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Development of specific PCR primers for a rapid and accurate identification of *Staphylococcus xylosus*, a species used in food fermentation

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Abstract

Twenty-seven *Staphylococcus* strains isolated from food and food environments were assigned to *Staphylococcus xylosus* by API-Staph system. But only seven isolates had similar patterns to this species when compared to the pulse-field gel electrophoresis patterns of 12 *S. xylosus* strains. To perform a rapid identification of the *S. xylosus* species, a random amplified polymorphic DNA product of 539-bp shared by all of the *S. xylosus* strains was used to design a pair of primers. These primers were species-specific for *S. xylosus* when tested by PCR on 21 staphylococci species. This specific PCR assay confirms the identification of the seven isolates identified by PFGE to *S. xylosus*. In conclusion, we developed specific PCR primers for a rapid and accurate identification of the *S. xylosus* species.

Keywords: PCR primers; Pulse-field gel electrophoresis; Random amplified polymorphic DNA; *Staphylococcus xylosus*

1. Introduction

Among the Coagulase Negative Staphylococci (CNS), *Staphylococcus xylosus* is a species isolated in many food products and is also used as starter for the manufacture of fermented sausages (Lücke and Hechelmann, 1987). It ensures colour formation during sausage ripening and contributes to aroma development (Berdagué et al., 1993; Talon et al., 1999). *S. xylosus* is one of the prevalent staphylococci species found in naturally fermented products and environment of traditional workshops manufacturing dry sausage without using starters (Montel et al., 1992; Rebecchi et al., 1998).

Phenotypic identification of *S. xylosus* strains isolated from food environments is problematic because of the limited number of stable discriminating characteristics to distinguish one staphylococcal species from another (Takahashi et al., 1997; Bascomb and Manafi, 1998; Rossi et al., 2001). Furthermore, there is a high heterogeneity within the *S. xylosus* species (Garcia-Varona et al., 2000; Villard et al., 2000). For these reasons, several typing methods based on molecular techniques have been used to improve the species determination of the *S. xylosus* strains: ribotyping analysis (De Buyser et al., 1989; Villard et al., 2000), PCR amplification of the 16S–23S spacer region (Villard et al., 2000; Rossi et al., 2001), pulse-field gel electro-

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phoresis (PFGE) (Snopkova et al., 1994; Di Maria et al., 2002) and random amplified polymorphic DNA (RAPD) (Rossi et al., 2001; Di Maria et al., 2002). All of these methods generated characteristic patterns of *S. xylosus* strains and can distinguish *S. xylosus* among different staphylococcal species. But none is suitable for a rapid and easy identification of this species among CNS isolated from food products. In this study, we present the development of PCR primers species-specific of *S. xylosus*. A RAPD protocol was used to find a common fingerprinting product of 539-bp shared by a panel of strains of *S. xylosus* but not encountered in other CNS. This DNA fragment was sequenced and used to design a pair of PCR primers suitable for the specific identification of the *S. xylosus* species.

2. Materials and methods

2.1. Bacterial strains

The 27 *Staphylococcus* strains studied were isolated from different food environments (Table 1). All of these strains were identified as *S. xylosus* by the API-Staph system (bioMérieux, Lyon, France). The *S. xylosus* type strain DSM 20266 (DSM: Deutsche Sammlung von Mikroorganismen) and 11 strains well identified to this species were used as references (Table 1).

Twenty-one *Staphylococcus* species were used to check the specificity of the *S. xylosus* PCR primers: *S. arlettae* CIP 103501 (CIP: Collection Institut Pasteur), *S. aureus* ATCC 25923 (ATCC: American Type Culture Collection), *S. aureus* CIP 65.8 T, *S. auricularis* DSM 20609, *S. capitis* CIP 104192 T, *S. caprae* CIP 104000 T, *S. carnosus* DSM 20501, *S. cohnii* DSM 20260, *S. epidermidis* DSM 20042, *S. equorum* DSM 20674, *S. haemolyticus* CIP 81.56T, *S. hominis* CIP 81.57, *S. intermedius* CIP 81.60 T, *S. kloosii* DSM 20676, *S. lentus* CIP 81.63T, *S. pasteuri* CIP 103540 T, *S. piscifermentans* CIP 103958 T, *S. saprophyticus* DSM 76125 T, *S. sciuri* CIP 81.62 T, *S. simulans* DSM 20322, *S. vitulinus* CIP 104850 T and *S. warneri* CIT 863 (CIT: Collection INRA, Theix).

