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3 *Streptococcus parauberis* associated with modified atmosphere packaged
4 broiler meat products and air samples from a poultry meat processing plant
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7 Joanna Koort^{a*}, Tom Coenye^b, Peter Vandamme^b, Johanna Björkroth^a
8

9 ^aDepartment of Food and Environmental Hygiene, Faculty of Veterinary Medicine,
10 P.O.Box 66, FIN-00014 University of Helsinki, Helsinki, Finland

11 ^bLaboratory of Microbiology, University of Gent, Gent, Belgium.
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20 *Corresponding author: Department of Food and Environmental Hygiene, Faculty of
21 Veterinary Medicine, P.O.Box 66, FIN-00014 University of Helsinki, Finland. Phone:
22 +358-9-19157119, e-mail: joanna.koort@helsinki.fi.

ABSTRACT

Lactic acid bacteria (LAB) isolated from marinated or non-marinated, modified atmosphere packaged (MAP) broiler leg products and air samples of a large-scale broiler meat processing plant were identified and analyzed for their phenotypic properties. Previously, these strains had been found to be coccal LAB. However, the use of a 16 and 23S rRNA gene RFLP database had not resulted in species identification because none of the typically meat-associated LAB type strains had clustered together with these strains in the numerical analysis of the RFLP patterns. To establish the taxonomic position of these isolates, 16S rRNA gene sequence analysis, numerical analysis of ribopatterns, and DNA–DNA hybridization experiments were done. The 16S rRNA gene sequences of three isolates possessed the highest similarities (over 99%) with the sequence of *S. parauberis* type strain. However, in the numerical analysis of HindIII ribopatterns, the type strain did not cluster together with these isolates. Reassociation values between *S. parauberis* type or reference strain and the strains studied varied from 82 to 97%, confirming that these strains belong to *S. parauberis*. Unexpectedly, most of the broiler meat-originating strains studied for their phenotypical properties did not utilize lactose at all and the same strains fermented also galactose very weakly, properties considered atypical for *S. parauberis*. This is, to our knowledge, the first report of lactose negative *S. parauberis* strains and also the first report associating *S. parauberis* with broiler slaughter and meat products.

KEYWORDS: *Sreptococcus parauberis*, MAP, Broiler meat, Lactose

1 INTRODUCTION

Approximately one-fourth of the food supply in the world is spoiled via microbes (Anonymous, 1985). Because the ability to spoil foods differs between, and even within, the bacterial species, the term “specific spoilage organism” (SSO) is used to describe the organisms typically spoiling certain products. In cold stored, vacuum or modified-atmosphere packaged (MAP) meat products, lactic acid bacteria (LAB) mainly from the genera of *Lactobacillus*, *Leuconostoc* or *Carnobacterium*, have been the predominant SSO (Borch *et al.*, 1996). Also in cold-stored, MAP, marinated broiler meat products, these genera usually dominate the microbial population in the end of shelf-life and cause spoilage (Susiluoto *et al.*, 2002; Björkroth *et al.*, 2000, 2005). During a study of developing spoilage LAB in marinated, MAP non-skinned broiler leg product, a group of unidentified gram-positive cocci was detected in fresh products (Björkroth *et al.*, 2005). In the numerical analysis of the 16 and 23S *Hind*III RFLP patterns, these isolates, named as Unidentified II (UII), clustered together but remained apart from the typically meat-associated LAB. Since these isolates possessed homofermentative glucose metabolism, they were considered to belong either to the genera of *Enterococcus*, *Lactococcus* or *Streptococcus*. More isolates with similar patterns were detected in air samples of a large-scale broiler meat processing plant and non-marinated, MAP broiler leg products of two large scale producers (Vihavainen *et al.*, submitted for publication). In this study, analysis of ribopatterns, 16S rRNA gene analysis, DNA-DNA hybridization and determination of phenotypic properties, was set to clarify the taxonomic position of these strains.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culturing. Thirteen isolates with similar *Hind*III –ribopatterns detected in the earlier studies were included in this study (Fig. 1). Nine of these were isolated in fresh (2 days after packaging), marinated MAP broiler leg products (Björkroth *et al.*, 2005), two in non-marinated MAP broiler leg products in the end of their shelf

lives and two in air samples from a broiler meat processing plant (Vihavainen *et al.*, submitted for publication). Three (332, 349 and 358) of these strains were selected to represent the different riboclusters in the 16S rRNA gene sequencing.

