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16S–23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification

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The 16S–23S rRNA internal transcribed spacer (ITS) region of several *Streptococcus thermophilus* strains and some related dairy streptococci, *S. macedonicus*, *S. salivarius* and *S. bovis*, was analysed by sequence analysis. All the *Streptococcus* species were easily discriminated on the basis of sequence variations principally located upstream and downstream of the region encompassing the double-stranded processing sites and the tRNA^{Ala} gene. Comparison between tRNA^{Ala} gene sequences highlighted a high level of sequence conservation among the *Streptococcus* species investigated despite their belonging to separated phylogenetic clusters, i.e. the *S. salivarius* and *S. bovis* rRNA groups. A low but significant degree of variability was detected among the *S. thermophilus* strains, allowing the identification of four different ITS sequences. Similarity analysis of the ITS sequences showed that the *Streptococcus* species were clustered in two main branches, one containing *S. macedonicus* and *S. bovis* strains, and one containing *S. thermophilus* and *S. salivarius* strains. With the aim of developing a rapid tool for the identification of the dairy streptococci species a multiplex ITS-SSCP analysis of two discrete regions within the ITS locus was carried out.

INTRODUCTION

Thermophilic dairy streptococci are routinely identified as *Streptococcus thermophilus* or as '*S. thermophilus*-like' micro-organisms. The latter show a similar or a slightly atypical carbohydrate fermentation pattern, compared with those of *S. thermophilus*, but are a different genotype. The taxonomic position of these anomalous strains has been clarified starting from the description of the species *Streptococcus macedonicus* and *Streptococcus waius* (Flint *et al.*, 1999; Tsakalidou *et al.*, 1998). These species are phylogenetically located in the *Streptococcus bovis* rRNA group while *S. thermophilus* is closely related to *Streptococcus salivarius.*

Within the bacterial chromosome, the ribosomal locus has often been investigated with taxonomic and/or phylogenetic purpose; the internal transcribed spacer (ITS) region located between the 16S and the 23S rRNA genes provides an excellent tool for a finer identification at the species/strain level, while ribosomal genes may lose resolution at the lower taxa level (Rodriguez-Valera & Garcia-Martinez, 2000). ITS regions show highly conserved sequences in the areas encompassing the tRNA genes and relatively stable regions located at the ends of the spacer, where secondary structures are formed by pairing with stretches upstream of the 16S rRNA gene and downstream of the 23S rRNA gene. ITS sequence analysis has been applied successfully for the identification and differentiation of several bacterial species (Ehrenstein *et al.*, 1996; Maes *et al.*, 1997; Tilsala-Timisjarvi & Alatossova, 1997; Vaneechoutte *et al.*, 1992) and a freely available spacer database has recently been developed (Rodriguez-Valera & Garcia-Martinez, 2000).

In this study, several strains of *S. thermophilus*, *S. macedonicus* and *S. waius* isolated from various sources, such as yogurt, whey, natural cheese starter, cheese and biofilms on stainless steel surfaces of dairy manufacturing plants, were analysed by sequencing the 16S–23S rRNA ITS. The genetic variability of the 16S–23S ribosomal ITS locus

Abbreviations: ITS, internal transcribed spacer; MIS, multiplex ITS-SSCP (analysis); SSCP, single-strand conformational polymorphism.

The GenBank accession numbers for the sequences determined in this work are given in the text.

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of thermophilic dairy streptococci has been investigated with the double aim of obtaining a detailed picture of this biotechnologically important group of lactic acid bacteria and finding an effective method of identifying them. The availability of rapid and simple methods for the identification of these dairy streptococci would facilitate the investigation of the microbial composition of traditional dairy products produced with raw milk without the use of selected starter cultures. In addition, a species/strain-specific multiplex ITS-SSCP analysis (MIS) of the regions upstream and downstream of the tRNA gene was carried out.

METHODS

Bacterial strains. All *Streptococcus* strains used in this work were routinely maintained at 4 °C after growth at 37 °C for 18 h in M17 broth (Difco). For long-term maintenance, stock cultures were stored in 20 % (v/v) glycerol, 80 % (v/v) M17 broth at −80 °C. The strains of lactic acid bacteria used in this work, their origin and some relevant characteristics are shown in Table 1.

DNA extraction and PCR experiments. Total bacterial DNA was extracted as previously described (Mora *et al.*, 2000) starting from 100 µl of M17 broth culture. A DNA 16S–23S rRNA ITS region amplification was performed in a volume of 100 µl containing: 3 µl bacterial genomic DNA solution, 10 µl 10 × PCR reaction buffer, 200 µmol l^{-1} of each dNTP, 2 mmol l^{-1} $MgCl_2$, 0·5 µmol l^{-1} of each primer (ITSF 5′-GTCGTAACAAGGTAGCCGTA-3′ and ITSR 5′-CAAGGCATCCACCGT-3′) and 0·5 U *Taq* DNA polymerase (Amersham-Pharmacia). The temperature profile was the following: 5 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; and 30 cycles consisting of 92 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min. A final extension at 72 °C for 7 min was performed. All amplification reactions were performed in a Gene Amp PCR System 2400 (Applera). Following amplification, 2 µl of product was analysed at 5 V cm^{-1} by agarose electrophoresis (1·5 % agarose gel, 0·2 µg ethidium bromide ml^{-1}) or polyacrylamide electrophoresis (6 %, acrylamide : bisacrylamide, 29 : 1, w/w) in TBE buffer (90 mmol l^{-1} Tris/borate, 2 mmol l^{-1} Na_2EDTA, pH 8) and imaged under UV light.

The 16S rRNA gene was amplified as previously described (Mora *et al.*, 2000). The amplification of the *sodA* gene was carried out in 100 µl containing: 3 µl bacterial genomic DNA solution obtained as described above, 10 µl 10 × PCR reaction buffer, 200 µmol l^{-1} of each dNTP, 2 mmol l^{-1} $MgCl_2$, 0·5 µmol l^{-1} of each primer (sodAF 5′-CGATGCAGAAACAATGACATT-3′, sodAR 5′-GGATTGTCTTG-GTTAGCTGT-3′, and sodAR1 5′-GGAGTATCTTGGTTAGCAGT-3′) and 0·5 U *Taq* polymerase (Amersham-Pharmacia). The primer set for the *sodA* gene was designed on the basis of the sequences reported by Poyart *et al.* (2002); sodAF, sodAR and sodAR1 were used together in the same PCR reaction to allow the amplification of *sodA* gene both in *Streptococcus infantarius* and in *Streptococcus lutetiensis* (previously *S. infantarius* subsp. *coli*) (Schlegel *et al.*, 2000). The temperature profile was the following: 94 °C for 2 min and 35 cycles consisting of 94 °C for 45 s, 58 °C for 35 s and 72 °C for 50 s. A final extension at 72 °C for 7 min was performed.

Table 1. Strains tested and their origin

Streptococcus strain	Origin and relevant characteristics
S. thermophilus	DSM 20617^T
S. thermophilus	A1, B1, C1, D1, E1, F1, G1, H1, I1, I2, L1, N1, N2, P1, Q1; strains isolated from yogurt (Mora *et al.*, 2002)
*S. thermophilus**	ST20, isolated from whey starter; ST24, isolated from Toma cheese; ST52, isolated from Provolone cheese; ST29, isolated from whey starter from Provolone cheese (Mora *et al.*, 2002)
S. thermophilus†	ST32, isolated from natural cheese starter; ST35, isolated from Crescenza cheese; ST69, isolated from whey starter (Mora *et al.*, 2002)
Streptococcus sp.	42†, 48† isolated from Grana cheese; A2.3, A2.7, A2.11, A2.9, B1.21, B1.23, B1.24, B1.18 isolated from Toma cheese
S. macedonicus	LMG 15061, sour mash (Tsakalidou *et al.*, 1998)
S. macedonicus	LMG 18487, LMG 18488^T Greek Kasseri cheese (Tsakalidou *et al.*, 1998)
S. macedonicus‡	ASG 38, ASG 62, isolated from Asiago cheese; MON30, isolated from Montasio cheese, Italy
'*S. thermophilus*-like'‡	ASG 19, ASG 69, isolated from Asiago cheese, Italy
'*S. thermophilus*-like'§	RALF 1, raw milk of Modicana cow race; RACF7, curd of Ragusano cheese; RAC2413, 'pasta filata' of Ragusano cheese; RCF15807, Ragusano cheese after 15 days of ripening
'*S. thermophilus*-like'	TA3.1, TA3.4, TA3.5, TA3.7, TA3.9, TB1.4, TB1.9, TB1.12, TB1.15 isolated from Italian Toma cheese
S. waius‖	NZRCC 21100^T, isolated from biofilm on stainless steel; T, TLST3 isolated from casein; 7c, 6/2, isolated from biofilm on stainless steel (Flint *et al.*, 1999)
S. bovis	DSM 20480^T
S. salivarius	DSM 20560^T

