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Simultaneous Detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from Meat by Use of a Multiplex PCR Assay Targeting the *carA* Gene[▽]

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Species-specific primers and a multiplex PCR assay were developed for the simultaneous identification and differentiation of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* based on the coamplification of different portions of the small subunit of the carbamoyl phosphate synthase gene (*carA*). The *carA* multiplex PCR was used to detect the presence of the three *Pseudomonas* species from beef, chicken, and pork samples and proved to be effective in showing their evolution during the storage of meat.

Several species of the genus *Pseudomonas* are very often recognized as the principal causative agents of the spoilage of fresh foods stored aerobically. Members of the *Pseudomonas fluorescens* group, along with the psychrotrophic *P. fragi*, *P. lundensis*, and *P. putida*, are usually involved in spoilage of milk, meat, and fish, even during storage at low temperatures. These bacteria are often isolated from spoiled meat (10, 12, 18).

The molecular detection and identification of microorganisms is widely used in food microbiology. However, only limited information is now available on the molecular detection of spoilage bacteria, and the development of appropriate strategies for their rapid identification and monitoring is needed. Some molecular approaches, such as ribotyping, PCR amplification of the 16S-23S rRNA gene spacer region, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, have been exploited for the analysis of the diversity of *Pseudomonas* isolates from foods (4, 13, 19, 21).

The molecular identification of *Pseudomonas* is often difficult and controversial. The sequence analysis of the 16S rRNA gene is widely employed for the identification of bacteria; however, this region is not satisfactorily discriminating between the species of *Pseudomonas*. Phylogenetic studies have highlighted that inferred phylogenies based on the 16S rRNA gene lack resolution at the intrageneric level because of its low rate of evolution (1, 15, 20). In recent studies on the spoilage-related microbiota of beef, we also realized that it was difficult to achieve an unequivocal identification of *Pseudomonas* at the species level, even though variable regions of the 16S rRNA gene were analyzed (7, 17). Several authors have evaluated the use of alternative sequences for the identification and phylogenetic studies of *Pseudomonas* spp. For this purpose, the sequences of the *carA*, *recA*, *gyrB*, *fliC*, and *rpoD* genes of *Pseudomonas* species have been determined (2, 11, 20). The sequences of the carbamoyl phosphate synthase gene small subunit (*carA*) of several *Pseudomonas* spp. of environmental origin have been determined by Hilario et al. (11). However, the *carA* sequences for species of food interest, such as *P. fragi* and *P. lundensis*, were not considered.

In this study, the *carA* gene sequence was used as a target in order to design species-specific primers to selectively and simultaneously detect *P. fragi*, *P. lundensis*, and *P. putida* from meat.

***carA* gene sequencing and primer design.** The *Pseudomonas* strains used in this study are listed in Table 1. They were cultivated aerobically on nutrient agar (Oxoid, Milan, Italy) at 20°C and stored in nutrient broth with 20% glycerol at −20°C. DNA extraction was carried out from a loopful of grown culture on nutrient agar plates according to the method of Marmur (14).

The *carA* gene of *P. fragi* DSM3456 and that of *P. lundensis* DSM6252 were amplified by using primers and conditions reported by Hilario et al. (11); the PCR products (700 bp) were sequenced by using a deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). *carA* sequence accession numbers were determined for *P. fragi* DSM3456^T and *P. lundensis* DSM6252^T (see below). Sequence alignment between the determined *carA* genes and the previously sequenced *carA* genes (11) was performed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S.A., Olivet Cedex, France). The alignment is shown in Fig. 1, where the sequence heterogeneity used for a species-specific primer design is highlighted. Three different forward primers were designed for the specific amplification of fragments of the *carA* gene of *P. fragi*, *P. lundensis*, and *P. putida*. A specific identification of *P. fluorescens* would also be of interest in food microbiology. However, in our case it was impossible to design a species-specific probe targeting all the biotypes of *P. fluorescens* because they showed very high sequence variability within the sequence of the *carA* gene (Fig. 1).

Multiplex PCR amplification of the *carA* gene. Multiplex PCR amplifications were performed in a programmable heating incubator (MyCycler; Bio-Rad, Milan, Italy). Each mixture (final volume, 50 μl) contained 20 ng of each template DNA, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 5 μl of 10× PCR buffer (Invitrogen, Milan, Italy), and 2.5 U of *Taq* polymerase. The PCR condi-

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TABLE 1. *Pseudomonas* strains used in this study and identification by species-specific *carA* PCR assay

Species	Strain	Source[a]	Origin	Reference	*carA* PCR result for:		
					P. putida	*P. fragi*	*P. lundensis*
P. fragi	DSM3456^T	DSMZ			−	+	−
P. lundensis	DSM6252^T	DSMZ			−	−	+
P. putida	DSM291^T	DSMZ			+	−	−
P. aeruginosa	DSM50071^T	DSMZ			−	−	−
P. aeruginosa	DSM27853	DSMZ			−	−	−
P. agarici	DSM11810^T	DSMZ			−	−	−
P. chlororaphis	DSM50082	DSMZ			−	−	−
P. cichorii	DSM50259^T	DSMZ			−	−	−
P. costantinii	DSM16734^T	DSMZ			−	−	−
P. flavescens	DSM12071^T	DSMZ			−	−	−
P. fluorescens	DSM50091	DSMZ			−	−	−
P. fluorescens	DSM50108	DSMZ			−	−	−
P. fluorescens	DSM50415	DSMZ			−	−	−
P. marginalis	DSM13124^T	DSMZ			−	−	−
P. putida biotype A	DSM50208	DSMZ			+	−	−
P. putida biotype B	DSM50222	DSMZ			−[c]	−	−
P. stutzeri	DSM5190^T	DSMZ			−	−	−
P. syringae	DSM1241	DSMZ			−	−	−
P. syringae	DSM10604	DSMZ			−	−	−
P. fragi	PMK37	DTA	Cheese	16	−	+	−
P. lundensis	PMK52	DTA	Cheese	16	−	−	+
P. putida	PMK32	DTA	Cheese	16	−	−	−
Pseudomonas sp.	L1114	DFST	Fish	19	−	+	−
Pseudomonas sp.	L414	DFST	Fish	19	−	+	−
Pseudomonas sp.	L47	DFST	Fish	19	−	+	−
Pseudomonas sp.	L514	DFST	Fish	19	−	−	−
Pseudomonas sp.	L128	DFST	Fish	19	−	−	−
Pseudomonas sp.	L1110	DFST	Fish	19	−	−	−
Pseudomonas sp.	25P	DSA[b]	Meat		−	−	−
P. fragi	24P	DSA	Meat		−	+	−
P. fragi	26P	DSA	Meat		−	+	−
P. fragi	27P	DSA	Meat		−	+	−
Pseudomonas sp.	28P	DSA	Meat		−	−	−
Pseudomonas sp.	29P	DSA	Meat		−	−	−
P. fragi	30P	DSA	Meat		−	+	−
Pseudomonas sp.	33M	DSA	Meat		−	−	−
Pseudomonas sp.	39M	DSA	Meat		−	−	−
Pseudomonas sp.	1P2	DSA	Cheese		−	−	−
Pseudomonas sp.	6P2	DSA	Cheese		−	−	+
P. fluorescens	PSEflu4	DISTAM	Milk		−	−	−
P. fluorescens	PSEflu24	DISTAM	Salad		−	−	−
P. fluorescens	PSEflu13	DISTAM	Salad		−	−	−
P. fluorescens biovar C	PSEflu14	DISTAM	Salad		−	−	−
P. fluorescens biovar G	PSEflu20	DISTAM	Salad		−	−	−
P. fluorescens	PSEflu22	DISTAM	Salad		−	−	−
P. fragi	1S 63	VSA	Fish	8	−	+	−
P. fragi	4S 72	VSA	Fish	8	−	+	−
P. fragi	F188	VSA	Meat	9	−	+	−
P. fragi	F271	VSA	Fish	9	−	+	−
P. fragi	101M5	VSA	Meat	9	−	+	−
P. lundensis	F31	VSA	Meat	9	−	−	+
P. putida biotype A	F385	VSA		9	−	−	−
P. putida biotype A	F164	VSA		9	+	−	−
P. putida biotype A	F196	VSA		9	+	−	−
P. putida biotype B	F292	VSA		9	−	−	−

