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Development of a Rapid Real-Time PCR Method as a Tool To Quantify Viable Photobacterium phosphoreum Bacteria in Salmon (Salmo salar) Steaks

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Abstract:

A specific real-time PCR quantification method combined with a propidium monoazide sample treatment step was developed to determine quantitatively the viable population of the Photobacterium phosphoreum species group in raw modified-atmosphere-packed salmon. Primers were designed to amplify a 350-bp fragment of the gyrase subunit B gene (*gyrB*) of P. phosphoreum. The specificity of the two primers was demonstrated by using purified DNA from 81 strains of 52 different bacterial species. When these primers were used for real-time PCR in pure culture, a good correlation (R^2 of 0.99) was obtained between this method and conventional enumeration on marine agar (MA). Quantification was linear over 5 log units as confirmed by using inoculated salmon samples. On naturally contaminated fresh salmon, the new real-time PCR method performed successfully with a quantification limit of 3 log CFU/g. A correlation coefficient (R^2) of 0.963 was obtained between the PCR method and classic enumeration on MA, followed by identification of colonies (290 isolates identified by real-time PCR or by 16S rRNA gene sequencing). A good correlation with an R^2 of 0.940 was found between the new PCR method and an available specific conductance method for P. phosphoreum. This study presents a rapid tool for producing reliable quantitative data on viable P. phosphoreum bacteria in fresh salmon in 6 h. This new culture-independent method will be valuable for future fish inspection, the assessment of raw material quality in fish processing plants, and studies on the ecology of this important specific spoilage microorganism.

1. Introduction

Modified atmosphere packed (MAP) fresh fish is increasingly popular in the Europe and widely sold in supermarkets as a chilled product. Compared to aerobically stored fresh fish, this kind of packaging, with typical head-space CO_2 concentrations of 20-60%, modifies the dominating microbiota mainly by reducing growth of aerobic Gram negative bacteria like *Pseudomonas* (1, 2). This results in an extended shelf-life which facilitates the chilled distribution and marketing of fresh MAP fish. However, this packaging allows the growth of CO_2-resistant bacteria including Gram positive lactic acid bacteria and the Gram negative *Photobacterium phosphoreum* (2-4). The latter has been identified as the specific spoilage organism (SSO) responsible for trimethylamine production and sensory spoilage of MAP cod (5, 6) and as the main spoilage bacterial species of several chilled marine fish including cod, garfish, halibut, saithe, salmon, and shrimp (7-15).

Multilocus analysis, based on 16S rDNA and on *gyr*B and *lux* ABFE genes, divided strains originally isolated and identified as *P. phosphoreum* into three distinct clades of *P. phosphoreum*, *P. iliopiscarium* and *P. kishitanii* (16, 17). The members of the *P. phosphoreum* species group have been reported as important for spoilage of raw MAP fish and they include both luminous and non-luminous variants (5, 18). It is therefore relevant to detect and enumerate this species group in fish products.

As no specific medium is available for enumeration of either the *P. phosphoreum* species group or the individual species in seafood, the control of these important spoilage bacteria is problematic. There is a specific conductance method for quantifying the *P. phosphoreum* species group in the fish samples (19) which is quantitative, sensitive, and selective. However, it is not very rapid with analyses taking 12 to 50 h depending on the bacterial concentration in the sample. Instruments required to run this conductance method are available, e.g. from SY-LAB in Austria, but they are far less widespread than real-time PCR thermocyclers. Therefore, the development of a rapid and specific real-time PCR quantification method for viable cells of the *P. phosphoreum* species group in fresh fish is clearly of interest. One of the limitations of PCR and real-time PCR methods for the quantification of bacteria in the food samples is that both live and dead cells are detected (20). To overcome this problem, propidium monoazide (PMA) can be used before DNA extraction. PMA is an intercalating DNA agent which enters dead cells and binds to DNA, inhibiting subsequent PCR amplification and thereby ensuring quantification of viable bacteria (21). PMA has been used in combination with several real-time PCR methods for quantification of viable bacteria in the food e.g. *Campylobacter* (22), *Listeria monocytogenes* (23), *Brochothrix thermosphacta* (24, 25), *Vibrio parahaemolyticus* (26, 27) and *Escherichia coli* O157:H7 (28).

In the present study, we describe a new rapid assay to quantify and identify the *P. phosphoreum* species group in raw salmon using real-time PCR. This new method has been validated for artificially inoculated and naturally contaminated fresh MAP salmon and compared to both the classic enumeration method on culture media followed by colony identification and the conductance method specifically developed for these bacteria. In the following, the term *P. phosphoreum* will be used to refer collectively to the closely related members of the *P. phosphoreum* species group.

2. Materials and methods

2.1. Raw salmon samples

To establish a standard curve on artificially contaminated fish, 24 samples of fresh salmon were inoculated with different concentrations of *P. phosphoreum* and analyzed. In order to evaluate the real-time PCR quantification method on naturally contaminated samples, it was compared with viable plate counting on 13 samples of MAP salmon steaks. These were obtained from a local French processing plant or purchased from a local supermarket and were packed with a head-space gas composition of approximately 50% CO_2 and 50% N_2. With the aim of comparing the real-time PCR method and a conductance method for quantification of *P. phosphoreum*, 26 naturally contaminated MAP salmon steaks were assessed. These samples were obtained from a Danish processing plant and were packed with a head-space gas composition of approximately 20% CO_2 and 80% N_2.

