Delimiting the genus *Staphylococcus* through description of *Macrococcus caseolyticus* gen. nov., comb. nov. and *Macrococcus equipercicus* sp. nov., *Macrococcus bovicus* sp. nov. and *Macrococcus carouselicus* sp. nov.

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Four species of the newly proposed genus *Macrococcus*, namely *Macrococcus caseolyticus* gen. nov., comb. nov. (formerly *Staphylococcus caseolyticus* Schleifer, Kilpper-Bälz, Fischer, Faller and Endl 1982, 19^{VP}), *Macrococcus equipercicus* sp. nov., *Macrococcus bovicus* sp. nov. and *Macrococcus carouselicus* sp. nov., are described on the basis of a phylogenetic analysis comparing 16S rRNA sequences, DNA–DNA liquid hybridization, DNA base composition, normalized ribotype patterns, macrorestriction pattern analysis and estimation of genome size using PFGE, cell wall composition, phenotypic characteristics and plasmid profiles. Compared with their closest relatives, members of the genus *Staphylococcus*, these organisms demonstrated significantly lower 16S rRNA sequence similarities (93.4–95.3%), higher DNA G+C content (38–45 mol%), absence of cell wall teichoic acids (with the possible exception of *M. caseolyticus*), unique ribotype pattern types and macrorestriction patterns, smaller genome size (approx. 1500–1800 kb) and generally larger Gram-stained cell size (1.1–2.5 µm in diameter). *Macrococci* can be distinguished from most species of staphylococci (except *Staphylococcus sciuri*, *Staphylococcus vitulus* and *Staphylococcus lentus*) by their oxidase activity. The four *Macrococcus* species can be distinguished from one another on the basis of DNA–DNA hybridization, ribotype pattern types, macrorestriction patterns and their phenotypic properties, including colony morphology, cell morphology, haemolysins, Staph Latex agglutination, acid production from a variety of carbohydrates, acetoin production, nitrate reduction, aesculin hydrolysis, and DNase and urease activities. The type species is *M. equipercicus*. The type strains of *M. equipercicus*, *M. caseolyticus*, *M. bovicus* and *M. carouselicus* are ATCC 51831^T (= DD 9350^T), ATCC 13548^T (= TDD 4508^T) (Schleifer et al. 1982), ATCC 51825^T (= DD 4516^T) and ATCC 51828^T (= DD 9348^T), respectively.

Keywords: *Macrococcus* gen. nov., *Macrococcus caseolyticus* gen. nov., comb. nov., *Macrococcus equipercicus* sp. nov., *Macrococcus bovicus* sp. nov., *Macrococcus carouselicus* sp. nov.

Abbreviation: SEM, scanning electron microscopy.

The EMBL accession numbers for the 16S rRNA sequences of *M. caseolyticus* ATCC 13548^T, *M. equipercicus* ATCC 51831^T, *M. carouselicus* ATCC 51828^T and *M. bovicus* ATCC 51825^T are Y15711–Y15714, respectively.

INTRODUCTION

Comparative chemical, biochemical and nucleic acid hybridization analyses of strains designated as '*Micrococcus caseolyticus*' ATCC 13548 and *Micrococcus varians* ATCC 29750 indicated that they are related to, but distinct from, other known members of the genus *Staphylococcus*, and for this reason they were placed in a new staphylococcal species named *Staphylococcus caseolyticus* (Schleifer *et al.*, 1982). These strains were isolated from the milk of cattle, but more recently several strains have been isolated from the abscesses of slaughtered lambs (De La Fuente *et al.*, 1992) and one from the milk of goats (De Buyser *et al.*, 1992).

In a preliminary investigation (Ballard *et al.*, 1995), we sampled the skin of 15 cattle, 25 goats, 14 horses, 10 ponies, 4 whales, 25 dolphins and meat products for the presence of *Staphylococcus caseolyticus*. This species was isolated from only three samples of raw beef and the skin of a pilot whale and so can still be thought of as a relatively uncommon species. Surprisingly, we discovered a group of three new species related to *Staphylococcus caseolyticus* living on the skin of cattle, horses and ponies. The group was found as large populations on 11 of the 14 horses and all of the ponies sampled in this study. This report is a description of these organisms based on a variety of phenotypic characters, cell wall composition, electrophoretic patterns of *EcoRI* restriction fragments labelled by hybridization with an rRNA operon from *Escherichia coli*, macrorestriction patterns of *SmaI* and *NotI* chromosome fragments, DNA-DNA liquid hybridization, DNA base composition and 16S rRNA sequences. Compared with staphylococci, these organisms demonstrated significantly lower 16S rRNA sequence similarities, higher DNA G+C content, absence of cell wall teichoic acids, larger cell size and smaller genome size, indicating that the new species and *Staphylococcus caseolyticus* should be placed into a separate, but related genus, which has been given the name *Macrococcus* gen. nov.

METHODS

Bacterial strains. The strains selected for the descriptions of *Macrococcus caseolyticus* (Schleifer *et al.* 1982) gen. nov., comb. nov., *Macrococcus equipercicus* sp. nov., *Macrococcus bovis* sp. nov. and *Macrococcus crouselicus* sp. nov., including their designations and sources, are listed in Table 1. Unless indicated otherwise, strains were isolated from the skin of mammalian hosts. The following type strains of staphylococci, bacilli and salinicocci were used for making various comparisons with macrococci: *Staphylococcus vitulus* ATCC 51145^T (= DD 756^T), *Staphylococcus lentus* ATCC 29070^T (= DD 4203^T), *Staphylococcus sciuri* subsp. *sciuri* ATCC 29062^T (= DD 4277^T), *Staphylococcus muscae* CCM 4175^T (= DD 4795^T), *Staphylococcus hyicus* ATCC 11249^T (= DD 887^T), *Staphylococcus chromogenes* ATCC 43764^T (= DD 851^T), *Staphylococcus gallinarum* ATCC 35539^T (= DD 854^T), *Staphylococcus equorum* ATCC 43958^T (= DD 853^T), *Staphylococcus kloosii* ATCC 43959^T (= DD 857^T), *Staphylococcus auricularis* ATCC 33753^T (= DD

872^T), *Staphylococcus aureus* ATCC 12600^T (= DD 10156^T), *Staphylococcus epidermidis* ATCC 14990^T (= DD 8726^T), *Staphylococcus hominis* ATCC 27844^T (= DD 2708^T), *Staphylococcus haemolyticus* ATCC 29970^T (= DD 855^T), *Staphylococcus capitis* ATCC 27840^T (= DD 873^T), *Staphylococcus saccharolyticus* ATCC 14953^T (= DD 860^T), *Staphylococcus saprophyticus* ATCC 15305^T (= DD 866^T), *Staphylococcus warneri* ATCC 27836^T (DD = 870^T), *Staphylococcus schleiferi* subsp. *schleiferi* ATCC 43808^T (= DD 861^T), *Staphylococcus intermedius* ATCC 29663^T (= DD 856^T), *Staphylococcus carnosus* DSM 20501^T (= DD 1091^T), *Bacillus subtilis* ATCC 6051^T (= DD 379^T), *Bacillus subtilis* 168 and *Salinicoccus roseus* ATCC 49258^T (= DD 12047^T).

Phenotypic characterization. The following characteristics were determined as described previously (Kloos & Schleifer, 1975; Schleifer & Kloos, 1975; Kloos *et al.*, 1976; Webster *et al.*, 1994; Kloos & Bannerman, 1995): Gram-stained cell morphology and cell arrangement, colony morphology and pigmentation, motility, anaerobic growth in thioglycollate semi-solid medium, catalase activity, acetylmethylcarbinol (acetoin) production, nitrate reduction, oxidase activity, pyrrolidonylarylamidase activity, aesculin hydrolysis, DNase activity, thermonuclease activity, ornithine decarboxylase activity, urease activity, staphylocoagulase activity, lysostaphin susceptibility, haemolysis of sheep, bovine and horse blood, and carbohydrate reactions. The presence of clumping factor and/or protein A was tested using the Staph Latex Kit (Remel). Antibiotic susceptibilities were determined by disk-diffusion testing, involving the incubation of cultures on tryptic soy agar (TSA; Difco) and Mueller-Hinton agar plates at 35 °C for 24 h in the presence of various antibiotic disks, followed by the measurement of diameters of zones of inhibition (haloes) (Woods & Washington, 1995). Colony size, pigmentation, lustre, profile and consistency were determined on freshly prepared P agar (Kloos *et al.*, 1991), TSA, and TSA plus 5% sheep blood plates following point inoculation, incubation for 72 h at 35 °C and storage at room temperature for an additional 2 d (Kloos & Schleifer, 1975; Kloos & Bannerman, 1995). Alkaline phosphatase, urease, β -galactosidase, β -glucosidase and β -glucuronidase activities, and arginine utilization were tested with the API STAPH-IDENT system (bioMérieux). Additional biochemical profile data were obtained by using the STAPH Trac and ID32 STAPH systems (bioMérieux Vitek).

Normalized ribotyping. Discrete and reproducible ribotype descriptions of strains resulted from a single method described previously in detail (Webster *et al.*, 1994; Bruce *et al.*, 1995; Hubner *et al.*, 1995). Briefly, strains were lysed with staphylolytic enzymes and SDS. The resulting solution was extracted with phenol and chloroform, and the DNA was precipitated with ethanol. The DNA was digested with *EcoRI*. The resulting fragments were separated using agarose-gel electrophoresis in a minigel apparatus. The size-separated fragments were immobilized on a membrane by electrophoretic transfer, denatured and probed. The *rrnB* ribosomal operon from *E. coli* (Brosius *et al.*, 1981), labelled by sulfonation, was used as a probe. Immobilized fragments selected by hybridization were detected with an anti-sulfonated DNA monoclonal antibody conjugated to alkaline phosphatase and a chemiluminescent substrate. Images of bands on the membrane were captured electronically and recorded. Computerized procedures were

used to extract the strain data from each lane and digitally process the bands to a normalized and reproducible characterization. Each strain was then represented in a database by a set of fragment bands. Sets from independently isolated strains that were indistinguishable within the experimental error were averaged and the mean value was stored as a ribotype pattern type.

Genomic DNA–DNA hybridization. Unlabelled and [^3H]thymidine-labelled total DNAs were isolated and purified by using a modification of the procedures of Brenner *et al.* (1969) for use with *Macrococcus* and staphylococcal species that are somewhat difficult to lyse (Kloos & Wolfshohl, 1979; Kloos *et al.*, 1997). DNA–DNA reassociation reactions were performed in duplicate or triplicate under stringent (70 °C) and optimal (55 °C) conditions (Kloos & Wolfshohl, 1979; Kloos, 1980, 1998). At the termination of reactions, double-stranded (hybridized) DNA was separated from unreacted, single-stranded DNA on hydroxyapatite by the batch procedure described by Brenner *et al.* (1969). The relative binding (or DNA relatedness) was determined by normalizing the amount of DNA bound to hydroxyapatite in heterologous reactions to that bound in the homologous reaction, where labelled and unlabelled DNA were from the same organism.

Phylogenetic analyses based on 16S rRNA sequences. The procedures for determining the 16S rRNA sequences of the *Macrococcus* species type strains have been described previously (Springer *et al.*, 1993). The new 16S rRNA sequences were added to an alignment of homologous primary structures available from public databases (Maidak *et al.*, 1996; Van de Peer *et al.*, 1996). Phylogenetic analyses were performed by applying distance matrix, maximum-parsimony and maximum-likelihood methods on different data sets. Distance matrix as well as maximum-parsimony analyses were performed using data sets comprising all available 16S rRNA primary structures from Gram-positive bacteria with a low DNA G + C content as well as reference sequences from selected representatives from each of the remaining major phylogenetic groups of bacteria. Maximum-likelihood methods (Spring *et al.*, 1996) were used to analyse subsets of about 50 sequences comprising the homologous primary structures from macrococci, staphylococci, *B. subtilis* and *Salinicoccus roseus*, and also selected outgroup references from other major phylogenetic groups. The data sets varied with respect to the reference sequences as well as the alignment positions. The variabilities of the individual alignment positions were determined and used as criteria to successively remove highly variable positions from the data set. This was done to recognize and minimize treeing artefacts resulting from alignment errors, database inconsistencies and 'false' identities (multiple base changes) in highly variable regions. The calculations and data analyses were performed using the ARB program package (Ludwig & Strunk, 1997).

Nucleotide sequence accession numbers. The EMBL accession numbers for the 16S rRNA sequences used in addition to those of the four macrococcal species are: *Staphylococcus sciuri* ATCC 29062^T, S83569; *Staphylococcus muscae* CCM 4175^T, S83566; *Staphylococcus auricularis* ATCC 33753^T, D83358; *Staphylococcus aureus* ATCC 12600^T, X68417, L37599; *Staphylococcus epidermidis* ATCC 14990^T, D83363; *Staphylococcus hominis* ATCC 27844^T, X66101, L37601; *Staphylococcus haemolyticus* ATCC 29970^T, D83367, L37600; *Staphylococcus capitis* ATCC 27840^T, L37599; *Staphylococcus saccharolyticus* ATCC 14953^T, L37602; *Staphylococcus saprophyticus* ATCC

15305^T, L37596; *Staphylococcus warneri* ATCC 27836^T, L37603; *Staphylococcus schleiferi* ATCC 43808^T, S83568; *Staphylococcus intermedius* ATCC 29663^T, D83369; *Staphylococcus carnosus* DSM 20501^T, Z26891; *B. subtilis* 168, K00637, M10606, X00007; and *Salinicoccus roseus* ATCC 49258^T, X94559.

PFGE of chromosome fragments. DNA was prepared according to a modification of the procedures of George & Kloos (1994). Cultures were grown in 5 ml Brain Heart Infusion (BHI; Difco) broth for 18 h at 35 °C. A 1 ml aliquot of the overnight culture was then added to a 1.5 ml microcentrifuge tube and centrifuged for 5 min in a Micro-Centrifuge (model 235A, Fisher Scientific). The cell pellet was resuspended in 1 ml TEN buffer (0.1 M Tris, 0.15 M NaCl, 0.1 M EDTA, pH 7.5) and centrifuged again for 5 min. The resulting cell pellet was resuspended in 300 µl EC lysis buffer (6 mM Tris/HCl, 1 M NaCl, 0.1 M EDTA, containing 0.5% Brij 58, 0.2% deoxycholate and 0.5% sodium lauroylsarcosine, pH 7.5), to which was then added 15 µl recombinant lysostaphin (Ambicin L; Aplin & Barrett) solution (1 mg ml⁻¹ in 20 mM sodium acetate, pH 4.5) and 5 µl chicken egg white lysozyme (Sigma) solution (50 mg ml⁻¹ in water). The treated cell suspension was vortexed and a 300 µl aliquot was transferred to 300 µl warm (55 °C) 2% low-gelling-temperature agarose (SeaPlaque, FMC) prepared with EC lysis buffer. The agarose/cell suspension was immediately vortexed and then transferred to a well of a 10-well sample plug mould (Bio-Rad). The agarose plug was cooled for 10 min at room temperature. The cooled agarose plug was removed from the mould with the aid of a small spatula and then transferred to 3 ml EC lysis buffer and incubated for 1–2 h at 37 °C until the plug was cleared (complete lysis). Following lysis, the EC lysis buffer was carefully decanted and replaced with 3 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.6). The agarose plug in TE buffer was incubated at 55 °C for 1 h. The TE buffer was decanted and replaced with 3 ml fresh TE buffer. The agarose plugs were stored at 4 °C for up to 1 month.

Digestion of total DNA by the restriction endonuclease *Sma*I (for *Staphylococcus* species and *M. caseolyticus*, which have a DNA G + C content of less than 40 mol%) and *Not*I (for *M. equiperdicus*, *M. bovis*, *M. carouzelicus*, *B. subtilis* and *Salinicoccus roseus*, which have a G + C content of greater than 40 mol%) was performed in a 1.5 ml microcentrifuge tube by using a 125 µl assay conducted according to the instructions of the manufacturer (New England Biolabs). A section (2 × 4 × 1.5 mm) of the agarose plug prepared as above was added to the assay tube and then incubated with shaking (150 r.p.m.) for 2 h at 25 °C for *Sma*I assays or 37 °C for *Not*I assays.

PFGE procedures, macrorestriction pattern analysis and genome size determinations were conducted as described previously (George & Kloos, 1994).

Plasmid profile analysis. The procedures for plasmid DNA isolation and the agarose gel electrophoresis of the purified plasmid preparations have been described previously (DeGuglielmo *et al.*, 1991), except that in the present study the lysing buffer contained both lysostaphin (500 µg ml⁻¹) and lysozyme (775 µg ml⁻¹) as the staphylococcal enzymes and electrophoresis was performed in horizontal agarose gels (10 cm × 15 cm × 3 mm, 20 wells).

