGCCAC-3') were performed in a final volume of 25 µl using the following temperature profile with a modified ramp at 50%: primary DNA denaturation step at 94 °C for 2 min followed by 5 cycles of 45 s at 94 °C, 45 s at 31 °C and 2 min at 72 °C; 35 additional cycles were carried out increasing the annealing temperature to 40 °C. For all the amplification cycles the final extension was continued for 7 min at 72 °C. The reproducibility of RAPD patterns was tested by repeating the analysis three times on

Table 1. Strains tested and their origin

Species	Strain	Origin
<i>S. macedonicus</i>	LMG 15061	Sour mash; Tsakalidu <i>et al.</i> (1998)
<i>S. macedonicus</i>	LMG 18487	Greek Kasseri cheese; Tsakalidu <i>et al.</i> (1998)
<i>S. macedonicus</i>	LMG 18488 ^T	Greek Kasseri cheese; Tsakalidu <i>et al.</i> (1998)
<i>S. macedonicus</i>	ASG 19*	Asiago cheese, Italy
<i>S. macedonicus</i>	ASG 38*	Asiago cheese, Italy
<i>S. macedonicus</i>	ASG 62*	Asiago cheese, Italy
<i>S. macedonicus</i>	ASG 69*	Asiago cheese, Italy
<i>S. macedonicus</i>	MON 30*	Montasio cheese, Italy
<i>S. waius</i>	NZRCC 20100 ^T	Biofilm on stainless steel; Flint <i>et al.</i> (1999)
<i>S. waius</i>	T	Casein; Flint <i>et al.</i> (1999)
<i>S. waius</i>	TLST2	Pasteurized milk
<i>S. waius</i>	TLST3	Pasteurized milk
<i>S. waius</i>	7c	Biofilm on stainless steel; Flint <i>et al.</i> (1999)
<i>S. waius</i>	6/2	Biofilm on stainless steel; Flint <i>et al.</i> (1999)
<i>S. bovis</i>	DSM 20480 ^T (ATCC 33317 ^T)	
<i>S. salivarius</i>	DSM 20560 ^T	
<i>S. salivarius</i>	ATCC 13419	
<i>S. equinus</i>	NCTC 10386	
<i>S. thermophilus</i>	ATCC 19258	
<i>S. thermophilus</i>	DSM 20617 ^T	
<i>L. lactis</i> subsp. <i>lactis</i>	IL1403	Le Bourgeois <i>et al.</i> (1992)

* Strains kindly provided by Dr Angioletta Lombardi, Veneto Agricoltura, Thiene, Italy.

the same DNA template and also using DNA extracted at different stages of growth.

All amplification reactions were performed in a Gene Amp PCR System 2400 (Applied). Following amplification, 5 µl of product was electrophoresed at 5 V cm⁻¹ (1.5–3 % agarose gel, 0.2 µg ethidium bromide ml⁻¹) in TAE buffer and photographed in UV light.

Computation of strain similarities. A computer similarity analysis of RAPD patterns was estimated by means of the Jaccard coefficient (Sneath & Sokal, 1973) and clustering of strains was based on the unweighted pair group method with arithmetic averages (UPGMA). The NTSYS-PC computer program (version 1.30; Rohlf, 1987) was used in the data analysis.

16S–23S ITS. The ITS amplified products of strains LMG 18488^T, LMG 15061, ASG 38, ASG 62 and MON 30, visualized by agarose gel electrophoresis, were excised from the gel using a Qiagen extraction kit and sequenced by using the dideoxy chain-termination method (Sanger *et al.*, 1977) using ABI Prism BigDye technology in an ABI Prism 310 DNA sequencer (Applied). ITSF and ITSr were used as sequencing primers. The sequences obtained were manually aligned with the published *S. waius*, *S. bovis* and *S. thermophilus* ITS sequences.

Determination of DNA–DNA relatedness. DNA–DNA homology was determined as described previously (Manachini *et al.*, 2000) by the optical renaturation rates method (Kurtzman *et al.*, 1979) with a model Response spectrophotometer equipped with an Advance Kinetics Graphic Version 1.3 thermoprogrammer (CIBA-Corning Diagnostics). For all samples tested the melting temperature (T_m) was calculated in 5 × SSC, the same salt concentration was used for the determination of DNA–DNA homology. The temperature of hybridization was 25 °C below the T_m previously calculated. The equation of Seidler & Mandel (1971) was used to calculate the extent of DNA–DNA reassociation.

