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3 # Use of *recA* gene sequence analysis for the identification of

4 # *Staphylococcus equorum* strains predominant on dry-cured

5 # hams

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23

24   Abstract

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26   Spanish dry–cured ham is an uncooked meat product highly appreciated due to its

27   characteristics flavour. In this study, we examined the accuracy of biochemical tests and

28   16S rDNA sequencing in the identification of 56 staphylococcal strains isolated during

29   industrial Spanish dry–cured ham processes. Important differences were observed

30   comparing genotypic and phenotypic data. *S. xylosus* was the prevalent species

31   identified by biochemical methods (87.5%), however, sequencing of the 16S rDNA

32   resulted in an unambiguous identification of *S. equorum* (73.2%) and *S. vitulinus* (8.9%)

33   strains. Reliable identification of meat staphylococci, mainly among *S. xylosus* and *S.*

34   *equorum* strains could be also achieved by means of *recA* gene sequence comparison.

35   Two degenerate primers previously described for lactic acid bacteria were used to

36   amplify an internal fragment of the *recA* gene. This fragment was amplified from twelve

37   staphylococcal type strains representing frequent meat species. The results indicated

38   that *recA* sequencing is an adequate method to discriminate among meat staphylococci.

39   In addition, *S. xylosus* and *S. equorum* strains could be more accurately discriminated

40   by *recA* sequencing than 16S rDNA or *sodA* sequencing. The *S. equorum* sequence

41   diversity showed at the intra–species level by *recA* gene sequencing confirmed the high

42   heterogeneity described among *S. equorum* strains.

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45   *Keyworks*: *Staphylococcus equorum*, Dry–cured ham, *Staphylococcus xylosus*, *recA*

46   gene, identification method

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1. Introduction

Spanish dry-cured ham, a valuable meat product in Spain, is a traditional intermediate-moisture meat product which is obtained from white and Iberian (black) breeds (Losantos et al., 2000). The method of preparation consists basically on four stages: obtention and preparation of pieces, salting, postsalting and drying-maturation. During the salting stage a microbial reservoir is created from microorganisms present in the salt, on the surface more than on deep tissues of hams and in the environment (Cornejo et al., 1992). Some of these microorganisms could develop several actions in the brine formed (reduction of nitrates to nitrites, proteolysis and lipolysis) contributing to the sensorial properties of the ham (Toldrá, 1998; Sondergaard and Stahnke, 2002). Hams are dried-matured at least for 110 days in drying chambers. During this period, water losses and different chemical and biochemical changes occur, resulting into the typical colour, flavour and taste of the ham (Toldrá, 1998).

Staphylococcaceae are the prevalent microbiological group in the processing of Spanish dry-cured hams (Carrascosa and Cornejo, 1991; Cornejo and Carrascosa, 1991; Cordero and Zumalacárregui, 2000). Coagulase-negative staphylococci are important microorganisms in meat products and they influence technological properties of the cured meat products. *Staphylococcus* spp. play a role in the development of aroma as well as flavour and colour, after muscle enzymes. Staphylococci also release lipases, and show nitrate reductase activity contributing to the development of the aroma and colour of the dry-cured ham. Identification methods based on biochemical tests, may sometimes be uncertain, complicated and time-consuming due to an increasing number of species that varied in few of the taxonomical characters. Moreover, new species of staphylococci are continually being described, making further identification tools

73 necessary. For a better identification, in the last years there has been an increasing

74 interest in the use of molecular methods in order to simplify characterization

75 procedures, to provide rapid and reliable identification, or to validate phenotypically

76 determined taxa. The comparison of results obtained by molecular techniques with

77 those of biochemical identification sometimes showed the unreliability of the traditional

78 identification methods (Sondergaards and Stahnke, 2002; Blaiotta et al., 2003).

79   The majority of molecular methods are based on the DNA sequence of the 16S

80 rDNA gene, from which it is possible to obtain important information for the detection,

81 identification and classification of microorganisms. However, 16S rDNA gene

82 sometimes is too conserved to be useful for species differentiation. Some less-conserved

83 genes, especially those under positive selection, have often been used for species

84 identification, and, in some cases, for bacterial typing. The choice of appropriate genes

85 may vary according to the species. As the precise species discrimination of

86 staphylococci is important in clinical and epidemiological studies, several gene

87 sequences such as the elongation factor Tu (*tuf*) (Martineau et al., 2001), RNA

88 polymerase B (*rpoB*) (Drancourt and Raoult, 2002; Mellmann et al., 2006), heat shock

89 protein (*hsp60*) (Goh et al., 1996; Kwok and Chow, 2003), glyceraldehyde-3-phosphate

90 dehydrogenase (*gap*) (Yugueros et al., 2000), a protein precursor which plays a role in

91 peptidoglycan biosynthesis (*femA*) (Vannuffel et al., 1999), superoxide dismutase A

92 (*sodA*) (Giammarinaro et al., 2005; Poyart et al., 2001) have been used in the

93 identification of staphylococcal species. However, like 16S rDNA, highly conserved

94 genes are not suitable for the discrimination of closely related species. The *recA* gene

95 has been proposed as a useful marker in inferring bacterial phylogeny and has been used

96 successfully to differentiate species of some bacterial genera. LAB species included in

97 the *L. casei* and the *L. plantarum* groups, and heterofermentative wine lactobacilli were

98 differentiated based on their *recA* sequences (Felis et al., 2001; Torriani et al., 2001,

99 Rodríguez et al., 2007).

100 In a previous work, staphylococcal strains were isolated during industrial Spanish

101 dry–cured ham processes and they were selected as possible starter cultures after several

102 *in vitro* tests. As these staphylococcal strains were only biochemically classified

103 (Cornejo and Carrascosa, 1991; Carrascosa and Cornejo, 1991), the aim of this study

104 was to classify these strains by genotypic methods and evaluate *recA* sequencing for the

105 discrimination of closely related meat staphylococcal species.

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108 2. Materials and methods

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110 *2.1. Strains and growth conditions*

111

112 In this work, a total of fifty–six coagulase–negative staphylococci were molecularly

113 identified by 16S rDNA sequencing. These strains were previously isolated during

114 industrial Spanish dry–cured ham processes. Thirty–five of them were isolated during an

115 industrial process using 100 days of ripening. Most of them, 31 strains, were previously

116 classified, using the API–STAPH system and other tests, as *Staphylococcus xylosus*, one

117 strain was classified as *S. capitis*, and three strains shared taxonomic characteristics

118 with *S. xylosus*, *S. capitis* and *S. sciuri* (Cornejo and Carrascosa, 1991). The additional

119 twenty–one staphylococcal strains were isolated during a process using 160 days of

120 ripening. Using the API–STAPH system and additional tests, 18 of them were

121 characterized as *S. xylosus* and the other three strains participated in taxonomic

122 characters with *S. xylosus* and *S. capitis* (Carrascosa and Cornejo, 1991). Type strains

123 from the staphylococcal species often isolated from meat products were purchased from

124 the American Type Culture Colecction (ATCC) or from the German Collection of

125 Microorganisms and Cell Cultures (DSMZ) (Table 1).

126 Staphylococcal strains were routinely grown in brain heart infusion (BHI, Difco,

127 France) and incubated at 37 ºC under aerobic conditions for 24 hours.

128

129 *2.2. Bacterial DNA extraction*

130

131 Bacterial chromosomal DNA was isolated from overnight cultures using a protocol

132 previously described (Vaquero et al., 2004). DNA precipitates were resuspended in an

133 appropriate volume of TE solution (10 mM Tris–HCl, pH 8.0; 1 mM EDTA; Sambrook

134 et al., 1989).

135

136 *2.3. PCR amplification and DNA sequencing of the 16S rDNA*

137

138 16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f

139 (5´–CAGGCCTAACACATGCAAGTC) and 1387r (5´–

140 GGGCGGWGTGTACAAGGC) as previously described (Marchesi et al., 1998). The

141 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR

142 **reaction was performed in 0.2 ml microcentrifuge tubes in a total volume of 25 μl**

143 containing aprox. 10 ng of template DNA (Landeta et al., 2007). The amplifications

144 products were purified on QIAquick spin Columns (Quiagen, Germany, Hilden) for

145 direct sequencing. DNA sequencing was carried out by using an Abi Prism 377[TM] DNA

146 sequencer (Applied Biosystems, USA). Sequence similarity searches were carried out

147 by comparing to sequences from type strains included on the Ribosomal Database

148 (http://rdp.cme.msu.edu).

149

150 *2.4. PCR amplification of a sodA gene fragment in S. equorum*

151

152   For the specific amplification of a *sodA* fragment in *S. equorum* strains, primers

153 SdAEqF (5´–GTGGAGGACACTTAAACCATT) and SdAEqR (5´–

154 CAATTTACCATCGTTTACAACTAG) were used (Blaiotta et al., 2004). These

155 primers target positions 173–194 and 363–340 of the *S. equorum sodA* gene, and,

156 therefore, they amplified a 193 bp gene fragment. The PCR amplification was

157 performed as described by Blaiotta et al. (2004). Briefly, a **total volume of 20 µl**

158 contained 25 ng of template DNA, 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM

159 MgCl$_2$, 250 **µM of each dNTP, each primer at a concentration of 0.5µM, and 1 U of**

160 Ampli*Taq* Gold DNA polymerase. The reaction was performed in a Gradient Eppendorf

161 thermocycler (Eppendorf, Germany, Hamburg) using the following cycling parameters:

162 initial 10 min for enzyme activation at 95 ºC followed by 30 cycles of 5 sec at 95 ºC and

163 25 sec at 61 ºC. Amplified products were resolved on a 2% agarose gel.

164

165 *2.5. PCR amplification and DNA sequencing of a recA gene fragment*

166

167   For the amplification of *recA* regions, 10 ng of chromosomal DNA was added to

168 **the 25 µl PCR mixture containing RecA**–up (5´–GARCAYGCNCTNGAYCC) and

169 RecA–down (5´–CCWCCWGKWGTHGTYTCNGG) oligonucleotides (Duwat et al.,

170 1992). These degenerate primers are based on well–conserved domains, approximately

171 120 amino acids apart, of the RecA proteins. The expected size of the amplicon was 360

172   pb. Fragments of the expected sizes were purified from 2% agarose gel and sequenced

173   (Rodríguez et al., 2007).

174

175   *2.6. Data analysis*

176

177   Sequence similarity searches were carried out using Basic local alignment search

178   tool (BLAST) (Altschul et al., 1997) on the EMBL/GenBank databases. Sequence

179   alignments and comparison were done with the program BioEdit and converted into

180   MEGA files with MEGA version 2.1 sotfware. Phylogenetic trees were constructed by

181   the neighbour–joining method. The percentage of bootstrap confidence levels for

182   internal branches, as defined by the MEGA program, was calculated from 10000

183   random resamplings.

184

185   3.   Results and discussion

186

187   *3.1. Molecular classification of Staphylococcus sp. strains isolated from dry-cured*

188   *hams*

189

190   The dry–cured ham is an uncooked meat product highly appreciated for its

191   characteristics flavour. The positive role of some staphylococci in dry–cured meat

192   products, due to their nitrate reductase, catalase, lipolytic and proteolytic activities also

193   contributes to the flavour characteristics of dry–cured hams. Although the number of

194   different species is relatively small, a remarkable variety of staphylococcal strains are

195   present on dry–cured hams. In a previous work, a total of 56 staphylococcal strains were

196   isolated during industrial Spanish dry–cured ham processes (Carrascosa and Cornejo,

197 1991; Cornejo and Carrascosa, 1991). These strains were taxonomically classified by

198 morphological and biochemical tests, including the API STAPH system, as 49 *S.*

199 *xylosus* strains, one *S. capitis* strain, and 6 strains which shared taxonomic

200 characteristics with *S. xylosus*, *S. capitis* and *S. sciuri* species.

201 Previously, in a screening for biogenic amine production by these strains, we

202 have found that a cadaverine and putrescine–producer strain, initially identified as *S.*

203 *xylosus* IFIJ47 by biochemical methods, was reclassified as *S. lugdunensis* IFIJ47 by the

204 sequencing of its 16S rDNA (Landeta et al., 2007). Therefore we decided to identify by

205 molecular methods all the 56 strains isolated from dry–cured hams. Only one strain was

206 correctly identified; *S. capitis* IFIJ12 was confirmed by the molecular method used.

