Complex genomic and phenotypic characterization of the related species *Staphylococcus carnosus* and *Staphylococcus piscifermentans*

Roman Pantůček,¹ Ivo Sedláček,² Jiří Doškař¹ and Stanislav Rosypal¹

Author for correspondence: Stanislav Rosypal. Tel: +420 5 41129549. Fax: +420 5 41211214.
e-mail: rosypal@sci.muni.cz

¹ Department of Genetics and Molecular Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

² Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Tvrdeho 14, 602 00 Brno, Czech Republic

On the basis of numerical analysis of 100 phenotypic features, the strains of two species, *Staphylococcus carnosus* and *Staphylococcus piscifermentans*, were differentiated into two separate phenons corresponding with the macrorestriction patterns of their genomic DNA, as well as with the results of ribotyping and PCR amplification of enterobacterial repetitive intergenic consensus sequences. One of the *S. carnosus* strains, the F-2 strain, was shown to be marginal, exhibiting the lowest genomic and phenotypic similarity to the *S. carnosus* type strain DSM 20501^T. Two of the strains studied (strains *S. carnosus* SK 06 and *S. piscifermentans* SK 05) were phenotypically convergent, forming a separate phenon. They were phenotypically similar, even though the genomic DNA of one of them was homologous with that of the *S. carnosus* type strain, whereas that of the other was homologous with the genomic DNA of the *S. piscifermentans* type strain. In such cases, fingerprinting methods (particularly macrorestriction analysis and ribotyping) served as important correctives, as they allow phenotypically convergent strains to be distinguished on the basis of their genomic profiles. The results of this paper support the proposal for the new species *Staphylococcus condimenti* as well as the new subspecies *Staphylococcus carnosus* subsp. *utilis*.

Keywords: *Staphylococcus carnosus*, *Staphylococcus piscifermentans*, PFGE, ribotyping, ERIC-PCR

INTRODUCTION

Strains of *Staphylococcus carnosus* were previously incorrectly classified in the genus *Micrococcus* (Niinivaara & Pohja, 1956) and later, tentatively, as belonging to the species *Staphylococcus simulans* (Fischer & Schleifer, 1980). On the basis of DNA–DNA hybridization relationships, biochemical reactions and cell wall composition, they were defined as a new species, *S. carnosus* (Schleifer & Fischer, 1982). The species *Staphylococcus piscifermentans* was defined by Tanasupawat *et al.* (1992) in strains isolated from fermented fish and soy sauce mash in Thailand. Both the species are used alone or in combination with

the other bacterial strains (*Staphylococcus xylosum* and strains of the genus *Pediococcus* and *Lactobacillus*) as starter cultures in food technology (Nychas & Arkoudelos, 1990; Hammes *et al.*, 1995; Hartmann *et al.*, 1995).

As an apathogenic organism, *S. carnosus* is also widely used as a Gram-positive recipient strain for molecular cloning (Götz, 1990; Halfmann *et al.*, 1993; Ayora & Götz, 1994; Connolly *et al.*, 1994; Wieland *et al.*, 1994; Heilmann *et al.*, 1996; Neubauer & Götz, 1996), as well as for the production of heterologous proteins (Demleitner & Götz, 1994; Pschorr *et al.*, 1994; Samuelson *et al.*, 1995; Strauss & Götz, 1996; Robert *et al.*, 1996; Thumm & Götz, 1997; Liljeqvist *et al.*, 1997). Different methods for the transformation of *S. carnosus* (Götz *et al.*, 1983; Götz & Schumacher, 1987; Augustin & Götz, 1990) and suitable cloning vectors for recipient *S. carnosus* strains (Keller *et al.*, 1983; Kreutz & Götz, 1984; Augustin *et al.*, 1992;

Abbreviations: ERIC-PCR, PCR amplification of enterobacterial repetitive intergenic consensus sequences; S_j , Jaccard's similarity coefficient; S_{SM} , simple matching similarity coefficient; TAE, Tris/acetate electrophoresis buffer; UPGMA, unweighted pair group method with averages.

Rosenstein *et al.*, 1992; Wieland *et al.*, 1995; Brückner & Götz, 1996; Brückner, 1997) were developed. Recently, the physical and genetic map of the *S. carnosus* genome was constructed (Wagner *et al.*, 1998) and several genes were characterized and sequenced (Kohlbrecher *et al.*, 1992; Witke & Götz, 1993, 1995; Meens *et al.*, 1994; Christiansen & Hengstenberg, 1996; Fast *et al.*, 1996).

According to Tanasupawat *et al.* (1991, 1992), the strains classified as species *S. piscifermentans* and *S. carnosus* are similar to each other in their determinative phenotypic characteristics. Therefore, these characteristics have only relative value for separating these two species. Furthermore, there are some differences between the characteristics of *S. carnosus* given in *Bergey's Manual of Systematic Bacteriology* and the characteristics found by Tanasupawat *et al.* (1992). On the other hand, in spite of the similarity in phenotypic characteristics, the species *S. piscifermentans* and *S. carnosus* could be differentiated from each other on the basis of DNA relatedness. The DNA homology values between *S. piscifermentans* and *S. carnosus* type strains were estimated to be 43% and those between *S. piscifermentans* and the other staphylococcal species were less than 25% (Tanasupawat *et al.*, 1992).

It follows from these data that a strategy for differentiation of *S. carnosus* from *S. piscifermentans* has not been complete up to the present. Since *S. carnosus* is used industrially as a starter culture, the boundary between the two species should be defined on the basis of a description including more phenotypic features in correlation with their genomic relatedness. We concentrated therefore on the following points: (i) to show whether these two species are phenotypically distinguishable when using a greater number of phenotypic features subjected to numerical analysis according to Sneath & Sokal (1973); (ii) to complement DNA-DNA hybridization analysis of genetic relatedness made by Tanasupawat *et al.* (1992) with DNA fingerprinting techniques [PFGE of macrorestriction fragments of genomic DNA, ribotyping and ERIC-PCR (PCR amplification of enterobacterial repetitive intergenic consensus sequences); so far these techniques have not been used in distinguishing these two species]; (iii) to show to what measure phenotypic and genomic characteristics correspond with each other; and (iv) to correlate results obtained in this paper with the proposal (Probst *et al.*, 1998) for the new species *Staphylococcus condimenti* including the strains previously identified as *S. carnosus* and the new subspecies *Staphylococcus carnosus* subsp. *utilis*.

METHODS

Bacterial strains. The bacterial strains (Table 1) were obtained as follows: those labelled as CCM were from the Czech Collection of Microorganisms (Brno, Czech Republic); those designated SK, F-2, M3 and TM 300 were from Professor F. Götz (Universität Tübingen, Tübingen, Germany); and the type strain *S. carnosus* DSM 20501^T was obtained from the German Collection of Microorganisms

and Cell Cultures (Braunschweig, Germany). The strain Mediph 2 was obtained from Medipharma CZ.

Biotyping. The tested cultures were examined for the following characters by key tube or plate tests as previously described (Barrow & Feltham, 1993; Hébert *et al.*, 1988; Kloos & Bannerman, 1994, 1995; Kloos & Schleifer, 1975): colony morphology and pigment; catalase activity; tube coagulase activity; clumping factor (Murex Diagnostica); growth in 15% NaCl; anaerobic growth in a thioglycollate medium; growth at 15 and 45 °C; novobiocin (1.6 µg) and furazolidone (100 µg) resistance; hydrolysis of DNA, Tween 80, gelatin, starch, casein and tyrosine; antibacterial sensitivity testing (bioMérieux discs: tetracycline 30 µg, oxacillin 1 µg, gentamicin 10 µg, imipenem 10 µg, erythromycin 15 µg, chloramphenicol 30 µg, piperacillin 100 µg, penicillin G 10 U). Additional biochemical profile data were obtained by using the ID32 Staph system and API 50CH and API ZYM systems (bioMérieux). The ID32 Staph system is based on detection of the activities of 26 tests of staphylococcal identification. The API 50CH test is strip-based and allows the study of bacterial carbohydrate metabolism; results are indicated by changes in the colour of the pH indicator in wells. The API ZYM system is a semi-quantitative micromethod designed for the detection of 19 enzymic activities. The sensitivity to polyvalent bacteriophages of the Twort species was estimated as described previously (Pantůček *et al.*, 1998).

Genomic DNA preparation and PFGE. DNA isolation for PFGE was performed according to the methods of Pantůček *et al.* (1996). For restriction enzyme cleavage, *Sma*I (Boehringer Mannheim) and *Apa*I (Promega) were used. Cleavage was performed with 8 U enzyme (for 1 × 1 × 5 mm agarose blocks) overnight. PFGE was performed with the CHEF-MAPPER system (Bio-Rad) in 1.2% (w/v) agarose gels (Qualex Gold Agarose; Angewandte Gentechnologie Systeme) at 14 °C in a 1 × Tris/acetate electrophoresis buffer (TAE buffer, 0.04 M Tris/acetate, 0.001 M EDTA, pH 8.2). A constant voltage 6 V cm⁻¹ was applied with an increasing pulse time of 1–45 s over a period of 34 h. As size markers, concatemers of pBR328 (5 kb ladder; Bio-Rad) and concatemers of bacteriophage λcI857Sam7 (Bio-Rad) were used. The gels were stained in ethidium bromide (0.5 µg ml⁻¹ in 1 × TAE buffer), destained in water and photographed under UV illumination (302 nm).

DNA isolation for ribotyping and PCR. Whole-cell DNA was prepared from overnight cultures in a 20 ml brain-heart infusion broth (Oxoid) at 37 °C. Bacterial cells were harvested by centrifugation for 15 min at 8000 g at 4 °C, washed in 10 ml NaCl-buffer (50 mM Tris/HCl pH 8.0, 2.5 M NaCl, 50 mM EDTA), resuspended in 3 ml standard PBS buffer (0.145 M NaCl, 0.01 M Na₂HPO₄, 0.0017 M KH₂PO₄, pH 7.4) containing lysostaphin (5 µg ml⁻¹; Sigma), lysozyme (0.2 mg ml⁻¹) and RNase (10 µg ml⁻¹) and the mixture was then incubated for 30 min at 37 °C. Lysis was completed by adding 3 ml 1% (w/v) SDS solution and incubating for 10 min at 37 °C. The samples were then treated with proteinase K (Merck), added to a final concentration 200 µg ml⁻¹, and incubated at 55 °C for 60 min. Purification of DNA was performed by phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform extractions and ethanol precipitation according to Maniatis *et al.* (1982).

Ribotyping. *Eco*RI (Boehringer Mannheim) digested DNAs (10 µg) were resolved by horizontal slab gel electrophoresis performed in 1.0% (w/v) agarose gels in 1 × TAE buffer at

Table 1. *S. carnosus* and *S. piscifermentans* strains under study

Strain	Other designation	Reference
<i>S. carnosus</i> DSM 20501 ^T	Strain no. 361	Schleifer & Fischer (1982)
<i>S. carnosus</i> CCM 3885	Strain no. 51	Schleifer & Fischer (1982)
<i>S. carnosus</i> CCM 3886	Strain no. 91	Schleifer & Fischer (1982)
<i>S. carnosus</i> CCM 4579	Isolated from dry sausage	
<i>S. carnosus</i> Mediph 2	Starter culture	
<i>S. carnosus</i> TM 300	Strain no. 311	Schleifer & Fischer (1982)
<i>S. carnosus</i> M3	Derived from TM 300	
<i>S. carnosus</i> SK 06	<i>S. carnosus</i> subsp. <i>utilis</i> SK 06	Tanasupawat <i>et al.</i> (1991); Probst <i>et al.</i> (1998)
<i>S. carnosus</i> F-2	' <i>Tetracoccus soyae</i> ' F-2, <i>Staphylococcus condimentii</i> F-2 ^T	Tanasupawat <i>et al.</i> (1991); Probst <i>et al.</i> (1998)
<i>S. piscifermentans</i> CCM 4345 ^T	Strain SK 03	Tanasupawat <i>et al.</i> (1992)
<i>S. piscifermentans</i> CCM 4346	Strain SK 14	Tanasupawat <i>et al.</i> (1992)
<i>S. piscifermentans</i> CCM 4347	Strain SK 16	Tanasupawat <i>et al.</i> (1992)
<i>S. piscifermentans</i> SK 02		Tanasupawat <i>et al.</i> (1992)
<i>S. piscifermentans</i> SK 05		Tanasupawat <i>et al.</i> (1992)

0.8 V cm⁻¹ for 16 h at room temperature. DNA fragments were transferred to nylon membranes (Boehringer Mannheim) by a capillary Southern blot procedure using 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Labelling of DNA probes from 16S and 24S rRNA of *Escherichia coli* (Boehringer Mannheim) with digoxigenin-11-dUTP (Boehringer Mannheim), hybridization and immunological detection (DIG DNA Labelling and Detection kit; Boehringer Mannheim) were performed as described by Bangsberg *et al.* (1995). DIG-labelled molecular mass markers II and VII (Boehringer Mannheim) were used as size standards.

PCR amplification of bacterial DNA. The primers ERIC 1R and ERIC 2 described by Versalovic *et al.* (1991) were synthesized on the Expedite 8909 Nucleic Acid Synthesis system (PerSeptive Biosystems) and used without further purification. The reaction mixtures (50 µl) consisted of 10 mM Tris/HCl pH 9.0, 50 mM KCl, 2.5 mM MgCl₂, 0.1 % Triton X-100 and 200 µM each of dNTPs. To each reaction, 1 µl 50 µM primer stock, 1 U *Taq* DNA polymerase (Promega) and template DNA (50 ng) in a 1 µl volume were added. PCR was performed using 40 cycles of amplification consisting of denaturation (1 min, 94 °C), annealing (1.5 min, 35 °C) and DNA chain extension (2 min, 74 °C) in a DNA thermal cycler (model GENE E; Techne).

After amplification, 20 µl mixture was mixed with 5 µl loading buffer (10 %, v/v Ficoll 400, 10 mM Tris/HCl pH 7.5, 50 mM EDTA and 0.25 % bromophenol blue) and electrophoresed in a 2.0 % (w/v) agarose gel (Quallex Gold Agarose) for 4 h at 5 V cm⁻¹ in 1 × TAE buffer. Molecular mass marker VI (Boehringer Mannheim) was used as a size standard. The gels were stained in ethidium bromide (0.5 µg ml⁻¹) and photographed under UV illumination (302 nm).

Numerical analysis of phenotypic tests. The results of phenotypic tests were examined by numerical analysis according to Sneath & Sokal (1973) using SPSS 7.5 for Windows. Both the simple matching similarity coefficient (S_{SM}) and Jaccard's similarity coefficient (S_J) were calculated. The dendrograms constructed on the basis of these

coefficients by the UPGMA (unweighted pair group method with averages) algorithm exhibited stable clusters. The S_J coefficient, which ignores negative matches and is suitable for weakly reacting organisms (Logan, 1994), was chosen for final presentation.

Numerical analysis of band patterns. Photographs of gels were scanned with the S-12 Scanner (UMAX Data Systems) equipped with Adobe Photoshop 4.0. Digitized gel images were analysed using GELCOMP 4.1 software (Applied Maths BVBA). Intra-gel inconsistencies and variations in electrophoresis conditions were corrected by computer alignment of the size standards added at regular intervals in separate tracks.

Macrorestriction patterns and ribotype patterns of DNAs isolated from individual strains were compared quantitatively by using the formula proposed by Upholt (1977) and Nei & Li (1979):

$$F_{xy} = 2n_{xy}/(n_x + n_y),$$

in which F_{xy} is the proportion of restriction fragments that are common to the strains compared (identical with the Dice similarity coefficient, S_D); n_x and n_y are the total number of restriction fragments observed in strains x and y, respectively; and n_{xy} is the number of restriction fragments shared by both strains x and y.

The levels of similarity between different PCR band patterns were calculated by using the area sensitive coefficient, taking into account the correspondence of bands expressed as the S_J coefficient, as well as the differences of the relative areas under each corresponding band (which is an indication of the DNA concentration of the corresponding band).

The relevance of various clustering algorithms was assessed on the basis of co-phenetic correlations comparing linkage distances with an input similarity matrix. The UPGMA algorithm showed the best co-phenetic correlations (95–98 %) and was, therefore, selected for constructing the dendrograms from the PFGE and ERIC-PCR data. The ribotype patterns were ordered by similarity matrix using the

Euclidean model of multidimensional scaling. All statistical evaluations were made using statistical package SPSS 7.5 for Windows.

RESULTS

Biotyping

Nine *S. carnosus* and five *S. piscifermentans* strains were subjected to numerical analysis of 100 phenotypic features. All strains were positive for catalase, arginine dihydrolase, acid phosphatase, α -glucosidase, esterase and esterase lipase, they reduced nitrate to nitrite, produced acid from D-glucose and D-fructose and grew at 15 °C. None of them produced acetoin, coagulase, lecithinase (egg-yolk reaction), acid from L- and D-arabinose, cellobiose, D-raffinose, erythritol, L- and D-xylose, adonitol, methyl β -xyloside, sorbose, rhamnose, dulcitol, inositol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, melibiose, inulin, amidon, arbutin, β -gentiobiose, glycogen, xylitol, D-lyxose, D-tagatose, L- and D-fucose, L- and D-arabitol, gluconate, or 2- and 5-ketogluconate. No hydrolysis of tyrosine, casein, starch, gelatin or elastin was observed. β -Glucuronidase, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, trypsin, chymotrypsin, arginine arylamidase, valine arylamidase and cystine arylamidase were negative in all strains. All strains exhibited the

same antibiogram. They were resistant to tetracycline and oxacillin and were sensitive to gentamicin, imipenem, erythromycin, chloramphenicol, piperacillin, penicillin G, novobiocin and nitrofurantoin. Variable reactions of both species are listed in Table 2.

