***Streptococcus waius* sp. nov., a thermophilic streptococcus from a biofilm**

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Thermophilic streptococci were isolated from biofilms on stainless steel samples exposed to pasteurized skimmed milk and from dairy products from a dairy manufacturing plant. The phenotypic characters of these isolates were distinct from those of other thermophilic streptococci of dairy origin (*Streptococcus thermophilus* and *Streptococcus bovis*). Genotypic data [restriction endonuclease analysis, ribotyping, random amplified polymorphic DNA (RAPD) profiles, DNA–DNA hybridization and G+C contents] support the classification of these isolates as a new species. The sequence of the 16S rRNA was compared with that of 29 species of streptococci and shown to be significantly different. The sequence of the 16S–23S rRNA intergenic spacer region also differed from published sequences of closely related species. A fluorescent *in situ* hybridization probe prepared to a specific part of the 16S rRNA gene sequence was able to distinguish the unknown isolates from reference isolates of *S. thermophilus* and *S. bovis*. It is proposed that these thermophilic streptococcal isolates from a dairy environment be classified in the genus *Streptococcus* as a new species, *Streptococcus waius* (from waiu, the New Zealand Maori word for milk). The type strain is 3/1^T (= NZRCC 20100^T).

Keywords: *Streptococcus thermophilus*, *Streptococcus waius*, biofilms, milk

INTRODUCTION

The genus *Streptococcus* consists of Gram-positive, spherical or ovoid cells that are typically arranged in chains or pairs. They are facultatively anaerobic, non-spore-forming, catalase-negative and homofermentative, and have complex nutritional requirements (Hardie & Wiley, 1994). The thermophilic streptococci are characterized by the ability to tolerate pasteurization (72 °C, 15 s) and grow at temperatures up to 52 °C (Bridge & Sneath, 1983). Biofilms of thermophilic streptococci on the stainless steel surfaces of dairy manufacturing plants threaten the quality of dairy products (Flint *et al.*, 1997). The release of these bacteria from biofilms has been recorded at 10⁶ cells ml⁻¹ (Driessen & Bouman, 1979). Thermophilic streptococci isolated from dairy environments are often *Streptococcus thermophilus* or are referred to as 'S. thermophilus-like' organisms. The specific iden-

tification of thermophilic streptococci causing problems in the manufacture of dairy products would assist in identifying the source of the contamination and in developing methods to control contamination in dairy manufacture.

The taxonomic status of *S. thermophilus* has varied because of the close relationship between these streptococci and *Streptococcus salivarius*. *S. thermophilus* became *S. salivarius* subsp. *thermophilus* (Farrow & Collins, 1984) until separate species status was re-proposed by Schleifer *et al.* (1991) on the basis of genetic and phenetic criteria. 16S rRNA sequence data have demonstrated that *S. thermophilus* is closely related to *S. salivarius* and *Streptococcus vestibularis*.

Streptococcus bovis is another thermophilic streptococcus found in milk. Classification of this organism was hindered by the biochemical heterogeneity of the strains. Genetic studies confirmed that *S. bovis* was indeed made up of several distinct 'species' (Coykendall & Gustafson, 1985; Farrow *et al.*, 1984), although full descriptions of these have not been published. 16S rRNA sequence data representing only one of the three homology groups of *S. bovis* described by Wood & Holzapfel (1995) are currently available

Abbreviations: FISH, fluorescent *in situ* hybridization; RAPD, random amplified polymorphic DNA.

The GenBank accession numbers for the partial 16S rRNA and complete 16S rRNA sequences and the 16S–23S intergenic spacer region sequence determined in this work are U96621, AF088900 and AF088899, respectively.

from the GenBank database. In addition, information from DNA–DNA hybridization has demonstrated a close relationship between *S. bovis* and *Streptococcus equinus* (Wood & Holzapfel, 1995).

In this study, we examined isolates of thermophilic streptococci from dairy products or biofilms resulting from the exposure of stainless steel to pasteurized skimmed milk. These organisms were classified using phenotypic observations (including biochemical profiles), restriction endonuclease analysis, ribotyping, random amplified polymorphic DNA (RAPD) analysis, DNA–DNA hybridization, G+C contents and comparison of the nucleic acid sequences of the 16S rRNA gene and the 16S–23S intergenic spacer region. Specific sequences were used to develop a fluorescent probe to identify these bacteria *in situ* using epifluorescence microscopy.