207 From the strains possessing an uncertain taxonomic identification, most of them were

208 classified as *S. vitulinus* (IFIJ4, IFIJ31, IFIJ36, IFIJ38, and IFIJ41), and one strain was

209 identified as *S. equorum* IFIJ5. From the 49 strains previously identified as *S. xylosus*,

210 40 were classified as *S. equorum*, two as *S. warneri* (IFIJ15 and IFIJ18), two *S. aureus*

211 (IFIJ13 and IFIJ32), and one *S. caprae* (IFIJ10), *S. epidermidis* (IFIJ24), and *S. hominis*

212 strain (IFIJ26) by the 16S rDNA sequencing. Two of the studied strains were not

213 staphylococci (*Rothia dentocaria* IFIJ17 and *Lactobacillus sakei* IFIJ40). The

214 unreliability of the traditional identification methods has been previously described in

215 studies that compare the results obtained by molecular techniques with those of

216 biochemical identification of staphylococci. Similarly to this work, Sondergaards and

217 Stahnke (2002) reported, for strains isolated from fermented meat products, a high

218 identification probability for *S. xylosus* according to API STAPH results and

219 identification as *S. equorum* by molecular analysis. Blaiotta et al. (2003) reported that

220 for strains of *S. xylosus* isolated from dry fermented sausages identification by both

221 biochemical and molecular methods agreed for only 52% of the isolates. In some cases,

222   strains of *S. equorum*, *S. lentus*, *S. vitulinus* and *S. saprophyticus* could be misidentified

223   as *S. xylosus* by using traditional identification methods only; whereas strains of *S.*

224   *xylosus* can be misidentified as *S. saprophyticus*, *S. sciuri*, *S. epidermidis* or *S.*

225   *intermedius.*

226   In addition to the unreliability of the traditional identification methods, the

227   results obtained in this work indicated a high incidence of *S. equorum* strains in dry–

228   cured hams. Cordero and Zumalacárregui (2000) found *S. equorum* strains from the salt

229   used in the Spanish dry–cured ham elaboration. Recent studies have shown that the

230   species *S. equorum* represented 49% of staphylococcal isolates from French naturally

231   fermented sausages (Corbière Morot–Bizot et al., 2006), 10 to 40% from southern

232   Italian sausages (Blaiotta et al., 2004; Mauriello et al., 2004), 23.5% from fresh

233   sausages (Rantsiou et al., 2005) and in 28.5% from food–related samples (Coton et al.,

234   2010). It is probable that the presence of *S. equorum* in fermented food has been under–

235   estimated for a long time because of its confusion with *S. xylosus* by phenotypical

236   methods (Meugnier et al., 1996; Blaiotta et al., 2004; Mauriello et al., 2004). The

237   development of molecular methods for the identification of staphylococci underlines the

238   significance of this species, initially isolated from healthy horses (Scheleifer et al.,

239   1984).

240

241   *3.2. Use of recA gene sequence for the identification and discrimination of S. equorum*

242   *strains*

243

244   As reported above, 40 *S. equorum* strains were misidentified as *S. xylosus* by

245   using biochemical methods, indicating that there are difficulties in the differentiation of

246   both staphylococcal species. Only a small number of key tests can help to discriminate

247   between both species: pigment production, anaerobic growth and growth at 30 ºC.

248   However, phenotypic discrimination between these two species remains difficult since

249   the presence or absence of these characteristics is not shared by more than 90% of the

250   strains belonging to each taxon (Kloos and Schleifer, 1984; Kloos, 1990). As the

251   incidence of *S. equorum* probably occurs with a major incidence to that already

252   reported, Blaiotta et al. (2004) described a rapid identification method for the

253   identification of *S. equorum* strains by a species–specific PCR assay targeting the gene

254   encoding the manganese–dependent superoxide dismutase (*sodA*). The primer set,

255   SdAEqF and SdAeqR, amplified a 193 bp internal *sodA* fragment only when DNA from

256   *S. equorum* was used as template. In order to validate the method in our laboratory, we

257   used the method on type strains representing the twelve species more often isolated

258   from meat products (Table 1) and also in eight of the *S. equorum* strains identified in

259   this study. As showed in Figure 1, there are several staphylococcal type strains that gave

260   a faint positive PCR amplification (*S. capitis* ATCC 27840[T], *S. carnosus* ATCC 51365[T],

261   and *S. cohnii* ATCC 29974[T]). It should be mention that an obvious and apparent

262   positive amplicon was only obtained in the *S. equorum* strains assayed (Figure 1). In

263   spite that this method was previously evaluated by using a total of 112 strains

264   representing 26 different species, and including 27 reference strains, and that we used

265   the same PCR conditions described by Blaiotta et al. (2004), in our laboratory the

266   method are not as specific as expected, and PCR optimization will be needed.

267   Moreover, the verification of the specificity of the PCR product by restriction

268   endonuclease analysis was suggested (Blaiotta et al., 2004). This unspecific result led us

269   to look for an alternative method for the detection of *S. equorum* strains.