The selection of type and reference strains in this study was based on the results of 16S rRNA gene sequence similarities. The *Streptococcus parauberis* type and reference strains used were LMG 12174^T and its duplicate LMG 14376^T, LMG 12173^R and its duplicate LMG 14377^R. In addition, strains RM212.1 and RA149.1, isolated from diseased turbot (Romalde *et al.*, 1999) were kindly provided by Dr. Jesús L. Romalde (Departamento de Microbiología y Parasitología, Facultad de Biología, and Instituto de Acuicultura, Universidad de Santiago de Compostela, Spain). *Streptococcus uberis* type strain DSM 20569^T was also included.

All the strains were cultured at 25°C either overnight in MRS broth (Difco, BD Diagnostic Systems, Sparks, MD) or for 5 days on MRS agar plates (Oxoid, Hampshire, United Kingdom). The plates were incubated under anaerobic conditions (Anaerogen, Oxoid, 9-13% CO₂ according to the manufacturer). All isolates were maintained in MRS broth (Difco) at -70°C.

2.2. DNA isolation and 16S rRNA gene sequence analysis. Chromosomal DNA for all DNA-based analyses was isolated as previously described by Björkroth and Korkeala (1996).

For the sequencing, the nearly complete 16S rRNA gene was amplified by PCR with a universal primer pair F8-27 (5'-AGAGTTTGATCCTGGCTGAG-3') and R1541-1522 (5'-AAGGAGGTGATCCAGCCGCA-3'). Sequencing of the purified (QIAquick PCR Purification Kit, Qiagen, Venlo, Netherlands) PCR product was performed bidirectionally by Sanger's dideoxynucleotide chain termination method using primers F19-38 (5'-CTGGCTCAGGAYGAACGCTG-3'), F926 (5'-AACTCAAAGGAATTGACGG-3'), R519 (5'-GTATTACCGCGGCTGCTG-3') and R1541-1522. Samples were run in a Global IR2 sequencing device with e-Seq 2.0 software (LiCor, Lincoln, NE) according to the manufacturer's instructions. The consensus sequences of these strains (created with AlignIR software, LiCor) and representative strains belonging to the same phylogenetic group (retrieved from GenBank, <http://www.ncbi.nlm.nih.gov>, using BLASTN 2.2.6, Altschul *et al.* (1997) were aligned and a phylogenetic tree was constructed using the

neighbour-joining method and BioNumerics 3.5 software package (Applied Maths, Sint-Martens-Latem, Belgium).

2.3. DNA–DNA hybridization. Strains used in these studies are shown in the Table 1. DNA–DNA hybridizations were performed with photobiotin-labeled probes in microplate wells as described by Ezaki *et al.* (1989) using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 35 °C in 50% formamide.

2.4. Ribotyping. *Hind*III and *Eco*RI enzymes were used for the digestion of DNA as specified by the manufacturer (New England Biolabs, Beverly, MA). Restriction enzyme analysis was performed as described previously (Björkroth and Korkeala, 1996) and Southern blotting was made using a vacuum device (Vacugene, Pharmacia). The cDNA probe for ribotyping was labeled by reverse transcription (AMV-RT, Promega and Dig Labelling Kit, Roche Molecular Biochemicals, Mannheim, Germany) as previously described by Blumberg *et al.* (1991). Membranes were hybridized at 58 -58°C overnight and the detection of the digoxigenin label was performed as recommended by Roche Molecular Biochemicals.

2.5. Numerical analyses of ribopatterns. Scanned (Hewlett Packard Scan Jet 4c/T, Palo Alto, CA) ribopatterns were analyzed using the BioNumerics 3.5 software package. The similarity between all pairs was expressed by the Dice coefficient correlation and UPGMA clustering was used for the construction of the dendrograms. Based on the use of internal controls, position tolerance of 1.5% was allowed for the bands.

2.6. Phenotypical tests. All isolates were Gram stained. For the phenotypical tests, eight strains (LMG 12174^T, LMG 12173^R, 332, 349, 358, 366, RM212.1 and RA149.1) were selected to represent different riboclusters and origins. Growth at different temperatures (4, 10, 37 and 40 °C) or in the presence of NaCl (2, 4 and 6.5 % w/v) was tested in MRS broth (Difco) incubated until growth was observed or otherwise at least for 21 days. Isolates were tested for their carbohydrate fermentation profiles by API 50 CHL (bioMérieux) and for other biochemical activities by API STREP identification systems (bioMérieux) according to the manufacturer's instructions. Haemolyses were tested on blood agar. Each test was carried out at least twice.