Table 1. List of *Macrococcus* strains and their sources

Species	Strain designation			Source
	DuPont	Kloos	ATCC	
<i>M. caseolyticus</i>	DD 4508		13548 ^T	Cow's milk
	DD 4509		29750	Cow's milk
	DD 4511	BT6	51834	Bovine tongue
	DD 4510	BL6		Bovine lips
	DD 4512	BH5		Bovine heart
	DD 4513	BH10		Bovine heart
	DD 7049			Food-processing factory
	DD 7087			Food-processing factory
	DD 7098			Food-processing factory
	DD 6075	GMWg16	51835	Pilot whale
<i>M. bovicus</i>	DD 4516	C2F4	51825 ^T	Holstein cow
	DD 4518	C2F1		Holstein cow
	DD 4517	C2F24	51826	Holstein cow
	DD 9344	H8a14	51827	Irish thoroughbred horse
	DD 9604	H9a16		Morgan horse
	DD 9603	H9b6		Morgan horse
	DD 9601	H9b7		Morgan horse
	DD 9618	H10a13		Anglo-Trakehner horse
	DD 9617	H10b15		Anglo-Trakehner horse
	DD 9860	H17h3		Shetland pony
<i>M. equiperficus</i>	DD 9350	H8h3	51831 ^T	Irish thoroughbred horse
	DD 9345	H8b6		Irish thoroughbred horse
	DD 9347	H8b12		Irish thoroughbred horse
	DD 9616	H9b2		Morgan horse
	DD 9615	H9b10	51832	Morgan horse
	DD 9840	H15b3	51833	Shetland pony
	DD 9846	H17h1		Shetland pony
	DD 9845	H17h2		Shetland pony
	DD 11639	H21a1		Shetland pony
	DD 11645	H21a9		Shetland pony
	DD 11640	H21h2		Shetland pony
	DD 11653	H21h3		Shetland pony
	DD 11643	H21L4		Shetland pony
	DD 11647	H22h4		Shetland pony
	DD 11648	H22h5		Shetland pony
	DD 11644	H22h7		Shetland pony
	DD 11646	H23b2		Shetland pony
	DD 11652	H23b6		Shetland pony
	DD 11650	H23b9		Shetland pony
	DD 11654	H23b10		Shetland pony
	DD 11651	H23b11		Shetland pony
	DD 11649	H23b12		Shetland pony
<i>M. carouselicus</i>	DD 9348	H8b16	51828 ^T	Irish thoroughbred horse
	DD 9341	H8a1	51829	Irish thoroughbred horse
	DD 9342	H8a4		Irish thoroughbred horse
	DD 9343	H8a10		Irish thoroughbred horse
	DD 9346	H8b7		Irish thoroughbred horse
	DD 9349	H8h1		Irish thoroughbred horse
	DD 9610	H9a7		Morgan horse
	DD 9608	H9a10		Morgan horse
	DD 9607	H9b3	51830	Morgan horse
	DD 9600	H9b9		Morgan horse

Table 1 (cont.)

Species	Strain designation			Source
	DuPont	Kloos	ATCC	
	DD 9602	H9b14		Morgan horse
	DD 9609	H9h9		Morgan horse
	DD 9613	H10a10		Anglo-Trakehner horse
	DD 9621	H10b1		Anglo-Trakehner horse
	DD 9851	H15b6		Shetland pony
	DD 9848	H15b14		Shetland pony
	DD 9855	H16L2		Shetland pony
	DD 11641	H23b13		Shetland pony

Cell wall analysis. Cell walls were purified with 4% SDS by using the procedures of Glauner *et al.* (1988). The cell wall peptidoglycan and teichoic acid analyses were performed as described previously (Schleifer & Kandler, 1972; Anderson *et al.*, 1977; Kaya *et al.*, 1985; Webster *et al.*, 1994). Phosphate levels were determined by the method of Ames (1966). Lipoteichoic acid analyses were performed as described for *Staphylococcus* species (Ruhland & Fiedler, 1990).

DNA base composition. The DNA G+C content was determined by the thermal denaturation method of Marmur & Doty (1962) using a Gilford Response Spectrophotometer with Response II Thermal Programming (Gilford Systems).

RESULTS AND DISCUSSION

Phylogenetic position and analysis of 16S rRNA sequences

Phylogenetically, *Staphylococcus caseolyticus* (Schleifer *et al.* 1982) and the related group of species previously designated as '*Staphylococcus equiperficus*', '*Staphylococcus bovicus*' and '*Staphylococcus carouselicus*' (Ballard *et al.* 1995) were separated from other recognized staphylococci to such an extent that they should be considered as members of a separate genus, herein designated *Macrococcus* gen. nov. The intergroup 16S rRNA sequence similarities were 93.4–95.3%, suggesting that macrococci represent the sister group of the staphylococci. The monophyletic status of both groups is well-documented in the unrooted tree of Fig. 1 and by overall intragroup 16S rRNA sequence similarities of $\geq 97.7\%$ for macrococci and $\geq 96.5\%$ for staphylococci. The sequence similarities of these two groups and *B. subtilis* and *Salinicoccus roseus*, their next shown relatives among the Gram-positive bacteria with a low DNA G+C content, were 90.5–92.9%. The data shown in Fig. 1 are based on at least 90% complete (in comparison with the 16S rRNA from *E. coli*) 16S rRNA primary structures from each of the representative species. All other staphylococcal

species not shown, but yet represented by complete or partial 16S rRNA sequence data in public (Maidak *et al.*, 1996; Van de Peer *et al.*, 1996) and other databases clearly clustered among the staphylococci group, i.e. *Staphylococcus arlettae*, *Staphylococcus caprae*, *Staphylococcus cohnii*, *Staphylococcus equorum*, *Staphylococcus hyicus*, *Staphylococcus kloosii*, *Staphylococcus lugdunensis*, *Staphylococcus piscifermentans* and *Staphylococcus xylosus*.

DNA–DNA hybridization and DNA base composition

The DNA relationships and DNA G+C content of selected *Macrococcus*, *Staphylococcus*, *Salinicoccus* and *B. subtilis* strains are shown in Table 2. Each of the *Macrococcus* species was represented by three strains, one of which was the type strain. The *Staphylococcus* species selected were those found living together with *Macrococcus* species on the same host species (i.e. artiodactyls, perissodactyls and cetaceans), and the more distantly related species *Staphylococcus epidermidis* that is occasionally found as transient populations on these hosts. In addition, *Staphylococcus auricularis* was selected for this study because it has been reported (Schleifer, 1986) that this species has an unusual teichoic acid like that proposed for *Staphylococcus caseolyticus*, i.e. a poly(*N*-acetylglucosaminyl-phosphate) type. As can be seen from the data, each proposed *Macrococcus* species formed a separate, well-defined DNA similarity group under optimal (55 °C) and stringent (70 °C) conditions. Strains of the same *Macrococcus* species demonstrated a relatively high level of DNA relatedness (mean relative binding of $78 \pm 6\%$ at 55 °C and $72 \pm 3\%$ at 70 °C, shown as bold numbers in the table), whereas strains of different *Macrococcus* species demonstrated a lower level of DNA relatedness (mean relative binding of $33 \pm 12\%$ at 55 °C and $14 \pm 3\%$ at 70 °C). The species *M. equiperficus*, *M. bovicus* and *M. carouselicus* were more closely related to one another than to *M. caseolyticus* and formed a species group (Kloos, 1980; Kloos & Schleifer, 1981), which herein is informally

Fig. 1. 16S rRNA tree reflecting the phylogenetic relationships of the type strains of *Macrococcus* species and a representative selection of *Staphylococcus* (*St.*) species, as well as *Salinicoccus* (*Sa.*) *roseus* and *B. subtilis*. The latter two organisms indicate the level of relatedness of the *Macrococcus*–*Staphylococcus* branch and the other Gram-positive bacteria with a low DNA G+C content. The tree is based on the results of a maximum-likelihood analysis. The tree topology was corrected according to the data obtained by applying distance and maximum-parsimony methods. Only sequence positions which shared identical nucleotides in at least 50% of all available sequences of macrococci and staphylococci were used to construct the tree. Bar, 5 substitutions per 100 nucleotides.

designated the *Macrococcus equiperficus* species group. The DNA relatedness of *Macrococcus* species to members of the *Staphylococcus sciuri* species group (*Staphylococcus sciuri*, *Staphylococcus lentus* and *Staphylococcus vitulus*) appeared to be slightly higher than to the other staphylococcal species (14 ± 3 vs $11 \pm 2\%$ at 55°C). Furthermore, of the four macrococcal species, *M. caseolyticus* appeared to be the most closely related to the *Staphylococcus sciuri* species group (19 ± 0.5 vs $13 \pm 1\%$ at 55°C). There was a good correlation between the results of DNA–DNA hybridization and the analysis of 16S rRNA sequences (Fig. 1) in that both methods suggested the genus *Macrococcus* is more closely related to *Staphylococcus sciuri* than to staphylococcal species outside the *Staphylococcus sciuri* species group.