270   It is well establish that DNA sequence could be used for differentiation and

271   phylogenetic analysis of bacterial strains. The main advantage of DNA sequencing–

272 based methods over DNA banding pattern methods is its high reproducibility because it

273 relies on unambiguous DNA sequences that can easily be stored in online databases and

274 compared among laboratories. The 16S rDNA gene is highly conserved among bacteria

275 and consequently, amplification and sequencing of the 16S rDNA gene is widely used

276 for identification and phylogenetic classification of prokaryotic species. However, the

277 interpretation of the 16S ribosomal DNA data may be complicated by the fact that

278 closely related species may have identical 16S rDNA sequences or, alternatively, that

279 divergent 16S rDNA sequences may exist within a single organism. Takahashi et al.

280 (1999) pointed out that closely related species of staphylococci could have nearly

281 identical 16S rDNA sequences, decreasing the discriminatory potential of this gene for

282 staphylococci. In this regard, the 16S rDNA sequence similarity has been shown to be

283 very high, 90 to 99%, in 29 *Staphylococcus* species (Kwok et al. 1999). *S. caprae* and *S.*

284 *capitis* cannot be distinguished by their 16S rDNA gene sequences (Taponen et al.

285 2006). Similarly some *Staphylococcus* taxa have the same 16S rDNA gene sequences in

286 variable regions V1, V3, V7, and V9, with identical sequences occurring in, e. g., *S.*

287 *vitulinus*, *S. saccharolyticus*, *S. capitis*, *S. aureus* and *S. caprae* (Taponen et al. 2006).

288   To solve this problem, it is possible to use alternative monocopy target

289 sequences which exhibit a higher divergence than those of the 16S rDNA. The choice of

290 appropriate genes may vary according to the species. Recently, partial sequencing of the

291 highly conserved and ubiquitous *hsp60* (Goh et al., 1996; Kwok and Chow, 2003), *tuf*

292 (Martineau et al., 2001) and *sodA* (Giammarinaro et al., 2005; Poyart et al., 2001) genes

293 had been found to be useful for identification and taxonomic classification of species of

294 the genus *Staphylococcus*.

295   The *recA* gene sequence analysis has been proposed as a method for inferring

296 relationships among very closely related species (Thompson et al. 2004; Payne et al.

297 2005; Rossi et al. 2006; Sepe et al. 2008). Previously, we have used this method to

298 identify closely related heterofermentative wine lactobacilli species (Rodríguez et al.

299 2007). In order to know if the partial sequence of the *recA* gene could be also an

300 adequate method for the identification of meat staphylococci, we used the previously

301 described degenerate primer RecA–up and RecA–down (Duwat et al., 1992). Simple

302 PCR and sequencing assays that utilize this single pair of primers were used to

303 characterize a 280 bp–long DNA internal fragment of the *recA* gene in 12

304 staphylococcal type strains. These primers amplified all the strains assayed, and, the

305 amplified fragments were subsequently sequenced. In addition, we compare the 16S

306 rDNA and *sodA* sequences from the same strains (Table 1 and Figure 2). Figure 2

307 shows the sequence identity matrix based on comparisons of the 16S rDNA, *sodA* and

308 *recA* gene sequences. Similarity values for 16S rDNA gene sequences among the

309 staphylococcal type strains analyzed ranged from 95 to 99% (Figure 2A). These values

310 are consistently higher than those from the *sodA* gene sequence (range 65–93%; Figure

311 2B). However, the similarity for the *recA* sequences ranged from 77 to 89% (Figure 2C)

312 indicating that *recA* sequencing is more discriminatory among the staphylococcal

313 species found in meat products as compared to the 16S rDNA or *sodA* sequences.

314 Results previously described have been shown that the identity of the 16S rDNA

315 sequence is very high (90–99%) in 29 staphylococcal species analyzed (Kwok et al.

316 1999); by contrast, the sequence similarity of the *hsp60* gene was 74 to 93% in 23

317 *Staphylococcus* species (Kwok et al. 1999), and for the partial *rpoB* gene sequence the

318 similarity was 72 to 94% in 29 staphylococcal species analyzed (Drancourt and Raoult,

319 2002). Therefore, *recA* sequencing showed higher discrimination than *hsp60* and the

320 *rpoB* genes among staphylococcal species commonly isolated from meat products.