The strains were grown at 37 °C on Brain Heart Infusion (BHI) agar or in BHI broth (Difco, Detroit, USA).

2.2. PFGE analysis

A suspension of staphylococcal culture at optical density of 1 at 600 nm was centrifuged and resuspended in 0.5 volume of TEE buffer (Tris 10 mM pH 9, EDTA 100 mM, EGTA 10 mM). The suspension of bacteria was mixed in a ratio of 1:1 with 2.0% low-melting-point agarose (GIBCO, Cergy Pontoise, France). Agarose plugs were incubated with 40 μg ml^{-1} of lysostaphin (Sigma, St. Quentin, France), 0.05% sarkosyl and 5 mg ml^{-1} of lysozyme (Eurobio, les Ullis, France) at 37 °C for 2 h. Then, they were lysed overnight in TEE buffer containing 1% of sodium dodecyl sulfate and 1 mg ml^{-1} of proteinase K (Eurobio) at 55 °C. Plugs were washed three times for 1 h each, once in TE buffer (Tris 10 mM pH 8, EDTA 1 mM) containing 20 mM of phenylmethylsulfonyl fluoride and twice in TE buffer. They were stored at 4 °C. DNA within half a plug was digested by 25 U of *Apa*I for 16 h at 25 °C (Promega, Lyon Charbonnières, France) or *Sma*I (Roche, Meylan, France) for 16 h at 25 °C. Pulse field gel electrophoresis was performed in 1% agarose using a CHEF-DRIII apparatus (Bio-Rad, Ivry, France) in 0.5 X Tris–borate–EDTA buffer pH 8.3 (TBE) at 6 V cm^{-1} at 14 °C. Pulse times increased from 10 to 30 s over 23 h. Lambda ladder (Bio-Rad) was used as molecular weight marker. Gels were stained with ethidium bromide and digitalized with the Gel Doc 2000 apparatus (Bio-Rad).

2.3. Preparation of lysates

Staphylococcus strains were grown 5 h at 37 °C in BHI broth under 200 rpm stirring. Fifty microliters of culture (optical density of 0.5 at 600 nm) were lysed in 200 μl of TES buffer (Tris 50 mM pH 8.0, EDTA 1 mM, Sucrose 25%) for 10 min at 95 °C. The lysates were stored frozen at − 20 °C.

2.4. RAPD amplification

The 10-mer primer 5 (5′AAC GCG CAA C 3′) was used (Wieser and Busse, 2000). The RAPD was carried out from 10 μl of staphylococcal lysate. DNA was amplified in 15 μl volume mix containing 1.3 μM primer, 200 μM of each deoxynucleotide triphosphate, 3 mM $MgCl_2$ and 1.25 unit of Taq DNA polymerase in

Table 1
Phenotypic and genotypic identifications of the *S. xylosus* strains and *Staphylococcus* strains isolated from food environments

