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Development of a real-time PCR method coupled with a selective pre-enrichment step for quantification of *Morganella morganii* and *Morganella psychrotolerans* in fish products

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ABSTRACT

Histamine fish poisoning is common and due to toxic concentrations of histamine often produced by Gram-negative bacteria in fin-fish products with a high content of the free amino acid histidine. The genus *Morganella* includes two species previously reported to cause incidents of histamine fish poisoning. *Morganella morganii* and *Morganella psychrotolerans* are both strong producer of histamine. However, little is known about the occurrence and critical stages for fish contamination with these bacteria. To elucidate contamination routes of *Morganella*, specific real-time quantitative PCR (RTi qPCR) methods for quantification of *M. morganii* and *M. psychrotolerans* have been developed. Selective primers amplified a 110 bp region of the *vasD* gene for *M. psychrotolerans* and a 171 bp region of the galactokinase gene for *M. morganii*. These primer-sets showed high specificity as demonstrated by using purified DNA from 23 other histamine producing bacteria and 26 isolates with no or limited histamine production. The efficiency of the qPCR reactions on artificially contaminated fish samples were 100.8% and 96.3% respectively. The limit of quantification (LOQ) without enrichment was 4 log CFU/g. A quantitative enrichment step with a selective medium was included and improved the sensitivity of the methods to a LOQ of below 50 CFU/g in seafood. RTi qPCR methods with or without enrichment were evaluated for enumeration of *Morganella* species in naturally contaminated fresh fish and lightly preserved seafood from Denmark. These new methods will contribute to a better understanding of the occurrence and histamine production by *Morganella* species in fish products, information that is essential to reduce the unacceptably high frequency of histamine fish poisoning.

1. Introduction

Histamine fish poisoning (HFP) is common worldwide and due to consumption of a range of dark-fleshed fin-fishes (Dalgaard et al., 2008). Between 2008 and 2010, HFP was responsible for 51% of all seafood related outbreaks of disease in France and in Europe and for 20% in United States (CDC, 2011; Helwigh et al., 2012; InVS (Institut de Veille Sanitaire), 2011). HFP is typically caused by consumption of fish muscle containing high concentration of histamine (>500 mg/kg) which can be observed in fish species with high level of histidine such as Scombridae (tuna, mackerel…), Clupeidae (herring, sardine…) and other species like *Coryphaena hippurus* (mahi-mahi) or *Belone belone* (garfish) (Hungerford, 2010). HFP is a relatively mild illness with allergy-like symptoms that appear some minutes to few hours after consumption of the food. They are mainly characterized by rash, diarrhea, nausea, headache, flushing and sweating (Prester, 2011).

Histamine in fish flesh is produced by decarboxylation of free histidine by bacterial decarboxylase. Gram-negative marine and enteric bacteria have been identified as the main bacterial groups responsible for HFP in fish products (Bjornsdottir et al., 2009; Dalgaard et al., 2008; Veciana-Nogués et al., 2004). Histamine producing bacteria (HPB) can be sub-divided into low and high producers of histamine based on the formation of histamine in a broth culture medium containing histidine. The high histamine producers include mesophilic species

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such as *Morganella morganii*, *Enterobacter aerogenes*, *Hafnia alvei*, *Raoultella planticola* and *Photobacterium damselae*, which are able to produce more than 1000 mg/l of histamine in tryptone soy broth supplemented with 2% histidine after 24–48 h of incubation at temperatures above 15 °C (Bjornsdottir et al., 2009). High histamine-producing and psychrotolerant bacteria have also been described, including *Photobacterium phosphoreum* (Kanki et al., 2004) and *Morganella psychrotolerans* (Emborg et al., 2006). Both species produce histamine at low temperature until 0 °C in fish products (Dalgaard et al., 2006; Emborg et al., 2005).

Whereas all *Morganella* isolates seem to be strong histamine producers, intra-species variability exists for other species including *P. phosphoreum* (Dalgaard et al., 2006; Emborg et al., 2005; Kim et al., 2002; Klausen and Huss, 1987). Both *M. morganii* and *M. psychrotolerans* have been isolated from fish products (fresh tuna, tuna in sauce, cold-smoked tuna) responsible for outbreaks (for a review, see Dalgaard et al., 2008). Despites those facts, there is a lack of data on prevalence of *Morganella* spp. in fish products, probably due to the absence of sufficiently sensitive and specific enumeration method. Previous experiments of detection of HPB have been performed on differential media (Mavromatis and Quantick, 2002; Tao et al., 2009) based on color modification of pH indicators once histidine is converted to histamine during bacterial growth. However, these methods are time consuming and unreliable (Bjornsdottir et al., 2009). Nowadays, molecular methods based on RTi qPCR are more reliable for detection, identification and quantification of bacteria but their lack of sensitivity when used with food products remains a problem (Postollec et al., 2011). Bjornsdottir-Butler et al. (2011a) developed a RTi qPCR method based on the primers of Takahashi et al. (2003) designed on the histidine decarboxylase gene to quantify Gram-negative and high HPB. That method has been set-up for mesophilic species but cannot detect *P. phosphoreum* or *M. psychrotolerans* (experiments in our laboratory, data not shown). More recently, RTi qPCR methods for quantification of *M. morganii* (Ferrario et al., 2012a, 2012b) and viable *P. phosphoreum* (Macé et al., 2013) in fish products have been proposed. However, in those studies the detection limit of RTi qPCR was typically between 3 and 4 log CFU/g in food products. This limit is too high to study the occurrence of *Morganella* spp. in fish products in which expected contamination level is most likely less than 100 CFU/g. To overcome this problem of the sensitivity of RTi qPCR methods, enrichment steps with selective media have been proposed, allowing quantification of 1–10 CFU/g for e.g. the pathogenic bacteria *Listeria monocytogenes* (O'Grady et al., 2008) and *Salmonella* (McGuinness et al., 2009) in different food products.

The objectives of the present study were to develop two specific and sensitive RTi qPCR methods for quantification of *M. morganii* and *M. psychrotolerans* in fish products. Selective primers for both species have been designed and evaluated against isolates of HPB and non-HBP isolates. These RTi qPCR methods used in combination with a new enrichment step reduced the LOQ and increase the methods field of application for fish products.

2. Materials & methods

2.1. