Biochemical and genomic characteristics of *Micrococcaceae* from French dry sausages

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SUMMARY : Forty five strains of *Micrococcaceae* isolated from french sausages were characterized by using API Staph system, by determinating their resistance to antibiotics, by measuring some biochemical activities (production of D and L lactate isomers and lipases). These strains were susceptible to lysostaphin and so were classified in the genus *Staphylococcus*. On the basis of their biochemical characteristics most of them were not easly identified to known species. To help their identification, DNA-DNA hybridizations were carried out. Some strains do not share any homology with species frequently found in meat products : *Staphylococcus xylosus* or *Staphylococcus carnosus*

INTRODUCTION

Non pathogenic *Micrococcaceae* take an important part in sausage production. They are used as starter cultures for their nitrate and nitrite reducing properties which are crucial in the formation and stability of cured meat color (LIEPE, 1983). They may also enhanced the flavour and aroma of the products (LUCKE, 1986).

The classification and identification of *Micrococcaceae*were somewhat confused in the past : the distinction of *Staphylococcus* from *Micrococcus*. was not always clear. SCHLEIFER and KLOOS (1975a) clearly defined the main characteristics for the distinction of the genera *Micrococcus* and *Staphylococcus* : G+C content, cell wall composition. For the routine identification of *Micrococcaceae* these authors recommanded to use lysostaphin susceptibility : all staphylococci are susceptible whereas most micrococci are resistant . Their studies lead to a new identification of *"Micrococcus candidus"*and *"Micrococcus caseolyticus "* respectively as members of the species *Staphylococcus epidermidis* and *Staphylococcus caseolyticus* (SCHLEIFER et *al.*, 1982)

At the present time, by using this lysostaphin susceptibility test, it is well recognized that most of the strains from meat origin belong to the genus *Staphylococcus* but it remains difficult to assigne them to a species. *S. carnosus, S. xylosus, S cohnii, S. hyicus, S. saprophyticus, S. warneri* and *S. sciuri* are often described to occur in meat products.(FISCHER and SCHLEIFER, 1980 ; SCHLEIFER and FISCHER, 1982 ; SEAGER et *al.*, 1986 ; NYCHAS and ARKOUDELOS, 1990). PIRONE and MANGANELLI (1990) also noted the occurence of strains not identifiable with recognized species.

In this study biochemical and physiological tests but also DNA-DNA hybridizations were used in an attemtp to identify strains of staphylococci isolated from French sausages.

MATERIALS AND METHODS

Bacterials strains :

The strains were isolated from French sausageson Chapman medium (Difco) or P. agar medium (KLOOS et *al.* 1974) . They were stored at -20°C in 50% glycerol in APT broth (Difco).

Biochemical characteristics :

Production of acid from carbohydrates, urease, arginine dihydrolase, phosphatase, nitrate reduction, and acetoin production were tested with API STAPH system according to the manufacturer's instruction (API System, Biomerieux).

Isomers of lactate formed from glucose in the supernatant fluids of 48 h cultures at 30°C in APT broth were determined enzymatically with L and D lactate dehydrogenases from Boehringer, Mannheim.

Antibiotic susceptibility was tested on P.agar medium with antibiotic disks (Diagnostics Pasteurs). Resistance to novobiocin (5ug/ml), lysostaphin (200ug/ml) and lysozyme (400 ug/ml) was performed as described by Schleifer and Kloos (1975b).

Esterase activities were determined on 2 naphtyl esters using commercial API ZYM system(API System, Biomerieux).

Lipase activity was revealed with tributyrin, triolein and pork fat according to KOUKER and JAEGER (1986).

Hemolytic activity was detected on P agar supplemented with 5% (Vol/Vol) sheep erythrocytes. Free coagulase was tested on rabbit plasma and deoxyribonuclease (DNase) production on DNase test agar (Difco).

Computer analysis

Seventeen characters were coded as positive or negative. The data were examined by Hierarchical Ascendant Classificatio (metric of KHI-2 ; criterial of aggregation : mean of the distance)with STAT.I.T.C.F. program.

DNA-DNA hybridization.

DNA was extracted and purified by the method of BRENNER et *al.*(1982). Native DNA was labelled *in vitro* by nick translation with deoxy (^{3}H)cytidine 5' phosphate nucleotide using the commercial kit Amersham.

DNA-DNA hybridizations were performed at 62°C following the S1 nuclease method described by GRIMONT et *al.* (1980).

RESULTS AND DISCUSSION

All strains have the following general properties :

- Cells are spherical, gram positive, non motile, catalase positive and are susceptible to lysostaphi (2OO µg/ml), thus they belong to the genus *Staphylococcus*.
- They reduce nitrate to nitrite, they produce acetyl methyl carbinol from pyruvate
- They produce acid aerobically from glucose and fructose but not from methyl glucoside and melibiose
- They hydrolyze naphtyl derivatives of butyrate, valerate, caproate, caprylate, nonanoate and caprat as also tributyrin.
- They are susceptible to chloramphenicol (30 µg), erythromycin (15 UI), furane (300 µg), neomyci (30 UI), oxacillin (5 µg) and penicillin (6 µg).
- All strains are negative for coagulase, DNase and hemolysin on sheep blood agar.

Since the characteristics listed above are identical for all the strains, they are omited for the calculation of similarities between strains

The forty five isolates could be grouped into 5 clusters according their phenotypic features (Table 1).

The two strains in cluster A appeared phenotypically identical to *S. carnosus* .

The strains in cluster E share many common characters with *S. xylosus*.