*Strains kindly provided by Dr Giorgio Giraffa, Istituto Sperimentale Lattiero Caseario, Lodi, Italy.

†Strains kindly provided by Dr Mauro Scarpellini, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, sezione Microbiologia Agraria Ambientale e Ecologica, Università di Milano, Italy.

‡Strains kindly provided by Dr Angiolella Lombardi, Veneto Agricoltura, Thiene, Italy.

§Strains kindly provided by Professor Sandra Torriani, Università degli Studi di Verona, Italy.

‖Strains kindly provided by Dr Steve Flint, New Zealand Dairy Research Institute, Palmerston North, New Zealand. NZRCC, New Zealand Dairy Research Institute Culture Collection, Palmerston North, New Zealand.

ITS sequence analysis. After amplification, ITS products were purified (NucleoSpin Extract, Machery-Nagel) and sequenced using ITSF as a sequencing primer and a model 310 automatic DNA sequencer (Applera) with fluorescent dideoxy chain terminators. ITS sequences were manually aligned with the published sequences of *Streptococcus* species. Phylogenetic reconstructions were done using Jalview software (http://www2.ebi.ac.uk/ ~ michele/jalview/contents.html).

SSCP analysis. To develop a species-specific assay, the amplified ITS regions of all *Streptococcus* strains was used in SSCP analysis. Two different SSCP analyses were carried out: (i) an analysis performed on the entire ITS amplified using the primer set ITSF-ITSR, and (ii) an analysis performed on ITS-16Sf and on ITS-23Sf fragments separately amplified using the primer sets ITSF-ITSscpR and ITSscpF-ITSR (see Fig. 3). BLASTN analysis (http://www.ncbi.nlm.nih.gov/BLAST) carried out on ITSscpR and ITSscpF sequences showed significant matches only for ITS region of species belonging to the genus *Streptococcus*.

The SSCP analyses were performed on MDE gels prepared as described by the manufacturer (FMC Bioproducts) or on a standard polyacrylamide gel. For all SSCP analyses, 2–4 μl of each PCR product plus 5 μl gel loading solution was denatured at 100 °C for 15 min and immediately cooled on ice. After 1 min, 3 μl of the solution was analysed by MDE or polyacrylamide gel electrophoresis in TBE buffer using a Miniprotean III apparatus (Bio-Rad). The gel was run at 5 V cm^{-1} in TBE buffer, stained in a solution containing 0·5 μg ethidium bromide ml^{-1} and photographed in UV light. The reproducibility of the method was evaluated by using amplified fragments obtained from three independent amplifications and repeating the electrophoresis on two independent gels.

RESULTS AND DISCUSSION

ITS amplification and sequence analysis

DNA was extracted from a total of 23 *S. thermophilus* strains, six *S. macedonicus* strains, five *S. waius* strains, and the type strains of *S. bovis* and *S. salivarius*, and used for the amplification of the 16S–23S rRNA ITS region. Length polymorphism analysis of amplified ITS regions has been used as an efficient tool for species differentiation by several authors (Mora *et al.*, 1997; Tyrrell *et al.*, 1997; Daffonchio *et al.*, 2000) but it was not applicable for the differentiation of several species of dairy streptococci including those analysed in this study. A single ITS amplification product of about 350 bp was detected in *S. thermophilus* and *S. salivarius* strains using a standard agarose gel electrophoresis as previously reported by Tilsala-Timisjärvi & Alatossava (1997) and by Moschetti *et al.* (1998). Furthermore, (data not shown) a 350 bp amplified fragment was also detected in *S. bovis*, *S. macedonicus* and *S. waius*, increasing the number of *Streptococcus* species showing identical ITS profiles and often isolated from cheeses produced with raw milk and natural starter (Andrighetto *et al.*, 2002; Tsakalidou *et al.*, 1998). Identical results were obtained for all '*S. thermophilus*-like' and *Streptococcus* sp. strains listed in Table 1.