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; DTA, Departamento de Tecnologia de Alimentos, INIA, Madrid, Spain; DFST, Department of Food Science and Technology, University of Athens, Athens, Greece; DSA, Dipartimento di Scienza degli Alimenti Università degli Studi di Napoli Federico II, Portici, Italy; DISTAM, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università di Milano, Milano, Italy; VSA, Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università di Milano, Milano, Italy.

[b] Strains previously identified by Microlog System 2 (Biolog, Hayward, CA) according to the manufacturer's instructions.

[c] A spurious PCR product of less than 200 bp was detected after amplification of genomic DNA from this strain.

tions are reported in Table 2; the reverse primer (11) was used at concentration of 0.6 μM in each PCR. The PCR products were run in 2% agarose electrophoresis gels for 45 min at 150 V. A 16S rRNA gene amplification was also performed prior to multiplex PCR using the conditions previously described (3). This amplification was used in order to check that the DNA was suitable for PCR amplification and to avoid false negatives.

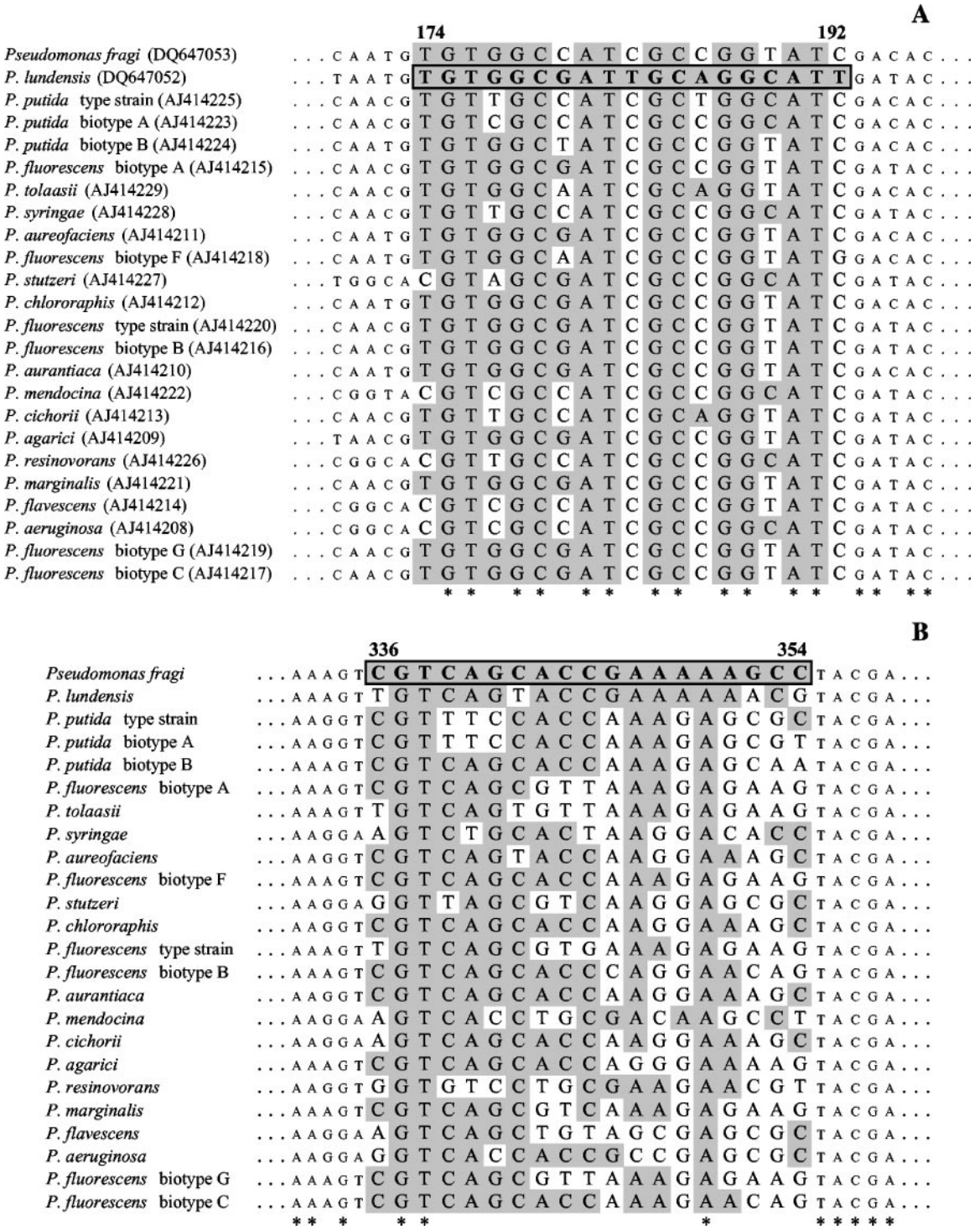

FIG. 1. Sequence alignment of variable portions of the *carA* gene sequences of selected *Pseudomonas* spp. (A) The accession numbers of the sequences used for the alignment are indicated. Variable sequences are highlighted in white, and consensus sequences are indicated by asterisks. The sequences used to design primers (Table 2) for the species-specific detection of *P. lundensis* (A), *P. fragi* (B), and *P. putida* (C) are boxed and reported in bold.

DNAs from the strains *P. fragi* DSM3456^T, *P. lundensis* DSM6252^T, and *P. putida* DSM291^T were used for the optimization of the PCR conditions. The species-specific assay was shown to give the specific amplification products in uniplex, duplex, and multiplex PCR assays involving the three *Pseudomonas* species (Fig. 2), suggesting the potential for a simultaneous detection and identification of the three spoilage bacteria. The detection limits for each species were found to be 1 ng