Strain	Origin	PFGE type		RAPD type	Amplification by PCR
		*Apa*I	*Sma*I		
S. xylosus DSM 20266	Type strain	A	*I*	a	+
S. xylosus C2a[a]	University of Tübingen	A2	*Ia*	a3	+
S. xylosus CIT M203	Starter culture	A1	*I*	a	+
S. xylosus CIT M505	Starter culture	ND[b]	*Ib*	a2	+
S. xylosus CIT S01-003	Starter culture	A3	*Ic*	a3	+
S. xylosus CIT S01-004	Starter culture	A4	*Id*	a1	+
S. xylosus CIT S01-005	Starter culture	A4	nd[c]	a1	+
S. xylosus CIT S01-006	Starter culture	A3	nd	a3	+
S. xylosus CIT S01-007	Starter culture	A3	nd	a3	+
S. xylosus CIT S01-008	Starter culture	A4	nd	a3	+
S. xylosus CIT 839	Starter culture	ND	*Ib*	a3	+
S. xylosus CIT 840	Starter culture	A5	*Ig*	a3	+
CIT S01-009	Dairy product	A6	*Ie*	a4	+
CIT S01-010	Dairy product	C	*II*	c	–
CIT S01-011	Dairy product	D	nd	d	–
CIT S01-012	Dairy product	E	nd	e	–
CIT S01-013	Dairy product	F	*III*	f	–
CIT 837	Dry sausage	B	*IV*	b	–
CIT S02-001	Dry sausage	A5	*If*	a3	+
CIT S02-002	Dry sausage	A5	nd	a3	+
CIT S02-003	Dry sausage	A5	nd	a3	+
CIT S02-004	Dry sausage	A5	*If*	a3	+
CIT S02-005	Dry sausage	A7	*Ih*	a3	+
CIT S02-006	Dry sausage	A5	*If*	a3	+
CIT S00-027	Dry sausage	L	*VII*	g	–
CIT S00-032	Dry sausage	I	*IX*	h	–
CIT S00-033	Dry sausage	I	*IX*	h	–
CIT S00-034	Dry sausage	J	*VI*	g	–
CIT S00-036	Dry sausage	J	*VI*	g	–
CIT S00-004	Environment[d]	G	*V*	g	–
CIT S00-005	Environment	H	nd	g	–
CIT S00-009	Environment	I	*IX*	h	–
CIT S00-010	Environment	J	*VI*	g	–
CIT S00-012	Environment	I	*IX*	h	–
CIT S00-014	Environment	I	*IX*	h	–
CIT S00-016	Environment	K	nd	i	–
CIT S00-018	Environment	H	nd	j	–
CIT S00-020	Environment	I	*IX*	h	–
CIT S00-041	Environment	M	nd	g	–

[a] Strain derivative of *S. xylosus* DSM20267, cured of its endogenous plasmid pSX267 (Götz et al., 1983).
[b] ND: non-determinable.
[c] nd: non-done.
[d] *Staphylococcus* strains isolated from the environment of a workshop manufacturing naturally fermented dry sausage.

1 × buffer according to the manufacturer's instructions (Promega). The samples were subjected to thermal cycling program: 5 min at 94 °C and then 40 cycles of 30 s at 94 °C for the denaturation step, 30 s at 35 °C for the annealing step, and 1 min 30 s at 72 °C for the extension step with a GenAmp® PCR system 9700 PE thermal cycler (Perkin-Elmer, Courtaboeuf, France). A final extension step of 7 min at 72 °C was performed. RAPD fragment fingerprints were

analysed by electrophoresis in 1.5% agarose gels in 1 × TBE buffer pH 8.3. The gels were visualized as described above. The sizes of the RAPD products were estimated by comparison with a 100-bp-molecular-size DNA ladder (Promega). RAPD assays were performed at least three times each to check reproducibility. Each reaction included a control tube without template DNA.

2.5. Synthesis of the specific primers

A 539-bp RAPD product amplified from *S. xylosus* CIT M203 DNA lysate was extracted and purified from the gel by using the QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). The purified fragment was then cloned into the PGEM-T easy vector system (Promega). Recombinant plasmids were isolated from transformed *Escherichia coli* TG1 strain by using the Qiagen plasmid mini kit (Qiagen). The presence of the 539-bp insert was confirmed by digestion of the plasmids with *Eco*RI (Roche). The 539-bp insert for the selected recombinant plasmid was labelled with the Dig-High Prime system (Roche) and used as a probe to hybridize a Southern blot of RAPD patterns. The hybridized probe was detected by the Dig luminescent detection kit (Roche). Both strands of the 539-bp DNA insert were sequenced with the 'BigDye terminator cycle sequencing ready reaction' kit with an automatic DNA sequencer 'ABI PRISM 310' (Perkin-Elmer). The 539-bp sequence was used to design a pair of primers specific of the *S. xylosus* species. These primers were designed by MWG-Biotech (Ebersberg, Germany).