The ITS sequence analysis carried out on *S. thermophilus* and related species showed that the length of their ITS between the 16S and the 23S rRNA genes ranged between 273 and 274 bp. All *Streptococcus* species were easily discriminated on the basis of sequence variations principally located upstream and downstream of the region encompassing the double-stranded processing sites and the $tRNA^{Ala}$ gene. Comparison between $tRNA^{Ala}$ gene sequences highlighted a high level of sequence conservation among *Streptococcus* species despite their belonging to different phylogenetic clusters, i.e. the *S. salivarius* and *S. bovis* rRNA groups. With regard to *S. thermophilus* strains, ITS sequence comparison highlighted single insertions/deletions and substitutions that allowed the identification of four different ITS sequences (Fig. 1), designated ITS-*St*-I, ITS-*St*-II, ITS-*St*-III and ITS-*St*-IV. The sequence type ITS-*St*-I of 274 bp showed 100 % similarity with the published ITS sequence of *S. thermophilus* ATCC 19987 (U32965) and was characteristic of the strains DSM 20617^T, ST29, ST32, ST69, E1, Q1, G1, N2, N1, D1 and C1; strains A1, H1, P1, I1, L1, ST20, ST24 and ST35 showed the sequence type ITS-*St*-II of 273 bp due to an adenine deletion in position 282 (Fig. 1). A single substitution in position 284, an insertion in position 286 and a deletion in position 282 allowed the identification of the sequence ITS-*St*-III for the strain ST52 while the sequence type ITS-*St*-IV of strains B1 and I2 was characterized by two substitutions, at positions 21 and 275. Sequence ITS-*St*-IV showed the highest similarity with the ITS sequence of *S. salivarius* DSM 20560^T (Fig. 2). Specifically, the substitution in position 264 (A instead of G) determined the lack of one of the two *Hae*III restriction sites that were reported to be species-specific for *S. thermophilus* strains by Moschetti *et al.* (1998). These authors also reported the presence of an anomalous *S. thermophilus* strain showing a *Hae*III ITS restriction profile identical to that obtained for *S. salivarius* but characterized by typical *S. thermophilus* phenotypic traits. As substantiated by our sequence data, *Hae*III restriction analysis highlights an ITS sequence polymorphism in *S. thermophilus* but fails to differentiate this species from the closely related *S. salivarius*.

A discrete level of ITS sequence variation was also observed between *S. salivarius* DSM 20560^T and the ITS sequence of *S. salivarius* ATCC 13419 obtained from the EMBL/GenBank database.

While *S. thermophilus* strains showed ITS sequence polymorphism that allowed the identification of four different ITS alleles, an extremely high level of sequence conservation (100 % sequence similarity) was detected among the *S. macedonicus* and *S. waius* strains. This high level of sequence similarity was unexpected considering the different geographical origin of the strains, i.e. New Zealand and Greece. Furthermore, ITS sequencing did not allow differentiation between *S. macedonicus* and *S. waius* strains according to our previous observation based on genotypic and phenotypic characterization which provided evidence that *S. macedonicus* and *S. waius* should be considered synonyms (Manachini *et al.*, 2002). ITS sequence variation was also detected within the *S. bovis* species, confirming the genetic heterogeneity of this taxon (Farrow *et al.*, 1984; Poyart *et al.*, 2002).