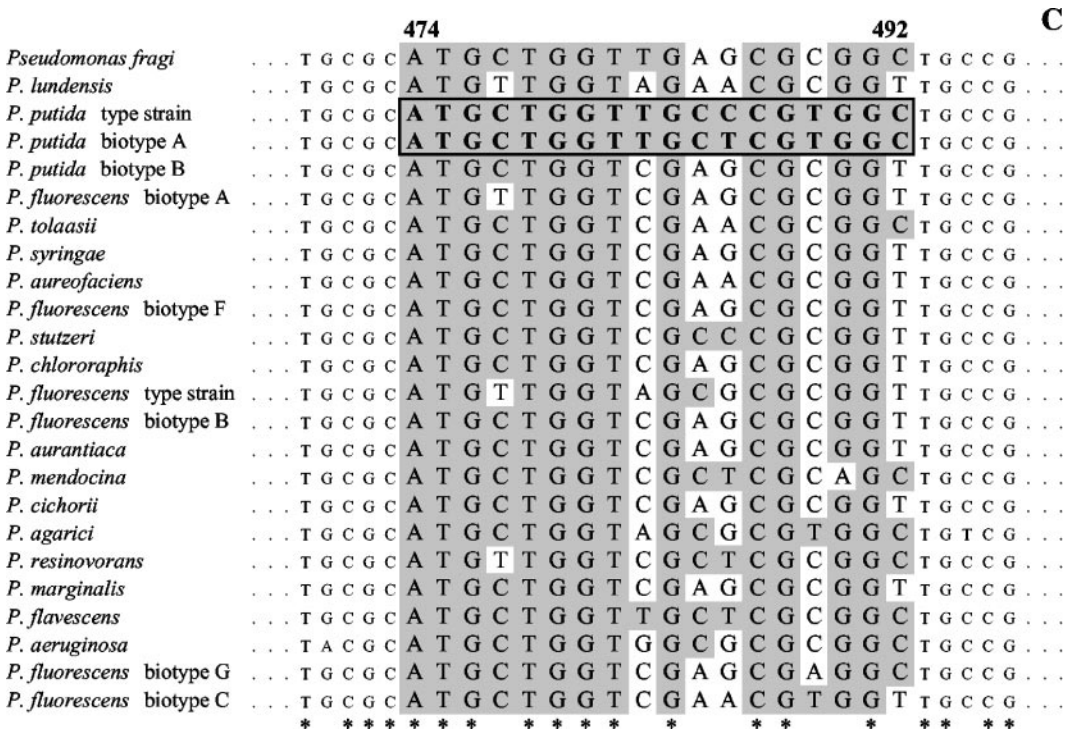

FIG. 1—*Continued*.

and 5 ng of DNA template per PCR for uniplex and multiplex PCR, respectively. The PCR products showed the expected sizes of 530, 370, and 230 bp for *P. lundensis*, *P. fragi*, and *P. putida*, respectively (Fig. 2).

The multiplex PCR assay was validated by using DNA extracted from different *Pseudomonas* species and strains, and the results are reported in Table 1. Species of *Pseudomonas* different from the ones targeted by the multiplex PCR gave no PCR product, confirming the specificity of the assay. The expected results were not obtained in only a few cases. Our forward primer for the specific amplification of the *P. putida carA* gene was designed on the basis of the *carA* sequences of *P. putida* DSM291^T and *P. putida* DSM50208 biotype A. The sequence of the primer differs from the sequence of biotype B in several nucleotides (Fig. 1). However, a spurious PCR product slightly shorter than 200 bp was obtained when the DNA from *P. putida* DSM50222 (biotype B) was used in our experiments. The *carA* gene sequence of *P. putida* DSM50222 was determined, and it did not show zones of possible annealing of the primers used (Fig. 1), indicating that the PCR product could be a genomic fragment outside the *carA* gene. Moreover, the DNAs of *P. putida* PMK32, F385, and F292, isolated in other studies and identified by biochemical tests, gave no PCR product, suggesting the possession of a different sequence in the primer alignment zone. Strains L414 and L47 were identified as *P. fragi* strains according to our assay (Table 1); however, they were previously isolated from fish and were reported as belonging to the same sodium dodecyl sulfate-polyacrylamide gel electrophoresis cluster as *P. lundensis* (19). In the above-described cases, the strains might have been misidentified in the previous characterization.

Detection of *P. fragi*, *P. lundensis*, and *P. putida* from meat. Twelve beef steak, 10 pork steak, and 11 chicken breast samples were used. Each meat sample was analyzed soon after the purchase and after 8 days of aerobic storage at 5°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 selective supplement (Oxoid); the plates were incubated at 20°C for 48 h. After plate counts, all the colonies present on the surface of each countable plate were collected in bulk as previously described (5) by suspending them in a suitable volume of quarter-strength Ringer's solution. The bulk cells were har-

TABLE 2. Specific oligonucleotide sequences of the *carA* gene used as forward primers in this study

Name	Sequence	Expected size[a]	Source	Concentration in multiplex PCR	PCR conditions (30×)
putF	5′-ATG CTG GTT GCY CGT GGC-3′	230 bp	This study	0.2 μM	10 s at 94°C
fraF	5′-CGT CAG CAC CGA AAA AGC C-3′	370 bp	This study	0.2 μM	10 s at 94°C
lunF	5′-TGT GGC GAT TGC AGG CAT T-3′	530 bp	This study	0.2 μM	20 s at 59°C

[a] Size of the PCR product obtained using the reverse primer for the amplification of the *carA* gene (11) at concentration of 0.6 μM in each PCR.