METHODS

Organisms. Thermophilic streptococci were isolated from biofilms formed on stainless steel exposed to pasteurized skimmed milk in the laboratory (strains 7c, 3/1 and 6/2) and from casein (strain T). *S. thermophilus* strains H, L3 and BCD7 were isolated from dairy manufacturing plants. These strains were compared with the reference *S. thermophilus* strain ATCC 19258 (type strain) and the reference *S. bovis* strain ATCC 33317. All thermophilic streptococci were cultured in M17 broth or on M17 agar (Difco) at 37 °C for 18 h.

Phenotypic characters. The four unknown isolates of thermophilic streptococci (T, 7c, 3/1 and 6/2) were compared with reference strains of *S. thermophilus* and *S. bovis*. Colony morphology on M17 agar and the Gram stain reaction of each isolate were observed. All isolates were tested for the ability to grow in and clot 10% (w/v) reconstituted skimmed milk at 45 °C. Growth was monitored in reconstituted skimmed milk from 20 to 60 °C using a gradient temperature incubator (Toyo Kagaku Sangyo) and using pH reduction to indicate growth.

Growth was monitored in M17 broth over a range of pH (4.0–10.0), in the presence of NaCl (1–7%, w/v) and bile (1–2%, w/v).

The API (LaBalle Les Grottes) ID 32S system was used in an attempt to identify the isolates according to their biochemical profiles. The API 50 CH system was used to provide more comprehensive information on carbohydrate metabolism.

The API ZYM system was used to compare the activity of 19 enzymes from cultures of each isolate.

Restriction endonuclease analysis. Genomic DNA was extracted using a procedure based on that of Marmur (1961). DNA samples (2 µg) were digested with *EcoRI* (Boehringer Mannheim) according to the manufacturer's instructions. The digested DNA was separated on a 1% (w/v) agarose gel.

Ribotyping. Digested DNA was transferred from agarose gels to Hybond-N⁺ membrane (Amersham) by the method of Southern (1975). The enhanced chemiluminescent method (ECL; Amersham) was used for DNA hybridization and detection according to the manufacturer's instructions. The rRNA gene probe was the PCR product amplified from the 16S rRNA gene as described below.

RAPD comparison. Randomly primed profiles of the strains were generated using the primer RP (5'-CAGCACCCAC-3') under the following conditions: 1 cycle of 94 °C for 3 min, 45 °C for 45 s and 72 °C for 1 min; 30 cycles of 94 °C for 45 s, 45 °C for 45 s and 72 °C for 1 min; 1 cycle of 94 °C for 45 s, 45 °C for 45 s and 72 °C for 5 min. Amplification products were separated on a 2% (w/v) agarose gel.

DNA–DNA hybridization. DNA (1 µg ml⁻¹, 100 µl) from *S. thermophilus*, *Streptococcus waius* and *S. bovis* was diluted in twofold serial dilutions in 10 × SSC (1.5 M NaCl, 0.15 M trisodium citrate) to provide concentrations of 0.125–1 µg DNA 200 µl⁻¹. Six replicate 200 µl samples of each dilution were loaded onto Nylon blotting membrane (Hybond-N⁺; Amersham) using a dot-blot filtration manifold system. The membrane was removed from the blotting apparatus, cut into six blocks of replicate samples and placed in denaturing solution (1.0 M NaOH, 3.0 M NaCl), followed by neutralizing solution (0.5 M Tris/HCl pH 7.5, 1.5 M NaCl) for 5 min, each at room temperature. The membrane pieces were placed between two sheets of blotting paper, air-dried and then baked in a vacuum oven at 80 °C for 2 h. The membranes were placed in glass hybridization tubes with 10 ml Rapid-hyb buffer (Amersham) pre-warmed to 65 °C and incubated for 1 h at 65 °C. Probes were prepared to the three strains (*S. thermophilus*, *S. waius* and *S. bovis*) by labelling with ³²P using the Rediprime DNA labelling system (Amersham). Two replicate membranes were incubated with one of the three ³²P-labelled probes at 65 °C for 12 h. The hybridization solution was removed and the membranes washed twice with 2 × SSC at room temperature for 15 min. The membranes were washed either under conditions of high stringency (0.1 × SSC at room temperature for 15 min followed by 0.1 × SSC at 45 °C for 15 min) or at lower stringency (two washes with 0.5 × SSC at room temperature for 15 min). The amount of ³²P bound to each DNA sample was determined using a Packard scintillation counter.

G+C contents. The melting temperature (*T*_m) of the DNA (20 µg ml⁻¹) was determined by monitoring the increase in optical density at 260 nm with increasing temperatures at 1 °C increments from 80 °C using a Gilford spectrophotometer model 260. The base composition of DNA was determined from its melting temperature (Marmur & Doty, 1962).

PCR amplification using 23S rRNA primers specific for *S. thermophilus*. A specific primer for *S. thermophilus*, 23S therm (5'-CATGCCTTCGCTTACGCT-3'), together with a universal primer, 23L (5'-CGCAAACCGACAACAGGTAG-3'), were used in a PCR to determine if the isolates were *S. thermophilus* by the production of a PCR product from the 23S rRNA gene (Schleifer *et al.*, 1995). PCR conditions used here and in the following assays were 1 cycle of 95 °C for 3 min, 55 °C for 45 s and 72 °C for 1 min; 40 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min; 1 cycle of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 5 min.

PCR amplification using 16S rRNA primer specific for *S. bovis*. The specific primer Bovis (5'-CATCTAACATGTGTTAAATGC-3') was used in conjunction with the universal primer Y1 (5'-TGGCTCAGAACGAACGCTGGC-CCG-3') (Young *et al.*, 1991) to amplify a product of approximately 190 bp from the 16S rRNA gene of *S. bovis* strains.

PCR amplification and sequencing of 16S rRNA. A series of fragments representing 1484 bp of 16S rRNA was amplified using a selection of PCR primers. The 16S rRNA fragments were purified for sequence analysis using the Wizard (Promega) system. The fragments were sequenced by the

Table 1. Biochemical test profile of *S. waius*, *S. thermophilus* and *S. bovis*

Data shown are positive results from 49 tests using the API 50 CH test kit, two replicates for each strain.

Test	<i>S. waius</i> 3/1 ^T	<i>S. waius</i> 6/2	<i>S. waius</i> 7c	<i>S. waius</i> T	<i>S. thermophilus</i> ATCC 19258 ^T	<i>S. bovis</i> ATCC 33317
Glycerol	+	—	—	—	—	—
D-Arabinose	—	—	—	—	—	+
Galactose	+	+	+	+	—	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+
Amygdalin	—	—	—	—	—	+
Arbutine	—	—	—	—	—	+
Aesculin	—	—	—	—	—	+
Salicin	+	+	+	+	—	+
Cellobiose	+	+	+	+	—	+
Maltose	+	+	+	+	—	+
Lactose	+	+	+	+	+	+
Melibiose	+	+	+	+	—	+
Saccharose	+	+	+	+	+	+
Inulin	—	—	—	—	—	+
D-Raffinose	+	+	+	+	—	+
Amidon	—	—	—	—	—	+
Glycogen	—	—	—	—	—	+
β-Gentiobiose	—	—	—	—	—	+

Table 2. Enzyme profiles of *S. waius*, *S. thermophilus* and *S. bovis*

Data shown are positive reactions from the API ZYM assay, two replicates for each strain.

Enzyme	<i>S. waius</i> 3/1 ^T	<i>S. waius</i> 6/2	<i>S. waius</i> 7c	<i>S. waius</i> T	<i>S. thermophilus</i> ATCC 19258 ^T	<i>S. bovis</i> ATCC 33317
Esterase C4	—	—	—	—	—	+
Esterase lipase C8	—	—	—	—	—	+
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+
Cystine arylamidase	+	+	+	+	+	+
Trypsin	—	—	—	—	—	+
Acid phosphatase	+	+	+	+	+	+
α-Galactosidase	+	—	+	+	+	+
β-Galactosidase	+	+	+	+	+	—
α-Glucosidase	+	—	+	—	—	+
β-Glucosidase	—	—	—	—	—	+

Massey University DNA Analysis Service. The sequences were compared with those in the Ribosomal Database Project (Maidak *et al.*, 1997) in an attempt to classify the unknown thermophilic streptococci. Sequences were aligned using the CLUSTAL-X program (Thompson *et al.*, 1997) and sequence homology was calculated with GeneDoc (Nicholas & Nicholas, 1997). Genetic distances were obtained from the alignments using the DNADIST program of the PHYLIP package (Felsenstein, 1989) which also enabled the con-

struction of a dendrogram with the neighbour-joining tree prepared using the Kimura two-parameter correction.

PCR amplification and sequencing of the 16S–23S rRNA intergenic sequence. The spacer region between the 16S and 23S rRNA was amplified using PCR primers 16S EXT (5'-GGGTGAAGTCGTAACAAGGTAGCC-3') and 23S EXT (5'-GCTCCTAGTGCCAAGGCATCCACC-3'). The sequence of this region was compared with the intergenic spacer region of closely related streptococci.

Preparation and use of a fluorescent probe for *in situ* hybridization. The specific sequence selected for the preparation of a fluorescent probe to the unknown isolates was a 22-mer containing 12 bp from the V2 region encoding 16S rRNA which were different from those of *S. thermophilus* and *S. bovis* (5'-GTCTCTAACATGTGTAAACAC-3') (Fig. 3). The specificity of this sequence was confirmed using this primer in conjunction with primer Y1 in PCR to amplify the nucleic acid containing these sequences from the 16S rRNA gene of the unknown isolates and known isolates of *S. thermophilus* and *S. bovis*. Fluorescent probes were synthesized by Oligos Etc. with either a rhodamine label or a fluorescein label at the 5'-end.

The protocol of Poulsen (1995) was used for fluorescent *in situ* hybridization (FISH). Stainless steel samples with cells of thermophilic streptococci attached were fixed in 4% (v/v) formaldehyde at 4 °C overnight and dehydrated in an ethanol series [50, 80 and 96% (v/v) ethanol, 3 min each]. A hybridization mix containing 40% (v/v) formamide and 2.5 ng probe μl^{-1} was added to the surface of the sample and incubated at 37 °C for 18 h. Following hybridization, the samples were washed three times with hybridization solution without the probe, and then with a washing solution (1 M Tris pH 7.2, 5 M NaCl). Samples were observed under epifluorescence microscopy using a Leitz Ortholux II microscope with an I_2 incident light filter, producing an excitation wavelength of 450–490 nm.

Replicate samples of stainless steel with the bacteria attached to the surface were stained with 0.001% acridine orange for 2 min and the total number of bacteria per cm^2 were counted using epifluorescence microscopy at 450–490 nm.

Fig. 1. EcoRI digestion of total DNA (a) and ribotype profiles (b). Lanes: 1, BRL 1 kb ladder; 2, *S. waius* 3/1^T; 3, *S. bovis* ATCC 33317; 4, *S. thermophilus* ATCC 19258^T.

RESULTS

Phenotypic characters

Colonies of thermophilic streptococcal unknown isolates T, 7c, 3/1 and 6/2 on M17 agar appeared as pinpoint colonies that were indistinguishable from the colonies of the reference strain of *S. thermophilus*. In Gram stains, the cells all appeared as small (0.7 μm

Fig. 2. RAPD profiles (a), and PCR products obtained from amplification of total bacterial DNA with primer combinations specific for *S. waius* (b), *S. thermophilus* (c) and *S. bovis* (d). Lanes: 1 and 11, BRL 1 kb ladder; 2, *S. waius* 3/1^T; 3, *S. waius* 6/2; 4, *S. waius* 7c; 5, *S. waius* T; 6, *S. thermophilus* ATCC 19258^T; 7, *S. thermophilus* H; 8, *S. thermophilus* L3; 9, *S. bovis* ATCC 33317; 10, negative control.

Table 3. DNA–DNA hybridization (% homology) of *S. waius* 3/1^T, *S. thermophilus* ATCC 19258^T and *S. bovis* ATCC 33317 at high and low stringency

Probe	High stringency			Low stringency		
	<i>S. waius</i>	<i>S. thermophilus</i>	<i>S. bovis</i>	<i>S. waius</i>	<i>S. thermophilus</i>	<i>S. bovis</i>
<i>S. waius</i>	100	9.6	22.3	100	10.3	32.3
<i>S. thermophilus</i>	7.5	100	9.1	10.3	100	9.9
<i>S. bovis</i>	23.7	9.5	100	25.2	10.2	100

Table 4. G + C contents for *S. thermophilus* ATCC 19258^T and *S. waius* 3/1^T determined from the DNA melting temperature

DNA source	<i>T_m</i> measured (°C)*	<i>T_m</i> published (°C)†	G + C measured (mol %) [‡]	G + C published (mol %) [†]
<i>S. thermophilus</i>	86.5	85.7	41.9	40.0
<i>S. waius</i>	83.5	—	34.6	—

* Standard deviation = 0.53.

† Data from Hardie (1986).

‡ Standard deviation = 1.4.

diameter) spherical cells in pairs, typical of *S. thermophilus*. All isolates were able to clot milk following 18 h incubation at 45 °C. The range and optimum growth temperatures for the unknown isolates were 24.5–52.4 °C and 38.8 °C, respectively. The range and optimum growth temperatures for *S. thermophilus* were 28.8–55.3 and 40.9 °C, respectively. The range and optimum growth temperatures for *S. bovis* were 27.0–45.9 °C and 35.9 °C, respectively.

Growth of the unknown isolates was detected over the pH range 4.0–10.0 and at salt concentrations up to 7%. The isolates were intolerant of bile at 1%.

The unknown isolates could not be identified using the API ID 32S test kit.

The reference strain of *S. thermophilus* produced a positive reaction in 6/49 tests of the API 50 CH test kit, compared with 20/49 tests for *S. bovis*. The unknown isolates (T, 7c and 6/2) produced 12/49 positive results and isolate 3/1 produced 13/49 positive results (Table 1).

