Letters in Applied Microbiology 2000, **31**, 368–373

Rapid differentiation of *Staphylococcus aureus* from staphylococcal species by arbitrarily primed-polymerase chain reaction

M.J. Benito, M.M. Rodríguez, M.G. Córdoba, E. Aranda and J.J. Córdoba

Higiene de los Alimentos, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain

183/00: received 12 May 2000, revised 21 July 2000 and accepted 28 July 2000

M.J. BENITO, M.M. RODRÍGUEZ, M.G. CÓRDOBA, E. ARANDA AND J.J. CÓRDOBA. 2000. An arbitrarily primed-polymerase chain reaction (AP-PCR) method was optimized to differentiate *Staphylococcus aureus* from other staphylococcal species, using DNA from crude cell extract. From the different assays carried out, the best resolution of the band patterns was obtained when the reaction mixture contained 200 μmol l^{-1} dNTPs, 200 ng primer, 1 U Taq DNA polymerase and 3 mmol l^{-1} $MgCl_2$ and the amplification conditions were: initial denaturation of 94 °C for 1 min, primer annealing of 30 °C for 1·5 min, DNA extension at 55 °C for 5 min and final extension at 55 °C for 5 min. The results of the characterization of the staphylococcal isolates by AP-PCR are in accordance with those of the biochemical identification by the API Staph System, time of analysis of the AP-PCR being only 6–7 h. Thus, this technique could be a useful method for microbial quality assurance.

INTRODUCTION

Staphylococcus aureus has consistently been one of the three most important micro-organisms responsible for food-borne disease (Bean *et al*. 1991).

Conventional methods for detection of *Staph. aureus* in foods require a minimum of 4–5 d to obtain presumptive results after initiation of sample analysis. More rapid, simple and sensitive methods, able to differentiate *Staph. aureus* from other staphylococcal species, are needed for microbial quality assurance. Rapid biochemical tests, such as the API Staph System (BioMérieux Vitek, Hazelwood, MO, USA) are faster and less laborious than conventional biochemical characterization, but take at least 24 h for appropriate identification (Pascoli *et al*. 1986).

An increasing number of reports have indicated that application of the polymerase chain reaction (PCR) to reliably and quickly detect pathogens in foods is a promising new diagnostic tool to monitor food safety. Arbitrarily primed-PCR (AP-PCR) could be appropriate since it involves amplification of genomic DNA with a single primer of arbitrary sequence (Welsh and McClelland 1990). The PCR products, after resolution on agarose gels, give rise to artificial fingerprinting of the genome. The analysis time of this method could be shortened if DNA crude cell extracts were used as template instead of purified DNA. An AP-PCR has been evaluated for the differentiation of staphylococcal species of animal origin (Matthews and Oliver 1994), although purified DNA was used as template.

The aim of this work has been the development of a fast and reliable protocol of AP-PCR to differentiate *Staph. aureus* from other staphylococcal species to be used in quality assurance in the food industry. DNA from crude cell extract was assayed as template in the AP-PCR to reduce the analysis time.

Correspondence to: J.J. Córdoba, Higiene de los Alimentos, Facultad de Veterinaria, Avda. de la Universidad s/n, 10.071 Cáceres, Spain (e-mail: jcordoba@unex.es).

MATERIALS AND METHODS

Bacterial strains

The culture collection strains used as standards to develop this method were: *Staph. aureus* NCTC 5664, *Staph. aureus* CCUG 9128, *Staph. epidermidis* ATCC 12228, *Staph. epidermidis* ATCC 14990 and *Staph. warneri* DSM 20316. Strains *Staph. xylosus* 270 and *Staph. xylosus* 205 from dry cured ham were also used as standards (Rodríguez *et al*. 1996).

In addition, 50 isolates obtained with contact plates of Baird Parker agar (Oxoid, Unipath, Basingstoke, UK) at different stages of cheese (M1–M20, M22 and M23) and meat processing (M24–M50 and M21) were used to test the AP-PCR method.

Biochemical identification

Both reference strains and isolates from foods were subcultured at 37 °C for 24 h in Agar P (Kloos *et al*. 1974). Pure isolates were first characterized by Gram and catalase tests and then by the API Staph System (BioMérieux Vitek). The results were interpreted according to the classification of Kloos and Schleifer (1986).