2.2. Bacterial strains and DNA extraction

Bacterial strains used in this study are listed in Table 1. *P. phosphoreum* strains were grown in brain heart infusion broth (BHI, Biokar Diagnostics, Beauvais, France) supplemented with 2.0% NaCl and incubated at 15°C for 24 h.

Photobacterium isolates from culture collections were grown in BHI (Biokar) with 2.0% NaCl between 15°C and 25°C depending on the strain and for 24 to 48 h. Other strains were grown in BHI (Biokar) at 20°C for 24-48 h except *Escherichia coli* ATCC 25922 /CIP 76.24, *Enterococcus faecalis* CIP 105042, *Enterococcus faecium* CIP 106742, *Staphylococcus aureus* ATCC 25923/CIP 76.25 and *Salmonella enterica* CIP 81.3 which were grown at 37°C and *Listeria innocua* CIP 80.11^T, *Listeria monocytogenes* CIP 78.35, and *Pseudomonas fluorescens* CIP 69.13^T which were grown at 30°C. *Vibrio* isolates from culture collections were grown in Marine Broth (Difco Laboratories, Detroit, MI, USA) at 20°C for 24 to 48 h except for *Vibrio parahaemolyticus* which was grown at 37°C. To validate primer specificity, a total of 27 isolates of *P. phosphoreum* were tested as targets for inclusivity tests while 54 non-*P. phosphoreum* isolates belonging to 49 different species were used for exclusivity testing (Table 1). These 81 strains were tested *in vitro* using genomic DNA extracted from 1.5 mL of bacterial cultures at a concentration of at least 7 log (CFU/ml) (data not shown). The chromosomal DNA of all bacterial isolates was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, S.A., Courtaboeuf, France).

2.3. Extraction of bacterial DNA from salmon flesh

For each salmon sample, a 30 g portion was aseptically weighed in a sterile stomacher plastic bag and 120 ml sterile peptone water (0.85% NaCl; 0.1% peptone) was added to obtain a five-fold dilution which was then homogenized for 2 min using a stomacher 400 (Seward Medical, London, UK). In order to separate the eukaryotic cells and DNA of salmon from the bacterial cells, 9 ml of homogenized suspension (previously treated with PMA for naturally contaminated samples) was filtered on a Nucleospin Plant L column (Macherey-Nagel, Hoerdt, France) by centrifugation at 11700 x g for 10 min at 4°C. The supernatant was carefully removed and the bacterial cell pellet was resuspended in 400 µl of enzymatic lysis solution (20 mM Tris-HCl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme, 11.6 U mutanolysine) in a 2 ml microtube and then incubated at 37°C for 1 h. Reagents used in this step were purchased from Sigma-Aldrich. A mechanical lysis step was then performed by addition of about 0.2 g of glass beads (150-200 µm diameter) and shaking for two-times 2 min in a bead beater MM200 (30 Hz) (BioSpec Products, Bartlesville, OK,

USA). Proteins were digested by proteinase K (20 mg/ml) during incubation at 56°C for 30 min and with 200 µl of AL buffer (DNeasy Blood and Tissue Kit, Qiagen). To pellet the glass beads, centrifugation at 9500 x g for 3 min was carried out and the supernatant was transferred into a 2 ml microtube to perform nucleic acid precipitation by addition of 200 µl of ice-cold absolute ethanol. DNA purification was carried out using a DNeasy Blood and Tissue Kit as described in the Qiagen instruction manual.

2.4. Propidium monoazide (PMA) treatment of samples

Prior to DNA extraction, a preliminary treatment with PMA (Biotium, Hayward, USA) was carried out in order to reduce amplification of DNA from dead cells. PMA was dissolved in 20% dimethyl sulfoxide to provide a stock solution of 2.5 mM. To obtain a final PMA concentration of 50 µM in samples, 20 µl of the PMA stock solution was added to 980 µl of *P. phosphoreum* pure culture or 180 µl of the PMA stock solution was added to 8820 µl of homogenized and naturally contaminated salmon. The tubes were then shaken and placed in the dark at room temperature for 5 min to allow the PMA to penetrate the dead cells and bind to DNA. Finally, to photo-activate the PMA, tubes were placed in crushed ice with their lids removed and exposed for 5 min to light from a 500 W halogen lamp positioned at a distance of 20 cm. They were shaken occasionally to ensure complete cross-linking of the available DNA (24).

2.5. Artificially contaminated pieces of salmon flesh

Salmon flesh was prepared and ionized as previously described by Macé et al. (29). A 60 g portion of ionized raw salmon cubes was aseptically weighed into a sterile plastic bag and 240 ml of sterile physiological saline solution (0.85% NaCl ,0.1% peptone) was added to obtain a 5-fold dilution. The suspension was homogenized for 2 min in a stomacher 400. Eight sterile plastic tubes were filled with 25 ml of the sterile salmon homogenate. Seven of them were inoculated with 1 ml of 10-fold serially diluted suspensions of *P. phosphoreum* CIP 102511, previously treated with PMA, and one non-inoculated tube was used as a control. The concentrations of *P. phosphoreum* in the salmon samples were prepared in order to range from approximately 7.7 to 1.7 log (CFU/g). DNA was extracted from each homogenate as described above. Concentrations of bacteria in the homogenates were determined by real-time PCR and viable counting in duplicate using Marine Agar (MA, Difco Laboratories, Detroit, MI, USA) incubated at 15°C 7 days. These conditions were used for *P. phosphoreum* growth in a previous study (30). The viable counts were expressed as the arithmetic mean of the two plate counts in log (CFU/g). This experiment was repeated three times independently.

