System. Appl. Microbiol. 22, 225–228 (1999)

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Identification of *Staphylococcus carnosus* and *Staphylococcus warneri* Isolated from Meat by Fluorescent *in situ* Hybridization with 16S rRNA-Targeted Oligonucleotide Probes

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Received December 2, 1998

Summary

16S rRNA targeted oligonucleotide probes were designed by sequence analysis of an rRNA database to discriminate *S. carnosus, S. warneri*, and *S. saprophyticus* species. After establishing hybridization conditions by RNA dot blot hybridization with reference species, our probes were shown to be specific. By *in situ* hybridization only S-S-S.carno-0440-a-A-23 and S-S-S.war-0180-a-A-23 can specifically detect *S. carnosus* and *S. warneri*, respectively. The detection of old cells of *S. carnosus* 833 was more limited by the permeabilisation than by the low rRNA content. One day old cells could be permeabilized with lysostaphin, whereas young cells were permeabilized with lysozyme.

Key words: *Staphylococcus* – 16S rRNA – fluorescence – oligonucleotide probe – *in situ* hybridization

Introduction

Dry sausage manufacturing involves lactic acid bacteria as well as staphylococci, which play an important role in the development of the sensorial qualities of the fermented products.

Staphylococcus xylosus and *Staphylococcus carnosus*, catalase positive cocci, are selected as starter culture for dry sausage fermentation. Other species, such as: *Staphylococcus simulans* (FISCHER and SCHLEIFER, 1980), *Staphylococcus saprophyticus, Staphylococcus warneri* (MONTEL et al., 1992), *Staphylococcus epidermidis* and *Staphylococcus aureus* (NYCHAS and ARKOUDELOS, 1990), can also be part of the natural flora of fermented meat products, but they are less desirable.

The meat industry needs to check rapidly the presence of inoculated starter cultures. *In situ* hybridization with oligonucleotide probes targeting rRNA seems to be a powerful tool for rapid and specific identification of individual microbial strains in pure culture, on colonies and in their environment (AMANN et al., 1995).

The aim of our study was to design 16S rRNA targeted oligonucleotide probes for the whole cell hybridization of *S. carnosus, S. warneri* and *S. saprophyticus* isolated from fermented meat products.

Source and strain numbers of staphylococci are listed in Table 2. DNA was extracted by the method of BRENNER et al. (1982). 16S rDNA was amplified by PCR with different primers and sequenced as described GODON et al. (1997). Partial sequences of 16S rDNA of *Staphylococcus caprae* (AF041362), *Staphylococcus equorum* (AF041363), *Staphylococcus kloosii* (AF041360), *Staphylococcus piscifermentans* (AF041359), *Staphylococcus sciuri* (AF041358) and *S. xylosus* (AF041357) as well as complete sequences of *S. carnosus* (AF041356) and *Staphylococcus pasteuri* (AF041361) were deposited in GenBank database (BENSON et al., 1993) under accession numbers indicated in parentheses. The oligonucleotide probes were designed following alignment of these sequences and those deposited in GenBank.

The oligonucleotide probes for dot blot hybridization were labeled at 5' ends with T4 polynucleotide kinase (Eurogentec, Seraing, Belgium) and [γ-^{32}P] ATP (Amersham, Les Ulis, France, (STAHL et al., 1988). The probe S-D-EUB-0338-a-A-18 was labeled with rhodamine and the other probes (Eurobio, France), were coupled with fluorescein for whole-cells hybridization.

Nucleic acids were extracted from *Staphylococcus* cells harvested in exponential phase as previously described (CHOMCZYNSKI and SACCHI, 1987). Concentrations of total RNA were determined with the orcinol method (DANIELS et al., 1994). The blots were hybridized

0723-2020/99/22/02-225 $ 12.00/0

at 43 °C and washed with SDS at the optimal washing temperature (Td) as described by RÖNNER and STACKEBRANDT (1994).