Identification by arbitrarily primed-polymerase chain reaction

Preparation of DNA crude cell extracts. DNA crude cell extracts were prepared from single colonies to be used as template. For this, the isolates were cultured at 37 °C for 18–24 h in nutrient agar (Oxoid) with 0·5 IU penicillin ml^{-1} to facilitate cell lysis (Silvestri and Hill 1965). Single colonies were then picked from agar plates and resuspended in 100 μl sterile pure water. The cell suspension was heated at 100 °C for 10 min, followed by a rapid freezing at −80 °C; 20 μl each of 10 $\mu g\ \mu l^{-1}$ lysozyme (Sigma Chemical Co., St. Louis, MO, USA) and 10 $\mu g\ \mu l^{-1}$ proteinase K (Sigma) were then added. The mixture was incubated at 55 °C for 20 min. To denature the above enzymes, the samples were incubated at 100 °C for 10 min; 10 μl of DNA extract were used for the PCR assays.

Polymerase chain reaction amplification. Amplification was performed in a Thermal Cycler (Biometra, Maidstone, UK) using a single primer of arbitrary nucleotide sequence (5′ GTAACGCC 3′) (Matthews and Oliver 1994). The PCR was performed in a 50 μl volume containing 5 μl 10 × buffer (10 mmol l^{-1} Tris-HCl, 50 mmol l^{-1} KCl, 0·1% Triton X-100) and 10 μl DNA extract. All assays were carried out in triplicate. To optimize the AP-PCR method different concentrations (Table 1) of Taq DNA polymerase (Finnzymes, Espoo, Finland), dNTPs (Boëhringer Mannhein, Mannhein, Germany), primer and magnesium chloride (Boëhringer Mannhein) were tested in the reaction mixture. Furthermore, several conditions of time and temperature of annealing, extension and final extension were assayed (Table 2). For all these, only one parameter was modified in every test. Once a factor was optimized, the selected value was used for the remaining tests.

Table 1 Concentrations assayed of the different ingredients used in the arbitrarily primed-polymerase chain reaction mixture

Components of reaction	Concentrations				
$MgCl_2$ (mmol l^{-1})	1·5	2	3	3·5	4
Taq DNA polymerase (U)	0·5	1	1·5	2	
Primer (ng μl^{-1})	4	5	6		
dNTPs (μmol l^{-1})	200	300	400		

Table 2 Amplification conditions tested for the optimization of arbitrarily primed-polymerase chain reaction to differentiate *Staphylococcus aureus* from other *Staphylococcus* species

Amplification stage			
Denaturation	Primer annealing	DNA extension	Final extension
94 °C, 1 min	30 °C, 1·5 min	72 °C, 1·5 min	72 °C, 3·0 min
	30 °C, 2·0 min	55 °C, 3·0 min	55 °C, 3·0 min
	30 °C, 1·0 min	55 °C, 5·0 min	55 °C, 5·0 min
	35 °C, 1·5 min	55 °C, 7·0 min	55 °C, 7·0 min
		45 °C, 7·0 min	45 °C, 7·0 min

Detection and analysis of polymerase chain reaction products. Amplified fragments were analysed by submerged gel electrophoresis in 1% agarose gels using 0·5 × Tris-borate-EDTA buffer. The gels were stained with ethidium bromide (0·5 $\mu g\ ml^{-1}$), the products visualized by u.v. transillumination and photographed. DNA molecular size markers of 10–0·5 kb from Pharmacia Biotech (Uppsala, Sweden) and of 2·2–0·15 kb from Boëhringer Mannheim were used to determine the size of the PCR products. Electrophoretic patterns were compared using ID Image Analysis Software (Kodak Digital Science, Rochester, NY, USA). Similarities between the profiles, based on band positions, were derived by the Pearson correlation coefficient (r). All bands, irrespective of their intensity, ranging between 3000 and 154 bp were considered for calculation of the similarity coefficients. A dendogram was constructed to reflect the similarities between strains in the matrix. Strains were clustered by the unweighted pair group method with arithmetic averages (UPGMA) (Struelens 1996).