The unknown isolates produced 5–7 reactions using the API ZYM test kit. *S. thermophilus* produced a similar set of reactions to the unknown isolates. *S. bovis* produced 10 reactions (Table 2).

Restriction endonuclease analysis, ribotyping and RAPD profiles

Digestion of total DNA and ribotyping produced profiles of digested fragments for the unknown isolates

that were different from *S. thermophilus* and *S. bovis* (Fig. 1). RAPD analysis also differentiated these organisms (Fig. 2).

DNA–DNA hybridization

DNA–DNA hybridization also differentiated the three organisms and confirmed the results from the biochemical data indicating a closer relationship between the unknown isolates and *S. bovis* than between the unknown isolates and *S. thermophilus* (Table 3).

G + C ratio

The G + C ratio for *S. waius* was 34.6 mol % (Table 4).

PCR amplification using 23S rRNA primers specific for *S. thermophilus* and 16S rRNA primers specific for *S. bovis*

No PCR product was produced from the four unknown isolates using the 23S rRNA primer combination specific for *S. thermophilus* or the 16S rRNA primer combination specific for *S. bovis* (Fig. 2). A PCR product was produced from all three of the *S. thermophilus* isolates tested with the primer combination for *S. thermophilus* (Fig. 2). A PCR product was also produced from the reference isolate of *S. bovis* using the primer combination specific for *S. bovis* (Fig. 2).

Table 5. Percentage homology of the 16S rRNA of *S. waius* compared with 29 other streptococci

Species	Accession no.	Homology (%)	Species	Accession no.	Homology (%)
<i>S. bovis</i>	X58317	94	<i>S. acidominimus</i>	X58301	82
<i>S. uberis</i>	U41048	93	<i>S. cecorum</i>	X54290	82
<i>S. thermophilus</i>	X68418	92	<i>S. pyogenes</i>	M58835	82
<i>S. salivarius</i>	M58839	91	<i>S. dysgalactiae</i>	X59030	82
' <i>S. milleri</i> '	X81023	89	<i>S. criceti</i>	X58305	82
<i>S. parasanguinis</i>	X53652	88	<i>S. oralis</i>	X58308	82
<i>S. mitis</i>	D38482	87	<i>S. pneumoniae</i>	X58312	81
<i>S. gordonii</i>	D38483	86	<i>S. equi</i>	X58314	81
<i>S. equinus</i>	X58318	85	' <i>S. criceti</i> '	X58316	81
<i>S. ratti</i>	X58314	84	<i>S. suis</i>	X59031	81
<i>S. sobrinus</i>	X58307	83	<i>S. macacae</i>	X58302	80
<i>S. vestibularis</i>	X58321	83	<i>S. porcinus</i>	X58317	80
<i>S. mutans</i>	X58303	83	<i>S. anginosus</i>	X58310	80
<i>S. agalactiae</i>	X59032	83	<i>S. pleomorphus</i>	M23730	78
<i>S. canis</i>	X59061	83			

16S rRNA and 16S–23S rRNA intergenic spacer region comparisons with published sequences

A comparison of 16S rRNA from the unknown isolates with published sequences of 29 different streptococci showed the unknown isolates to be most closely related to *S. bovis* (Table 5, Fig. 3).

The 16S–23S rRNA intergenic spacer region differed from the most closely related streptococci for which sequence data are available [*S. bovis* (GenBank accession no. U39766, 93.0% homology); *S. thermophilus* (U32965, 89.5% homology); and *S. salivarius* (X83760, 87.7% homology)] (Fig. 4).

The differences in homology of the 16S rRNA and the 16S–23S rRNA intergenic spacer region together with the biochemical and other molecular data, support the classification of the unknown isolates in a new species. We propose a new species with the name *Streptococcus waius*, after the New Zealand Maori word for milk, reflecting the origin of these isolates.

Species-specific oligonucleotide and FISH detection

An identical PCR product (Fig. 2) was produced from all four unknown isolates using a 22-mer primer from the variable V2 region of the genome encoding 16S rRNA (Fig. 3) in conjunction with the conserved primer Y1. No product was obtained when this primer combination was used with the *S. thermophilus* or *S. bovis* strains.

The fluorescein-labelled oligonucleotide specific for *S. waius* was able to be used to identify these unknown isolates attached to stainless steel in a FISH reaction. The *S. waius* oligonucleotide did not react with *S.*

thermophilus	1	TGGCTCAGGA	CGAACGCTGG	CGGCGTGCCT	AATACATGCA	AGTAGAACGC
salivarius		TGGCTCAGGA	CGAACGCTGG	CGGCGTGCCT	AATACATGCA	AGTAGAACGC
bovis		TGGCTCAGGA	CGAACGCTGG	CGGCGTGCCT	AATACATGCA	AGTAGAACGC
equinus						
waius		..GCTCAGGA	CGAACGCTGG	CGGCGTGCCT	AATACATGCA	AGTAGAACGC
thermophilus	51	TGAAGAGAGG	AGCTTGCTCT	TCTTGGATGA	GTTGCGAACG	GGTGAGTAAC
salivarius		TGAAGAGAGG	AGCTTGCTCT	TCTTGGATGA	GTTGCGAACG	GGTGAGTAAC
bovis		TGAAGACTTT	AGCTTGCTAA	AGTTGGAAGA	GTTGCGAACG	GGTGAGTAAC
equinus					GAACG	GGTGAGTAAC
waius		TGAAGACTTT	AGCTTGCTAG	AGTTGGAAGA	GTTGCGAACG	GGTGAGTAAC
thermophilus	101	GCCTAGGTAA	CCTGCCTNGT	AGCGGGGAT	AACTATTGGA	AACGATAGCT
salivarius		GCCTAGGTAA	CCTGCCTNGT	AGCGGGGAT	AACTATTGGA	AACGATAGCT
bovis		GCCTAGGTAA	CCTGCCTACT	AGCGGGGAT	AACTATTGGA	AACGATAGCT
equinus		GCCTAGGTAA	CCTGCCTACT	AGCGGGGAT	AACTNTTGA	AACGATAGCT
waius		GCCTAGGTAA	CCTGCCTATT	AGTGGGGAT	AACTATTGGA	AACGATAGCT
thermophilus	151	AATACCGCAT	AACAATGGAT	GACACATGTC	ATTTATTGTA	AAGGGGCAAT
salivarius		AATACCGCAT	AACAATGGAT	GACCATGTC	ATTTATTGTA	AAGGGGCAAA
bovis		AATACCGCAT	AACAGCATTT	AACACATGTT	AGATGCTTGA	AAGAGCAAT
equinus		NATACCGCAT	AACAGCATTT	AACACATGTT	AGATGCTTGA	AAGAGCAAT
waius		AATACCGCAT	AATAGTGTIT	AACACATGTT	AGAGACTTAA	AAGATGCAAT
			*****	*****	*****	
thermophilus	201	TGCTCCACTA	CAAGATGGAC	CTGCGTTGTA	TTAGCTAGTA	GGTGAGGTAA
salivarius		TGCTCCACTA	CAAGATGGAC	CTGCGTTGTA	TTAGCTAGTA	GGTGAGGTAA
bovis		TGCTCCACTA	GTAGATGGAC	CTGCGTTGTA	TTAGCTAGTT	GGTGAGGTAA
equinus		TGCTCCACTA	GTAGATGGAC	CTGCGTTGTA	TTAGCTAGTT	GGTGAGGTAA
waius		TGCTCCACTA	GTAGATGGAC	CTGCGTTGTA	TTAGCTAGTT	GGTGAGGTAA
thermophilus	251	TGGCTTACCT	AGGCGACGAT	ACATAGCCGA	CCTGAGAGGG	TGATCGGCCA
salivarius		CGGCTACCT	AGGCGACGAT	ACATAGCCGA	CCTGAGAGGG	TGATCGGCCA
bovis		CGGCTACCA	AGGCGACGAT	ACATAGCCGA	CCTGAGAGGG	TGATCGGCCA
equinus		AGGCTACCA	AGGCGACGAT	ACATAGCCGA	CCTGAGAGGG	TNATCGGCCA
waius		AGGCTACCA	AGGCGACGAT	ACATAGCCGA	CCTGAGAGGG	TGATCGGCCA
thermophilus	301	CACTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGAGGCA	GCAGTAGGG
salivarius		CACTGGGACT	GAGABABGGC	CCAGACTCCT	ACGGGAGGCA	GCAGTAGGG
bovis		CACTGGGACT	CCAGACTCCT	CCAGACTCCT	ACGGGAGGCA	GCAGTAGGG
equinus		CACTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGAGGCA	GCAGTAGGG
waius		CACTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGAGGCA	GCAGT....

Fig. 3. Alignment of part of the 16S rRNA gene sequences from *S. thermophilus* (GenBank X68418), *S. salivarius* (GenBank M58839), *S. bovis* (GenBank X58317), *S. equinus* (GenBank X58318) and *S. waius* sp. nov. The bases of *S. waius* that are underlined differ from one or more of the other species listed. Asterisks denote the 22-mer primer specific for *S. waius*.

thermophilus or *S. bovis* and therefore was able to differentiate *S. waius* from other thermophilic streptococci (Table 6).