Staphylococcus cells were fixed by the addition of paraformaldehyde, spotted on microscopic slides according to AMANN et al. (1990_a) and hybridized with fluorescent-labelled oligonucleotide probes (BEIMFOHR et al., 1993). Before blotting, fixed cells (10^9/ml) were permeabilized for 20 min with 200 µl of lytic enzyme solution at room temperature. The following enzymes were tested: (i) lysozyme (Sigma) at 10 mg/ml; (ii) lysostaphin (Sigma) at 1.6 to 3 µg/ml for cells in stationary phase. Enzymatic treatments were stopped as described by AMANN et al. (1990_a). Different hybridization stringencies adjusted by various concentrations of formamide ranging from 0% to 50% (v/v) in intervals of 5% were tested. Fluorescence was detected with a Polyvar microscope (Reichert-Jung) fitted for epifluorescence microscopy with filter sets. Hybridization efficiencies of the probes were compared qualitively by the comparison of the brightness of rhodamine-stained cells.

Probe specificity by dot blot hybridization

The sequences of the oligonucleotide probes are indicated in Table 1. The results of dot blot hybridization are given in Table 2. *S. carnosus* can be differentiated from other species of *Staphylococcus* by hybridization with the probes S-S-S.carno-0163-a-A-22 and S-S-S.carno-0440-a-A-23. Indeed, RNA of 12 *Staphylococcus carnosus* strains gave a positive signal, and there was no hybridization signal with other strains, even if some strains (*S. kloosii, S. piscifermentans*) reveal similar sequences. The probe S-S-S.war-0180-a-A-23 hybridized with 6 *S. warneri* strains and with *S. auricularis*. It was surprising as the probe has theoretically 2 mismatches with the sequence of *S. auricularis*. Nevertheless this probe could be used to identify *S. warneri* in meat products, since the species *S. auricularis* has never been found in meat products. The probe S-S-S.sapro-0517-a-A-26 gave a positive signal for a washing temperature of 50 °C with 6 *S. saprophyticus* strains but also with *Staphylococcus arlettae* and *S. cohnii*. The specificity could not be increased by washing at 52 °C because at this temperature the hybridization with *S. saprophyticus* was suppressed.

Optimizing whole-cell hybridization of staphylococci

S. carnosus 833 cells from the exponential phase were permeabilized with lysozyme and hybridized with the probe S-D-EUB-0338-a-A-18. They gave the most intense fluorescent signal, whereas the fluorescence intensity of cells decreased in stationary phase (10 hours) and late-stationary phase (9 day old) (Fig. 1). Old cells (9 and 12 days) showed a weaker fluorescence and were heterogeneous in size. The intracellular rRNA content was three times lower for cells in stationary phase than for cells in exponential phase. The decrease in RNA content could explain the decrease in fluorescent cells. Indeed DELONG et al. (1989) demonstrated that the amount of a rRNA-specific probe that binds to *E. coli* was correlated to the cellular rRNA content and growth rates. However, the correlation did not apply to carbon-starved *Vibrio sp.* strain CCUG15956 cells (FLÄRDH et al., 1992) or *Desulfovibrio gigas* strain ATCC19364 cells growing at a slower rate (AMANN et al., 1990_b). Under these conditions *Vibrio sp.* and *D. gigas* retained quite large numbers of ribosomes.

Nine and 12 day old cells of *S. carnosus*, treated 20 min with lysostaphin (3 µg/ml) were fluorescent after *in situ* hybridization. Whereas, 6 h old cells were lysed and were not detectable. Consequently the accessibility of probe targets is dependent on the permeability of the cells. Our results confirm that Gram-positive organisms are particularly difficult to permeabilize. BEIMFOHR et al.