T_m values for *S. macedonicus* and *S. waius* were also compared using the LightCycler (Roche) method of Xu *et al.* (2000).

PFGE. The procedure used to prepare genomic DNA in agarose blocks has been described by Kelly *et al.* (1993). DNA embedded in agarose was digested with the enzymes *Bss*HII, *I-CeuI* or *SmaI* (New England Biolabs), loaded into the wells of 1 % agarose gels (pulsed field certified agarose; Bio-Rad) and run at 200 V for 20 h at 14 °C in 0.5 × Tris-borate buffer using a CHEF DR II PFGE apparatus and model 1000 mini chiller (Bio-Rad). Pulse times used were 1–20 s for *Bss*HII, 1–30 s for *SmaI* and 5–50 s for *I-CeuI*. To determine the size of the largest fragments from *I-CeuI* digests, gels were prepared from 0.8 % chromosomal grade agarose (Bio-Rad) and run with the pulse time ramped between 150 and 300 s. DNA prepared from *Lactococcus lactis* subsp. *lactis* IL1403 (Le Bourgeois *et al.*, 1992) and *Saccharomyces cerevisiae* PFG markers (New England Biolabs) were used as size standards.

RESULTS AND DISCUSSION

Phenotypic traits

Phenotypic analysis of *S. macedonicus* and *S. waius* strains listed in Table 1 was carried out in comparison with *S. thermophilus* DSM 20617^T, *S. salivarius* DSM

20560^T and *S. bovis* DSM 20480^T (Table 2). *S. macedonicus* and *S. waius* strains were all able to grow in M17 broth in the presence of 4 % NaCl, while only *S. waius* strains showed growth in up to 6.5 % NaCl. No growth was detected in the presence of 4 % NaCl for *S. bovis* DSM 20480^T or *S. salivarius* DSM 20560^T. *S. macedonicus* and *S. waius* strains were able to produce acid from D-glucose, galactose, D-fructose, D-mannose, sucrose, cellobiose and maltose. All *S. macedonicus* and *S. waius* strains, except strain LMG 15061 were also able to utilize raffinose and melibiose. These properties have been linked to a transposable element in *L. lactis* (Kelly *et al.*, 1998) and if similarly arranged in *S. macedonicus* and *S. waius* could be relatively easily lost from a strain. All *S. macedonicus* and *S. waius* strains were negative when tested for acid production from glycogen and they failed to produce the blackening reaction in medium containing aesculin. The ability of *S. macedonicus* and *S. waius* to utilize galactose, maltose and cellobiose allowed their differentiation from *S. thermophilus* strains. Exopolysaccharide production was evaluated on ruthenium red milk where *S. macedonicus* and *S. waius* strains grew as white colonies.

In conclusion, the phenotypic characteristics analysed allowed an easy discrimination of *S. macedonicus* and *S. waius* strains from the closely related *S. thermophilus*, *S. salivarius* and *S. bovis*. It was not possible to detect phenotypic differences between *S. macedonicus* and *S. waius* strains with the exception of a moderately higher halotolerance shown by *S. waius* strains.

Genotypic characterization

The genetic characterization of *Streptococcus* species was carried out by comparing the 16S rDNA and ITS sequences, and evaluating the DNA–DNA homology values. Moreover, the genetic variability among *S. macedonicus* and *S. waius* strains was analysed by RAPD-PCR and PFGE experiments.

Phylogenetic analysis. The published 16S rDNA sequences of *S. macedonicus* LMG 18488^T and *S. waius* NZRCC 20100^T were compared to each other using the Sequence Similarity Matrix tool of the Ribosomal Database Project II (Maidak *et al.*, 2001). The 16S rDNA sequence similarity between these two species was 99.5 %. Likewise the 16S–23S rDNA ITS of *S. macedonicus* LMG 18487^T, LMG 15061, ASG 38, ASG 62 and M30 and the published ITS of *S. waius* NZRCC 20100^T (Flint *et al.*, 1999) were found to be identical. Sequence comparison showed that the main differences among the analysed species were located upstream and downstream of the highly conserved tRNA^{Ala} gene. No ITS sequence variation was observed among *S. macedonicus* and *S. waius* dairy strains isolated from different geographical areas, specifically Northern Italy, Greece and New Zealand.

