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Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers

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ABSTRACT

The functional diversity of a population of sixty-five different strains of *P. fragi* isolated from fresh and spoiled meat was studied in order to evaluate the population heterogeneity related to meat spoilage potential. The strains were characterized for the proteolytic activity at 4 °C on beef sarcoplasmic proteins and only 9 strains were found to be proteolytic. An iron-dependent growth behaviour was shown when each strain was grown in citrate medium containing either myoglobin, haemoglobin or iron chloride as iron sources. Increase of maximum population and μ_{max} in presence of different iron sources was registered. The release of volatile organic compounds (VOC) by each strain in beef during aerobic storage at 4 °C was evaluated by GC–MS. A considerable variability of occurrence of each molecule in the GC–MS profiles obtained by the different strains was observed ranging from 3% to 79% although the strains showed a high degree of similarity. In particular, ethylhexanoate, ethyloctanoate, ethylnonenoate, ethyldecanoate, 1-octen-3-ol, 3-octanone, 4-methylthiophenol, and 2-pentylfurane were produced by more than 50% of the strains. Representative strains were used to spoil meat in the same conditions used for the VOC analysis and the samples were evaluated by a sensory panel. The results of the sensory analysis indicated that the different strains could significantly affect the odour of meat and strains characterized by production of esters gave fruity odours to the spoiled meat. However, the similarity of strains based on the sensory profiles does not necessarily match the similarity shown in VOC profiles. *P. fragi* has a significant role in the microbial ecology of meat and the influence of meat-related sources of iron on the growth behaviour of many different strains suggests that meat can be an ecological niche for *P. fragi*. Regardless of the proteolytic and lipolytic capacities shown *in vitro*, different molecular types of *P. fragi* can release odour active volatile molecules and play a similar overall role as spoilage agents of meat.

1. Introduction

Pseudomonas fragi is a psychrotrophic, Gram-negative bacterium that is able to grow at temperatures ranging between 2 °C and 35 °C. This species of *Pseudomonas* is often used as source of lipases (Pabai et al., 1995; Tran and Crout, 1998). The esterases produced by *P. fragi* have been used in the biotechnological production of fruity aromas for the development of flavour in a wide variety of food products (Lamer et al., 1996). *P. fragi* can play a role as a direct plant growth promoter for the production of indole acetic acid and solubilization of phosphate (Selvakumar et al., 2009), as corrosion inhibitor of steel surfaces (Jayaraman et al., 1998) and potential agent of bioremediation (Adelowo et al., 2006). However, the food environment is the most acknowledged source of this microorganism where it significantly contributes to spoilage processes. *P. fragi* is involved in the spoilage of fresh food products stored in refrigerated conditions. Traditionally recognized as milk spoiler (Pereira and Morgan, 1957; Wiedmann et al., 2000; Marchand et al., 2009a), it has been lately recognized as one of the principal agents of meat spoilage (Labadie, 1999) and is very frequently isolated from fresh and spoiled meat products (Ercolini et al., 2007, 2009a; Arnaut-Rollier et al., 1999). The fresh meat environment is a particularly suitable substrate for the growth of *P. fragi*. Beyond the nutritional value of meat components, this psychrotrophic species is favored by the strict chill chain applied to the fresh meat production from slaughtering through portioning until distribution is completed.

Spoilage can be defined as any change in a food product that makes it unacceptable to the consumer from a sensory point of view (Gram et al., 2002). Apart from physical damage, oxidation and color change, the other spoilage symptoms are ascribable to undesired growth of microorganisms to unacceptable levels and also to the activity of endogenous enzymes. In the case of meat, microbial spoilage leads to development of off odours and often slime formation, which make the product undesirable for human consumption (Jackson et al.,

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1997). The development of organoleptic spoilage is related to microbial consumption of meat nutrients, such as sugars and free amino acids and the release of undesired volatile metabolites. Therefore, the deterioration depends on the contaminating microbial populations and storage conditions. The microbial populations associated with the meat environment are known as belonging to the groups of *Enterobacteriaceae*, lactic acid bacteria, *Brochothrix thermosphacta* and pseudomonads (Borch et al., 1996; Labadie, 1999; Ercolini et al., 2006).

Unlike other environments, the meat ecosystem is rarely studied at strain level and therefore it is often unknown how homogeneous or diverse are biotypes of the same species. Such information can be important in assessing the populations responsible for meat spoilage and to better know the species in order to handle their contamination and prevent their growth and spoilage activity by using the most appropriate storage conditions. Among the pseudomonads, *P. fragi* is by far one of the most threatening species in the spoilage of meat and the study of its diversity, ecology and spoilage behaviour in meat at low temperatures is of utmost importance to understand its actual role in the meat environment as a first step for a well thought out control of spoilage dynamics. The current available studies on the development of microbial populations in meat are either merely based on viable counts or focus on one or few species occurrence and dynamics. There is a complete lack of information on the diversity and spoilage related activities of *Pseudomonas* populations usually developing in meat, including a considerable number of strains of the same species. Do different strains of the same species have the same spoilage behaviour while growing in meat? How heterogeneously can a population of biotypes of *P. fragi* behave in the meat environment? In addition, a better knowledge on the spoilage development by certain species needs to be investigated *in situ*.

The aim of this work was to investigate the molecular diversity and the spoilage-related activities of a population of *P. fragi* strains isolated from fresh and spoiled meats in order to understand how functionally close the members of the same species are in matter of spoilage potential in the complex meat environment.

2. Materials and methods

2.1. P. fragi isolation and identification

Thirteen beefsteak, 11 pork steak and 12 chicken breast samples were the isolation sources of the *P. fragi* strains used in this study. The samples were collected in local butcher shops within 1 month of the same winter season (2007). Each meat sample was analyzed soon after the purchase and after 8 days of aerobic storage at 4 °C, when the meat presented objective signs of spoilage. Samples (25 g) were homogenized in quarter strength Ringer's solution (Oxoid). Decimal dilutions were prepared and plated in triplicate on Pseudomonas Agar with cetrimide-fucidin-cephaloridine (CFC) selective supplement (Oxoid); the plates were incubated at 25 °C for 48 h. After performing viable counts, colonies present on the countable plates were randomly isolated and purified on *Pseudomonas* agar plates. The purified isolates were preliminary characterized by microscopic observations and Gram, catalase and oxidase reactions. Working cultures were maintained in Tryptone Soy Broth (TSB, Oxoid) with 25% glycerol at −20 °C.

DNA extraction from 264 isolates was carried out from a loopful of grown culture on PCA according to Marmur (1961); after the extraction, the DNA was quantified by using the Nanodrop 1000 (Thermo Scientific, Milano, Italy). The identification was performed by a previously described species-specific PCR assay targeting the *carA* gene (Ercolini et al., 2007) by using only the primer couple for the identification of *P. fragi*. DNA from *P. fragi* DSM3456 was used as positive control.

2.2. Molecular typing by RAPD-PCR and rep-PCR and cluster analysis

Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was performed by using the primer XD5 (5′-CTGGCGGCTG-3′) previously shown to have a good discriminatory potential for strains of the same species of both yeast and bacteria (Di Maro et al., 2007; Ercolini et al., 2008, 2009a,b). Further fingerprinting was obtained by using repetitive extragenic palindromic PCR (rep-PCR) with the primer BOXA1R (CTACGGCAAGGCGACGCTGACG) (Versalovic et al., 1994) and the couple of primers REP1R-I (IIIICGICGICATCIGGC) and REP2-I (ICGICTTATCIGGCCTAC) (Versalovic et al., 1991). The molecular typing was only performed for 103 strains identified as *P. fragi* as above described. PCR reactions were carried out in 25 µl of reaction mix containing 1× Taq Polymerase-Buffer (Invitrogen, Milano, Italy), 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.4 mM, 1.00 µM of the primer, 2.5 U of Taq Polymerase and 20 ng of the extracted DNA. PCR was carried out in a programmable heating incubator using an initial denaturation step at 94 °C for 1 min followed by 40 cycles of 1 min at 94 °C, 1 min of annealing at a specific temperature, and 2 min at 72 °C/cycle. Finally, a 7 min extension period at 72 °C was performed. The annealing temperatures were 31 °C for XD5, 50 °C for BOX and 40 °C for REP primers.

The amplified products were resolved by electrophoresis on 1.5% (w/v) agarose-TBE at 7 V/cm for 2.5 h. 1 kb DNA Ladder Plus (Invitrogen) was used as molecular weight marker. The gels were acquired using a gel doc apparatus (Bio-Rad).

A database of fingerprints was created by using the software Bionumerics version 5.1 (Applied Maths, Sint Marten Latem, Belgium). A combined data matrix of all the fingerprints obtained using the 3 different PCR conditions was obtained and a dendrogram of similarity was retrieved by using the Dice coefficient and unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992). On the basis of the molecular typing 65 strains were selected as representative of the various similarity groups and used for further characterization.

2.3. Proteolytic and lipolytic activity and screening for the presence of the aprX gene

Beef sarcoplasmic proteins were extracted as described by Mauriello et al. (2002). The proteolytic activity of the 65 *P. fragi* strains was determined according to Villani et al. (2007).

The lipolytic activity was tested on Spirit Blue Agar plates supplemented with a mixture of olive oil and Tween 80 according to the supplier's instructions (Sigma, Milano, Italy) and using *Staphylococcus aureus* ATCC25923 as positive control. Duplicate plates were inoculated with streaks of the *P. fragi* strains and incubated for 3 days at 20 °C and 4 °C, respectively. After incubation, lipolytic activity was detected by the formation of a clear zone around the colony (Immanuel et al., 2008).

A PCR assay was performed using the DNA extracted from all the *P. fragi* strains for the assessment of the presence of genes encoding for alkaline metalloprotease. The PCR amplification for the detection of the *aprX* gene was carried out with primer and conditions recently described by Marchand et al. (2009b) and using DNA from *P. fluorescens* DSM50091 as positive control.

2.4. Growth in presence of different iron sources

In order to evaluate the effect of the presence of iron on the growth of *P. fragi*, the strains used in this study were grown in succinate medium (Meyer and Abdallah, 1978) and in succinate medium containing either 0.04% haemoglobin (Sigma), 0.04% myoglobin (Sigma) or 2 mM $FeCl_3$ according to. Strains were inoculated in triplicate in a 0.2 ml volume of each medium. Growth curves were