The DNA G+C content of 41–45 mol% for members of the *M. equiperficus* species group is higher than that of recognized *Staphylococcus* species (Kloos & Schleifer, 1986; Kloos *et al.*, 1991; Kloos, 1998) (Table 2) and these results further support the inclusion of these species in a separate genus. The DNA G+C content of 38–39 mol% reported for *M. caseolyticus* (Schleifer *et al.*, 1982) was shared by several of the staphylococcal species and this feature is consistent with the results of DNA–DNA hybridization, suggesting that this species is more closely related to staphylococci than are members of the *M. equiperficus* species group.

Normalized ribotype patterns

An ensemble of ribotype pattern types sharing common features and not fitting into any known species

structure potentially constitutes an undescribed species. This statement is dependent on knowledge of the polymorphic pattern structures surrounding the newly recognized species. With this in mind, we determined the ribotype patterns of approximately 2500 strains representing 31 species of *Staphylococcus* (Hubner *et al.*, 1993; Cole *et al.*, 1994; Webster *et al.*, 1994) and compared them with patterns of strains representing the new *Macrococcus* species and *M. caseolyticus* (Tables 1, 2). When sorted by similarity analysis, ribotype pattern groups representing strains of *M. equiperficus*, *M. bovicus*, *M. carouzelicus* and *M. caseolyticus* appeared closer to one another than to any of the *Staphylococcus* species. Each of the above four species representations (Fig. 2) had a unique combination of conserved fragment sizes detected by hybridization with the labelled rRNA operon probe. All of the pattern types of *M. caseolyticus* contained bands at 0.76, 1.05, 1.3, 2.3 and 3.9 kbp; all *M. equiperficus* patterns contained bands at 1.1, 2.3, 2.6, 4.2 (except dd 11648) and 10 kbp; all *M. bovicus* patterns contained bands at 1.1 (except dd 09617), 1.3, 2, 2.4 (except dd 04518) and 4 (except dd 09344) kbp; and all *M. carouzelicus* patterns contained bands at 1.05, 1.3, 1.7, 2.4 and 8 kbp. In support of the transfer of *Staphylococcus caseolyticus* to the genus *Macrococcus*, all of the above four species demonstrated conserved fragments of 1.05–1.1 kbp and 9.5–10 kbp, whereas some *Staphylococcus* species demonstrated a slightly smaller conserved fragment of 1.0 kbp and only occasionally had a fragment of about 10 kbp (Fig. 2). Furthermore, several of the lower *Staphylococcus* species, such as *Staphylococcus simulans*, *Staphylococcus carnosus*, *Staphylococcus schleiferi*, *Staphylo-*

Table 2. Results of hybridization of *Macrococcus*, *Staphylococcus*, *Salinicoccus* and *Bacillus* DNA with [methyl-³H]thymidine-labelled DNA from each of the *Macrococcus* species under optimal (55 °C) and stringent (70 °C) conditions

Genus and species	Strain	DNA G + C (mol %)	Percentage relative binding with labelled DNA from:							
			<i>M. caseolyticus</i> DD 4508 ^T		<i>M. equipercicus</i> DD 9350 ^T		<i>M. bovicus</i> DD 4516 ^T		<i>M. carouselicus</i> DD 9348 ^T	
			55 °C	70 °C	55 °C	70 °C	55 °C	70 °C	55 °C	70 °C
<i>M. caseolyticus</i>	DD 4508 ^T	38	100	100	28	10	21	11	17	13
	DD 4511	ND	74	69	29	10	24	9	18	9
	DD 6075	ND	73	73	24	16	21	10	18	10
<i>M. equipercicus</i>	DD 9350 ^T	45	25	14	100	100	40	15	36	18
	DD 9615	ND	27	16	80	72	44	14	41	17
	DD 9840	45	26	17	71	69	42	17	28	17
<i>M. bovicus</i>	DD 4516 ^T	44	30	17	32	17	100	100	51	15
	DD 4517	42	24	15	37	14	80	68	54	ND
	DD 9344	ND	22	13	34	11	72	76	47	16
<i>M. carouselicus</i>	DD 9348 ^T	41	25	11	47	21	58	18	100	100
	DD 9341	41	25	10	37	12	50	ND	89	74
	DD 9607	ND	23	9	37	17	54	16	81	74
<i>Staphylococcus vitulus</i>	DD 756 ^T	34	19	9	13	8	13	9	15	8
<i>Staphylococcus lentus</i>	DD 4203 ^T	34	19	5	12	7	11	6	12	9
<i>Staphylococcus sciuri</i>	DD 4277 ^T	37	18	8	12	7	12	9	13	7
<i>Staphylococcus muscae</i>	DD 4795 ^T	40	13	5	11	ND	14	ND	11	ND
<i>Staphylococcus hyicus</i>	DD 887 ^T	33	15	4	12	ND	11	ND	10	ND
<i>Staphylococcus chromogenes</i>	DD 851 ^T	34	12	6	9	ND	11	ND	9	ND
<i>Staphylococcus gallinarum</i>	DD 854 ^T	35	12	7	12	ND	10	ND	14	6
<i>Staphylococcus equorum</i>	DD 853 ^T	35	16	8	11	ND	10	ND	9	ND
<i>Staphylococcus kloosii</i>	DD 857 ^T	36	17	6	10	ND	10	ND	12	8
<i>Staphylococcus auricularis</i>	DD 872 ^T	39	11	ND	9	ND	9	ND	10	ND
<i>Staphylococcus aureus</i>	DD 10156 ^T	33	14	ND	10	ND	9	ND	13	9
<i>Staphylococcus epidermidis</i>	DD 8726 ^T	34	12	ND	ND	ND	9	ND	12	7
<i>Salinicoccus roseus</i>	DD 12047 ^T	49	11	ND	12	ND	10	ND	10	ND
<i>B. subtilis</i>	DD 379 ^T	43	10	ND	10	ND	9	ND	9	ND

ND, Not determined.

coccus delphini, *Staphylococcus chromogenes*, *Staphylococcus lentus*, *Staphylococcus vitulus* and *Staphylococcus sciuri*, did not even have a fragment in the size range of 0.8–1.3 kbp. Each of the DNA similarity groups examined in Table 2 manifested a different ribotype pattern.

Macrorestriction pattern analysis and estimation of genome size

The restriction endonuclease *Sma*I (recognition site: 5' ...CCCGGG...3') is commonly used to digest the chromosomes of staphylococci for macrorestriction

Fig. 2. Diversity of normalized ribotype pattern types found within each of the *Macroccoccus* species and a representative ribotype pattern type of selected species of the related genera *Staphylococcus* (*St.*) *Salinicoccus* (*Sa.*) and *Bacillus*. Certain *Macroccoccus* pattern types are likely to represent many strains, as shown by the strain count.

pattern analysis (Lina *et al.*, 1992; George & Kloos, 1994; Bannerman *et al.*, 1995). It cleaves the *Staphylococcus* chromosome into about 7–30 fragments,

depending upon the particular species and strain. This enzyme cleaved the chromosomes of *M. caseolyticus* strains into 29–34 fragments, most of which were well

Fig. 3. Macrorestriction pattern analysis of *Sma*I-digest fragments of chromosomes of *M. caseolyticus* (a), and *Not*I-digest fragments of chromosomes of *M. carouzelicus* (b), *M. equipercicus* (c) and *M. bovicus* (d), separated by PFGE.

separated following PFGE (Fig. 3). The ten strains produced eight different macrorestriction patterns. Three strains (DD 7087, DD 7049 and DD 7098) isolated from the same food-processing factory produced a very similar pattern. These strains could be distinguished only on the basis of their plasmid profile (data not shown) and they probably represent relatively recent subclones. *Sma*I-digest fragments of sizes 2.9, 3.0, 3.2, 9.5, 23, 40, 45, 68, 99 and 108 kb are highly conserved among *M. caseolyticus* strains. Based on the sum of the sizes of *Sma*I-digest fragments, the estimated size of the *M. caseolyticus* genome is 1714 ± 93 kb (Table 3).