RESULTS AND DISCUSSION

Biochemical identification using the API Staph System offered the expected characterization for the standard strains (Table 3). All of the isolates tested were cocci, Gram positive and catalase positive. According to the API Staph System, seven of the isolates (M14, M15, M16, M17, M30, M31 and M42) were characterized as *Staph. aureus*, 10 as *Staph. xylosus* (M1, M2, M5, M6, M8, M10, M11, M12, M22 and M23), two as *Staph. epidermidis* (M4 and M7), three as *Staph. warneri* (M13, M19 and M20), 18 as *Staph. cohnii* (M21, M24, M25, M27, M28, M29, M34,

Table 3 Biochemical identification using the API Staph System from the 50 Gram-positive, catalase-positive cocci isolated from the cheese industry and slaughterhouse and from the reference *Staphylococcus* species

Isolates	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE	Species
M14, M15, M16, M17, M30, M31, M42	+	+	+	+	+	+	+	−	−	+,	−/+	+	−	−	+,	−	+	+,	−	*Staph. aureus*
M1, M2, M5, M6, M8, M10, M11, M12, M22, M23	+	+	+,	+,	+	+	+	+,	+,	+,	+	+	+,	+	+	+,	+	+,	+,	*Staph. xylosus*
M4, M7	+	+	+	+	+	−	−	−	−	+	+	+	−	−	+	−	+/−	+	+	*Staph. epidermidis*
M13, M19, M20	+	+	+	+	−	+	+	−	−	−	+	+	−	−	+	−	+/−	+	+	*Staph. warneri*
M21, M24, M25, M27, M28, M29, M34, M35, M36, M37, M38, M44, M45, M46, M47, M48, M49, M50	+	+	−	+	−	+,	−	−	−	+	+	+	−	−	−	−	−	−	−	*Staph. cohnii*
M33, M39, M40, M41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	*Staph. lentus*
M3, M9	+	+	+/−	+	+	+	+	+/−	−	−	+	+	−	−	+	−	+/−	−	+	n.i.
M18	+	+	+	+	+	−	−	−	−	+	+	+	−	−	+	−	−	+	+	n.i.
M26	+	+	+	+	+	+	−	−	+	−	−	+	+	+/−	+	−	+	+	−	n.i.
M32	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	n.i.
M43	+	+	+	+	+	+	−	−	+	−	+	+	+	−	+	−	+/−	−	+	n.i.
Staph. aureus NCTC 5664	+	+	+	+	+	+	+	−	−	+	+	+	−	−	+	−	+	+	+	
Staph. aureus CCUG 9128	+	+	+	+	+	+	+	−	−	+	+	+	−	−	+	−	+/−	+	+	
Staph. xylosus 270	+	+	+/−	+	+	−	+/−	−	−	+	+	+	−	+/−	+	−	+/−	−	+	
Staph. xylosus 205	+	+	+	+	+	−	−	−	−	+	+	+	−	+/−	+	−	+	−	+	
Staph. epidermidis ATCC 12228	+	+	+	+	+	+	+/−	−	−	+	+	+	−	−	+	−	−	+	+	
Staph. epidermidis ATCC 14990	+	+	+	+	+	−	−	−	−	+	+	+	−	−	+	−	+	+	+	
Staph. warneri DSM 20316	+	+	+	+	+/−	−	+/−	−	−	+	+	+	−	−	+	−	−	+/−	+	

+, Strains positive; −, strains negative; +,, strains variable; +/−, strains weakly positive; n.i., not identified.

M35, M36, M37, M38, M44, M45, M46, M47, M48, M49 and M50) and four as *Staph. lentus* (M33, M39, M40 and M41). However, six isolates (M3, M9, M18, M26, M32 and M43) could not be identified.

From the different conditions evaluated for the AP-PCR of the different standard strains using DNA crude cell extracts, the highest number of amplification bands was obtained when the reaction mixture contained 200 μmol l^{-1} dNTPs, 200 ng primer, 1 U Taq DNA polymerase and 3 mmol l^{-1} $MgCl_2$ (data not shown). For all these parameters, the most critical factor was the concentration of $MgCl_2$ in the reaction mixture. Thus, very low resolution of the patterns was observed at 1·5 and 4 mmol l^{-1} $MgCl_2$. The concentration of Mg^{2+} affects PCR yield (Innis and Gelfand 1990), increasing the stringency of the primer annealing or improving DNA-polymerase performance during amplification.