In the dendrogram constructed from the S_j values, the strains clustered into the following three separate phenons (Fig. 1): phenon 1 (S_j values of 70–100%), includes only the strains of *S. carnosus* clustered around their type strain DSM 20501^T; phenon 2 (S_j values of 85–100%), includes only the strains of *S. piscifermentans* clustered around their type strain CCM 4345^T; phenon 3 (S_j values of 78–100%), includes the strains *S. carnosus* SK 06 and *S. piscifermentans* SK 05 which did not cluster around any type strains mentioned above.

From data given in Table 2, it can be seen that phenon 1 (*S. carnosus*) and phenon 2 (*S. piscifermentans*) may be differentiated from each other based on the following phenotypic features: acid production from mannose, mannitol and sucrose, β -glucosidase, hydrolysis of aesculin, urease, hydrolysis of Tween 80, pyrrolidonyl arylamidase and sensitivity to phages of the Twort species. Strain *S. carnosus* F-2 has several biochemical features (urease, hydrolysis of Tween 80 and pyrrolidonyl arylamidase) that are similar to those of *S. piscifermentans*. Phenon 3 is significantly different

Table 2. Variable reactions of *S. carnosus* and *S. piscifermentans*

Test: 1, clumping factor; 2, anaerobic growth in thioglycollate medium; 3, growth at 45 °C; 4, growth in 15% NaCl; 5, sensitivity to polyvalent bacteriophages of the species Twort; 6, hydrolysis of DNA; 7, hydrolysis of Tween 80; 8, urease; 9, hydrolysis of aesculin; 10, acid from mannose; 11, acid from maltose; 12, acid from lactose; 13, acid from trehalose; 14, acid from mannitol; 15, acid from ribose; 16, β -galactosidase; 17, pyrrolidonyl arylamidase; 18, acid from sucrose; 19, acid from *N*-acetylglucosamine; 20, acid from turanose; 21, acid from glycerol; 22, acid from galactose; 23, β -glucosidase; 24, acid from melezitose; 25, acid from salicin; 26, acid from sorbitol; 27, alkaline phosphatase; 28, leucine arylamidase; 29, naphthol AS-BI phosphohydrolase; +, positive reaction; w, weak reaction; –, negative reaction.

Strain	Test																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
<i>S. carnosus</i>																													
DSM 20501 ^T	–	–	w	w	+	w	–	–	–	+	–	+	+	+	w	+	+	–	w	–	–	+	–	–	–	w	+	–	w
CCM 3885	–	–	+	+	+	w	–	–	–	+	–	+	+	+	w	+	+	–	w	–	–	+	–	–	–	w	+	w	w
CCM 3886	–	–	w	w	+	+	–	–	–	+	–	+	+	+	w	+	+	–	w	–	–	+	–	–	–	w	+	–	w
CCM 4579	–	–	–	+	+	+	–	–	–	+	–	+	+	+	w	+	+	–	w	–	–	+	–	–	–	w	+	–	w
Mediph 2	–	w	–	+	+	+	–	–	–	+	–	+	+	+	w	+	+	–	w	–	–	w	–	–	–	w	+	w	w
M3	–	–	–	+	+	w	–	–	–	+	–	+	–	+	w	+	+	–	w	–	–	w	–	–	–	w	+	–	w
TM300	–	–	–	+	+	w	–	–	–	+	w	+	+	+	–	+	+	–	w	–	–	w	–	–	–	w	+	–	w
SK 06	–	w	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
F-2	–	+	+	+	+	+	+	+	–	+	–	+	+	+	w	+	–	–	+	–	–	–	w	–	–	w	+	–	–
<i>S. piscifermentans</i>																													
CCM 4345 ^T	w	–	+	+	–	+	+	+	w	–	+	+	+	–	w	+	–	+	+	+	–	+	+	+	–	–	w	w	–
CCM 4346	w	+	+	w	–	+	+	+	–	+	+	+	+	–	w	+	–	+	+	+	–	+	+	+	–	–	w	w	–
CCM 4347	w	+	+	–	–	w	+	+	+	–	+	+	+	–	w	+	–	+	+	+	w	+	+	–	+	–	–	–	–
SK 02	–	+	+	w	–	+	+	+	+	–	–	+	+	–	+	–	–	w	+	–	–	+	+	+	+	–	+	w	–
SK 05	–	+	–	w	–	–	–	–	–	–	–	–	+	–	–	–	–	w	w	–	–	–	–	–	–	–	–	–	–

Fig. 1. Dendrogram based on the numerical analysis of 100 phenotypic traits. It shows the strains clustered into three phenons. The dendrogram was constructed by the UPGMA method with S_j coefficients.

from both phenons 1 and 2, being biochemically inactive in almost all features. Biochemical profiles from strains F-2, SK 05 and SK 06 exhibited low discrimination and they have not been precisely defined at the species level.