2.6. Conventional PCR amplification

Amplifications were performed from 5 μl of staphylococcal lysates. The 20 μl-PCR mixture contained 0.2 μM (each) of the two *S. xylosus*-specific primers XYL F 5′-AAC GCG CAA CGT GAT AAA ATT AAT G-3′ (annealing positions 1–25) and XYL R 5′-AAC GCG CAA CAG CAA TTA CG-3′ (520–539), 200 μM of each deoxyribonucleoside triphosphate (Eurobio), 1.5 mM $MgCl_2$ and 1.25 U of *Taq* DNA polymerase in 1 × buffer according to the manufacturer's instructions (Promega). PCR was performed under the following conditions: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 30 s at 57 °C, 45 s at 72 °C and a final hold of 7 min at 72 °C. Five microliters of the PCR reaction mixture was analysed by electrophoresis through a 1.5% agarose gel as described above.

2.7. Nucleotide sequence accession number

The nucleotide sequence of the *S. xylosus*-specific DNA fragment is available from GenBank as accession no. AY157732.

3. Results

3.1. PFGE analysis of the S. xylosus strains and food isolates

All of the *S. xylosus* strains and the *Staphylococcus* strains isolated from food environments and identified by the API-Staph system as *S. xylosus* were analysed by PFGE. Among the 39 strains typed by PFGE with *Apa*I, 13 different patterns (A–M) were obtained (Table 1, Fig. 1). The *Apa*I PFGE profile of the *S. xylosus* type strain (DSM 20266) was designated A and the profiles of the strains that showed similar PFGE patterns from the one of the type strain were designated A1–A7 (Table 1). Nine of the eleven *S. xylosus* strains used as references gave a similar PFGE profile with the type strain pattern (A1–A5), the two other strains being nontypable by *Apa*I PFGE. Amongst the *Staphylococcus* strains isolated from food environments, only 7 on 27 strains showed identical or closely similar *Apa*I PFGE patterns with those of the *S. xylosus* strains (A5–A7). If these results are interpreted on the basis of guidelines issued previously (Tenover et al., 1995), these seven strains belong to the *S. xylosus* species. The other strains showed different PFGE patterns with those of the *S. xylosus* strains and were designated B–M (Table 1). It appeared that these 20 isolates identified by the API-Staph system as *S. xylosus* could not belong to the *S. xylosus* species. The *Apa*I PFGE analysis revealed a high diversity within the *S. xylosus* strains and the food isolates.

Among the 27 strains typed by *Sma*I PFGE, the *S. xylosus* reference strains CIT M505 and CIT 839, nontypable by *Apa*I analysis, were clearly similar to the *S. xylosus* type strain (Table 1). Concerning the isolates tested, five strains closely similar to *S. xylosus* by *Apa*I PFGE were also similar to the *S. xylosus* type