2.6. Primer design and real-time PCR assay

Alignment of the gyrase subunit B (*gyr*B) gene DNA sequence from *P. phosphoreum* and closely related species was performed using the CLC DNA workbench 6.5 (CLC bio, Aarhus, Denmark) and the BioEdit sequence alignment software (31) (Figure 1). Primers specific to the *P. phosphoreum* species group were designed in a conserved region so that they excluded other *Photobacterium* and the closely related *Vibrio fischeri* (Figure 1). The forward primer MO627 (5´-TACTGTTGAAGTGGCGAT-3´) matched position 461-478 and the reverse primer MO628 (5´- TCTGCTGGGCTTTCTAAT-´3) matched position 794-811 of the *P. phosphoreum* type strain *gyr*B gene (ATCC 11040^{T}/CIP 102111^{T}, accession number AY455875.1). The primers amplified a 350 bp specific fragment. Primer specificity was tested *in silico* using the Basic Local Alignment Search Tool program (BLAST) (Figure 1) and Primer BLAST (32). The hybridization temperature of the primers was optimized using the

temperature gradient test on a Chromo4 real-time PCR Detection System (Bio-Rad Laboratories, Marnes La Coquette, France). Real-time PCR amplification was performed using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Marnes La Coquette, France) in 15 µl reaction volumes containing 7.5 µl of 2x IQ SYBR Green Supermix; 2.5 µM of each primer (Fisher Bioblock Scientific, Illkirch, France) and 2 µl of template DNA or 1 ng for the specificity test on the pure strains listed in Table 1. The amplification reaction was conducted using the Chromo4 real-time PCR Detection System (Bio-Rad Laboratories). The optimum cycling parameters were: 94°C hold-temperature for 3 min for initial denaturation followed by 35 cycles of amplification (94°C for 30 sec, 58°C for 1 min and 72°C for 1 min) followed by a final extension at 72°C for 5 min. A melting curve between 70°C and 90°C was determined after the last amplification cycle and at a temperature transition rate of 0.5°C/s. The background limit was set at 0.02 when the cycle threshold (*Ct*) values were determined. All amplification reactions were run in triplicate in three independent assays. The *P. phosphoreum*-specific PCR product had a melting temperature of 84°C and a response was considered positive only when this was observed. Real-time PCR assays were interpreted as positive for *P. phosphoreum* if the determined *Ct* values were ≤ 27.5 for DNA from pure cultures or ≤ 31 for DNA from inoculated fish samples. Higher *Ct* values were considered out of the range for this assay. These cut-off values were determined as the lowest observed *Ct* for the non-*P. phosphoreum* isolates when evaluating specificity (Table 1).

2.7. Standard curves and amplification efficiency

Two different calibration curves were constructed to determine the sensitivity and efficiency of the real-time PCR method. A standard curve was obtained by using purified genomic DNA from *P. phosphoreum* CIP 102511^T, extracted from serial dilutions of a bacterial suspension. The culture was diluted in sterile peptone water (0.85% NaCl; 0.1% peptone) to obtain concentrations ranging from 1 to 8 log (CFU/ml).

The other standard curve was obtained from bacterial DNA extracted from independent triplicate pieces of salmon flesh previously inoculated with 10-fold serially diluted concentrations of the *P. phosphoreum*-type strain. The different bacterial concentrations (log CFU/g) were plotted against the corresponding *Ct* values. The slope of the linear relation of this curve was used to determine the amplification efficiency (E) by applying the following equation: $E = 10^{-1/slope} - 1$ (33).

2.8. Comparison of real-time PCR and plate counting for quantification of *P. phosphoreum* in naturally contaminated salmon

A total of 13 samples of MAP salmon steaks with a head-space gas composition of approximately 50% CO_2 and 50% N_2 was obtained from a local French processing plant or purchased from a local supermarket. The samples were stored at 4°C for 3 days and then at 8°C for 7 days after a break of 2 hours at 20°C, according to a French shelf-life protocol (34). At different times during the storage period, bacterial DNA was extracted from salmon samples and *P. phosphoreum* was quantified using the real-time PCR method described above. The *Ct* values obtained by real-time PCR were used to calculate the concentration (log CFU/g) present in each sample, based on the standard curve established with artificially contaminated pieces of salmon flesh. All the real-time PCR reactions were carried out in triplicate and the mean values were used. Results were compared to viable counts on MA (Difco), followed by determination of the ratio of *P. phosphoreum* in the samples. This was done by testing about 20 colonies per countable plate using real-time PCR (see next section). The concentration of *P. phosphoreum* was then calculated by multiplying the viable counts on MA and the proportion of *P. phosphoreum* isolates for each tested sample. The

correlation between the concentrations obtained by plate counts and real-time PCR quantification of *P. phosphoreum* was determined by linear regression.

2.9. Identification of bacterial isolates from MA plates

MA is a non-selective medium used for total viable counting so identification was required to determine the proportion of *P. phosphoreum* colonies on this medium for each naturally contaminated sample. From each of the 13 MAP salmon samples, about 20 colonies were randomly selected from MA plates of the highest dilution showing growth. The 290 isolates collected were purified twice on BHI agar (Biokar) supplemented with 2.0% NaCl. In order to determine whether or not they belonged to the *P. phosphoreum* species group, all 290 isolates were analyzed by the real-time PCR assay described above and following extraction of chromosomal DNA using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). They were thus classified as *P. phosphoreum* or non-*P. phosphoreum* isolates. To confirm these results, from the 290 isolates, 50 from 4 different salmon samples were identified by partial 16S rDNA gene sequencing as described in a previous study (11). In brief, the partial nucleotide sequence (about 600 bp) of the amplified 16S rRNA gene was determined with an automated sequencer (Beckman Coulter Genomics, Takeley, UK) using the primer SP1 (35) for the 50 strains. The resulting sequences were then submitted to BLAST available at the National Center for Biotechnology Information (NCBI, Bethesda, USA, http://www.ncbi.nlm.nih.gov/) for representation of sequence and similarity searches in the GenBank database.