In relation to the effects of PCR conditions, the best band patterns were obtained at an initial denaturation of 94 °C for 1 min, primer annealing of 30 °C for 1·5 min, DNA extension at 55 °C for 5 min and final extension at 55 °C for 5 min (data not shown). Annealing at 30 °C for 1 min or at 35 °C resulted in a reduction in the number of amplification products as compared with 30 °C for 1·5 min. In addition, low temperature of extension (55 or 45 °C) was critical for amplification, since low resolution of the band patterns was observed at 72 °C. Probably when low temperature of amplification is used deannealing of the primer is minimized and higher yield of amplification bands is achieved. The possible decrease in the efficiency of Taq DNA polymerase during amplification at 55 °C is compensated for by increasing the time of this step to 5 min. The resolution of the band patterns at 45 °C extension temperature was not improved as compared with 55 °C.

Typical band patterns obtained by AP-PCR from the standard strains under the selected conditions are shown in Fig. 1. The same results were always observed in triplicate assays (data not shown). Several species-specific bands could be observed: strains *Staph. aureus* NCTC 5664 and CCUG 9128 showed nine primary fragments from 1744 to 389 bp; *Staph. xylosus* 270 and 205 showed similar band profiles from 770 to 150 bp and *Staph. epidermidis* ATCC 12228 and *Staph. epidermidis* ATCC 14990 also had similar band patterns with common primary fragments from 800 to

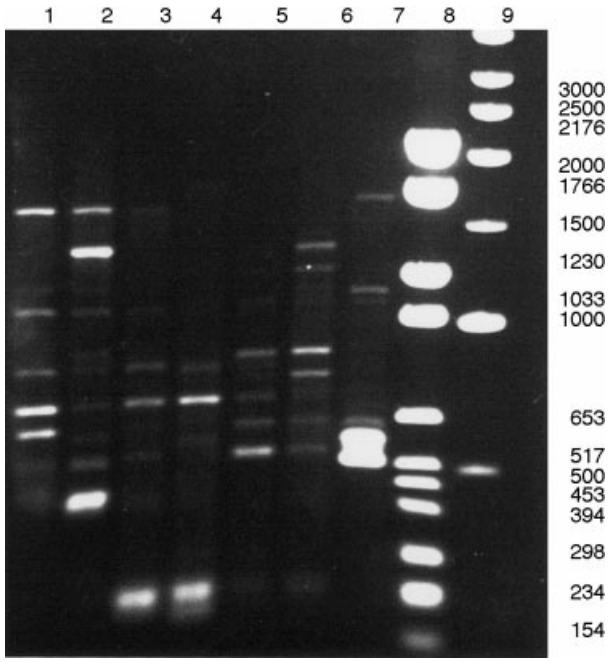

Fig. 1 Agarose gel electrophoresis of the arbitrarily primed-polymerase chain reaction products of crude cell extracts of *Staphylococcus aureus* NCTC 5664 (lane 1), *Staph. aureus* CCUG 9128 (lane 2), *Staph. xylosus* 270 (lane 3), *Staph. xylosus* 205 (lane 4), *Staph. epidermidis* ATCC 12228 (lane 5), *Staph. epidermidis* ATCC 14990 (lane 6) and *Staph. warnerii* DSM 20316 (lane 7); DNA marker 2·2–0·15 kb (Boëhringer Mannheim) (lane 8); DNA marker 10–0·5 kb (Pharmacia Biotech) (lane 9)