DNA–DNA reassociation experiments and G + C content. To confirm the close genetic relationship of *S. macedonicus* and *S. waius* strains, DNA–DNA hybrid-

Table 2. Metabolic traits of *S. macedonicus*, *S. waius*, *S. thermophilus* and *S. bovis*

Data in parentheses were obtained in this study; ND, not determined; +, positive; –, negative; v, variable among the analysed strains; w, weak growth.

Characteristic	<i>S. macedonicus</i> *	<i>S. waius</i> †	<i>S. thermophilus</i> DSM 20617 ^{T†}	<i>S. bovis</i> DSM 20480 ^{T†}	<i>S. salivarius</i> DSM 20560 ^T
Glycerol	–	v	–	–	(+)
Glycogen	–	–	–	+	(+)
D-Arabinose	(–)	–	–	–	(+)
Galactose	(+)	+	–	+	(+)
D-Glucose	(+)	+	+	+	(+)
D-Fructose	(+)	+	+	+	(+)
D-Mannose	(+)	+	+	+(–)	(+)
N-Acetylglucosamine	–(+)	+	+	+	(+)
Aesculin	(–)	–	–	+	(+)
Salicin	(v)	+	–	+	(+)
Inulin	–	–	–	+	(+)
Cellobiose	+	+	–	+	(+)
Maltose	+	+	–	+	(+)
Lactose	(+)	+	+	+	(+)
Melibiose	(v)	+	–	+	(+)
Sucrose	(+)	+	+	+	(+)
D-Raffinose	(v)	+	–	+	(+)
Growth in presence of NaCl					
4%	(+)	+	–	–	(–)
6.5%	(w)	+	–	–	(–)
7%	(–)	+	–	–	(–)
Production of:					
Acetoin	+	(+)	+	(–)	(–)
Exopolysaccharide	(+)	(+)	(–)	(–)	ND

* Based on the description of Tsakalidu *et al.* (1998).

† Based on the descriptions of Flint *et al.* (1999) and Hardie (1986).

ization experiments were carried out among some representative strains of each species. Values of 90–100% were obtained between *S. waius* NZRCC 20100^T, *S. macedonicus* LMG 18487^T and *S. macedonicus* LMG 15061. Furthermore, a DNA–DNA reassociation value of 40% was found between *S. macedonicus* DSM 18487^T and the closest phylogenetic neighbour *S. bovis* DSM 20480^T. These data confirm that *S. macedonicus* (Tsakalidu *et al.*, 1998) and *S. waius* (Flint *et al.*, 1999) belong to the same DNA–DNA homology group, well separated from the closest phylogenetically related species, *S. bovis*.

Melting curve comparisons using the LightCycler (Roche) produced G + C values that were consistently lower than the published values, with 32.0 mol% for *S. macedonicus* and 33.6 mol% for *S. waius*, supporting the similarity between the two organisms. The G + C content of *S. bovis* DSM 20480^T (ATCC 33317^T) used as reference was 34.7 mol%.

RAPD-PCR analysis and computation of strain similarity. The genetic variability of *S. macedonicus* and *S. waius* strains and related *Streptococcus* species was analysed by RAPD analysis using the OPI02-mod primer. As

shown in Fig. 1, *S. macedonicus* and *S. waius* strains formed a cluster characterized by two main amplified fragments of about 600 and 810 bp and by a weaker amplified fragment of 250 bp. Amplified fragments ranging from 380 to 600 bp, and from 1400 to 1450 bp allowed the identification of different genotypes among the *S. macedonicus/waius* homology group.

The RAPD analysis using the AP2 primer supported the above results, confirming the similarity between *S. macedonicus* and *S. waius* (data not shown).