2.10. Comparison of real-time PCR with a conductance method for quantification of *P. phosphoreum* in naturally contaminated salmon

A total of 26 MAP salmon steaks with a head-space gas composition of approximately 20% CO_2 and 80% N_2 was obtained from a Danish processing plant. The samples were divided into two batches which were stored at 2°C or 4°C, for 9 days, respectively. Each day, the concentration of *P. phosphoreum* was determined by using both an available specific conductance method (19) and the new real-time PCR method described above. An Mx3000P^{TM} thermocycler (Stratagene, AH diagnostics, Aarhus, Denmark) was applied and a standard curve was developed for the real-time PCR method when used with this instrument. The correlation between the results obtained by the two methods was determined by linear regression.

2.11. Linear regression and statistical analysis

Linear regression was performed using Fig. P software (v. 2.98, Biosoft, Cambridge, UK). ANOVA, the Fisher LSD test (95.0%), the Kruskal-Wallis Test and the Mood Test on medians were all performed on the non-pairwise *P. phosphoreum* concentration data obtained from the real-time PCR and viable count methods on naturally contaminated samples. ANOVA was used to compare variance within and between results obtained from both methods for 13 samples. The Fisher LSD test (95.0%) was used to measure variability in samples by displaying the standard deviation of means, the Kruskal-Wallis Test was used to compare medians and the Mood Test was used to analyze the distribution of data around medians. These tests were carried out using STATGRAPHICS Centurion XVI version 16.1.11 software (Statistical Graphics Corp).

3. Results

3.1. Specificity of the real-time PCR assay

Using Primer BLAST (32), the selected primers, MO627 and MO628, targeted only *P. phosphoreum*-related species and, by using the BLASTn program, they showed 100% maximum identity with *P. phosphoreum*-related species. The primers developed were specific to *P. phosphoreum*-related species and all 27 culture collection strains studied were correctly identified (Table 1). The threshold of the assay was set at a fluorescent background value of 0.02 resulting in *Ct* values ranging from 9 to 14 for the 27 target isolates with a *Ct* mean value of 10.3 ± 2.3. The melting temperature calculated at the end of each real-time PCR assay was 84°C. *Ct* values above 27.5 were obtained for all the 54 non-*P. phosphoreum* isolates and these values were considered out of the specificity range. The *Ct* threshold obtained was 27.5 for DNA from pure cultures and 31.0 for bacterial DNA extracted from salmon flesh.

The specificity of these primers, when tested against a large collection of non-target bacteria (Table 1) including other species of *Photobacterium* and other bacterial species frequently encountered in seafood products, shows that they can be used for specific detection of *P. phosphoreum*.

3.2. Sensitivity and quantification limits of the assay

The detection and quantification limits of the PCR assay when used for pure cultures were determined by analyzing the genomic DNA of 10-fold serial dilutions of a *P. phosphoreum* CIP 102511^T overnight culture at 15°C. The linear relation determined over a 5 log (CFU/ml) range from 2.2 to 7.2 Log (CFU/ml) was *Ct* = -3.15 * viable count (log CFU/ml) + 34.6 (R^2=0.999) which indicates an efficiency of 108% (Figure 2). The quantification was considered specific up to a *Ct* value of 27.5 (specificity limit described above) corresponding to 2.3 log (CFU/ml).

Sensitivity was then investigated by using sterile salmon flesh artificially contaminated with decreasing amounts of *P. phosphoreum* CIP 102511^T. The linear quantification (R^2 of 0.998) was determined from 3 to 8 log (CFU/g) achieving about 5 log of magnitude. The equation was *Ct* = - 3.06 * viable count (log CFU/g) + 40.73 corresponding to an efficiency of about 112% (Figure 2).

On this salmon model, the quantification was specific with a limiting *Ct* value of 31 enabling *P. phosphoreum* to be enumerated in concentrations between 3 and 8 log CFU/g.

Bacterial counts in non-inoculated ionized salmon flesh were below the enumeration threshold (0.7 log (CFU/g)) (data not shown). Thus, ionized flesh was validated as not contaminated.

3.3. Enumeration of *P. phosphoreum* in naturally contaminated MAP salmon steaks

A total of 13 MAP salmon steaks was analyzed to confirm the accuracy of real-time PCR for *P. phosphoreum* quantification. *P. phosphoreum* was detected in the range of 3.3 to 7.7 log (CFU/g) by real-time PCR (Table 2). Using MA plate counting and identification of colonies, *P. phosphoreum* viable counts varied from 2.9 to 7.6 log (CFU/g) for the 13 samples analyzed (Table 2). A good correlation was obtained (R^2 = 0.963) between real-time PCR and enumeration of *P. phosphoreum* by viable counting on the MA followed by identification

of colonies (Figure 3). The root mean square error (RMSE) was 0.34 log (CFU/g). Compared to plate counting, the relative accuracy of the real-time PCR method varied from the 87.7 to 115.3% of the *P. phosphoreum* detected on the MA (Table 2). The ANOVA, Fisher Test, Kruskal-Wallis Test and Mood Test statistical analyses revealed that the differences between plating and real-time PCR results were not significant ($P > 0.05$). The suitability of the real-time PCR assay for identifying *P. phosphoreum* isolates was also confirmed by partial sequencing of the 16S rRNA gene for 50 isolates from 4 of the 13 salmon samples. The identification results were exactly the same for both methods confirming the specificity of real-time PCR for *P. phosphoreum* detection (Table 3).