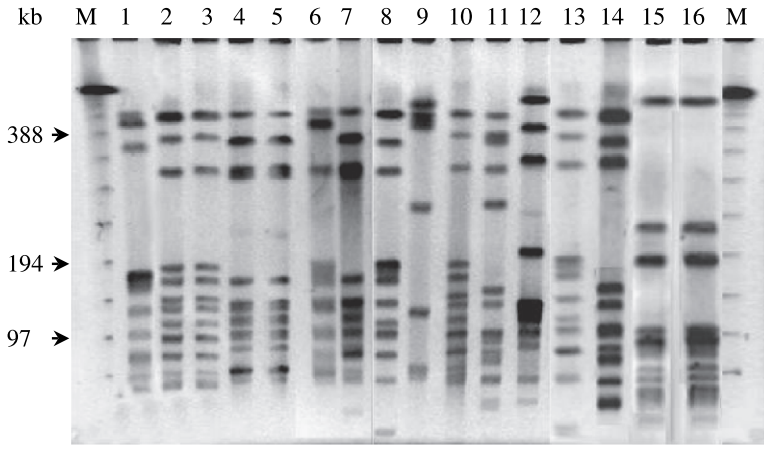

Fig. 1. PFGE data obtained for *Staphylococcus* strains with *Apa*I. Lanes M: DNA size Lambda standard (Bio-Rad); lane 1: *S. xylosus* DSM 20266; lane 2: *S. xylosus* CIT S01-006; lane 3: *S. xylosus* CIT S01-007; lane 4: *S. xylosus* CIT S01-008; lane 5: *S. xylosus* CIT S01-005; lane 6: *S. xylosus* C2a; lane 7: *S. xylosus* CIT S01-004; lane 8: *S. xylosus* CIT 840; lane 9: CIT 837; lane 10: *S. xylosus* CIT S01-003; lane 11: CIT S01-009; lane 12: CIT S01-010; lane 13: CIT S02-004; lane 14: CIT S02-005; lane 15: CIT S00-009; lane 16: CIT S00-014.

strain by *Sma*I PFGE analysis. The 14 remain isolates were not similar to *S. xylosus* by *Sma*I analysis, confirming the results obtained with *Apa*I analysis.

3.2. Isolation of a S. xylosus specific DNA fragment by RAPD

The primer 5 used for the RAPD analysis was selected among ten different RAPD primers for its ability to generate distinct and reproducible RAPD profiles among the different strains of *Staphylococcus* studied (data not shown). Control assays in which no template DNA was present yielded no detectable amplified product. The RAPD assays underlined the diversity of fingerprints among the 19 *S. xylosus* strains (type and reference strains, and isolates confirmed as *S. xylosus* by PFGE analysis) (Table 1). These 19 strains exhibited five related profiles of two to six fragments of variable intensity ranging from about 540–1400 bp (Fig. 2). The 20 other isolates exhibited different and various patterns (Table 1, Fig. 2). Thus, RAPD assays and PFGE analysis yielded

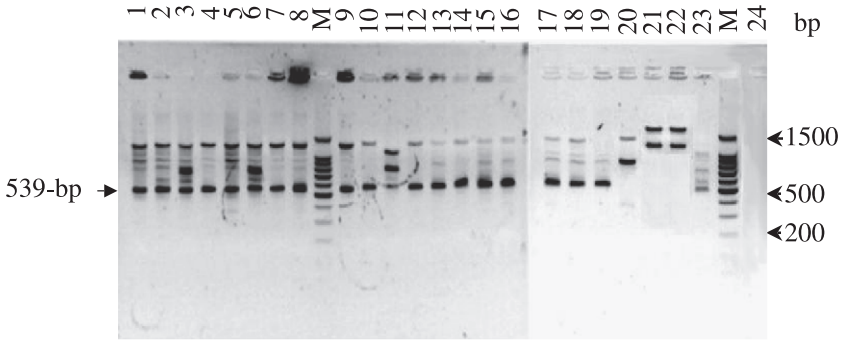

Fig. 2. RAPD analysis of *Staphylococcus* strains. Lanes M: 100-bp-molecular-size DNA ladder (Promega); lane 1: *S. xylosus* CIT S01-004; lane 2: *S. xylosus* CIT M505; lane 3: *S. xylosus* CIT M203; lane 4: *S. xylosus* C2a; lane 5: *S. xylosus* CIT S01-005; lane 6: *S. xylosus* DSM 20266; lane 7: *S. xylosus* CIT S01-006; lane 8: *S. xylosus* CIT S01-007; lane 9: *S. xylosus* CIT S01-003; lane 10: *S. xylosus* CIT S01-008; lane 11: CIT 837; lane 12: *S. xylosus* CIT 839; lane 13: *S. xylosus* CIT 840; lane 14: CIT S02-001; lane 15: CIT S02-002; lane 16: CIT S02-003; lane 17: CIT S02-004; lane 18: CIT S02-005; lane 19: CIT S01-009; lane 20: CIT S00-018; lane 21: CIT S00-032; lane 22: CIT S00-033; lane 23: CIT S01-013; lane 24: negative control. The *S. xylosus* common 539-bp fragment is highlighted.

concordant results. However, PFGE was more discriminant than RAPD.