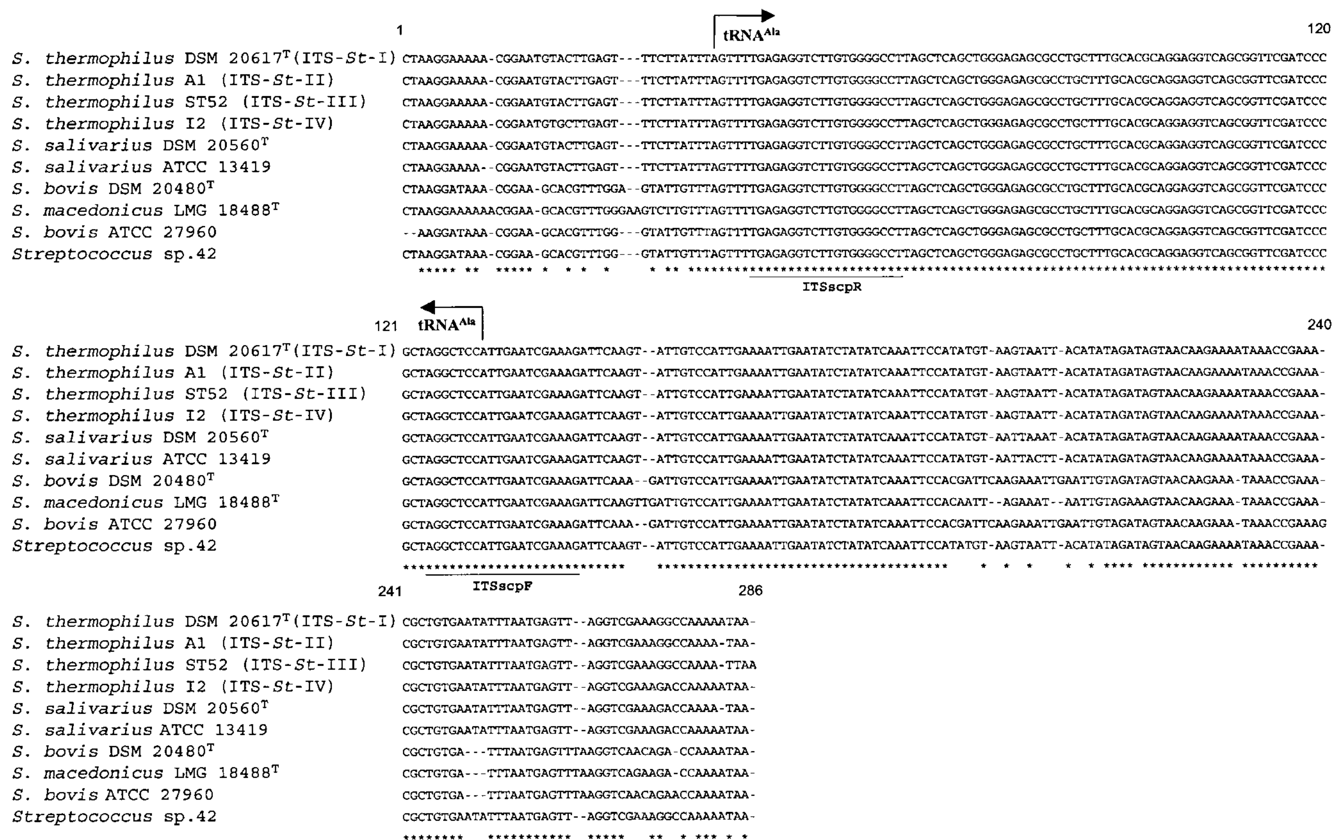

Fig. 1. 16S–23S rRNA ITS sequence alignment of *Streptococcus* species. The $tRNA^{Ala}$ gene is delimited by arrows. Target sequences of primers ITSscpF and ITSscpR are underlined. The ITS sequence types detected in *S. thermophilus* strains are indicated in parentheses after the strain names. The ITS sequence accession numbers are the following: *S. thermophilus* sequence type ITS-*St*-I (U32965), *S. thermophilus* sequence type ITS-*St*-II (AJ439455), *S. thermophilus* sequence type ITS-*St*-III (AJ439456), *S. thermophilus* sequence type ITS-*St*-IV (AJ439457), *S. salivarius* DSM 20560^T (AJ439458), *S. salivarius* ATCC 13419 (X83760), *S. bovis* DSM 20480^T (AJ439459), *S. bovis* ATCC 27960 (U39766), *Streptococcus* sp. 42 (AJ439460), *S. macedonicus*/*waius* (AF088899).

The phylogenetic analysis based on ITS sequences (Fig. 2) showed that the dairy *Streptococcus* species investigated clustered into two main branches, one grouping *S. macedonicus* and *S. bovis*, and one grouping *S. thermophilus* and *S. salivarius* strains. Further investigation should be carried out to verify if the phylogenetic diversity of *S. thermophilus* strains may reflect some relevant phenotypic traits of this species.