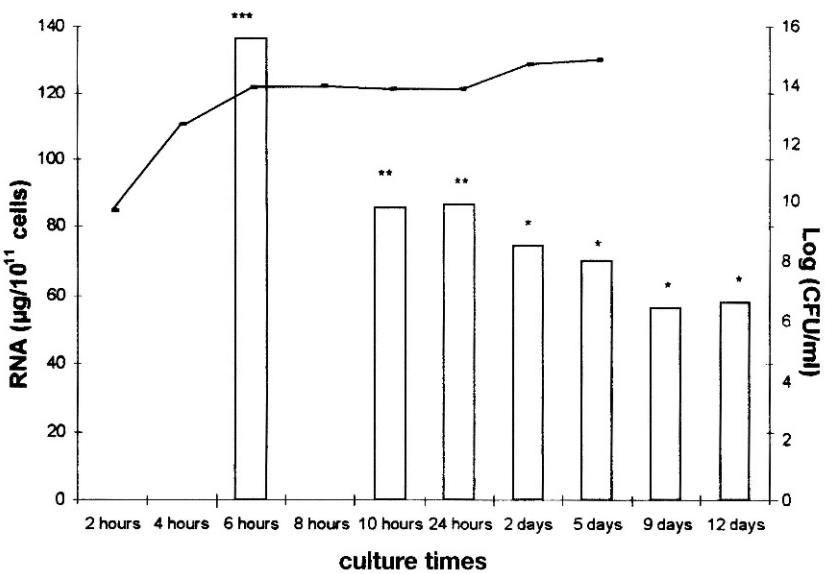

Fig. 1. RNA content per 1011 *S. carnosus* 833 cells (stippled bars) as function of growth phase (cfu solid line).
S. carnosus 833 cells were fixed with paraformaldehyde 4% and treated with 10 mg/ml lysozyme. Intensity resulting from binding of the Eubacterial probe S-D-EUB-0338-a-A-18:
*** Strong fluorescence, ** good fluorescence, * poor fluorescence

Table 1. Oligonucleotide probes, washing temperature for dot blot hybridization (Td), temperature and optimal formamide concentration for specific whole-cell hybridization.

probe	Sequence (5–3)	Target group	Td	whole-cells hybridization	
				Temperature	Formamide
S-D-EUB-0338-a-A-18[a]	GCTGCCTCCCGTAGGAGT	Eubacterial	54 °C	46 °C	15%
S-S-S.carno-0163-a-A-22	TTGCAGAACCATGCGGTTC	*S. carnosus*	45 °C	NS	NS
S-S-S.carno-0440-a-A-23	GTGCGCATAGTTACCTACG	*S. carnosus*	50 °C	46 °C	20%
S-S-S.war-0180-a-A-23	CAGCAAAGCCGCCTTTCAC	*S. warneri*	48 °C	46 °C	15%
S-S-S.sapro-0157-a-A-26	TCACTTTAGAACCATGCGG TTCCAAA	*S. saprophyticus*	50 °C	NS	NS

Probe nomenclature is based on the oligonucleotide probes database (ALM et al., 1996).
[a] AMANN et al., 1990_a
NS – no signal whatever the temperature and formamide concentration

Table 2. Specificity of the oligonucleotide probes by dot blot and whole-cells hybridization.