All the RAPD patterns of the *S. xylosus* strains (type and reference strains and isolates confirmed as *S. xylosus* by PFGE analysis) showed a common fragment of 539 bp (Fig. 2). This fragment was absent from the RAPD patterns of the other staphylococcal species tested (data not shown). This potential *S. xylosus*-specific DNA fragment was amplified from *S. xylosus* CIT M203, gel purified and cloned into pGEM-T. This fragment hybridized with the 539-bp RAPD product of all of the *S. xylosus* strains (data not shown). Subsequently, the sequence of both strands of this fragment was determined. The analysis of this sequence revealed that a part of the 539-bp fragment, corresponding to the position 293–535, presented 61% similarity, at the amino acid level, to a hypothetical regulatory protein of *S. aureus*, PfoR. One set of PCR primers, XYL F and XYL R, derived from this sequence was designed.

3.3. PCR assays

Specificity tests performed with the 21 *Staphylococcus* species showed that the selected PCR primers amplified only DNA from the *S. xylosus* strains (type and reference strains and isolates confirmed as *S. xylosus* by PFGE analysis) (Table 1, Fig. 3). The negative PCR results were not attributable to PCR inhibitors as all cell lysates were amplified by the universal bacterial primers for the 16S rDNA gene (Greisen et al., 1994) (data not shown).

4. Discussion

It is important to accurately identify *S. xylosus* because this species is involved in the quality of fermented products. Of the 27 CNS isolated from food environments and identified as *S. xylosus* by Api-Staph system, only seven were identified to this species by PFGE analysis and specific PCR. The remaining 20 strains were misidentified. PFGE analysis and specific PCR confirmed that they did not belong to the *S. xylosus* species. This result is in agreement with Goh et al. (1996) that observed that 7 of 20 clinical isolates of CNS were misidentified by commercially systems based on phenotypic characterization. Coppola et al. (2000) obtained 82% of staphylococcal food isolates unidentified by the API-Staph system. Finally, Rossi et al. (2001) encountered problems with phenotypic identification of *S. xylosus* strains isolated from naturally fermented sausage. In contrast, Garcia-Varona et al. (2000) and Di maria et al. (2002) obtained concordance between phenotypic and genotypic identifications. Although phenotypic methods are easy to use, they are often insufficient for an accurate identification of the CNS species such as *S. xylosus*.

The introduction of molecular methods has improved the accuracy of the identification at the species