321   We also showed the phylogenetic relationships of the staphylococcal species

322   based on the *recA* gene sequence (Figure 3). All the staphylococcal species were

323   unambiguously differentiated by the comparative analysis of the short fragment of the

324   *recA* gene. The phylogenetic relationships inferred from the partial *recA* sequence were

325   in agreement with those previously derived from 16S ribosomal DNA sequence. Based

326   on the neighbour joining tree created from the *recA* partial sequences, the most closely

327   related staphylococcal species to *S. equorum* species is *S. xylosus*. Figure 2 also shows

328   that *S. equorum* strains showed the higher similarity to *S. xylosus* strains: 98% identity

329   by the 16S rDNA, 92% by the *sodA* gene, and only 86% by the *recA* gene. Therefore, an

330   additional advantage of the partial *recA* sequencing is that these two closely–related

331   species are more discriminated by the *recA* gene than by the *sodA* gene or 16S rDNA.

332   These results demonstrate the usefulness of this method for rapid and accurate species

333   identification of staphylococcal meat isolates and confirm that the *recA* gene constitutes

334   an efficient alternative target sequence for differentiating closely related meat

335   staphylococcal species.

336   The usefulness and accuracy of any bacterial species identification system

337   depends on the distinction between intraspecific variation and interspecific divergence

338   in the selected gene. Some less conserved genes have often been used for bacterial

339   typing. Poyart et al. (2001) demonstrated that the *sodA* sequences of strains from the

340   same species isolated from food or clinical samples displayed less than 1.5% divergence

341   from the sequence of the corresponding type strain. Due to the sequence diversity

342   showed at the intra–species level by *recA* gene sequencing, the observed degree of

343   heterogeneity (0–2%) was used to know the intraspecies genetic diversity in

344   *Lactobacillus hilgardii* species (Rodriguez et al., 2007). In order to known the diversity

345   among *S. equorum* strains, we sequenced a 280 bp *recA* DNA fragment, excluding

346   regions of primer annealing, from ten *S. equrom* strains isolated from dry–cured ham

347   (IFIJ2, IFIJ14, IFIJ19, IFIJ21, IFIJ22, IFIJ28, IFIJ30, IFIJ42, IFIJ55, and IFIJ56). *S*

348   *equorum* strains presented eleven polymorphic sites, showing a 96–100% identity

349   among them. These polymorphic sites defined five different alleles of the *recA* gene

350   (Figure 4). Most of these alleles were represented just by one or two strains (alleles 1, 2,

351   4, and 5). However, allele 3 was present in six out ten strains (*S. equorum* IFIJ2, IFIJ19,

352   IFIJ28, IFIJ30, IFIJ55, and IFIJ56). Five alleles on only ten strains indicated a high

353   heterogeneity among the *S. equorum* strains isolated from dry–cured hams. This high

354   diversity among *S. equorum* strains has been previously described. Corbière Morot–

355   Bizot et al. (2006) detected only 8 PFGE profiles among 208 *S. equorum* isolates, and

356   Leroy et al. (2009) found 52 distinct PFGE profiles among 118 *S. equorum* strains or 28

357   PFGE profiles among 71 *S. equorum* strains (Leroy et al., 2010) collected from a small–

358   scale processing units manufacturing naturally dry fermented sausages.

359

360   In this work, we have demonstrated the usefulness of the partial sequence of the

361   *recA* gene as a confirmation method for the identification of meat staphylococcal

362   isolates. The use of *recA* analysis should not replace 16S analysis but should rather be

363   seen as a second confirmatory test for species differentiation. From a practical point of

364   view, as this method used the same primer set which has been successfully employed

365   for the identification of lactic acid bacteria, a food microbiology laboratory, could used

366   the same method for the reliable identification of lactic acid bacteria as well as

367   staphylococcal meat strains.

