Food Microbiology 54 (2016) 142–153

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Identification, enzymatic spoilage characterization and proteolytic activity quantification of *Pseudomonas* spp. isolated from different foods

L. Caldera [a, b], L. Franzetti [a], E. Van Coillie [b, *], P. De Vos [c], P. Stragier [c], J. De Block [b], M. Heyndrickx [b, d]

[a] DeFENS, Department of Food, Environmental and Nutritional Science, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy
[b] Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Brusselsesteenweg 370, 9090 Melle, Belgium
[c] Laboratory of Microbiology, Ghent University, K.L. Ledeganckstraat, 35, 9000 Ghent, Belgium
[d] Department of Pathology, Bacteriology, and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
10 September 2015
Accepted 12 October 2015
Available online 22 October 2015

Keywords:
Pseudomonas spp.
Identification
Enzymatic activity
Protease
aprX gene

ABSTRACT

Sixty-six putative *Pseudomonas* strains isolated from different food matrices (ready-to-eat vegetables, meat, milk and dairy products) were examined for their phenotypic features and enzymatic spoilage activities. Their genotype was studied by BOX-PCR, *Pseudomonas* specific 16S PCR, *aprX* and housekeeping genes sequencing (16S rRNA gene, *gyrB* and *rpoB*). The majority of the isolates are very versatile as shown by their wide ranges in growth temperature (4–45 °C), pigment production and production of enzymes. The BOX-PCR clustering showed a high genetic diversity among the isolates and phylogenetic analysis of the *rpoB* gene allowed a first putative identification at the species level. Thirteen isolates were provisionally classified as *Pseudomonas gessardii*-like, but probably belong to a yet unknown *Pseudomonas* species in the *Pseudomonas fluorescens* group.

Protease-activity was qualitatively and quantitatively verified. A large variation in proteolytic activity measured in UHT-milk was observed amongst the protease positive isolates. Several isolates provisionally classified as *P. gessardii*-like showed the highest activities. An *aprX* gene based phylogenetic dendrogram showed five different groups and two sub-groups, for which a correlation with the matrix of origin could be demonstrated. An insertion of 15 bp was observed in the *aprX* gene sequences of isolates of mainly dairy origin.

1. Introduction

The genus *Pseudomonas* comprises a heterogeneous group of microorganisms of the Pseudomonadaceae. It includes ubiquitous Gram negative, aerobic, non-fermentative, catalase and oxidase positive, mesophilic and psychrotolerant, non-spore forming rods. For identification of *Pseudomonas*, comparative 16S rRNA gene sequence analysis is used as taxonomic frame, but the high conservative nature of ribosomal genes makes it not suitable for *Pseudomonas* species differentiation (Janda and Abbott, 2007; Mellmann et al., 2008). Sequencing of several less conservative housekeeping genes was gradually included to obtain a more discriminatory phylogenetic evaluation of *Pseudomonas* isolates (Ait Tayeb et al., 2005; Mulet et al., 2010; Yamamoto and Harayama, 1995).

Pseudomonas members are adapted to various conditions and therefore are found in a wide range of niches as soil, water, plants, animal tissues, foods, etc. (Franzetti and Scarpellini, 2007) thanks to their complex enzymatic systems. Members of this genus are frequently implicated in the degradation and spoilage of a wide range of foods deriving from plants or animals. It is important to notice that many enzymes produced by *Pseudomonas* members, in particular the proteases, are heat-resistant; that results in consolidation of activity also after heat treatment processes used to eradicate microorganisms in certain matrices. This phenomenon is very important in UHT milk and dairy products in which the protease can cause coagulation and instability phenomena,

* Corresponding author. Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Brusselsesteenweg 370, 9090 Melle, Belgium.
E-mail address: Els.VanCoillie@ilvo.vlaanderen.be (E. Van Coillie).

http://dx.doi.org/10.1016/j.fm.2015.10.004
0740-0020/

respectively (Datta and Deeth, 2001).

The mechanisms of these spoilage processes are not completely known but are strain dependent and related to temperature and environmental conditions (Chabeaud et al., 2001; Woods et al., 2001; Nicodème et al., 2005). However, it is known that an important protease responsible for spoilage is an extracellular alkaline metallo-protease belonging to the AprX protein family, which has been extensively studied especially in *Pseudomonas aeruginosa* (Okuda et al., 1990; Duong et al., 2001; Blevesa et al., 2010). The *aprX* gene, which encodes this protein, is located in an operon that contains also the *lipA* gene, coding for a lipase, a protease inhibitor (*inh*), a secretion system (*aprDEF*) and two autosecreted serine-protease homologues (*prtAB*) (Woods et al., 2001). In previous work, it has been shown that *apr*X sequences from milk pseudomonads are heterogeneous and can be classified in four different groups and that AprX mediated proteolytic activity can vary widely among species and strains (Marchand et al., 2009a, b).

The aims of this work are *i)* a tentative molecular identification and enzymatic and phenotypic characterization of *Pseudomonas* isolates from different food products (ready-to-eat vegetables, meat, milk and dairy products); and *ii)* a study of the protease-activity of the isolates, in terms of quantification of the extracellular proteolytic activity, and genetic heterogeneity of the *aprX* gene. This information will help in identifying the most important *Pseudomonas* species and their spoilage enzymes in different food products, in particular AprX, which can cause spoilage in cold stored food products.