3.4. Comparison of real-time PCR and a conductance method for quantification of *P. phosphoreum* in fresh MAP salmon

P. phosphoreum was detected in the range of 2.5 to 8.5 log (CFU/g) by real-time PCR and from 1 to 8 log (CFU/g) by the conductance method. In one of the 26 samples tested, no *P. phosphoreum* was detected by either of these two methods. Three samples presenting *P. phosphoreum* concentrations from 1 to 2 log (CFU/g) were quantified by the conductance method only (data not shown). One sample was quantified by the real-time PCR method but was out of range with a concentration of about 2.5 log (CFU/g). However, this count was well correlated with the conductance method (2.7 log (CFU/g)) so this result was used for the correlation curve. Based on data for the remaining 22 samples, a high correlation with an R^2 of 0.940 and an RMSE of 0.49 log (CFU/g) was obtained (Figure 4).

4. Discussion

Microbiological quality control of fresh seafood products remains a challenge due to their high sensitivity to microbial contamination and growth. By improving good hygiene practices in processing plants and by using MAP, the shelf-life of fresh fish can be increased by several days (2, 4). The detection and quantification of SSO can support and document efforts with good hygiene practices and thereby contribute to increasing quality and reducing product losses. In recent years, culture-independent molecular methods like real-time PCR have been increasingly applied to detect and quantify target microorganisms in the food (36, 37). Several real-time PCR methods have been developed to enumerate or detect pathogenic bacteria in the seafood products including *Vibrio parahaemolyticus* (26, 38) and mesophilic Gram negative histamine-producing bacteria (39, 40). Spoilage bacteria like *Pseudomonas* (41) and *Brochothrix thermosphacta* (24) have also been enumerated by these methods. In this work, real-time PCR was chosen for our objective of quantifying the principal SSO of MAP fish, *P. phosphoreum,* since the specific enumeration of this bacterium is difficult using classic methods. To our knowledge, this is the first report to present a rapid molecular quantification tool for *P. phosphoreum* using real-time PCR. Moreover, the use of PMA allowed exclusive quantification of viable cells (21, 42) and, consequently, only the *P. phosphoreum* cells active in spoilage.

When real-time PCR was previously used for the enumeration of bacteria in a meat or fish matrix, linear quantifications were reported over a range of at least 5 log, and quantification limits without enrichment varied from about 2 to 3 log (CFU/g) (22, 24, 25, 37, 43, 44) as obtained in the present study.

When enumeration by viable counting and real-time PCR are compared, a relative accuracy of 100% indicates total agreement between the two methods. The relative accuracy values obtained in the present study on the naturally contaminated salmon samples (88 to 115%) are better than those reported in the several earlier studies for other bacteria (24, 25) and

similar to those obtained by Rodríguez-Lázaro et al. (45) for the quantification of *Listeria monocytogenes* (89 to 116%). Martin et al. (2006) (43) presented better relative accuracy and higher R^2 values for *Lactobacillus sakei* with less variability (91 to 98%) but these results were based on only 7 tested samples compared to 13 in the present study. The correlation curve obtained here on 13 samples gave an R^2 value of 0.963, which is higher than the correlation coefficients reported for several real-time PCR methods applied to more than 10 samples of meat or seafood products (R^2 values ranging from 0.798 to 0.911) (22, 24, 25, 41). This confirms the reliability of our new real-time PCR method dedicated to the quantification of *P. phosphoreum*.

The accuracy of this method was validated by comparison with the available specific conductance method. In fact, the correlation curve obtained by plotting the quantification results of the 22 tested MAP salmon samples showed a high R^2 of 0.940. The RMSE values obtained by comparing real-time PCR with the viable count (0.34 log (CFU/g)) or conductance method (0.49 log (CFU/g)) are lower than the currently accepted error value of 0.5 to 0.8 log for plate counting on solid matrices (46). With a quantification limit of 3 log (CFU/g), the new real-time PCR method is slightly less sensitive than the available conductance method which detects less than 1 log (CFU/g) (19). However, among the naturally contaminated samples, only a few (3 out of the 26 samples tested) were out of range of the real-time PCR method and could only be quantified by the conductance method. The major advantage of the real-time PCR method is its rapidity; it provides results in less than 6 hours (including DNA extraction and real-time PCR analysis), which is much faster than the available conductance method, requiring 12-50 hours (19), or the viable counting method, requiring more than a week as colonies must be identified.

Reducing the detection level is not required for *P. phosphoreum* as much as for pathogenic bacteria, since this organism induces spoilage rejection of products at a high population density e.g. above 6.5-7 log CFU/g (5-7, 29). This species is also known as a histamine producer and is involved in histamine food poisoning (47, 48) due to the consumption of cold-smoked tuna, dried sardines and fresh tuna. Nevertheless, histamine poisoning has never been reported following salmon consumption as the histidine content of salmon flesh is too low for toxic concentrations of histamine to be formed (8, 49). The real-time PCR method could thus be adapted for *P. phosphoreum* quantification in other fish, including transformed products such as cooked tuna, with an appropriate DNA extraction method. In this case also, detection from 3 log CFU/g is sufficient because histamine is produced when *P. phosphoreum* concentrations are above about 6 log (CFU/g) (8, 48, 49). Consequently, this real-time PCR method should provide a powerful tool to evaluate the contamination of seafood products by *P. phosphoreum* before spoilage appears or histamine is formed.