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525

526

527 Figure captions

528

529 Fig. 1. *S. equorum* specific PCR amplification of a *sodA* gene fragment in several

530 staphylococcal strains. (A) Amplification on type strains from the staphylococcal

531 species isolated frequently on meat products: *S. aureus* ATCC 12600^T (1), *S.*

532 *epidermidis* ATCC 14990^T (2), *S. capitis* ATCC 27840^T (3), *S. hominis* ATCC 27844^T

533 (4), *S. xylosus* ATCC 29971^T (5), *S. simulans* ATCC 27848^T (6), *S. carnosus* DSM

534 4600^T (7), *S. vitulinus* ATCC 51615^T (8), *S. cohnii* ATCC 29974^T (9), *S. sciuri* ATCC

535 29062^T (10), *S. equorum* ATCC 43958^T (11), and *S. hyicus* ATCC 11249^T (12). (B)

536 Amplification of several *S. equorum* strains isolated from dry-cured ham: *S. equorum*

537 ATCC 43958^T (1) (positive control), *S. xylosus* ATCC 29971^T (2) (negative control),

538 and *S. equorum* IFIJ2 (3), IFIJ14 (4), IFIJ21 (5), IFIJ28 (6), IFIJ30 (7), IFIJ42 (8),

539 IFIJ55 (9), and IFIJ56 (10). A 100 bp ladder marker was included on the middle of each

540 gel.

541

542 Fig. 2. Pairwise comparison of the 16S rDNA (A), *sodA* (B) and *recA* (C) gene

543 sequences from the *Staphylococcus* type strains analyzed in this study. The percentage

544 of identical nucleotides between genes is shown. The accession numbers of the analysed

545 sequences are indicated on Table 1.

546

547 Fig. 3. Phylogenetic tree based on the partial *recA* sequence and showing the

548 relatedness of the twelve staphylococcal type strains analyzed in this study. The

549 phylogenetic tree was constructed by the neighbour-joining method. The *recA* partial

550 sequences were obtained from the representative type strain of each species and

551 deposited on the EMBL database (Table 1).

552

553   Fig. 4. Polymorphic sites in the partial *recA* sequences of some *S. equorum* strains

554   analyzed in this study. The accession numbers for the five different alleles obtained are:

555   allele 1 (FN554822), allele 2 (FN554823), allele 3 (FN554824), allele 4 (FN554825),

556   and allele 5 (FN554826). Each of the sites where the sequence of one or more of the

557   genes differ is shown (only sites that differ are shown). Numbering of the polymorphic

558   sites (vertical format) is according to the position on the 280 bp *recA* DNA fragment

559   sequenced. The number of strains possessing each allele is indicated in parenthesis.

Table 1

Table 1

Table 1. Partial gene sequences from several *Staphylococcus* species

Staphylococcus type strain		Accession no.		
		16S rRNA	*sodA*	*recA*
S. aureus	ATCC 12600[T]	D83357	EU652773	FN554701
S. capitis	ATCC 27840[T]	FN554713	AJ343896	FN554702
S. carnosus	ATCC 51365[T]	AB009934	AJ343899	FN554703
S. cohnii	ATCC 29974[T]	D83361	AJ343902	FN554704
S. epidermidis	ATCC 14990[T]	D83363	AJ343906	FN554705
S. equorum	ATCC 43958[T]	AB009939	AJ343907	FN554706
S. hominis	ATCC 27844[T]	L37601	AJ343911	FN554707
S. hyicus	ATCC 11249[T]	D83368	AJ343913	FN554708
S. sciuri	ATCC 29062[T]	AJ421446	AJ343929	FN554709
S. simulans	ATCC 27848[T]	D83373	AJ343930	FN554710
S. vitulinus	ATCC 51615[T]	AB009946	AJ343931	FN554711
S. xylosus	ATCC 29971[T]	D83374	AJ343933	FN554712

Figure 1

Figure 1
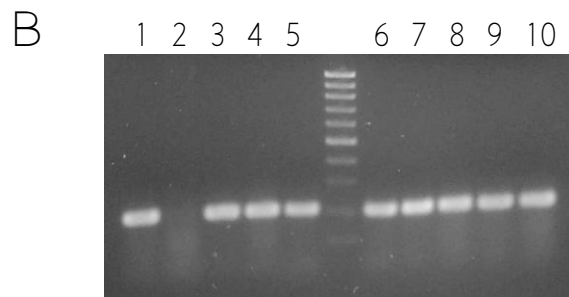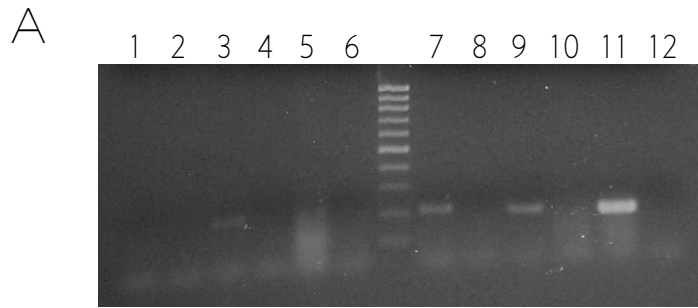
Figure 2