*Sma*I cleaved the chromosomes of members of the *M. equipercicus* species group into a very large number of small fragments, many of which were overlapping and not clearly distinguishable by PFGE. Several other

restriction endonucleases that recognize GC hexanucleotide sequences (*Nae*I, *Nar*I and *Sac*II) and GC octanucleotide sequences (*Not*I, *Fse*I and *Asc*I) were tested for their ability to produce definable macrorestriction patterns. Of these enzymes, the restriction endonuclease *Not*I (recognition site: 5' ... GCGGC-CGC ... 3') was most satisfactory for members of the *M. equipercicus* species group in that it cleaved their chromosome into a relatively small number of fragments, most of which were well-separated by PFGE. This enzyme cleaved the chromosomes of 21 of the 22 strains of *M. equipercicus* into 33–37 fragments (Fig. 3). These strains produced 19 different macrorestriction patterns. The chromosome of strain DD 9347 appeared to be only partially cleaved at two sites by *Not*I, as indicated by the presence of a single faint band migrating in the gel. It is quite possible that some strains of macrococci produce site-specific DNA

Table 3. Comparison of the genome sizes of *Macrococcus* species with those of *Staphylococcus* and *Salinicoccus* species

Genus and species	No. of strains	Genome fragments produced by:	Estimated genome size (kb)
<i>M. caseolyticus</i>	10	<i>Sma</i> I	1714 ± 93
<i>M. equiperdicus</i>	20	<i>Not</i> I	1536 ± 91
<i>M. bovicus</i>	8	<i>Not</i> I	1753 ± 64
<i>M. carouzelicus</i>	16	<i>Not</i> I	1706 ± 95
<i>Staphylococcus aureus</i>	26	<i>Sma</i> I	2741 ± 232
<i>Staphylococcus epidermidis</i>	13	<i>Sma</i> I	2365 ± 124
<i>Staphylococcus capitis</i>	26	<i>Sma</i> I	2365 ± 122
<i>Staphylococcus caprae</i>	13	<i>Sma</i> I	2584 ± 146
<i>Staphylococcus hominis</i>	26	<i>Sma</i> I	2372 ± 137
<i>Staphylococcus schleiferi</i>	10	<i>Sma</i> I	2283 ± 171
<i>Staphylococcus sciuri</i>	9	<i>Sma</i> I	2697 ± 162
<i>Salinicoccus roseus</i>	1	<i>Not</i> I	2041

methylnses inhibiting digestion by *Not*I, for this enzyme is sensitive to certain types of site-specific methylation. Many of the *Not*I-digest fragments of *M. equiperdicus* strains were highly conserved, especially those of sizes 2, 3.2–3.8, 4.2, 6.5, 7, 8, 8.5, 10, 12, 14, 15, 16, 18, 22, 25, 28, 34, 36, 48, 57, 79 and 88 kb. The estimated size of the *M. equiperdicus* genome is 1536 ± 91 kb (Table 3), though it may be slightly larger since the number of discrete fragments present in several of the intensely stained bands is uncertain.

*Not*I cleaved the chromosomes of nine of the ten strains of *M. bovicus* into 14–17 well-separated fragments (Fig. 3). The nine strains produced eight different macrorestriction patterns. The chromosome of strain DD 9603 appeared to be only partially cleaved at two sites by *Not*I, as indicated by the presence of a single faint band following PFGE. *Not*I-digest fragments of sizes 4.9, 12, 19, 28, 44 and 97 kb were highly conserved among *M. bovicus* strains. The estimated size of the *M. bovicus* chromosome is 1753 ± 64 kb (Table 3).

The chromosomes of 16 of the 17 strains of *M. carouzelicus* were cleaved by *Not*I into 12–16 well-separated fragments (Fig. 3). The 16 strains produced 15 different macrorestriction patterns. The chromosome of strain DD 9608 appeared to be cleaved by *Not*I at only two sites, as indicated by the presence of a single, well-defined band following PFGE. *Not*I-digest fragments of sizes 6.9, 11, 16, 26, 39, 56 and

100 kb were highly conserved among *M. carouzelicus* strains. The estimated size of the *M. carouzelicus* chromosome is 1706 ± 95 kb (Table 3). As can be seen from the data in Table 3, the genome sizes of *Macrococcus* species are significantly smaller than those of the *Staphylococcus* species for which there have been calculations of genome size.

Cell wall composition

The cell-wall peptidoglycan and teichoic acid composition were determined for several strains of each of the *Macrococcus* species, including *M. caseolyticus* DD 4508^T and DD 4509 determined previously (Schleifer *et al.*, 1982), DD 4510 and DD 6075; *M. equiperdicus* DD 9350^T, DD 9615 and DD 9840; *M. bovicus* DD 4516^T, DD 4518 and DD 9344; and *M. carouzelicus* DD 9348^T, DD 9341 and DD 9607. The peptidoglycan type for macrococci is L-Lys-Gly₃₋₄, L-Ser, a basic type that is also found among several staphylococcal species (Schleifer, 1986; Kloos, 1998). Some differences were noted in the levels of L-serine in the peptidoglycans of certain macrococcal species, for example, *M. caseolyticus* strains exhibited the highest levels [1.2–1.3 mol (mol glutamic acid)⁻¹] and *M. bovicus* exhibited the lowest levels [0.44–0.58 mol (mol glutamic acid)⁻¹]. The cell wall teichoic acid type of *M. caseolyticus* strain DD 4508^T has been reported to be poly(*N*-acetylgalactosaminylphosphate), where the *N*-acetylaminosugar residues form an integral part of the

Fig. 4. Phase-contrast photomicrographs of Gram-stained cells of (a) the type strain of *M. carouzelicus*, (b) the type strain of *Staphylococcus sciuri* subsp. *sciuri*, (c) a mixture of the type strains of *M. carouzelicus* (larger 'glowing' cells in clusters) and *Staphylococcus aureus* (small blue-outlined cells occurring singly and in pairs, small clusters and short chains), and (d) a mixture of the type strains of *M. carouzelicus* (larger 'glowing' cells in clusters) and *Staphylococcus sciuri* subsp. *sciuri* (smaller 'glowing' cells occurring singly and in pairs and small clusters). Cells were grown in TS broth for 18 h at 35 °C. Bar, 4 µm.

polymer chain (Schleifer, 1986). However, since only very low amounts of phosphorus [$0.45 \mu\text{mol (mg cell wall)}^{-1}$] and glycerol could be detected in the cell wall upon reinvestigation of the type strain, it appears that any teichoic acid present in *M. caseolyticus* would be in an amount much lower than that found in the cell walls of staphylococci. The teichoic acid of *M. caseolyticus* strain DD 6075 also contained *N*-acetylgalactosamine, whereas strain DD 4509 contained *N*-acetylglucosamine as the *N*-acetylaminosugar residue. The cell walls of members of the *M. equiperficus* species group did not contain detectable levels of teichoic acids, as indicated by the absence of both phosphorus and polyol residues. By contrast, the cell walls of all currently recognized *Staphylococcus* species contain teichoic acids (Endl *et al.*, 1983; Schleifer, 1986; Kloos, 1998). As for the genus *Staphylococcus* (Ruhland & Fiedler, 1990), lipoteichoic acids were found to be

present at the cell surface of the type strains of *M. caseolyticus*, *M. equiperficus*, *M. bovis* and *M. carouzelicus*. The glycosyl residue of the glycolipid is gentiobiosyl, which is the same glycosyl as that found in staphylococci. The fatty acid substitution was not investigated.

Phenotypic characterization

Cells of macrococci were Gram-positive and 1.1–2.5 µm in diameter, depending on the species and growth medium. They were non-motile and non-spore-forming, and were arranged mostly in pairs and tetrads, and occasionally single or arranged in short chains. Cells of *M. equiperficus* and *M. carouzelicus* were usually larger than those of the other macrococcal species. When grown in TS broth, the Gram-stained cells of macrococci were approximately 2.5–4 times the

Fig. 5. Scanning electron micrographs of the type strains of (a) *M. caseolyticus*, (b) *M. equipercicus*, (c) *M. bovicus* and (d) *M. carouselicus*. Magnification $\times 13320$.

diameter of *Staphylococcus aureus* cells and 1.5–2.5 times the diameter of *Staphylococcus sciuri* cells. The difference in cell size between the two genera was less remarkable when cells were grown in BHI broth or on TSA, TSA plus 5% sheep blood, BHI agar or P agar. Cells of *M. equipercicus* were arranged predominantly in tetrads. We found serendipitously that Gram-stained cells of macrococci, when viewed under the phase-contrast microscope, appeared much larger than staphylococci and their cells reflected a glowing pink- or orange-brown colour (Fig. 4). Scanning electron microscopy (SEM) revealed that the surface of *M. caseolyticus* cells was quite smooth, whereas the surface of *M. bovicus* and *M. carouselicus* cells was somewhat irregular, and numerous small piliform projections were present on the surface of *M. equipercicus* cells (Fig. 5).

Macrococci are marginally facultative anaerobes. Growth was much better under aerobic conditions than under anaerobic conditions. Members of the *M. equipercicus* species group do not grow significantly in the anaerobic portion of a thioglycollate semi-solid medium; however, some strains of *M. caseolyticus* grow slowly in the anaerobic portion of this medium.