ITS-SSCP analysis

On the basis of the ITS sequence polymorphism detected, SSCP analyses on the entire ITS, and on a discrete region within the ITS locus, were carried out with the aim of developing a species-specific assay. The SSCP analysis of the entire amplified ITS failed to differentiate streptococcal species (data not shown) due to the high molecular mass of the amplified fragment (about 350 bp) and the limited level of sequence divergence present among the species analysed. Two new sets of primers were then designed for the amplification of the regions upstream and downstream of the highly conserved $tRNA^{Ala}$ gene. The new experimental design focused on the amplification of the most variable regions within the ITS locus by the exclusion of the conserved tDNA (Fig. 3). The amplified fragments obtained from the region upstream and downstream of the $tRNA^{Ala}$ gene, designated ITS-16Sf and ITS-23Sf, showed dimensions of about 110 bp and 190 bp respectively, according to the sequence data. The SSCP analysis of ITS-16Sf and ITS-23Sf showed better discriminatory power than the use of the entire ITS. Comparison of the SSCP patterns of the ITS-16Sf and ITS-23Sf fragments showed the latter region to be the most effective in addressing the identification at species level, allowing the differentiation of most of the *Streptococcus* species analysed. Interestingly, the ITS-23Sf fragment generated three single-strand electrophoretic signals, suggesting sequence variation, not detected in the sequence analysis, in the ITS region downstream of the

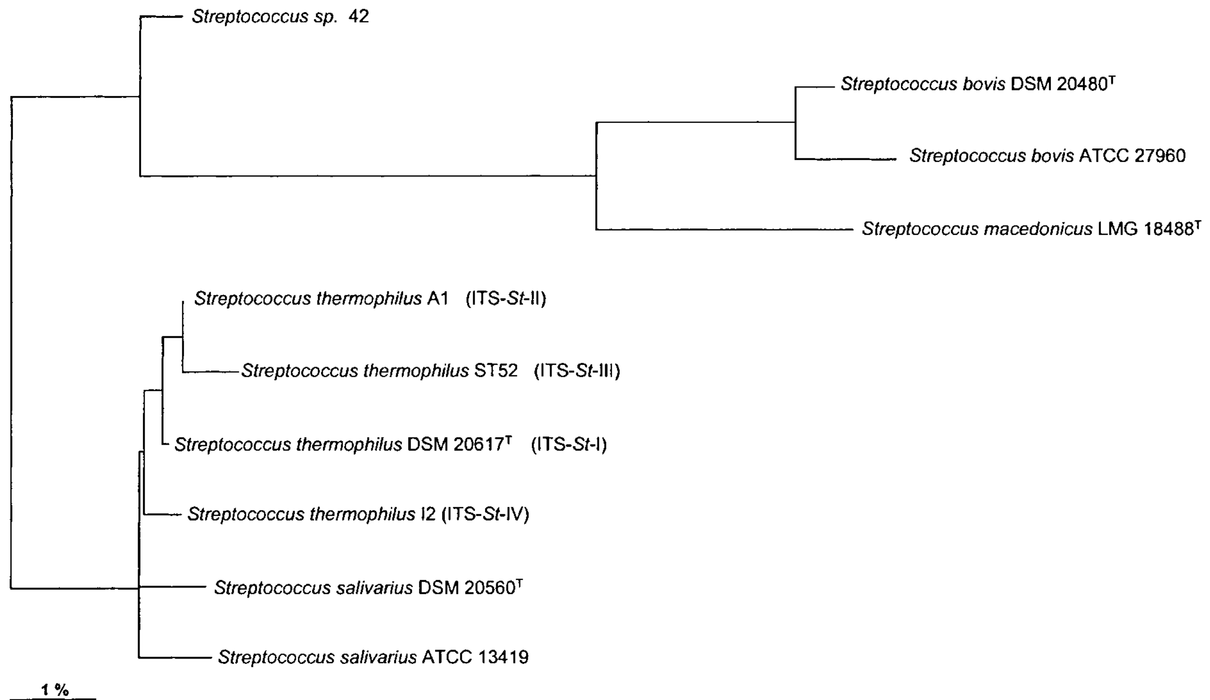

Fig. 2. Neighbour-joining tree based on the 16S–23S rRNA ITS sequences, showing relationships among *Streptococcus* species. The ITS sequence types detected in *S. thermophilus* strains are indicated in parentheses after the strain names.

tRNA^{Ala} gene present in the different copies of the ribosomal operon of the bacterial chromosome. *Streptococcus* sp. 42 and 48 showed an ITS-23Sf SSCP pattern identical to that obtained for all *S. thermophilus* strains except B1 and I2. The SSCP of the ITS-16Sf fragment allowed the identification of *S. macedonicus* strains but failed to differentiate between *S. salivarius* and *S. thermophilus*. Furthermore, an identical ITS-16Sf SSCP profile was shown by *S. bovis* DSM 20480^T and *Streptococcus* sp. 42 and 48 (data not shown).