Figure 2

A

No.	Species	% identity with 16S rRNA gene no.											
		1	2	3	4	5	6	7	8	9	10	11	12
1	*S. aureus*	ID[a]											
2	*S. capitis*	98	ID										
3	*S. carnosus*	97	97	ID									
4	*S. cohnii*	97	97	97	ID								
5	*S. epidermidis*	98	99	97	97	ID							
6	*S. equorum*	97	96	96	98	97	ID						
7	*S. hominis*	97	97	97	97	98	97	ID					
8	*S. hyicus*	97	97	97	97	97	96	97	ID				
9	*S. sciuri*	97	96	96	97	97	96	97	96	ID			
10	*S. simulans*	96	96	97	97	97	96	96	96	96	ID		
11	*S. vitulinus*	96	96	96	96	96	96	96	96	99	95	ID	
12	*S. xylosus*	97	97	97	98	97	98	97	96	97	96	96	ID

B

No.	Species	% identity with *sodA* gene no.											
		1	2	3	4	5	6	7	8	9	10	11	12
1	*S. aureus*	ID[a]											
2	*S. capitis*	75	ID										
3	*S. carnosus*	75	82	ID									
4	*S. cohnii*	76	84	82	ID								
5	*S. epidermidis*	76	90	82	86	ID							
6	*S. equorum*	74	86	82	89	84	ID						
7	*S. hominis*	75	87	82	89	88	87	ID					
8	*S. hyicus*	71	82	77	78	80	77	80	ID				
9	*S. sciuri*	66	75	71	74	75	74	75	73	ID			
10	*S. simulans*	74	86	90	83	83	85	84	76	73	ID		
11	*S. vitulinus*	65	77	74	74	75	75	75	73	93	73	ID	
12	*S. xylosus*	74	86	81	91	84	92	88	78	74	83	76	ID

C

No.	Species	% identity with *recA* gene no.											
		1	2	3	4	5	6	7	8	9	10	11	12
1	*S. aureus*	ID[a]											
2	*S. capitis*	85	ID										
3	*S. carnosus*	86	85	ID									
4	*S. cohnii*	87	85	85	ID								
5	*S. epidermidis*	86	87	82	85	ID							
6	*S. equorum*	86	86	84	86	83	ID						
7	*S. hominis*	85	82	85	83	83	85	ID					
8	*S. hyicus*	82	81	80	81	82	82	81	ID				
9	*S. sciuri*	87	87	85	85	87	84	85	82	ID			
10	*S. simulans*	86	85	88	82	83	84	85	78	88	ID		
11	*S. vitulinus*	82	84	81	83	85	82	82	81	89	82	ID	
12	*S. xylosus*	83	83	83	84	85	86	84	77	83	85	80	ID

[a] ID, identical

Figure 3

Figure 3

Figure 4

Figure 4

```
                    Polymorphic site
                         position
                    ─────────────────
recA                00000111222
                    45569046018
                    10242959440
allele 1 (type)     TGCTGGAGGTT
allele 2 (1)        .......A...
allele 3 (6)        .ATAAATACCC
allele 4 (2)        CATAAATACCC
allele 5 (1)        CATAAATTCTC
```
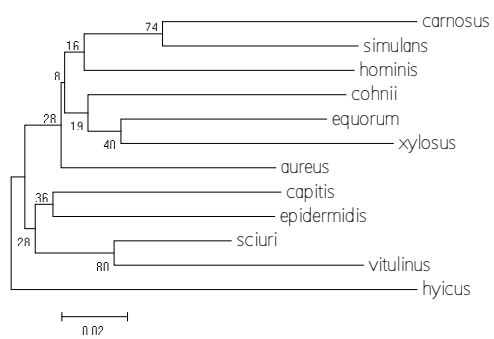
Research Highlights

1. Important differences were observed comparing identification of meat staphylococci by phenotypic and genotypic data.
2. *S. equorum* was the prevalent species identified by molecular methods.
3. Reliable identification of meat staphylococci could be achieved by means of *recA* gene sequence comparison.
4. *S. xylosus* and *S. equorum* strains could be more accurately discriminated by *recA* sequencing than 16S rDNA or *sodA* sequencing.