Macrococci were positive for catalase and oxidase (Microdase disk test) activities, resistant to the 0.04 U bacitracin (Taxo A) disk and lysozyme ($25 \mu\text{g ml}^{-1}$), and susceptible to the 100 μg furazolidone disk. They were negative for staphylocoagulase, alkaline phosphatase, ornithine decarboxylase, β -glucuronidase and β -galactosidase activities and arginine utilization, and did not produce acid from D-mannose, D-melezitose, D-xylose, L-arabinose, D-turanose, D-sorbitol, D-cellobiose, salicin and D-raffinose. Members of the *M. equipercicus* species group were susceptible to lyso-staphin (10 μg applied as a drop to the inoculated agar surface), whereas *M. caseolyticus* was slightly resistant to this enzyme.

Macrococci were susceptible to a wide range of antibiotics and did not exhibit the resistance profiles characteristic of most staphylococcal species. Using the breakpoints established for disk-diffusion testing of *Staphylococcus aureus*, all macrococcal strains tested were susceptible to penicillin G, erythromycin, clindamycin, tetracycline, ciprofloxacin, rifampin, trimethoprim-sulfamethoxazole, gentamicin, kanamycin, streptomycin, vancomycin and chloramphenicol. *M. equipercicus* strain DD 11653 was resistant to

oxacillin, and strains DD 11644, DD 11648 and DD 11649 showed intermediate resistance to oxacillin; all other macrococci examined in this study were susceptible to this antibiotic. The mechanism for oxacillin resistance in these organisms is unknown. The gene *mecA* appeared to be absent in the above strains since amplification products were not detected in PCR using a set of primers capable of detecting *mecA* homologues in staphylococci (Kloos *et al.*, 1998). *M. equipercicus* strain DD 11651 and *M. bovicus* strain DD 9860 were resistant to lincomycin. Members of the *M. equipercicus* species group were resistant to novobiocin (5 µg, disk inhibition zone diameters of 12–16 mm), on the basis of the breakpoint of 16 mm established for *Staphylococcus saprophyticus* (Kloos & Bannerman, 1995). *M. caseolyticus* strains exhibited inhibition zone diameters of 12–18 mm with novobiocin disks, classifying them as either resistant or susceptible.

Characters that were variable in one or more of the *Macrococcus* species are listed in Table 4. Several of the phenotypic characters were expressed differently among the species and therefore had taxonomic value. For example, acetoin was produced by all of the strains of *M. caseolyticus*, but was not detected in the other species. *M. caseolyticus* reduced nitrates, whereas *M. equipercicus* and all but one strain of *M. bovicus* and *M. carouselicus* failed to reduce nitrates. *M. carouselicus* exhibited a moderate to strong DNase activity. Only one strain of *M. equipercicus* had detectable DNase activity, and this was weak. Most strains (86%) of *M. equipercicus* exhibited moderate to strong urease activity, whereas none of the *M. caseolyticus* and *M. carouselicus* strains and only two of the *M. bovicus* strains had detectable urease activity. Most strains (80%) of *M. caseolyticus* exhibited pyrrolidonylarylamidase activity. Members of the *M. equipercicus* group did not exhibit this enzyme activity. *M. bovicus* and most strains (95%) of *M. equipercicus* produced acid aerobically from D-mannitol, but *M. caseolyticus* failed to produce acid from this carbohydrate. Most strains (94%) of *M. carouselicus* produced only low levels of acid from β-D-fructose, whereas the other species produced noticeably higher levels of acid from this carbohydrate. *M. caseolyticus* produced acid from maltose, but *M. carouselicus* did not produce acid from this carbohydrate. *M. bovicus* and most strains of *M. caseolyticus* (90%) demonstrated a partial haemolysis (greening) of horse blood in TSA following 24–48 h incubation at 35 °C. Haemolysis was not observed with *M. equipercicus* and most strains (94%) of *M. carouselicus*. On the basis of phenotypic characters, *M. caseolyticus* is the most distinguishable of the macrococcal species. The lower phenotypic similarity was supported by the genotypic data separating this species from the *M. equipercicus* species group.

Plasmid profiles

An examination of plasmid carriage was prompted by the results of antibiograms showing the absence among macrococci of antibiotic resistances (e.g. to penicillin

G, erythromycin, clindamycin, tetracycline, gentamicin, kanamycin, streptomycin and chloramphenicol) commonly determined by plasmid-borne genes in most staphylococcal species and other related bacteria (Lyon & Skurray, 1987; Novick, 1989; Archer & Climo, 1994; Kloos, 1998). Consequently, we considered the possibility that macrococci either do not carry plasmids or carry them infrequently. However, most of the macrococci carried one or more cryptic plasmids, including all of the *M. caseolyticus* strains, 95% of *M. equipercicus* strains, 94% of *M. carouselicus* strains and 70% of *M. bovicus* strains. The macrococci demonstrated a mean number of 4 ± 3 different plasmids per strain (based on the recognition of plasmid bands suspected of representing covalently closed circular forms). Most strains (86%) of *M. equipercicus* had 3 and 5 kb plasmids that produced a noticeable series of structural forms, i.e. a multi-form ladder, on agarose gels. Several strains of the other macrococcal species had plasmids of these same sizes.

Description of *Macrococcus* gen. nov.

Macrococcus (Ma.cro.coc'cus. Gr. adj. *macrus* large; Gr. masc. n. *kokkos* a grain or berry; M.L. masc. n. *Macrococcus* a large coccus).

The description below is based on data collected on the species *Staphylococcus caseolyticus* (Schleifer *et al.*, 1982; Endl *et al.*, 1983; Schleifer, 1986) and data from this study. Cells are spherical or coccoid, Gram-positive, non-motile, non-encapsulated and do not produce endospores. They are 1.1–2.5 µm in diameter and are larger than the cells of *Staphylococcus* species when comparisons are made of cells grown on the same medium. Metabolism is mainly respiratory; growth is chemoorganotrophic and only marginally facultatively anaerobic. They are catalase- and oxidase-positive, staphylocoagulase-, ornithine-decarboxylase-, alkaline-phosphatase-, β-glucuronidase- and β-galactosidase-negative, non-halophilic and mesophilic. They are resistant to bacitracin and lysozyme, and susceptible to furazolidone. The peptidoglycan type is L-Lys-Gly₃₋₄, L-Ser, and only one of the four macrococcal species, i.e. *Macrococcus caseolyticus*, appears to contain cell-wall teichoic acid. Lipoteichoic acid is present. The genome size range is approximately 1500–1800 kb. The G+C content of the DNA is 38–45 mol % [as determined by the thermal denaturation method (Marmur & Doty, 1962)]. The type species is *Macrococcus equipercicus*. It was selected as the type species in place of *M. caseolyticus*, the first described species of the group, because it is the most typical of the cluster of four species now recognized.

Description of *Macrococcus caseolyticus* (Schleifer *et al.* 1982) comb. nov.

Macrococcus caseolyticus (ca.se.o.ly'ti.cus. L. n. *caseus* cheese; Gr. adj. *lyticus* able to loose; M.L. adj. *caseolyticus* casein-dissolving).

Table 4. Variable characteristics of *Macrococcus* species

Characteristic	Percentage of strains positive (and weakly positive)			
	<i>M. caseolyticus</i> (n = 10)	<i>M. equiperdus</i> (n = 22)	<i>M. bovicus</i> (n = 10)	<i>M. carouelicus</i> (n = 18)
P agar colony diameter $\geq$ 4 mm	20	95	80	83
TSA colony diameter $\geq$ 6 mm	80	95	20	94
Anaerobic growth	0 (50)	0	0	0
Colony pigmentation: orange	0	95 (5)	70	0 (67)
Colony lustre: glistening	100	23	100	100
Haemolysis (horse blood)	0 (90)	0	0 (100)	0 (6)
Acetoin production	100	0	0	0
Aesculin hydrolysis	0 (20)	45 (23)	10	94
Nitrate reduction	100	0	10	6
DNase	40	0 (5)	30 (10)	100
Urease	0	86	20	0
Pyrrolidonylarylamidase	80	0	0	0
β -Glucosidase	0	0 (5)	50	6
Staph Latex agglutination	90	0 (5)	30	33
Lysostaphin resistance*	50 (50)	0	0 (10)	0
Novobiocin resistance†	80 (20)	100	100	100
Oxacillin resistance‡	0	5 (14)	0	0
Acid (aerobic) produced from:				
D-Mannitol	0	95	100	6 (33)
Maltose	100	32 (41)	50 (20)	0
Sucrose	40	0 (9)	0 (20)	67
Glycerol	90	100	100	0 (50)
D-Ribose	80	0 (5)	10	0
α -Lactose	60	0	0	0
β -D-Fructose	100	100	100	0 (94)
D-Trehalose	100	32 (18)	50 (10)	61 (33)

* Growth response on the surface of a TSA plate in the presence of a 0.05 ml drop of distilled water containing 10 μ g lysostaphin. Positive, resistant, confluent growth; weakly positive, intermediate resistance, reduction in growth in the area of the drop; negative, susceptible, no growth.