An efficient *Streptococcus* species identification was achieved by MIS analysis of ITS-16Sf and ITS-23Sf fragments as shown in Fig. 4. All the species analysed showed unique SSCP profiles and a strain-specific SSCP profile was detected for *S. thermophilus* I2 which easily discriminated it from the other *S. thermophilus* strains by the single-strand conformation polymorphism generated from the ITS-23Sf fragment. The species-specific signature profiles were identified in the SSCP profile of the ITS-16Sf and/or ITS-23Sf fragments depending on the species. *Streptococcus thermophilus* strains were differentiated from *S. salivarius* by the SSCP profile generated from the ITS-23Sf fragment and from *Streptococcus* sp. 42 and 48 by the SSCP profile generated from the ITS-16Sf fragment, while *S. macedonicus* strains were discriminated from *S. bovis* and *S. thermophilus* by the SSCP profile of both the amplified fragments (Fig. 4). In conclusion, the MIS approach, designed with the aim of reducing the influence of the highly conserved tDNA^{Ala}, is effective for the identification of dairy and non-dairy streptococcal species.

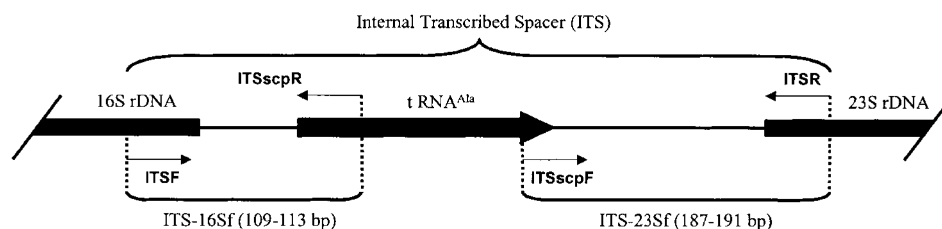

Fig. 3. Schematic representation of the 16S–23S rRNA ITS of dairy streptococci and position of the primers used in the MIS approach.