However, a lower detection level could be useful in the case of its use in combination with predictive microbiology models, for example by using the Seafood Spoilage and Safety Predictor (http://sssp.dtuaqua.dk) which includes models for growth of *P. phosphoreum* in fresh MAP cod, plaice and salmon (50). To improve the sensitivity of the new real-time PCR method, an enrichment step in broth, prior to the PCR assay, could be used e.g. in the same way as applied for the available conductance method for sensitive enumeration of *P. phosphoreum* (19). Enrichment steps in the broth or food samples have already been used to enhance the sensitivity of real-time PCR methods, both for qualitative and quantitative detection of the target bacteria (27, 44, 51). In fact, we tested this approach by using quantitative enrichment in *Photobacterium phosphoreum* differential medium ―PPDM" at 15°C (19). Unfortunately, this enrichment medium seemed to inhibit the real-time PCR reaction. Thus, on slightly contaminated samples with less than 3 log CFU/g of *P. phosphoreum*, this real-time PCR method is currently only suitable for qualitative purposes.

In conclusion, this method is now applicable to enumerating *P. phosphoreum* in salmon and, according to preliminary tests in progress on cod, it could also be used for other fish.

Thanks to the PMA treatment step, it could be used by industry to detect and quantify viable *P. phosphoreum* contamination in production plants and thus target processing steps which need to be controlled. This method could also be used for shelf-life testing of new packaged products, to quantify the growth of this very important fish spoiler, and in shelf-life determination as previously discussed for the available conductance method (4, 13).

Moreover, the development of new methods to quantify food spoilage organisms, like *P. phosphoreum* or other bacterial species (24, 25, 41, 51), could assist in the establishment of more accurate microbial hygienic criteria than those usually applied (e.g. total flora count). This would help to reduce food losses and to improve sustainable development in the food production. *Photobacterium* spp. have recently been reported as part of beef and pork spoilage microbiota (52, 53). Thus, this new quantitative method could clearly be used by the scientific community as a complementary tool to study *P .phosphoreum* distribution and abundance in bacterial populations.

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Tables

Table 1: Bacterial isolates used to develop and evaluate the specificity of a real-time PCR assay for detection of the *P. phosphoreum* species group

Bacterial species	Strain	Source	Number of strains	Real-time PCR results
Photobacterium phosphoreum group				
P. phosphoreum	CIP 102511[T]	Fish	1	+[a]
P. phosphoreum	DSM 2167	Hake (*Merluccius capensis*)	1	+
P. iliopiscarium	DSM 9896	Pyloric ceca of herring	1	+
P. kishitanii	LMG 23890	Light organ of *Physiculus japonicus*	1	+
P. phosphoreum	Oniris/Ifremer Collection	Spoiled salmon steak or fillets under MAP	23	+
Other *Photobacterium* species				
P. leiognathii	CIP 66.5[T]	Light organ of teleostean fish (*Leiognathus equula*)	1	-[b]
P. angustum	CIP 75.10[T]	Seawater at depth of 750 m	1	-
P. damselae ssp. piscicida	CIP 103910	White perch	1	-
P. damselae ssp. damselae	DSM 7482	Fish ulcer	1	-
P. indicum	LMG 22857	Marine mud	1	-
P. lypolyticum	LMG 23071	Light organ of teleostean fish (family *Leiognathidae)*	1	-
P. rosenbergii	LMG 22223	Tissue extract of bleached coral	1	-
Non-Photobacterium strains				

Aeromonas hydrophila	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Aeromonas salmonicida ssp. salmonicida	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Buttiauxela gaviniae	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Brochothrix campestris	DSM 4712	Soil	1	-
Brochothrix thermosphacta	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Carnobacterium maltaromaticum	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
C. maltaromaticum	NCDO 2762	Kidney of adult cutthroat trout	1	-
Carnobacterium divergens	NCDO 2763	Vacuum-packed minced beef	1	-
C. divergens	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Escherichia coli	ATCC 25922 CIP 76.24	Clinical isolate	1	-
Enterococcus faecalis	CIP 105042	Poultry cecum	1	-
Enterococcus faecium	CIP 106742	Dairy products	1	-
Hafnia alvei	Oniris/Ifremer Collection	Spoiled salmon fillet under MAP	1	-
H. alvei	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Listeria monocytogenes	CIP 78.35	Spinal fluid	1	-
Lactococcus lactis	CNRZ 1075	Origin not available	1	-
Lactococcus piscium	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Lactobacillus raffinolactis	Oniris/Ifremer Collection	Spoiled salmon fillet under MAP	1	-
Lactobacillus curvatus	Oniris/Ifremer Collection	Cold smoked salmon	1	-
Lactobacillus fuchuensis	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Lactobacillus sakei	NBIMCC 3453	NBIMCC	1	-
Leuconostoc gasicomitatum	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Listeria innocua	CIP 80.11^T	Bovine brain	1	-
Moellerella wisconsensis	Oniris/Ifremer Collection	Spoiled salmon fillet under MAP	1	-
Morganella morganii ssp. morganii	CIP A231	A case of summer diarrhea	1	-
Morganella psychrotolerans	Oniris/Ifremer Collection	Spoiled salmon fillet under MAP	1	-
M. psychrotolerans	CIP 109403	Cold smoked tuna	1	-