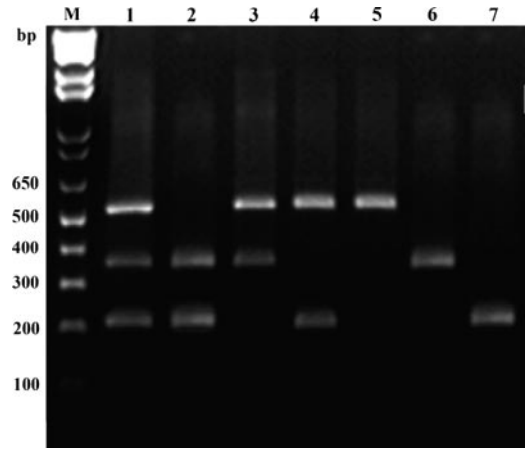

FIG. 2. Results of multiplex (lane 1), duplex (lanes 2 to 4), and uniplex (lanes 5 to 7) PCR obtained using mixtures of DNA templates from *P. lundensis* DSM6252^T (lane 5), *P. fragi* DSM3456^T (lane 6), and *P. putida* DSM291^T (lane 7). M, 1-kb Ladder Plus (Invitrogen).

vested with a sterile pipette and stored by freezing at −20°C. For DNA extraction, 100 μl of bulk suspension (optical density at 600 nm [absorbance], 1) was centrifuged at 17,000 × *g* for 5 min, and the pellet was washed in 100 μl of TE buffer (10 mM Tris, 0.1 mM EDTA), centrifuged at 17,000 × *g* for 5 min, resuspended in 20 μl of sterile water, and boiled for 10 min. After boiling, the samples were centrifuged for 1 min (17,000 × *g*), and 1 μl of the supernatant was employed in the multiplex PCR assays as described above.

Most of the samples showed *Pseudomonas* loads higher than 10^4 CFU g^{-1} at time zero increasing to a range of 10^7 to 10^9 CFU g^{-1} after 8 days of aerobic storage at 5°C (Table 3). The multiplex PCR was performed on the bulk cells from the countable plates in order to investigate the occurrence of our target species as dominant bacteria in the spoilage process. The analysis of bulk cells from countable plates has been often used for a rapid identification of mixed microbial species from food without the need for isolation (5, 6, 7). In this case, the initial contamination of the beef samples as revealed by multiplex PCR of bulk colonies was imputable to *P. fragi* in all the cases except for sample N (Table 3). When none of the species was detected (samples G and N), the viable counts were probably given by a *Pseudomonas* species different from the ones targeted by our assay. Fifty percent of the chicken breast samples at time zero were contaminated by *P. fragi*. After spoilage, all the chicken samples were contaminated by *P. fragi* and *P. putida*, while *P. lundensis* was found in samples O, Q, R, and S

TABLE 3. Viable counts of *Pseudomonas* spp. and results of *carA* multiplex PCR assays in meat samples at time zero and after aerobic storage at 5°C for 8 days