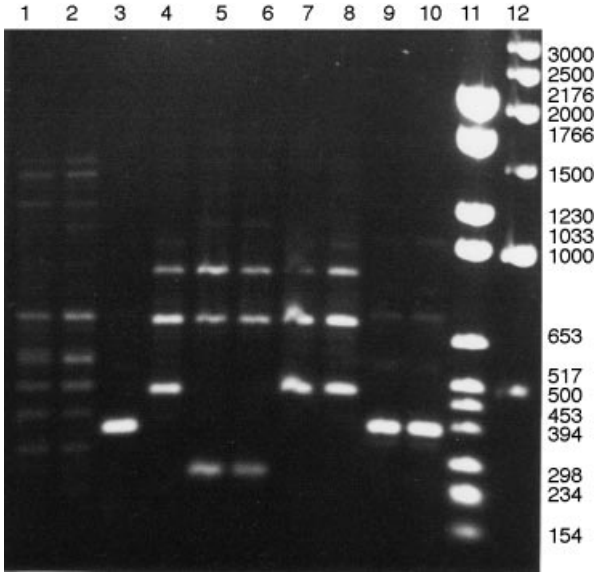

Fig. 2 Agarose gel electrophoresis of the arbitrarily primed-polymerase chain reaction products of crude cell extracts of strains M31 (lane 1), M32 (lane 2), M33 (lane 3), M34 (lane 4), M35 (lane 5), M36 (lane 6), M37(lane 7), M38 (lane 8), M39 (lane 9) and M40 (lane 10). Lane 11, DNA marker 2·2–0·15 kb (Boëhringer Mannheim); lane 12, DNA marker 10–0·5 kb (Pharmacia Biotech)

390 bp. Matthews and Oliver (1994) obtained a lower number of amplification fragments using the same primer and purified DNA from different *Staphylococcus* spp. The conditions of primer extension used here (low temperature and long time) seem to favour the yield of amplification fragments, increasing the possibility of the discrimination between species. Thus, the AP-PCR method here developed allows the *Staph. aureus* strains used to be distinguished from other staphylococcal species.

The optimized method was used to test 50 isolates obtained from food-related environments (Fig. 2). The similarities between the tested strains were plotted in a dendogram (Fig. 3). All isolates identified by the API Staph System as *Staph. aureus* were included in group 1 by the AP-PCR method. Similarly, those identified as *Staph. xylosus* by the API Staph System were classified in group 2, *Staph. epidermidis* in group 3, *Staph. warneri* in group 4, *Staph. conhii* in group 5 and *Staph. lentus* in group 6. Five of the six isolates not identified by the API Staph System were classified together with previous groups by AP-PCR (e.g. M32 to group 1; M3, M9 and M26 to group 2; M43 to group 6). Finally, M18 was not grouped with other strains by AP-PCR.

Thus, the AP-PCR assayed gave the same general result as biochemical identification by the API Staph System, although some isolates that could not be characterized by the biochemical method were tentatively characterized by the AP-PCR.

A high level of intraspecies variation was shown in the AP-PCR analysis, which allowed the typing of different isolates belonging to the same species. Thus, isolates characterized as the same species were separated in different subgroups (Fig. 3). Similar results have been reported when some AP-PCRs have been assayed to differentiate staphylococcal species (Belkum *et al.* 1995). This ability to subtype the different isolates could be useful in identifying the source of microbial contamination in the food industry.

Since the AP-PCR assayed works well with crude cell extracts, there is no need for DNA purification. Thus, isolated colonies of *Staph. aureus* can be differentiated from other staphylococcal species in just 6–7 h. Given that this method has proved to be efficient in separating *Staph. aureus* from other food-related staphylococci, it could be very useful in testing microbial isolates from the food industry. The AP-PCR analysis performed directly on crude cell extracts could be a rapid and useful method for microbial quality assurance.