3. RESULTS

3.1. 16S rRNA gene sequence comparison and DNA–DNA hybridization studies.

In the BLAST analysis, the 16S rRNA gene sequences of the isolates 332, 349 and 358 possessed the highest similarities (99.6, 99.9 and 99.8 %, respectively) with the corresponding sequence of *S. parauberis* DSM 6631^T(AY584477). Figure 2 shows the distance matrix tree based on the 16S rRNA gene similarities, the accession numbers of 16S rRNA gene sequences used/deposited to GenBank are also shown in the figure. Table 1 presents the DNA–DNA hybridization results. Reassociation values between *S. parauberis* type (LMG 14376) or reference (LMG 12173) strain and the strains originating from poultry and turbot varied from 82 to 97%.

3.2. Numerical analyses of ribopatterns. Fig. 1 shows the dendrograms and banding patterns based on *Hind*III (Fig. 1a) and *Eco*RI (Fig. 1b) digestions, together with the dendrogram obtained by combining the unweighted pattern information of both *Hind*III and *Eco*RI ribotypes into one numerical analysis (Fig. 1c). In the numerical analysis of *Hind*III ribopatterns, the unknown strains were located into one cluster with three subclusters including also the *S. parauberis* reference strain (LMG 12173 and its duplicate LMG 14377) and the two strains (RA149.1, RM212.1) from turbot. The pattern similarity level within these clusters was 82%. *S. parauberis* type strain LMG 12174 and its duplicate LMG 14376 possessed identical patterns and was separated from the *S. parauberis* cluster by a pattern similarity level of 53%. However, in the *Eco*RI and combined *Hind*III/*Eco*RI pattern analyses, the unknown strains, *S. parauberis* reference strains and the two strains from turbot clustered together with *S. parauberis* type strain (pattern similarities 69 and 70%, respectively).

3.3. Phenotypic properties. All isolates were Gram-positive cocci. All the isolates studied for their phenotypical properties grew at 10 and 37°C; none grew at 40°C. Strains RM212.1, RA149.1 and LMG 12173 grew also at 4°C, although the growth of strain LMG 12173 was slower than at the other temperatures. All these isolates grew in the presence of 2 and 4% NaCl but none in the presence of 6.5% NaCl. On blood agar, α -haemolysis was detected.

Basically, all strains showed carbohydrate fermentation profiles and biochemical activities typical to *S. parauberis* (Williams and Collins, 1990). However, the positive reactions were sometimes weak or delayed, especially with amygdalin, arbutine, galactose, mannitol, ribose, salicine and sorbitol. Unlike *S. parauberis* usually does, strains 332, 349 and 366 did not produce acid from lactose and only strain LMG 12174 reacted positively in the test for alkaline phosphatase.

4. DISCUSSION

The results of the present study clearly show that the formerly unidentified LAB strains detected in the MAP broiler meat products and broiler meat processing plant air belong to *S. parauberis* species.

Formerly, *S. parauberis* was not distinguished from *S. uberis* species. On the basis of DNA-hybridization studies, *S. uberis* was first divided into genotypes I and II (Garvie and Bramley, 1979; Collins *et al.*, 1984) and then, in 1990, the genotype II was classified as a separate species and nominated as *S. parauberis* (Williams and Collins, 1990). However, the differentiation between *S. uberis* and *S. parauberis* can still be problematic. Especially the conventional microbiological methods are not reliable, and the identity of clinical isolates is mainly expressed as “*S. uberis/S. parauberis*” in veterinary diagnostics. Therefore, both *S. parauberis* and *S. uberis* have been regarded as pathogens causing bovine mastitis, but their relative incidence is usually unknown. To overcome this problem, several molecular based methods (Bentley *et al.*, 1993; Harland *et al.*, 1993; Riffon *et al.*, 2001; Alber *et al.*, 2004) have been developed to differentiate within *S. uberis* and *S. parauberis*. By the means of these new techniques, *S. parauberis* has shown to be far more uncommon in bovine mastitis than previously thought (Bentley *et al.*, 1993; Alber *et al.*, 2004). However, controversial to the role in mastitis, its significance for pisciculture has increased. In 1996, *S. parauberis* was the first time reported causing streptococcosis in cultured turbot (Doménech *et al.*, 1996). Since that, it has caused several outbreaks in fish farms and severe economical losses at least in Spain (Romalde *et al.*, 1999).