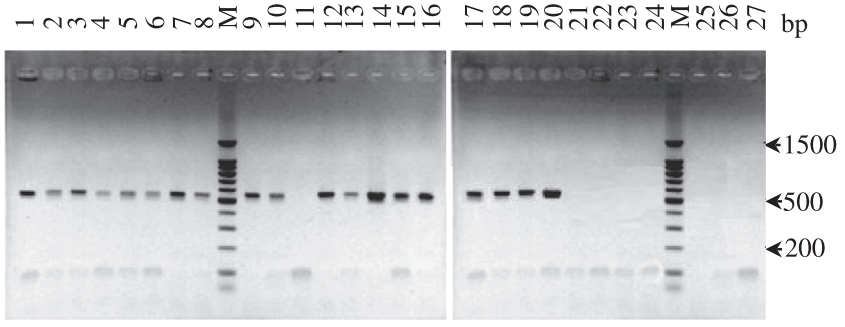

Fig. 3. PCR amplification with the *S. xylosus*-specific primer pair XYL F and XYL R. Lanes M: 100-bp-molecular-size DNA ladder (Promega); lane 1: *S. xylosus* DSM 20266; lane 2: *S. xylosus* C2a; lane 3: *S. xylosus* CIT M203; lane 4: *S. xylosus* CIT M505; lane 5: *S. xylosus* CIT S01-003; lane 6: *S. xylosus* CIT S01-004; lane 7: *S. xylosus* CIT S01-005; lane 8: *S. xylosus* CIT S01-006; lane 9: *S. xylosus* CIT S01-007; lane 10: *S. xylosus* CIT S01-008; lane 11: CIT 837; lane 12: *S. xylosus* CIT 839; lane 13: *S. xylosus* CIT 840; lane 14: CIT S02-001; lane 15: CIT S02-002; lane 16: CIT S02-003; lane 17: CIT S02-004; lane 18: CIT S02-005; lane 19: CIT S02-006; lane 20: CIT S01-009; lane 21: CIT S01-010; lane 22: CIT S01-011; lane 23: CIT S00-033; lane 24: *S. carnosus* DSM 20501; lane 25: *S. saprophyticus* DSM 76125 T; lane 26: *S. sciuri* CIP 81.62 T; lane 27: *S. hominis* CIP 81.57.

level. For instance, PFGE analysis allowed the clustering within CNS species (Snopkova et al., 1994). It is widely used for typing and subtyping the CNS strains such as *S. epidermidis*, *S. haemolyticus*, *S. schleiferi* and for the *S. aureus* strains (Linhardt et al., 1992; Kumari et al., 1997; Kluytmans et al., 1998; Mulvey et al., 2001). We have performed PFGE analysis in order to check the phenotypic identification of 27 staphylococci isolated from food environments. On top of the fact that this technique revealed the lack of accuracy of the API-Staph system, it showed a wide diversity among the CNS strains and in particular among the *S. xylosus* species, as shown by Snopkova et al. (1994) and Di Maria et al. (2002). But as PFGE is time-consuming, we focused on genotypic methods which could allow the development of rapid tools for specific identification of the *S. xylosus* strains. On this aim, RAPD seemed to be suitable. This method emphasized the wide heterogeneity among the *S. xylosus* strains, as previously described with the same approach (Rossi et al., 2001; Di Maria et al., 2002). Because of the wide heterogeneity among the *S. xylosus* strains and the difficulty to interpret RAPD patterns, RAPD cannot be used for a rapid identification of this species. But RAPD allowed the identification of a 539-bp fragment shared by all of the *S. xylosus* strains but by none of the other CNS type species studied. Thus, specific PCR primers were designed from the 539-bp RAPD product and can be used to identify all of the *S. xylosus* strains. The results obtained with PFGE and RAPD analyses and specific PCR were well correlated.

Until now no specific primers have been developed for the identification of *S. xylosus* strains isolated from food and food environments. Some species-specific primers are described for the direct identification of *Staphylococcus* species of clinical interest such as *S. epidermidis*, *S. aureus* and *S. saprophyticus* (Martineau et al., 1996, 1998, 2000). Forsman et al. (1997) developed PCR using primers designed in the 16S–23S rDNA spacer region for the identification of some staphylococcal species involved in bovine mastitis. Martineau et al. (2001) developed a *tuf*-based PCR assay for the identification of *S. epidermidis*, *S. aureus*, *S. haemolyticus*, *S. hominis* and *S. saprophyticus* but it needs to be coupled with hybridization with species-specific probes. Poyart et al. (2001) described PCR primers designed from the *sod*A gene in order to characterize 40 CNS species, including the *S. xylosus* species. But this discriminative approach needs sequencing of all the *sod*A amplified products to allow the identification. Consequently, it is not suitable for a rapid identification of a wide collection of strains isolated from food environments.

In conclusion, we developed a pair of specific primers for the *S. xylosus* species which allows a rapid and accurate identification of this species within *Staphylococcus* isolated from food environments.

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