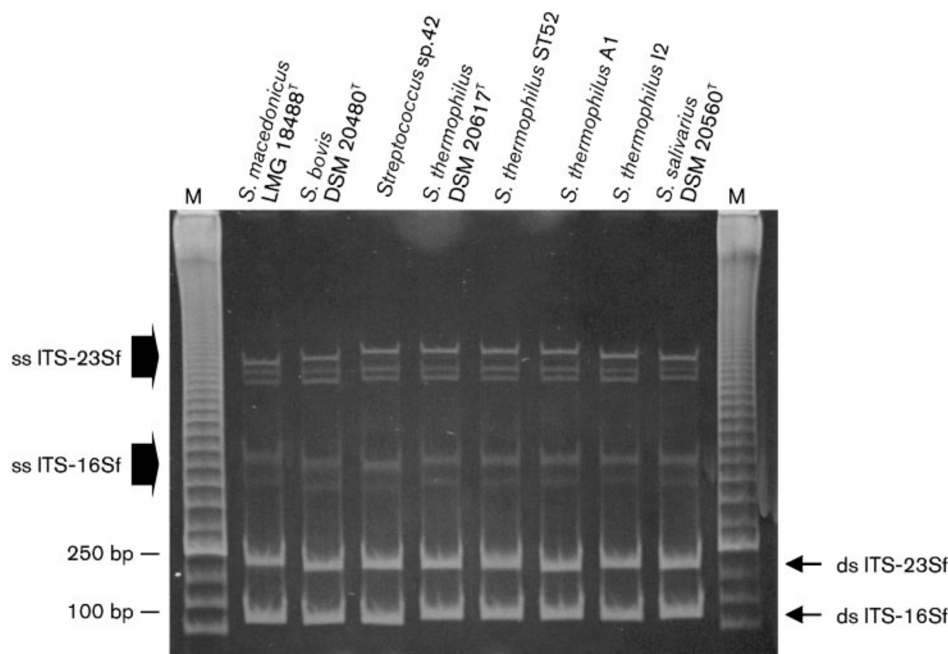

Fig. 4. MIS profiles of amplified ITS-16Sf and ITS-23Sf fragments of dairy streptococci. The molecular species present in the SSCP analysis are indicated by arrows. ds, double strand of ITS-16Sf or ITS-23Sf amplified fragments; ss, single strand of ITS-16Sf or ITS-23Sf amplified fragments. M, DNA molecular mass marker 50 bp ladder (Amersham Biosciences).

Application of MIS analysis for the identification of taxonomically undefined *Streptococcus* strains

With the aim of verifying the efficacy of MIS analysis as an identification tool for dairy streptococci, all '*S. thermophilus*-like' and *Streptococcus* sp. strains listed in Table 1 isolated from Italian traditional cheese were tested. The results obtained by MIS analysis were confirmed by direct sequencing of the entire ITS and when necessary by sequence analysis of the 16S rRNA and *sodA* genes.

Among 25 *Streptococcus* strains, 8 were identified as *S. thermophilus* (A2.3, A2.7, A2.11, A2.9, B1.21, B1.23, B1.24, B1.18) while 15 were identified as *S. macedonicus* (ASG19, ASG69, RALF 1, RACF7, RAC2413, RCF15807, TA3.1, TA3.4, TA3.5, TA3.7, TA3.9, TB1.4, TB1.9, TB1.12, TB1.15), underlining the wide presence of this species in dairy products. The taxonomic position of *Streptococcus* sp. 42 and 48, which showed a MIS profile different from the other strains tested, was investigated by sequencing the 16S–23S rRNA ITS, the 16S rRNA gene and a discrete region from the manganese-dependent superoxide dismutase gene (*sodA*). Interestingly, *Streptococcus* sp. strains 42 and 48 isolated from cheese showed an ITS sequence with 100 % similarity to *S. thermophilus* strains in the region downstream of the $tRNA^{Ala}$ gene and 99 % similarity with *S. bovis* DSM 20480^T in the region upstream of the $tRNA^{Ala}$ gene (Fig. 1). The phylogenetic analysis based on ITS sequences showed that strains 42 and 48 clustered in the *S. bovis*/*S. macedonicus* branch (Fig. 2). Further analysis was carried out by sequencing the first 600 bp of the 16S rRNA gene of strain 42 (AJ439568). The sequence obtained was compared with those present in the EMBL database using the BLASTN service and the results obtained showed 100 % identity with the small ribosomal gene of *S. lutetiensis* (previously *S. infantarius* subsp. *coli*) $HDP90246^T$ (AF429763) (Poyart *et al.*, 2002) and *S. bovis* ATCC 27960 (AB002481), and 99 % identity with *S. infantarius* HDP 90056^T (AF429762). Because of the high sequence similarity existing between the 16S rRNA genes of *S. lutetiensis*, *S. infantarius* and *S. bovis*, the identification of strains 42 and 48 was subsequently addressed by sequencing a discrete region of the *sodA* gene as recently suggested by Poyart *et al.* (2002). Specifically, 268 bp of the coding region of the amplified *sodA* gene (AJ439567) was compared with the sequences present in the EMBL database; the results obtained showed 99 % identity with the *sodA* gene sequence of *S. infantarius* CIP 103233^T (AJ297184), while lower levels of identity (88 %, 86 %) were obtained with the homologous gene of *S. lutetiensis* CIP56.23 (AJ297212) and *S. bovis* ATCC 33317 (Z95896) respectively. According to the *sodA* sequence analysis, strains 42 and 48 were therefore classified as *S. infantarius*. This species has been isolated from infant faeces, human clinical samples and dairy products (Schegel *et al.*, 2000; Poyart *et al.*, 2002) – as for strains 42 and 48, which were isolated from Italian Grana cheese. In this context, the reason for the presence of *S. infantarius* in dairy products should be investigated with the aim of clarifying if this species has a technological role in milk transformation or if it is the result of occasional contamination.

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