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waius      CTAAGGAAAAACGGAA--GCACGTTTGGGAAGTCTTGTAGTTTGTAGAGGTCTTGTGG
bovis      --AAGGATAAA--CGGAA--GCACGTTTGGGTA--TTGTTTGTAGTTTGTAGAGGTCTTGTGG
thermophilus CTAAGGAAAA--CGGAATGTAC--TTGAGTT-TCTTATTTAGTTTGTAGAGGTCTTGTGG
salivarius CTAAGGAAAA--CGGAATGTAC--TTGAGTT-TCTTATTTAGTTTGTAGAGGTCTTGTGG
          *****
waius      GGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTGAGCGGTTTCGATCCC
bovis      GGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTGAGCGGTTTCGATCCC
thermophilus GGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTGAGCGGTTTCGATCCC
salivarius GGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTGAGCGGTTTCGATCCC
          *****
waius      GCTAGGCTCCATTGAATCGAAAGATTCAAGTTGATTGTCCATTGAAAATTGAATATCTAT
bovis      GCTAGGCTCCATTGAATCGAAAGATTCAAA--GATTGTCCATTGAAAATTGAATATCTAT
thermophilus GCTAGGCTCCATTGAATCGAAAGATTCAAGT--ATTGTCCATTGAAAATTGAATATCTAT
salivarius GCTAGGCTCCATTGAATCGAAAGATTCAAGT--ATTGTCCATTGAAAATTGAATATCTAT
          *****
waius      ATCAAATTCACAATT--AGAAAT--AATTGTAGAAAGTAACAAGAAA-TAAACCGAAA-
bovis      ATCAAATTCACGATTCAAGAAATTGAATTGTAGATAGTAACAAGAAA-TAAACCGAAAG
thermophilus ATCAAATTCATATGT--AAGTAATT-ACATATAGATAGTAACAAGAAA-TAAACCGAAA-
salivarius ATCAAATTCATATGT--AATTACTT-ACATATAGATAGTAACAAGAAA-TAAACCGAAA-
          *****
waius      CGCTGTGA--TTTAATGAGTTTAAGGTCAGAAGA-CCAAAATAA
bovis      CGCTGTGA--TTTAATGAGTTTAAGGTCACAGAACCAAAATAA
thermophilus CGCTGTGAATATTTAATGAGTT--AGGTCGAAAGGCCAAAATAA
salivarius CGCTGTGAATATTTAATGAGTT--AGGTCGAAAGGCCAAAATAA
          *****

```

Fig. 4. Alignment of the 16S–23S rRNA intergenic spacer region of *S. waius*, *S. bovis*, *S. thermophilus* and *S. salivarius*. Asterisks indicate common bases.

Table 6. Detection of *S. waius* using FISH

Data shown are mean values, three replicates for each strain. Standard deviation = 0.42.

Species	Isolate number	log ₁₀ Cells cm ⁻²	
		FISH probe	Acridine orange
<i>S. waius</i>	7c	4.5	5.2
<i>S. bovis</i>	ATCC 33317	No detection	4.3
<i>S. thermophilus</i>	ATCC 19258 ^T	No detection	3.8

DISCUSSION

The four dairy unknown isolates used in this study appeared by cell shape (Gram stain) and colony morphology to be identical to other thermophilic streptococci. All were able to grow and clot milk at 45 °C and therefore were similar to other thermophilic streptococci (Wood & Holzapfel, 1995). They differed from *S. thermophilus* and *S. bovis* in their range and optimum temperatures for growth, with the results for the unknown isolates lying between those for the two reference cultures. *S. thermophilus*, *S. bovis* and the unknown isolates all grew over the pH range 4.0–10.0 and were all intolerant of bile at 1%. *S. thermophilus* and the unknown isolates were more salt-tolerant (up to 7% NaCl) than *S. bovis* (up to 6% NaCl).

The biochemical profiles of this group of isolates were sufficiently different from those of both *S. thermophilus* and *S. bovis*, the two thermophilic streptococci associated with milk and cattle, to suggest that they may belong to another species. Enzyme profiles distinguished between the unknown isolates and *S. bovis* but profiles of the unknown isolates and *S. thermophilus* were similar.

Supportive evidence for a separate species was obtained by the differentiation of the unknown isolates from *S. thermophilus* or *S. bovis* using restriction

endonuclease analysis, ribotyping and RAPD profiles. DNA–DNA hybridization confirmed the biochemical data that showed that the unknown isolates were more closely related to *S. bovis* than *S. thermophilus*. The percentage homology values between *S. thermophilus*, *S. bovis* and the unknown isolate 3/1 (7.5–32.3%) were less than reported for the closely related thermophilic streptococci, *S. thermophilus* and *S. salivarius* (61–100%) (Farrow *et al.*, 1984), which therefore supports the classification of this isolate as a new species.

The G + C ratio for this new organism is 34.6 mol%. The G + C values for streptococci lie between 34 and 46 mol% (Hardie, 1986). The G + C contents of thermophilic streptococci of dairy origin, *S. thermophilus* and *S. bovis*, are 40 and 36–39 mol% respectively (Hardie, 1986). The difference in *T_m* between *S. waius* and *S. thermophilus* was 3 °C, compared with a difference of 1.6 °C between the species *S. thermophilus* and *S. bovis*. The standard deviation for the determination of the *T_m* has been reported as ±0.4 °C (Marmur & Doty, 1962), and in our trials it was ±0.53 °C.