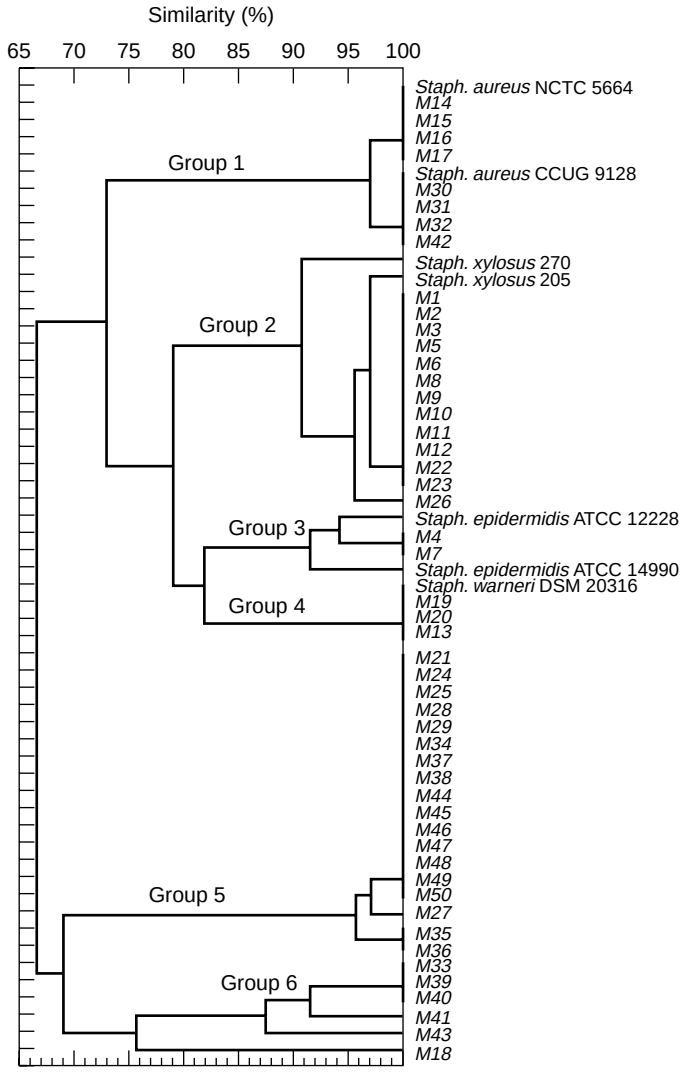

Fig. 3 Dendrogram obtained by using arbitrarily primed-polymerase chain reaction on crude cell extracts from *Staphylococcus* species used as standards and staphylococcal isolates from the cheese industry (M1–M20, M22 and M23) and slaughterhouse (M24–M50 and M21)

ACKNOWLEDGEMENTS

This work was supported by a grant from the Spanish Comisión Interministerial de Ciencia y Tecnología (ALI98–0253). The authors thank the Spanish Ministerio de Educación y Cultura for supplying a grant to M.J. Benito.

REFERENCES

Bean, N.H., Goulding, J.S., Daniels, M.T. and Angulo, F.J. (1991) Surveillance for Foodborne Disease Outbreaks – United States. *Journal of Food Protection* **60**, 1265–1286.

Belkum, A.V., Kluytmans, J., Leeuwen, W.V., Bax, R., Quint, W., Peters, E., Fluit, A., Vandenbroucke-Grauls, Ch., Brule, A., Koeleman, H., Melchers, W., Meis, J., Elaichouni, A., Vaneechoutte, M., Moonens, F., Maes, N., Struelens, M., Tenover, F. and Verbrugh, H. (1995) Multicenter evaluation of

arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **33**, 1537–1547.

Innis, M.A. and Gelfand, D.H. (1990) Optimization of PCRs. In *PCR Protocols: a Guide to Methods and Application* ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. pp. 3–12. New York: Academic Press.

Kloos, W.E. and Schleifer, K.H. (1986) The genus *Staphylococcus*. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2 ed. Sneath, P.H.A., Mair, N.S., Sarpe, M.E. and Holt, J.G. pp. 1015–1035. Baltimore: Williams and Wilkins.

Kloos, W.E., Tornabene, T.G. and Schleifer, K.H. (1974) Isolation and chracterizationof micrococci from human skin, including two new species: *Micrococcus lylae* and *Micrococcus kristinae*. *International Journal of Systematic Bacteriology* **24**, 79–101.

Matthews, K.R. and Oliver, S.P. (1994) Differentiation of *Staphylococcus* species by polymerase chain reaction-based DNA fingerprinting. *Journal of Food Protection* **57**, 486–489.

Pascoli, L., Chiaradia, V., Mucignat, G. and Santani, G. (1986) Identification of Staphylococci by the API STAPH, Sceptor, Rosco and Simplified Lyogroup Systems. *European Journal of Clinical Microbiology* **5**, 669–671.

Rodríguez, M., Núñez, F., Córdoba, J., Bermúdez, E. and Asensio, M.A. (1996) Gram-positive cocci from dry cured Iberian ham and their enterotoxigenic potential. *Applied and Environmental Microbiology* **62**, 1897–1902.

Silvestri, L.G. and Hill, L.R. (1965) Agreement between deoxyribonucleic acid base composition and taxometric classification of Gram-positive cocci. *Journal of Bacteriology* **90**, 136–140.

Struelens, M.J. (1996) Consensus guidelines for appropriate use and evaluation of microbiologic epidemiological typing systems. *Clinical Microbiology and Infection* **2**, 2–11.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213–7218.