Even though there are some taxonomy and population studies dealing with *Streptococcus* species in poultry (Cox *et al.*, 1983; Devriese *et al.*, 1991; Messier *et al.*, 1993; Turtura and Lorenzelli 1994; Kurzak *et al.*, 1998; Collins *et al.*, 2002), *S. parauberis* has not been detected in poultry and this is, to our knowledge, the first report of its association with poultry meat products and their processing environment. The habitats of *S. parauberis* may be more diverse than previously considered because sequences possessing high similarity with its 16S rRNA gene have been detected in swine manure by a culture-independent PCR approach (Whitehead and Cotta, 2004).

Even though two strains of this study originate from late shelf life broiler meat products, the definite role of *S. parauberis* in spoilage of these products remains unclear. In previous studies, lactococci have been the only homofermentative cocci associated with spoiled meat products (Barakat *et al.*, 2000; Sakala *et al.*, 2002). *S. parauberis* did not predominate the population in modified atmosphere packaged broiler legs (Björkroth *et al.*, 2005), but almost all strains studied (except LMG 12174^T) were able to grow at 10°C, and some (RM212.1, RA149.1 and LMG 12173^R) even at 4°C clearly showing the psychotropic nature of these strains. This variability in the growth temperatures was not reported in the original species description (Williams and Collins, 1990).

Diversity not reported before was also seen in the utilization of lactose when only the strains of bovine origin, LMG 12174^T and LMG 12173^R, fermented it clearly. The strains of other origins fermented lactose only weakly or not at all. The non-lactose-fermenting strains (332, 349 and 366) were also poor in fermenting galactose. The utilization of galactose is known to often be linked to that of lactose also through the gene-level operation systems. At least in the LAB having the PEP-PTS (phosphoenolpyruvate-phosphotransferase), a lactose transport system leading to the Embden-Meyerhof sugar metabolism pathway, the genes for enzymes needed in the efficient utilization of galactose via the tagatose 6P- pathway are also included in the PTS coding lac operon. The lack or impaired function of this operon leads to the inability to metabolize lactose, and may also convert the galactose metabolism from Embden-Meyerhof to far less efficient Leloir pathway (Jagusztyn-Krynicka *et al.*, 1992; de Vos and Vaughan, 1994; Chen *et al.*, 2002; Vaughan *et al.*, 2003). The existence of PEP-PTS in *S. parauberis* is unknown and requires further studies of the metabolism of the species.

Our study shows that *S. parauberis* strains possess more varied characteristics than previously considered. Not only were they associated with a new habitat but some strains were psychotropic and non-lactose utilizing. Further studies with higher numbers of strains of different origins are needed to evaluate the variability within *S. parauberis* and the possible link between these newly described traits and the ecology of this species.

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FIGURE CAPTIONS

Figs. 1. (a), (b) and (c) present numerical analysis of 16 and 23S RFLP patterns (ribotypes) generated by HindIII, EcoRI and an analysis combining the information of both restriction enzymes, respectively. Numerical analyses of the patterns are presented as dendrograms, left side of the HindIII and EcoRI banding patterns possesses high molecular weights, < 23 kbp, and right side >1000 bp.

Fig. 2 Phylogenetic tree based on homologies of almost entire 16S rRNA gene sequences (at least 1400 bp) of *Streptococcus parauberis* and its phylogenetic neighbours. Bootstrap probability values from 500 trees resampled are given at the branch points.

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Table 1. Results of DNA-DNA reassociation experiments.

Strain	DNA-DNA reassociation % with						
	LMG 12173 ^R	LMG 14376 ^T	358	332	RA149.1	RM212.1	366
LMG 14376	91	100					
358	82	85	100				
332	82	ND ^a	86	100			
RA149.1	94	94	85	94	100		
RM212.1	97	ND	ND	ND	97	100	
366	85	ND	ND	ND	85	86	100
349	86	ND	90	76	85	85	99

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359 LMG 12173^R, *S. parauberis* reference strain; LMG 14376^T, *S. parauberis* type strain; ND ^a, not determined.

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