Strains of cluster B are sensitive to novobiocin. The properties shown in Table 2 distinguish them from the named species o staphylococci susceptible to novobiocin described by SCHLEIFER (1986).

The strain M2 belonging to this group exhibit no relatedness to *S. carnosus*, *S. saprophyticus*, *S. simulans.*, *S. xylosus* (Table4) It is likely that this strain could be assigned to *S. warneri ;* in fact only production of acid from mannose differentiate them. This cannot b conclusive until proven by DNA-DNA hybridization.

Cluster C is the largest cluster in which strains are particularly heterogeneous in their carbohydrate fermentation pattern, productio of D lactate. This diversity make them difficult to identify with recognized species. On the basis of their resistance to novobiocin thes strains are closed to *S. sciuri, S. saprophyticus* and *S. cohnii*. Nevertheless their characteristics do not fit totaly the description of thes species like it is shown in Table3. There are only two properties distinguishing clusters C and D : arginine dihydrolase and urease.

Strain M31 belonging to this cluster was labelled and hybridized with other strains of the clusters C, B, D and with know *Staphylococcus* species.

Strain M31 show a high homology (75%) with the type strain *S. saprophyticus (*D.S.M. 20229) but not with other reference specie tested(Table 4), and thus M31 may belong to *S. saprophyticus*. Strains 848, 282, 843, classified in cluster C displayed a high homolog (65-89 %) with strain M31 suggesting they may also be allocated to *S. saprophyticus*. On the other hand on the basis of DNA-DN hybridization results strains 822 may be misclassified in this cluster C. This cluster probably do not represent an homogeneous genomi group.

Strains M2, 863, 851 from respectively clusters B and D are less than 20% homologous with strain M31(Table4). These value show that cluster C is really separated from clusters B and D.

CONCLUSION

The greatest care must be taken in identification of *Staphylococcus* strains from clusters B, D and some strains of cluster C. The remain unidentified and further investigations by DNA-DNA hybridization seem necessary .It may be a technological interest that thes strains hyrolyse pork fat .

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TABLE 1 : Discriminating characteristics of the five clusters .

Cluster Nb of isolates	A 2	B 9	C 19	D 5	E 10
Susceptibility to :					
Lysozyme	+/-	-	- (12)	-	+
Novobiocin	+	+	-	-	-
Acid production from :					
Lactose	-	-	+	+	+
D-Mannitol	+	-	+ (10)	+	+
D-Mannose	+	+	+ (16)	+	+
Maltose	-	+	+	+	+
N-Acetyl-glucosamine	+	- (6)	+ (10)	+	+
Raffinose	-	-	-	-	- (7)
Sucrose	-	+	+	+	+
Trehalose	+/-	+ (7)	+	+	+
Xylitol	-	-	+ (10)	-	-
Xylose	+/-	-	-	-	+
Production of :					
D-Lactate	+	+	+ (15)	+	-
Arginine dihydrolase	+	+	-	+	-
Urease	-	+	+	-	+
Pork fat hydrolysis	-	+	+	+	-

( ) number of strains positive or negative

TABLE 2 : A comparison of the characteristic properties of the Staphylococcus of cluster B with those of other novobiocin susceptible Staphylococcus

	Acid (aerobically) from :					Production of		
	Fructose	Maltose	D-Mannose	Sucrose	Trehalose	D-Lactate	Arginine dihydrolase	Urease
Cluster B	+	+	+	+	+	+	+	+
*S. simulans**	+	-	+/-	+	+	+	+	+
*S. warneri**	+	+	-	+	+	+	+/-	+
*S. carnosus**	+	-	+	-	+	+	+	-
*S. caprae**	-	+	+	-	+	-	+	+
*S. capitis**	+	-	+	(+)	-	-	+/-	-
*S. epidermidis**	+	+	+	+	-	-	+	+
*S. haemolyticus**	+	+	-	+	+	+	+	-
*S. hyicus**	+	-	+	+	+	-	+	+
*S. saccharolyticus**	+	-	(+)	-	-	-	+	nd

* Data from Bergey's Manual of Systematic Bacteriology

TABLE 3 : Comparison of the characteristics of clusters C and D of Staphylococcus with other novobiocin resistant Staphylococcus

	Cluster C	Cluster D	*S. sciuri**	*S. saprophyticus**	*S. cohnii**
Susceptibility to :					
Lysozyme	- (12)	-	-	-	-
Acid from :					
Lactose	+	+	-	+/-	-
D-Mannitol	+ (10)	+	+	-	+
Sucrose	+	+	+	+	-
Production of :					
D-Lactate	+ (15)	+	-	-	
Arginine dihydrolase	-	+	-	-	-
Urease	+	-	-	+	+/-
Nitrate reduction	+	+	+	+/-	-

( ) number of strains positive or negative

* Data from Bergey's Manual of Systematic Bacteriology

TABLE 4 : DNA-DNA hybridization results

Source of unlabelleted DNA Group	isolates n°	% of relatedness with labelled DNA from : M31	M2
C	M31	100	/
C	843	84	/
C	848	89	/
C	861	24	/
C	822	12	/
C	282	65	/
B	M2	10	100
B	863	5	40
D	851	18	/
B	855	33	/
S. carnosus DSM 20501		9	24
S. xylosus DSM 20266		nd	17
S. saprophyticus DSM 20229		75	7
S. cohnii DSM 20260		11	nd
S. hyicus		15	nd
S. simulans DSM 20322		5	13
S. sciuri DSM 20345		20	nd
S. caseolyticus DSM 20597		5	11

nd : non determined