† Disk containing 5 μ g; positive, resistant, zone of inhibition $\geq$ 16 mm; weakly positive, intermediate resistance, zone of inhibition 17–18 mm.

‡ Disk containing 1 μ g; positive, resistant, zone of inhibition $\geq$ 10 mm; weakly positive, intermediate resistance, zone of inhibition 11–12 mm.

The description below is based on characteristics previously described (Schleifer *et al.*, 1982) and this paper. A total of ten strains were examined (Table 1), including strains DD 4508^T (= ATCC 13548^T) and DD 4509 (= ATCC 29750) isolated from cow's milk, and an additional eight strains isolated in 1992 from the skin of a whale and from raw beef and other meat products. Colonies grow to 3 ± 1 mm in diameter on P agar and 7 ± 1 mm in diameter on TSA. They are

slightly convex, entire, butyrous, glistening and opaque, and may be unpigmented (grey-white) or have a pale yellow pigmentation. Growth in the anaerobic portion of a semi-solid thioglycollate medium ranges from no detectable growth to weak, delayed growth. A small amount of L-(+)-lactic acid is produced from glucose under anaerobic conditions. Growth is good at NaCl concentrations up to 10%. Optimum growth temperature is 35 °C. The cytochromes of strains DD

4508^T and DD 4509 are *aa*₃, *c*₅₄₉, *c*₅₅₃, *b*₅₆₀ and *b*₅₆₅, and *aa*₃, *c*₅₄₉, *c*₅₅₄, *b*₅₅₈ and *b*₅₆₄, respectively. Both of these strains produce a class II fructose-1,6-diphosphate aldolase. The cell wall contains only small amounts of phosphorus and glycerol and appears to have an atypical teichoic acid of the type poly(*N*-acetylgalactosaminylphosphate) [or poly(*N*-acetylglucosaminylphosphate)]. Acetoin is produced and nitrates are reduced. Acid is produced aerobically from maltose, β -D-fructose and D-trehalose. Acid is not produced from D-mannitol. Urease and β -glucosidase activities are negative. Characteristics of *M. caseolyticus* that are general properties of the genus are listed above. The variable characteristics for this species are listed in Table 4. The API STAPH-IDENT profile is 0400. Major ID32 STAPH profiles are 066130300 (30%), 062130300 (30%) and 066130101 (30%). The G + C content of the DNA is 38–39 mol%. This relatively uncommon species appears to have a preference for artiodactyl (e.g. cattle, sheep and goats) and cetacean (e.g. whale) hosts and may be found in their milk and meat products.

Amended description of the type strain. Type strain is ATCC 13548^T (Schleifer *et al.* 1982) (= DD 4508^T), isolated from cow's milk, which was previously designated the type strain of *Staphylococcus caseolyticus* (Schleifer *et al.* 1982). It has all of the general properties of the species and genus described above and also the following characteristics. Cells are spherical, 1.1–2 μ m in diameter, have a smooth surface (SEM), and occur singly, in pairs, short chains and clusters. Colonies on P agar and TSA are circular, 4–5 mm in diameter, entire, low convex, and possess a smooth and glistening surface and a sticky consistency. They are grey-white with slight cream-yellow pigmentation near the colony edge. Cells are facultatively anaerobic and show delayed weak growth in the anaerobic portion of thioglycollate medium. No haemolysis of sheep, horse and bovine blood. Moderate pyrrolidonylarylamidase activity. Positive latex agglutination. No DNase activity and aesculin hydrolysis. Acid produced aerobically from D-glucose, β -D-fructose, α -lactose, D-ribose, D-galactose, D-trehalose and maltose. No acid produced from D-mannitol, sucrose and glycerol. Susceptible to marginally resistant to novobiocin. Slightly resistant to lysostaphin. Type dd 04508 ribotype pattern. Class II fructose-1,6-diphosphate aldolase. Cytochromes *aa*₃, *b* and *c* are present. Cell wall peptidoglycan L-Lys-Gly₄, L-Ser. The presumed teichoic acid contains glucose and *N*-acetylgalactosamine and only small amounts of *N*-acetylglucosamine and glycerol. Lipoteichoic acid is present. G + C content of DNA is 38 mol%.

Description of *Macrococcus equipercicus* sp. nov.

Macrococcus equipercicus (equi.per'ci.cus. L. gen. n. equi of a horse. M.L. adj. *equipercicus* pertaining to a

horse named Percy, from which this species was first isolated).

The description below is based on the characteristics of 22 strains (Table 1) isolated in 1993–1995 from the skin of horses and ponies. Colonies grow to 6 $\pm$ 2 mm in diameter on P agar and 6 $\pm$ 1 mm on TSA. They are convex, entire, butyrous, dull to slightly glistening and opaque, and have a light- to medium-orange pigmentation. Growth is not detected in the anaerobic portion of a semi-solid thioglycollate medium. Growth is good at NaCl concentrations up to 7.5%. Optimum growth temperature is 35 °C. Culture growth does not cause haemolysis of horse, bovine or sheep blood. The cell wall does not contain teichoic acid. Acetoin is not produced and nitrates are not reduced. DNase, pyrrolidonylarylamidase and β -glucosidase activities are negative, except that strain DD 9350^T is weakly positive for DNase and β -glucosidase activities. Staph Latex agglutination is negative, except that strain DD 11654 is weakly positive for this test. All strains are resistant to novobiocin and susceptible to lysostaphin. Acid is produced aerobically from glycerol and β -D-fructose. All strains, except DD 9347, produce acid from D-mannitol. Acid is not produced from α -lactose and, with the exception of strain DD 9347, is not produced from D-ribose. Characteristics of *M. equipercicus* that are general properties of the genus are listed above. The variable characteristics for this species are listed in Table 4. The major API STAPH-IDENT profile is 2000 (68%). There are ten different ID32 STAPH profiles; the most common ones begin with the digits 17 (27%) or 35 (18%) in the nine-digit profile, although no specific profile is represented by more than 14% of the strains. The major ribotype pattern types are dd 11639 (41%) and dd 09345 (23%). The G + C content of the DNA is 45 mol%. This species has a preference for perissodactyls (e.g. horses and ponies) and is commonly found as large populations on the skin of these mammals.

Description of the type strain. Type strain is ATCC 51831^T (= DD 9350^T), isolated from the skin of an Irish thoroughbred horse. It has all of the general properties of the species and genus described above and also the following characteristics. Cells are spherical, 1.3–2.3 μ m in diameter, have an irregular surface (SEM) exhibiting numerous small piliform projections, and occur predominantly in tetrads. Colonies on P agar and TSA are circular, 8 mm in diameter, entire, convex, and with a dull matt surface and butyrous consistency. Light- to medium-orange pigmentation. Marginally facultatively anaerobic; no visible growth in anaerobic portion of thioglycollate medium. No haemolysis of sheep, horse and bovine blood. Positive aesculin hydrolysis and urease activity. Weakly positive DNase and β -glucosidase activities. Negative latex agglutination. Acid produced aerobically from D-trehalose, D-mannitol, glycerol and β -D-fructose. Acid produced weakly from maltose. No acid produced from sucrose, α -lactose and D-ribose. Susceptible to oxacillin. Type dd 09345 ribotype pattern.

Cell wall peptidoglycan is L-Lys-Gly₃₋₄, L-Ser. No detectable teichoic acid. Lipoteichoic acid is present. G+C content of DNA is 45 mol %.

Description of *Macrococcus bovicus* sp. nov.

Macrococcus bovicus (bov.ic'us. Gr. n. *bou* cow. L. gen. n. *bovis* of a cow. M.L. adj. *bovicus* pertaining to a bovine or cow, from which this organism was first isolated).

The description below is based on the characteristics of ten strains (Table 1) isolated in 1992–1994 from the skin of a cow, a pony and several horses. Colonies grow to 4±1 mm in diameter on P agar and TSA. They are slightly convex, entire, butyrous, glistening and opaque, and have a pale-yellow to medium-orange pigmentation. Growth is not detected in the anaerobic portion of a semi-solid thioglycollate medium. Growth is good at NaCl concentrations up to 7.5%. Optimum growth temperature is 35 °C. Culture growth causes a partial haemolysis (greening) of horse and bovine blood. The cell wall does not contain teichoic acid. Lipoteichoic acid is present. Acetoin is not produced. Pyrrolidonylarylamidase activity is negative. All strains are resistant to novobiocin and susceptible to lysostaphin and oxacillin. Acid is produced aerobically from D-mannitol, glycerol and β-D-fructose. Acid is not produced from α-lactose. Characteristics of *M. bovicus* that are general properties of the genus are listed above. The variable characteristics of this species are listed in Table 4. Major API STAPH-IDENT profiles are 0200 (30%), 4500 (20%) and 4600 (20%), and major ID32 STAPH profiles are 062200000 (20%), 062310200 (20%) and 072300200 (20%). The major ribotype pattern type is dd 09344 (40%). The G+C content of the DNA is 42–44 mol %. This species appears to have a preference for perissodactyls (e.g. horses and ponies) and artiodactyls (e.g. cattle).