Sample	CFU (g^{-1}) at[a]:		*carA* multiplex PCR assay result (bulk cells from countable plates) at[b]:	
	Time zero	8 days	Time zero	8 days
A (beef)	2.80×10^7	4.30×10^8	*P. fragi*	*P. fragi*
B (beef)	1.45×10^5	2.20×10^9	*P. fragi*	*P. fragi*
C (beef)	3.80×10^5	1.32×10^9	*P. fragi*	*P. fragi*
D (beef)	1.40×10^4	3.56×10^8	*P. fragi*	*P. fragi*
E (beef)	1.20×10^6	1.45×10^9	*P. fragi*	*P. fragi*
F (beef)	4.81×10^7	3.32×10^9	*P. fragi*	*P. fragi*
G (beef)	1.20×10^7	9.40×10^8	*P. fragi*	−
H (beef)	1.74×10^4	2.12×10^8	*P. fragi*	*P. fragi*
I (beef)	1.27×10^5	3.50×10^8	*P. fragi*	*P. fragi*
L (beef)	2.81×10^4	8.80×10^8	*P. fragi*	*P. fragi*
M (beef)	4.81×10^3	6.10×10^8	*P. fragi*	*P. fragi*
N (beef)	1.17×10^6	4.20×10^8	−	−
O (chicken)	1.12×10^6	3.80×10^8	*P. fragi*	*P. fragi, P. putida*, *P. lundensis*
P (chicken)	3.64×10^6	1.43×10^8	*P. fragi*	*P. fragi*, *P. putida*
Q (chicken)	1.65×10^6	6.50×10^8	*P. fragi*	*P. fragi*, *P. putida*, *P. lundensis*
R (chicken)	4.50×10^5	3.10×10^8	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*, *P. lundensis*
S (chicken)	1.43×10^6	2.60×10^8	−	*P. fragi*, *P. putida*, *P. lundensis*
T (chicken)	1.04×10^6	2.54×10^7	*P. fragi*	*P. fragi*, *P. putida*
U (chicken)	8.30×10^4	3.60×10^8	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
V (chicken)	8.20×10^4	1.06×10^9	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
W (chicken)	2.64×10^4	6.50×10^7	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
X (chicken)	1.22×10^6	1.10×10^9	*P. fragi*	*P. fragi*, *P. putida*
Y (chicken)	2.20×10^4	1.34×10^9	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
M1 (pork)	3.45×10^6	7.80×10^9	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
M2 (pork)	1.52×10^4	3.90×10^9	*P. fragi*, *P. putida*, *P. lundensis*	*P. fragi*, *P. putida*
M3 (pork)	7.60×10^4	7.20×10^9	*P. fragi*, *P. putida*	*P. fragi*, *P. putida*
M4 (pork)	5.60×10^4	3.40×10^9	*P. fragi*, *P. putida*	*P. fragi*
M5 (pork)	2.11×10^6	6.00×10^9	*P. fragi*, *P. putida*	*P. fragi*
M6 (pork)	3.60×10^6	1.53×10^9	*P. fragi*, *P. putida*, *P. lundensis*	*P. fragi*, *P. putida*
M7 (pork)	5.50×10^4	1.19×10^9	*P. fragi*, *P. putida*	*P. fragi*
M8 (pork)	5.60×10^5	1.95×10^9	*P. fragi*, *P. putida*	*P. fragi*
M9 (pork)	6.50×10^4	7.90×10^8	*P. fragi*, *P. putida*	*P. fragi*
M10 (pork)	2.72×10^5	2.61×10^9	*P. fragi*, *P. putida*, *P. lundensis*	*P. fragi*

[a] Values are expressed as the means based on triplicate plating. Standard deviations were always lower than 20% of the means.
[b] A dash indicates the absence of the targeted species on the countable plates; it does not implicate their absence at a lower concentration.

(Table 3). Most of the pork samples were contaminated by both *P. fragi* and *P. putida*, while *P. lundensis* was found at time zero only in samples M2, M6, and M10. After 8 days, 60% of the pork samples were spoiled by *P. fragi* only (Table 3).

The evolution of the *Pseudomonas* species during meat storage (Table 3) is the result of the competition between the targeted species and between them and other microorganisms developing during the spoilage. The interpretation of such results in further research could provide important insights into the microbial ecology associated with the storage of fresh foods. From the results of this study, it appears that *P. fragi* can play a significant role in the spoilage of the three different kinds of meat. This is in agreement with other reports (10, 18); even though this microorganism is often associated with the spoilage of several foods, it has been recognized that meat may be its ecological niche for several reasons, including its need for iron and its peculiar system for proteolytic enzyme release (12). As far as we know, this is the first molecular assay developed for the identification of *P. fragi*, *P. lundensis*, and *P. putida*. The *carA* multiplex PCR assay can give a rapid diagnosis of the possible spoilage-causing agents with a direct analysis of colonies from selective media. A direct identification could be very useful to recognize the *Pseudomonas* spp. occurring during meat storage in studies of shelf life determination and improvement. A rapid and reliable identification of *Pseudomonas* species can be fundamental for a better understanding of the microbial ecology associated with meat spoilage.

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers for the *carA* gene sequences are as follows: DQ647053 for *P. fragi* DSM3456^T, DQ647052 for *P. lundensis* DSM6252^T, and EF363547 for *P. putida* DSM50222.

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