species	S-S-S.war-0180-a-A-23		S-S-S.carno-0440-a-A-23		S-S-S.carno-0163-a-A-22		S-S-S.sapro-0157-a-A-26	
	dot blot	whole-cells	dot blot	whole-cells	dot blot	whole-cells	dot blot	whole-cells
S. carnosus DSM 20501[T]	–	–	+	+	+	–	–	ND
m379[a]-833[b]-834[b]-836[b]-m427[c] m430[c]-m431[c]-m433[c]-m434[c]-m435[c]	–	–	+	+	+	ND	ND	ND
S. warneri DSM20316[T]	+	+	–	–	–	ND	ND	ND
854[d]-863[d]-859[d]-864[d]751[d]-752[d]	+	+	–	–	ND	ND	ND	ND
S. saprophyticus DSM20299[T]-750[d]	–	ND	–	ND	–	ND	+	–
GM41-GM51-GM52-GM55	ND	ND	ND	ND	ND	ND	*	ND
S. arlettae CIP103501	–	–	–	–	ND	ND	+	ND
S. aureus DSM20231[T]	–	–	–	–	ND	ND	–	ND
S. auricularis DSM20609	+	–	–	–	ND	ND	–	ND
S. capitis CIP8153	–	–	–	–	ND	ND	–	ND
S. caprae DSM20608	–	–	–	–	ND	ND	–	ND
S. caseolyticus ATCC13548[T]	–	–	–	–	ND	ND	–	ND
S. chromogenes DSM20454	–	–	–	–	ND	ND	–	ND
S. cohnii DSM20260	–	–	–	–	ND	ND	+	ND
S. epidermidis DSM20044[T]	–	–	–	–	–	ND	–	ND
S. equorum DSM20674[T]	–	–	–	–	ND	ND	–	ND
S. felis DSM4916[T]	–	–	–	–	ND	ND	–	ND
S. gallinarum CIP103604	–	–	–	–	ND	ND	–	ND
S. haemolyticus DSM20263[T]	–	–	–	–	ND	ND	–	ND
S. hyicus DSM20459[T]	–	–	–	–	–	ND	–	ND
S. intermedius DSM20373[T]	–	–	–	–	ND	ND	–	ND
S. kloosii DSM20676[T]	–	–	–	–	ND	ND	–	ND
S. lentus CIP8163	–	–	–	–	ND	ND	–	ND
S. lugdunensis DSM4804	–	–	–	–	ND	ND	–	ND
S. muscae DSM7068	–	–	–	–	ND	ND	–	ND
S. pasteuri DSM51129	–	–	–	–	ND	ND	–	ND
S. piscifermentans m377[a]–m378[a]	–	–			–	ND	–	ND
S. schleiferi DSM4807	–	–	–	–	ND	ND	–	ND
S. sciuri DSM20345[T]	–	–	–	–	ND	ND	–	ND
G. simulans DSM20322[T]	–	–	–	–	–	ND	–	ND
S. vitilus ATCC51161	–	–	–	–	ND	ND	–	ND
S. xylosus DSM20266[T]	–	–	–	–	–	ND	–	ND

+ hybridization signal; – no hybridzation signal; ND not done; T type strain.
CIP: Institut Pasteur Collection, DSM: Deutsche Sammlung von Mikroorganismen, GM: INRA Paris-Grignon, ATCC: American Type Collection
[a] Culture Collection Center Tokyo University of Agriculture, [b] Society Texel, [c] University of Bath, [d] INRA Theix Collection.

(1993) demonstrated that cells of *Streptococcus salivarius* were only slightly permeabilized by lysozyme treatment, whereas after treatment with mutanolysin at least part of the cells could be detected by *in situ* hybridization.

Specific detection of *S. carnosus, S. warneri* and *S. saprophyticus* by fluorescence *in situ* hybridization

Whole cells of *S. carnosus, S. warneri, S. saprophyticus* and *S. xylosus* in exponential phase were hybridized with their fluorescent-labeled oligonucleotides (Table 2). Hybridization of *S. carnosus* with S-S-S.carno-0163-a-A-22 did not give a positive signal independent of they formamide concentration. At temperature hybridization of 46 °C, 20% formamide concentration, the probe S-S-S.carno-0440-a-A-23 have a good signal intensity with *S. carnosus* cells only. The probe S-S-S.war-0180-a-A-23 at 46 °C with 15% formamide concentration was optimal to discriminate target cells *S. warneri*. No signal was detected for any of the other *Staphylococcus* species, even for *S. auricularis*, which was not discriminated from *S. warneri* by dot blot hybridization.

S-S-S.sapro-0157-a-A-26 did not hybridize with *S. saprophyticus* strains independent of the formamide concentration. However, this probe gave a positive signal by dot blot hybridization. The denatured state of the rRNA may be the reason for the positive reaction. The present study shows that the newly designed probes can be used for a rapid identification of *S. carnosus* and *S. warneri* and should be tested in fermented meat products.

Acknowledgements

We would like to thank Françoise Irlinger for helpul collaboration. We are grateful to Emmanuelle Zumstein and Patrick Dabert for their help in *Staphylococcus* sequencing.

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