2. Materials and methods

2.1. Microbial isolates

A total of 66 isolates of putative *Pseudomonas* spp. were examined for phenotypic and genotypic characteristics. Sixteen of these were isolated between 1996 and 2005 (Franzetti and Scarpellini, 2007) and were obtained from the collection of freeze-dried cultures kept in the food microbiology laboratory of DeFENS, Milan; the others were isolated between 2009 and 2013 from different food matrices during their declared shelf life in refrigerated conditions. The origin of the isolates was: 24 from ready-to-eat vegetables, 10 from meat products (patty and speck), 23 from dairy products (soft cheese and mozzarella), 9 from milk. Seven type and reference strains, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH were included as well. All the strains are reported in Table 1. They were maintained on Tryptic Soy Agar (TSA) slopes at 5 °C and frozen at −24 °C in Tryptic Soy Broth (TSB) with 20% of glycerol.

2.2. Identification of the isolates

2.2.1. Preliminary classification

Each isolate was observed by optical microscopy for its morphology, motility (480×), and Gram stain (1200×). The preliminary biochemical characterization was based on the catalase and oxidase tests, and the oxidative/fermentative metabolism (OF test, Hugh and Leifson, 1953).

2.2.2. Molecular identification

Total DNA of each strain was extracted as described by Mora et al. (2000). If not specified otherwise, the different PCR assays were performed in a mixture of 25 μl containing approximately 50 ng of bacterial genomic DNA, 2.5 μl of 10 × PCR reaction buffer (Amersham Pharmacia Biotech, Italy), 200 μM of each dNTP, 2 mM of $MgCl_2$, each primer (Table 2) at a concentration of 0.5 μM and 0.5 U of Taq Polymerase (Amersham Pharmacia Biotech). After amplification, 8 μl of each sample was subjected to gel electrophoresis on 2% Seakem LE agarose gel in 0.5 × Tris-Acetate-EDTA buffer, at 100 V for 30 min, and the banding pattern was visualized under ultraviolet light after 15 min staining in ethidium bromide (EtBr) (2 μg ml^{-1}).

2.2.2.1. BOX-PCR. The amplification was conducted in a mixture of 25 μl containing 50 ng genomic DNA, 5 μl of 5 × Gitschier − buffer (83 mM $(NH_4)_2SO_4$, 335 mM Tris−HCl pH 8.8, 33.5 mM $MgCl_2$, 33.5 μM EDTA and 150 mM ß-mercapto-ethanol), 1.28 mM of each dNTP, 0.4 μl of BSA (10 mg/ml; Roche, Switzerland), 2.5 μl DMSO (100%; Sigma−Aldrich, Italy), BOX-A1R primer at a concentration of 1 μM and 1.5 U of Red Diamond Taq (Eurogentec, Belgium). The thermal program was 7 min at 95 °C; 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 65 °C for 8 min; final extension at 65 °C for 16 min. Four μl of each amplicon was subjected to agarose (1.5% Seakem LE agarose) gel electrophoresis (120 V for 4 h at room temperature) in 1 × Tris-Borate-EDTA buffer and the banding pattern was visualized after EtBr staining (30 min). The resulting fingerprints were analysed by BioNumerics 6.6 software package (Applied Maths Inc., Sint-Martens-Latem, Belgium). Similarities were calculated using Pearson correlation and used for clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. Based on in house experience 60% similarity was used as intra-species delineation level.

2.2.2.2. Allocation of the isolates at the genus level. In order to confirm the allocation of the isolates to the genus *Pseudomonas*, two 16S rRNA segments were considered. Firstly a *Pseudomonas* specific 16S rRNA gene fragment (Calisti, 2008) was amplified with the following thermal program: 2 min at 94 °C; 35 cycles of 95 °C for 10 s, 62.4 °C for 30 s, 72 °C for 30 s; final extension at 72 °C for 7 min. Secondly a universal 16S rRNA portion was amplified using universal primers (Lane et al., 1985) and the following thermal program: 2 min at 94 °C; 5 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min; 35 cycles of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min and final extension at 72 °C for 2 min. The obtained amplicons were sequenced with 16S forward primer using a 310 automatic DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the sequences were elaborated by Chromas 2.13 software (Technelysium Pty Ltd. Helensvale, Queensland, Australia).

2.2.2.3. RpoB analysis. For *rpoB* amplification, the following thermal profile was used: 1.5 min at 94 °C, 32 cycles of 94 °C for 10 s, 50 °C for 20 s, 72 °C for 50 s and final extension at 72 °C for 5 min. Sequencing was performed with LAPS primer using a ABI 3730 XL DNA Sequencer (Applied Biosystems) and the sequences were elaborated by Kodon 3.6 software (Applied Maths Inc.).

2.2.2.4. GyrB analysis. For *gyrB* amplification the following program was used: 2 min at 94 °C; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 7 min. The obtained amplicons were sequenced with UP1S primer using a ABI 310 Automated DNA Sequencer (Applied Biosystems) and Chromas 2.13 software (Technelysium Pty Ltd., Helensvale) was used to analyse the sequences.

2.2.2.5. Putative identification based on molecular data. All available sequence data of type strains of *Pseudomonas* species were either retrieved from NCBI sequence database or from the in house LMG (Lab. of Microbiology, Ghent University) database for 16S rRNA, *gyrB* and *rpoB* genes. For 16S rRNA, pairwise comparison was conducted using BLAST against the NCBI database with a cut-off level of 98%. *RpoB* sequences or alternatively *gyrB* sequences