Macrorestriction analysis using PFGE

Genomic DNAs of all strains were cleaved by restriction endonucleases *Sma*I and *Apa*I. In *S. carnosus*, *Sma*I cleaves the DNA into 17–23 restriction fragments (3–910 kb) and in *S. piscifermentans*, the DNA is cleaved into 26–27 fragments (4–750 kb). Restriction endonuclease *Apa*I cleaves the DNA of *S. carnosus* into 12–17 fragments (5–600 kb) and that of *S. piscifermentans* into 19–24 fragments (16–440 kb). Seven *S. carnosus* strains maintain the same macrorestriction patterns when either *Sma*I or *Apa*I is used (Fig. 2a, b).

The similarity values (F_{xy} values) in macrorestriction patterns of different strains of *S. carnosus* and *S. piscifermentans* were used to group these strains into so-called species restriction groups. The species restriction group included the type strain and the strains resembling the type strain, in terms of the macrorestriction pattern of their genomes, within the range of similarity 25–100% (Snopková *et al.*, 1994). The similarity value of 25% was considered to be the lowest for defining the species restriction group, because the relationship between F_{xy} and the amount of DNA sequence divergence is curvilinear, so that small errors at low values of F_{xy} result in large errors in the estimation of DNA sequence divergence (Dowling *et al.*, 1990). For this reason, it was suggested (Kessler & Avise, 1985) that simple fragment comparison should not be used where less than 25% of the fragments are shared. The species restriction groups that were estimated by Snopková *et al.* (1994) are in good accordance with the results obtained by DNA–DNA hybridization studies of coagulase-negative

staphylococci published previously (Schleifer & Kroppenstedt, 1990; Kloos *et al.*, 1992).

The dendrogram based on numerical analysis of *Apa*I genomic macrorestriction patterns of *S. carnosus* and *S. piscifermentans* strains consists of two clusters. The clusters correspond to the species restriction groups *S. carnosus* and *S. piscifermentans* since each of them includes strains with macrorestriction patterns similar to that of their type strain. The level of similarity in the cluster of *S. carnosus* strains under study is 34%, whereas for *S. piscifermentans* strains, the level is 54% (Fig. 2a).

The dendrogram based on *Sma*I genomic macrorestriction patterns (Fig. 2b) gives practically the same results as that based on *Apa*I. Two clusters, i.e. *S. carnosus* and *S. piscifermentans*, with their appropriate type strains were obtained, each being separated from the other on the level of 32% similarity. In both the dendrograms based on the *Apa*I and *Sma*I macrorestriction analysis, strain *S. carnosus* F-2 occupied the marginal branch (at the level of 35% similarity).

On the whole, the *S. carnosus* and *S. piscifermentans* clusters based on the genomic macrorestriction patterns correspond to phenons 1 and 2. Such a correspondence does not exist for strains SK 05 and SK 06. Even if these strains are phenotypically similar (phenon 3), they differ significantly from one another in their genomic DNA, SK 06 and SK 05 having macrorestriction patterns similar to those of the type strains *S. carnosus* DSM 20501^T and *S. piscifermentans* CCM 4345^T, respectively.

Ribotyping

On the basis of *Eco*RI ribotype patterns (Fig. 2c), the strains *S. carnosus* and *S. piscifermentans* were divided into three clusters. The clusters were formed on the level of 65% similarity used by De Buyser *et al.* (1992) for separating different species of the genus *Staphy-*

Fig. 2. Cluster analysis of gel images, which were digitized by scanning procedures. Schematic representation of band patterns for each of the DNA fingerprinting techniques and size of DNA fragments expressed in kbp or bp respectively, are given. The dendrograms were constructed by the UPGMA method. (a) Dendrogram showing the degree of similarity of *Apal* macrorestriction patterns of the genomic DNAs. (b) Dendrogram showing the degree of similarity of *SmaI* macrorestriction patterns of the genomic DNAs. (c) Three-dimensional ordination plot showing the clustering of *EcoRI* ribotype patterns on the basis of their similarity coefficients F_{xy} . (d) Dendrogram showing the degree of similarity of PCR fingerprints as generated by amplification of genomic DNA by primers ERIC 1R and ERIC 2 derived from enterobacterial repetitive intergenic consensus sequences.