Proteus mirabilis	CIP A235	Origin not available	1	-
Pseudomonas fluorescens	CIP 69.13^{T}	Pre-filter tanks	1	-
Pseudoalteromonas sp.	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Psychrobacter sp.	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Salmonella enterica	CIP 81.3	Feces	1	-
Serratia proteamaculans	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Serratia quinivorans	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Serratia sp.	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-
Shewanella putrefaciens	ATCC 8071	Surface of tainted butter	1	-
Shewanella baltica	Oniris/Ifremer Collection	Spoiled salmon fillet under MAP	1	-
S. baltica	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Staphylococcus aureus	ATCC 25923 CIP 76.25	Clinical isolate	1	-
Vibrio anguillarum	CIP 63.36^{T}	Ulcerous lesion of cod (*Gadus morhua*)	1	-
Vibrio fischeri	DSM 2168	Fish	1	-
Vibrio orientalis	CIP102891^{T}	Seawater	1	-
Vibrio parahaemolyticus	CIP 75.2^{T}	Shirasu food poisoning	1	-
Vibrio sp.	Oniris/Ifremer Collection	Spoiled shrimp under MAP	1	-
Vagococcus fluvialis	CIP 102976^{T}	Chicken feces	1	-
Vagococcus fluvialis/carniphilus	Oniris/Ifremer Collection	Trout	1	-
Yersinia intermedia	Oniris/Ifremer Collection	Spoiled salmon steak under MAP	1	-

[a] +: Amplification product with a Tm of 84°C obtained in the method range of use.

[b] - : No PCR-product obtained in the method range of use.

Table 2: Viable counts on Marine Agar and real-time PCR enumeration of *P. phosphoreum* in naturally contaminated MAP salmon steaks (log CFU/g) and their relative accuracy

Sample	Real-time PCR (log CFU/g)	Ct values [a]	Viable count (log CFU/g)[b]	Relative accuracy (%)[c]
1	3.32	30.57 ± 0.56	2.88	115.3
2	5.37	24,29 ± 0.77	5.49	97.8
3	7.13	18.89 ± 0.14	7.03	101.4
4	7.34	18.27 ± 0.17	7.38	99.5
5	3.47	30.11 ± 0.10	3.57	97.2
6	3.85	28.93 ± 0.47	4.42	87.1
7	7.06	19.11 ± 0.30	7.06	100
8	7.66	17.28 ± 0.20	7.61	100.7
9	7.47	17.85 ± 0.21	7.31	102.2
10	3.84	28.96 ± 0.19	4.54	84.7
11	5.36	24.32 ± 0.16	5.60	95.6
12	7.40	18.06 ± 0.17	7.20	102.8
13	6.76	20.02 ± 0.32	7.29	92.8

[a.] Mean and standard deviation of three Ct values.

[b.] Viable counts were determined using Marine Agar and identification of colonies.

[c.] Degree of correspondence between the results obtained with a conventional plating technique and with a real-time PCR assay. The relative accuracy is expressed as the percentage of numbers of log (CFU/g) calculated by the real-time PCR assay versus the conventional method (45).

Table 3: Identification of 50 isolates from MA plates using 16S rDNA gene partial sequencing and *P. phosphoreum* identification by real-time PCR method.

Sample	Isolate	Species or genera identification by 16S rDNA gene partial sequencing	*P. phosphoreum* identification using real-time PCR method
1	1	*Serratia fonticola*	-[a]
	2	*Pseudomonas* sp.	-
	3	*Pseudomonas* sp.	-
	4	*Pseudomonas* sp.	-
	5	*Pseudomonas* sp.	-
	6	*Acinetobacter* sp.	-
	7	*Pseudomonas* sp.	-
	8	*Pseudomonas* sp.	-
	9	*Pseudomonas* sp.	-
	10	*Pseudomonas* sp.	-
	11	*Shewanella* sp.	-
	12	*Pseudomonas* sp.	-
	13	*Myroides odoratimimus*	-
	14	*Acinetobacter* sp.	-
	15	Non-workable sequence	-
	16	*Shewanella* sp.	-
	17	*P. phosphoreum*	+[b]
	18	Non-workable sequence	-
	19	*Pseudomonas* sp.	-
	20	*Pseudomonas* sp.	-
2	1	*P. phosphoreum*	+
	2	*P. phosphoreum*	+
	3	*P. phosphoreum*	+
	4	*P. phosphoreum*	+
	5	*P. phosphoreum*	+
	6	*P. phosphoreum*	+
	7	*P. phosphoreum*	+
	8	*P. phosphoreum*	+
	9	*P. phosphoreum*	+
	10	*Shewanella* sp.	-
3	1	*P. phosphoreum*	+
	2	*P. phosphoreum*	+
	3	Non-workable sequence	+
	4	*P. phosphoreum*	+
	5	*P. phosphoreum*	+

	6	*Shewanella* sp.	-
	7	*P. phosphoreum*	+
	8	*Shewanella* sp.	-
	9	*P. phosphoreum*	+
	10	*P. phosphoreum*	+
4	1	*Shewanella* sp.	-
	2	Non-workable sequence	+
	3	Non-workable sequence	+
	4	*P. phosphoreum*	+
	5	*P. phosphoreum*	+
	6	*P. phosphoreum*	+
	7	*P. phosphoreum*	+
	8	*P. phosphoreum*	+
	9	*P. phosphoreum*	+
	10	*Photobacterium* sp.	+

[a] +: Amplification product with a Tm of 84°C obtained in the method range of use.