A UPGMA dendrogram (Fig. 2) derived from similarity coefficients calculated by the Jaccard method on the RAPD patterns, showing the relationship among the analysed strains, revealed that *S. macedonicus* and *S. waius* were well separated from *S. bovis* DSM 20480^T at a similarity level less than 5%. New Zealand *S. waius* strains were clustered together at a similarity level of 71.2% and they were separated from the nearest *S. macedonicus* European isolates at a similarity level of 52%. *S. macedonicus* strains isolated from cheeses from northern Italy and from Greek dairy products showed a high degree of diversity and were clustered in different groups. Interestingly, the

Fig. 1. Agarose gel electrophoresis showing the RAPD profiles obtained from *S. macedonicus* and *S. waius* strains and related streptococci using the OPI02-mod primer. Lanes: 1–5: *S. waius* NZRCC 20100^T, TLST3, TLST2, 7C and T, respectively; 6–10: *S. macedonicus* LMG 15061, LMG 18488^T, LMG 18487, M30 and ASG 62, respectively; 11, *S. bovis* DSM 20480^T; 12, *S. thermophilus* DSM 20617^T. M, molecular mass marker (GeneRuler 1 kb DNA Ladder; MBI Fermentas).

only vegetable-associated *S. macedonicus* strain, LMG 15061, showed the lowest similarity value with the other strains of the same species (about 40 %).

PFGE analysis. PFGE data (Fig. 3) support the hypothesis that *S. macedonicus* and *S. waius* are the same species, since the banding patterns for digests with *Bss*HII and *Sma*I (which recognize random sites

around the genome) are similar. The *Bss*HII digests provide additional evidence that *S. macedonicus*/*S. waius* are more closely related to *S. bovis* than they are to *S. thermophilus*.

DNA from strain LMG 18487 was not cut by *Sma*I, which suggests that the *Sma*I cutting site could be blocked by methylation and that this strain could carry a restriction-modification system. DNA from some *L. lactis* strains also fails to be cut with *Sma*I and most of these strains encode a methylase associated with the *Scr*FI restriction-modification system (Davis *et al.*, 1993; Ward & Kelly, unpublished data). Restriction-modification or other bacteriophage defence mechanisms have not previously been reported for *S. macedonicus* or *S. waius*, but their presence in dairy environments implies that they are vulnerable to bacteriophage attack in the same way as other dairy bacteria.

The intron-encoded endonuclease I-*Ceu*I recognizes a 19 bp sequence specific to all 23S rRNA genes and so produces a number of restriction fragments which correspond to the number of rRNA operons on the chromosome. In accordance with the 16S rRNA data, the *S. macedonicus* and *S. waius* strains examined each have six copies of the rRNA genes and show the same *rrn* genome organization. The chromosome size of the eight strains ranged between 2150 and 2250 kb. This is considerably larger than the *S. thermophilus* chromosome which has previously been estimated at ~ 1850 kb and which may have five or six copies of the rRNA genes (Roussel *et al.*, 1997).

Fig. 2. UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method (S_j , shown on the scale at the bottom), showing the relationship among *S. macedonicus*, *S. waius* and related dairy streptococci analysed by RAPD using primers OPI02-mod and AP2.

Fig. 3. PFGE analysis of *Streptococcus* species. Genomic DNA cut with *Bss*HII (a), *Sma*I (b) or *I-Ceu*I (c). Lanes: 1–3, *S. macedonicus* LMG 15061, LMG 18487 and LMG 18488^T, respectively; 4–8, *S. waius* 3/1, 7c, T, TLST2 and TLST3, respectively; 9, *S. bovis* ATCC 33317^T; 10, *S. equinus* NCTC 10386; 11, *S. salivarius* ATCC 13419; 12, *S. thermophilus* ATCC 19258; 13, *L. lactis* subsp. *lactis* IL1403.

Revision of the taxonomic position of *S. waius*

In the present study, phenotypic and genotypic analysis carried out on *S. macedonicus* and *S. waius* strains suggest that they should be ascribed to the same species. Both the 16S and the ITS sequences showed

extremely high correlation between the two previously described taxa. Likewise, DNA–DNA reassociation values above 80 % among *S. macedonicus* and *S. waius* strains confirmed that they belong to the same homology group. Moreover, the weak degree of variability observed in phenotypic characteristics (raffinose and melibiose fermentation) does not justify the allocation as two different species. We therefore suggest the reclassification of *S. waius* (Flint *et al.*, 1999) as *S. macedonicus* (Tsakalidu *et al.*, 1998).

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