lococcus. The results are as follows: cluster 1, including the same strains of *S. carnosus* as shown in macrorestriction analysis (Fig. 2a, b) except for strain F-2 (strains DSM 20501^T, CCM 3885, CCM 4579, Mediph 2, TM 300 and M3 are identical on the basis of ribotyping and the similarity of strains belonging to this species was higher than 90 %); cluster 2, including the same strains of *S. piscifermentans* as shown in macrorestriction analysis (Fig. 2a, b) [the ribotype patterns of *S. piscifermentans* are more heterogeneous (72 % similarity) than those of *S. carnosus* and only two ribotypes are the same (CCM 4345^T and CCM 4346)]; and cluster 3, represented only by strain F-2 and separated from the other *S. carnosus* strains at the level of 52 % similarity. The strain has some *Eco*RI ribotype motifs in common with *S. piscifermentans* (3.2, 10 and 14 kb fragments).

ERIC-PCR typing

The highest discrimination ability and reproducibility of PCR was reached when primers ERIC 1R and ERIC 2 were both used. From the dendrogram (Fig. 2d), it follows that eight strains of *S. carnosus* formed one cluster on the level of 56 % similarity. On the other hand, strains of *S. piscifermentans* formed a heterogeneous cluster on the level of 24 % similarity. Strain F-2 formed a separate cluster on the level of 13 % similarity. A separate cluster on the level of 5 % similarity was also formed by strain SK 05.

DISCUSSION

DNA analysis was shown to be an important and useful tool in molecular taxonomy. In staphylococci, it started with differentiation on the basis of DNA base composition of the family *Micrococcaceae* in two genetically distant genera *Micrococcus* and *Staphylococcus* (Silvestri & Hill, 1965; Rosypal *et al.*, 1966). Nucleic acid hybridization studies have resulted in a marked revision of the genus *Staphylococcus* and in the description of new species (Schleifer & Kroppenstedt, 1990; Kloos *et al.*, 1992). In recent years, different DNA fingerprinting techniques, which provide indirect access to DNA sequence polymorphisms, have also been used in the study of the staphylococcal genome (Tenover *et al.*, 1994; Kloos & Bannerman, 1995). The importance of DNA fingerprinting by PFGE as a tool for the differentiation of coagulase-negative staphylococci was emphasized by Lina *et al.* (1992), Linhardt *et al.* (1992), Snopková *et al.* (1994) and George & Kloos (1994). Moschetti *et al.* (1997) used PFGE of *Sma*I macrorestriction fragments to analyse strains of *S. xylosus* isolated from fermented sausages. Ribotyping, a method based on rRNA gene restriction site polymorphism, was developed for differentiation of *Staphylococcus* species and subspecies (El Solh *et al.*, 1990; De Buyser *et al.*, 1989, 1992). It was used for distinguishing a newly described *Staphylococcus* species and subspecies from previously described taxa (Chesneau *et al.*, 1993; Webster *et al.*,

1994; Kloos *et al.*, 1997), for the routine identification of isolates (Chesneau *et al.*, 1992) and for detecting intraspecies differences (Hesselbarth & Schwarz, 1995; Grattard *et al.*, 1993; Meugnier *et al.*, 1993). Another approach for intraspecies strain fingerprinting is ERIC-PCR (Versalovic *et al.*, 1991; Lupski & Weinstock, 1992). This method was suitable for discriminating strains of a variety of *Eubacteria* including *Staphylococcus aureus* (Struelens *et al.*, 1993; van Belkum *et al.*, 1994, 1995; van Leeuwen *et al.*, 1996).

Typing systems based solely on phenotypic tests have limitations because phenotypic traits are inconsistently expressed (Popovic *et al.*, 1993) or some species can be distinguished by only a limited number of stable biochemical tests (Chesneau *et al.*, 1992). Additionally, in a few cases our results did not correspond with the species description or data from references (Schleifer & Fischer, 1982; Tanasupawat *et al.*, 1991; Holt *et al.*, 1994), e.g. acid from glycerol, mannose, lactose and trehalose and β -galactosidase, α -glucosidase and acetoin production were not uniform (Table 2).