The inability to produce a PCR product with a primer combination specific for *S. thermophilus* or *S. bovis* rRNA genes confirmed that these organisms could not be classified as either of these bacteria. Sequence

Fig. 5. Dendrogram showing the relationship between *S. waius* and 29 other streptococci.

analysis of the 16S rRNA showed that although there was the expected similarity between sequence data for these isolates and 29 other streptococci, the unknown isolates formed a separate group. Bentley *et al.* (1991) used comparative analysis of partial 16S rRNA sequences to categorize 24 *Streptococcus* species and found that *S. thermophilus*, *S. salivarius* and *S. vestibularis* formed one distinct group, and that *S. bovis* and *S. equinus* formed another distinct group. This is shown in the dendrogram constructed by aligning the 16S rRNA sequences of 29 different streptococci (Fig. 5). The alignment of the 16S rRNA sequence of our unknown isolates, which we have named *S. waius*, fits between these two groups. *S. waius* appears to be most similar to the *S. bovis/equinus* group; however it does possess some similarity to the *S. thermophilus/salivarius* group. This is supported by the biochemical and DNA–DNA hybridization data. The intergenic 16S–23S rRNA spacer region of *S. waius* is sufficiently different from the closely related species (*S. bovis*, *S. thermophilus* and *S. salivarius*) to provide additional support for naming this organism as a separate species.

Strains represented by *S. bovis* and *S. equinus* have been found to be very heterogeneous (Wood & Holzapel, 1995). Genetic and biochemical studies led to the description of two new species – *Streptococcus alactolyticus* and *Streptococcus saccharolyticus* (Wood

& Holzapel, 1995). Our unknown isolates do not match the phenotypic or genotypic profiles for either of these species. There are 12 bp differences in the most variable (V2) region of the 16S rRNA gene between *S. waius* and the most closely related streptococcal species (*S. equinus*) for which 16S rRNA data are available (Fig. 3). This compares with a 2 bp difference between the known species *S. equinus* and *S. bovis* and a 3 bp difference between *S. salivarius* and *S. thermophilus* in the same region.

In the present study, the phenotypic and molecular data suggest that the unknown isolates should be categorized as a new species. The four dairy unknown isolates used in this study were isolated at different times from different sources and it is therefore unlikely that they belong to a clone from an identical source. This is supported by the RAPD profiles and the biochemical data where strain variations are apparent. The ability of the FISH probe to differentiate these new isolates from other thermophilic streptococci reinforces their separation into a distinct species.

Description of *Streptococcus waius* sp. nov.

Streptococcus waius (*waius*. New Zealand Maori n. *waiu* milk; N.L. gen. n. *waius* of milk).

Cells are Gram-positive, non-motile, non-spore forming, 0.7 µm diameter cocci in pairs or short chains. When the organism is grown on M17 agar at 37 °C for 48 h, colonies are 0.5 mm in diameter, circular, convex, translucent and smooth. The organisms are homo-fermentative, facultatively anaerobic and catalase-negative. The organisms will clot milk at 45 °C with an optimum temperature for growth of 38.8 °C, but are capable of growing over a range of 24.5–52.4 °C. They will grow over the pH range 4.0–10.0 and are tolerant of salt up to 7% NaCl. They are intolerant of bile at 1%. The bacteria ferment galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, salicin, cellobiose, maltose, lactose, melibiose, saccharose and D-raffinose. The bacterium produces the following enzymes: leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase. The DNA G+C content is 34.6 mol%. The habitat is milk and dairy products. The type strain is 3/1^T and is lodged in the New Zealand Reference Culture Collection (NZRCC 20100^T) at the New Zealand Dairy Research Institute, Palmerston North, New Zealand.

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