Description of the type strain. Type strain is ATCC 51825^T (= DD 4516^T), isolated from the skin of a Holstein cow. It has all of the general properties of the species and genus described above and also the following characteristics. Cells are spherical, 1.2–2.1 µm in diameter, have a slightly irregular surface (SEM), and occur singly and in pairs, tetrads and short chains. Colonies on P agar and TSA are circular, 3–5 mm in diameter, entire, slightly convex, and with a glistening surface and butyrous consistency. Medium-orange pigmentation. Marginally facultatively anaerobic; no visible growth in anaerobic portion of thioglycollate medium. Partial haemolysis (greening) of horse and bovine blood. Positive DNase activity and latex agglutination. Negative aesculin hydrolysis, nitrate reduction and urease activity. Acid produced aerobically from D-trehalose, D-mannitol, glycerol and β-D-fructose. No acid produced from sucrose, α-lactose, maltose and D-ribose. Type dd 04516 ribotype pattern. Cell wall peptidoglycan is L-Lys-Gly₃, L-Ser. No detectable teichoic acid. Lipoteichoic acid is present. G+C content of DNA is 44 mol %.

Description of *Macrococcus carouselicus* sp. nov.

Macrococcus carouselicus (car.ou.sel'i.cus. M.L. adj. *carouselicus* pertaining to a carousel or merry-go-round, which has carousel horses).

The description below is based on the characteristics of 18 strains (Table 1) isolated in 1993–1995 from the skin of horses and ponies. Colonies grow to 5±2 mm in diameter on P agar and 7±1 mm on TSA. They are slightly convex, entire, butyrous, glistening and opaque, and have a cream- to light-orange pigmentation. Growth is not detected in the anaerobic portion of a semi-solid thioglycollate medium. Growth is good at NaCl concentrations up to 7.5%. Optimum growth temperature is 35 °C. Culture growth usually does not cause haemolysis of horse and sheep blood. The cell wall does not contain teichoic acid. Lipoteichoic acid is present. Acetoin is not produced and, with the exception of strain DD 9346, nitrates are not reduced. All strains, except DD 9341, hydrolyse aesculin. DNase activity is positive. All strains, except DD 9607, are negative for β-glucosidase activity. Pyrrolidonylarylamidase activity is negative. All strains are resistant to novobiocin and susceptible to lysostaphin and oxacillin. All strains, except DD 9341, weakly produce acid aerobically from β-D-fructose. Acid is not produced from maltose, D-ribose and α-lactose. Characteristics of *M. carouselicus* that are general properties of the genus are listed above. The variable characteristics of this species are listed in Table 4. The major API STAPH-IDENT profile is 0000 (94%) and major ID32 STAPH profiles are 050100000 (28%), 070100000 (28%) and 010100000 (22%). The major ribotype pattern types are dd 09342 (28%) and dd 09349 (17%). The G+C content of the DNA is 41 mol %. This species has a preference for perissodactyls (e.g. horses and ponies) and is commonly found as large populations on the skin of these mammals.

Description of the type strain. Type strain is ATCC 51828^T (= DD 9348^T), isolated from the skin of an Irish thoroughbred horse. It has all the general properties of the species and genus described above and also the following characteristics. Cells are spherical, 1.4–2.5 µm in diameter, have a slightly irregular surface (SEM), and occur in pairs, tetrads, short chains and clusters. Colonies are 4–5 mm in diameter on P agar and 6–7 mm in diameter on TSA, circular, entire, slightly convex, and with a glistening surface and butyrous consistency. Cream-orange pigmentation. Marginally facultatively anaerobic; no visible growth in anaerobic portion of thioglycollate medium. No haemolysis of sheep, horse and bovine blood. Positive aesculin hydrolysis and latex agglutination. Negative nitrate reduction and β-glucosidase activity. Acid produced aerobically from sucrose. Acid produced weakly from D-trehalose, glycerol and β-D-fructose. No acid produced from D-mannitol, maltose, D-ribose and α-lactose. Type dd 09342 ribotype pattern. Cell wall peptidoglycan is L-Lys-Gly₃₋₄, L-Ser. No detect-

Table 5. Characteristics useful for differentiating *Macrococcus* species from the related genus *Staphylococcus* and from each other

The phylogenetic relationship of the genus *Macrococcus* to the genus *Staphylococcus* was estimated on the basis of 16S rDNA sequences. +, 90 % or more of the strains are positive; ±, 90 % or more of the strains are weakly positive; d, 11–89 % of the strains are positive; –, 90 % or more of the strains are negative. Parentheses indicate a delayed response.

Characteristic	<i>M. caseolyticus</i>	<i>M. equipercicus</i>	<i>M. bovicus</i>	<i>M. carouelicus</i>	<i>Staphylococcus</i>
For differentiating genera					
Cell diameter ≥ 2 mm	+	+	+	+	–
Cell wall teichoic acid	+	–	–	–	+
Oxidase (Microdase disk)	+	+	+	+	– > +†
DNA G + C > 40 mol %	–	+	+	+	–
<i>NotI</i> digestion of DNA‡	±	+	+	+	–
Genome size ≥ 2000 kb	–	–	–	–	+
Dull orange pigmentation	–	d	–	–	–
For differentiating species					
Haemolysis (horse blood)	(±)	–	(±)	–	
Acetoin production	+	–	–	–	
Aesculin hydrolysis	(d)	d	–	+	
Nitrate reduction	+	–	–	–	
DNase	d	–	d	+	
Urease	–	d	d	–	
Pyrrolidonylarylamidase	d	–	–	–	
Staph Latex agglutination	+	–	d	d	
Acid (aerobic) produced from:					
D-Mannitol	–	+	+	(d)	
Maltose	+	d	d	–	
Sucrose	d	–	(d)	d	
Glycerol	+	+	+	(d)	
D-Ribose	d	–	–	–	
α-Lactose	d	–	–	–	
β-D-Fructose	+	+	+	(±)	

* *Staphylococcus auricularis* and presumably *M. caseolyticus* have a poly(*N*-acetylglucosaminylphosphate) type of teichoic acid, where the *N*-acetylaminosugar residues form an integral part of the polymer chain (Endl *et al.*, 1983; Schleifer *et al.*, 1982).

† *Staphylococcus sciuri*, *Staphylococcus lentus* and *Staphylococcus vitulus* of the *Staphylococcus sciuri* species group are oxidase-positive (Schleifer, 1986; Webster *et al.*, 1994; Kloos *et al.*, 1997).

‡ *NotI* digestion of chromosomal DNA yields: +, 12–36 fragments; ±, 5–6 fragments; –, 0–4 fragments.

able teichoic acid. Lipoteichoic acid is present. G + C content of DNA is 41 mol %.

Distinguishing characteristics

The phenotypic and DNA characteristics that are useful for distinguishing the genera *Macrococcus* and *Staphylococcus*, and the *Macrococcus* species from each other are summarized in Table 5. Macrococci can be distinguished from staphylococci on the basis of their larger Gram-stained cell size and positive oxidase activity (with the exception of members of the oxidase-

positive *Staphylococcus sciuri* species group), and, with the availability of appropriate electrophoresis equipment, by ribotype pattern, larger number of chromosome fragments produced by digestion with *NotI* and smaller genome size. *M. caseolyticus* can be readily distinguished from the other macrococcal species on the basis of its ability to produce acetoin and reduce nitrates, and the combined characteristics of positive Staph Latex agglutination, partial haemolysis of horse blood, acid production from maltose, glycerol and β-D-fructose, negative urease activity and failure to produce acid from D-mannitol. Most strains of *M.*

caseolyticus demonstrate pyrrolidonylarylamidase activity and produce acid from D-ribose. *M. equiperdus* can be distinguished by the combined characteristics of acid production from D-mannitol, glycerol and β -D-fructose, negative Staph Latex agglutination and failure to produce acid from sucrose. Most *M. equiperdus* strains demonstrate strong urease activity. *M. bovicus* can be distinguished by the combined characteristics of acid production from D-mannitol, glycerol and β -D-fructose, partial haemolysis of horse blood and negative aesculin hydrolysis. Most strains of *M. bovicus* produce colonies on TSA that are smaller than those of the other species. *M. carougelicus* is unique in that it either fails to produce acid or produces acid only weakly from glycerol and produces acid only weakly from β -D-fructose, and it can be further distinguished by the combined characteristics of positive aesculin hydrolysis and DNase activity, negative urease activity and failure to produce acid from maltose.

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