[b] - : No PCR-product obtained in the method range of use.

Figures

Figure 1: Sequence alignment of the gyrase subunit B gene (gyrB) from Photobacterium species and Vibrio fischeri. The accession numbers of the sequences used for the alignment are indicated. Sequences used for primer design are boxed.

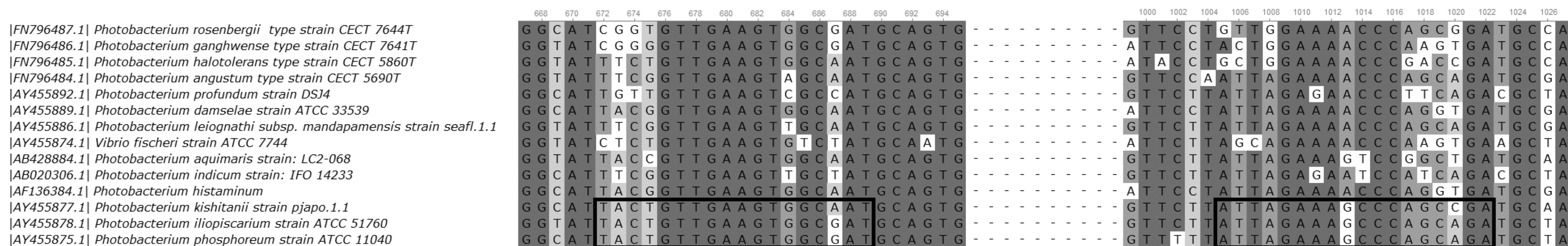

Figure 2: Standard curves for real-time PCR quantification of P. phosphoreum diluted in physiological saline solution (□) or in artificially contaminated salmon (●). Viable counts were determined using Marine Agar. The average efficiency of the real-time amplification was 108% with physiological saline and 112 % with artificially contaminated salmon.

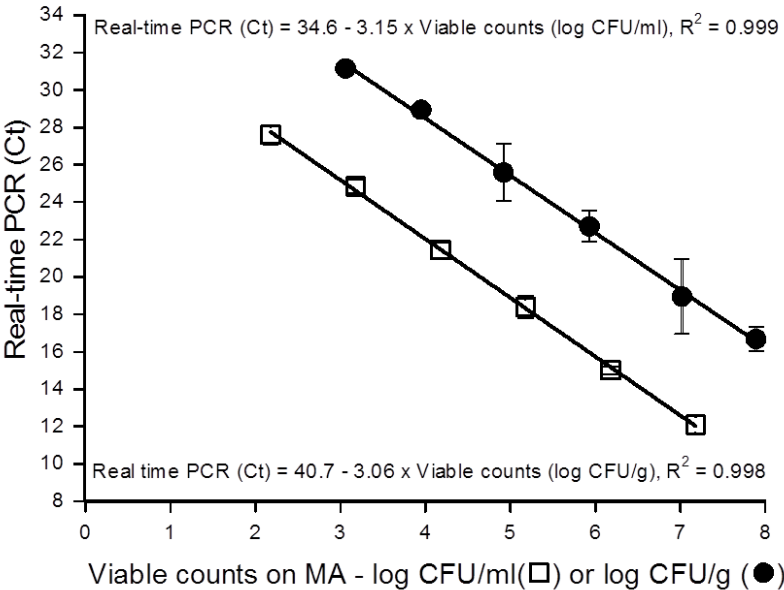

Figure 3: Correlation between concentrations of *P. phosphoreum* determined by real-time PCR and viable counting in 13 samples of naturally contaminated fresh MAP salmon steaks.

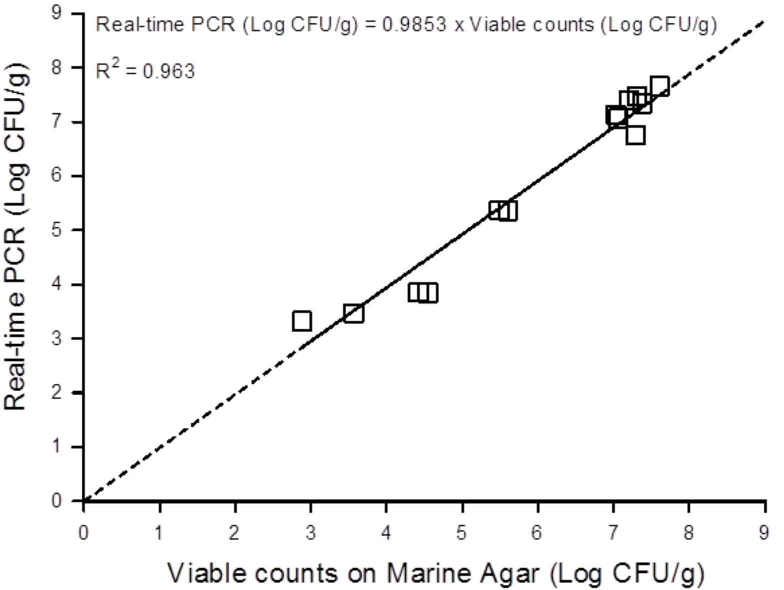

Figure 4: Correlation curve between *P. phosphoreum* enumeration by the specific conductance method and real-time PCR on 22 samples of naturally contaminated MAP salmon steaks.