In this paper, we have used the fingerprinting methods in conjunction with numerical analysis of 100 phenotypic traits of the species *S. carnosus* and *S. piscifermentans*. The results we obtained lead to the conclusion that strains *S. carnosus* and *S. piscifermentans* form two separate phenons (except for strains *S. carnosus* SK 06 and *S. piscifermentans* SK 05) corresponding with clusters formed on the basis of different DNA fingerprinting techniques. The two clusters, each involving strains that are similar both in their phenotypic traits and their genomic DNA, correspond with the definition of the species *S. carnosus* proposed by Schleifer & Fischer (1982) and *S. piscifermentans* proposed by Tanasupawat *et al.* (1992). The results of DNA fingerprinting methods reported in our paper are also in accordance with DNA hybridization of the same strains made by Tanasupawat *et al.* (1991, 1992).

Strains SK 06 and SK 05 are evidently phenotypically convergent, since genomic analysis used in our paper unambiguously showed that the genome of SK 06 is similar to that of the type strain *S. carnosus* DSM 20501^T and that of SK 05 is similar to that of the type strain *S. piscifermentans* CCM 4345^T. As reported by Tanasupawat *et al.* (1991), the genome homology estimated by hybridization of DNA of strain SK 06 with DNA of the type strain of *S. carnosus* was 100 %. On the other hand, the genome homology estimated by hybridization of the DNA of strain SK 05 with DNA of the type strain of *S. piscifermentans* was 80 %. Thus, the classification of these strains in a separate species based only on their mutual phenotypic similarity would be misleading. On the basis of their physiological properties as well as on the basis of reaction with specific 23S rRNA-directed probes, Probst *et al.* (1998) placed strain SK 06 in a new subspecies of *S. carnosus* (Schleifer & Fischer, 1982). They designated

the new subspecies *S. carnosus* subsp. *utilis* (Probst *et al.*, 1998). Our results support this classification.

The results obtained by the use of different DNA fingerprinting methods indicate collectively that the genome of strain F-2 is not similar to that of the type strain of *S. piscifermentans*. On the other hand, this strain exhibits genomic similarity to the type strain of *S. carnosus*, even if this similarity is significantly lower in comparison with that of the other strains of *S. carnosus*. Its separate position in the group of *S. carnosus* strains is supported by the following facts. (i) The similarity coefficient in phenotypic features of strain F-2 in relation to the type strain *S. carnosus* DSM 20501^T is as high as 70%. In spite of the fact that it has some phenotypic characteristics common to the other strains of *S. carnosus*, classification with this species made solely on the basis of determinative traits has not been successful. (ii) Tanasupawat *et al.* (1991) estimated the DNA homology of strain F-2 with the type strain of *S. carnosus* to be 69% and with that of *S. piscifermentans* 48%. On the other hand, Probst *et al.* (1998) estimated that the DNA homology of strain F-2 is 58% with the *S. carnosus* type strain and 51% with the *S. piscifermentans* type strain. According to the phylogenetic definition of a species proposed by Wayne *et al.* (1987), the species generally should include strains with approximately 70% or greater DNA–DNA relatedness. (iii) The similarity of the F-2 genome in relation to the genome of the type strain *S. carnosus* DSM 20501^T, based on *Sma*I and *Apa*I macrorestriction patterns, is 32%, i.e. near the lowest value (25%) of similarity in the macrorestriction patterns recommended by Snopková *et al.* (1994) and Pantůček *et al.* (1996) for the species restriction group. (iv) The similarity coefficient in terms of the *Eco*RI ribotype of strain F-2 in relation to the type strain DSM 20501^T ($F_{xy} = 53.8\%$) is lower than the lowest intra-taxon value (65%) proposed by De Buyser *et al.* (1992) in their identification scheme. (v) Additionally, the results obtained by ERIC-PCR indicate a very low similarity between F-2 and all strains of *S. carnosus*.

From our results and also from those reported by Probst *et al.* (1998), it follows that strain F-2 is phenotypically and genetically the most distant from the type strain *S. carnosus* DSM 20501^T of all the *S. carnosus* strains under study. This is in accordance with the proposal reported by Probst *et al.* (1998) that this strain should be classified as a new species, *S. condimentii*.

On the whole, it can be said that the strains under study, with the exception of strain F-2, grouped together with their type strains into clusters, the composition of which was shown to be stable even if different DNA fingerprinting techniques were used. It can be expected that the restriction map constructed by Wagner *et al.* (1998) will also completely hold for the strains *S. carnosus* DSM 20501^T, CCM 3885, CCM 3886, CCM 4579, Mediph 2 and M3, which all have 100% similarity to *S. carnosus* TM 300.

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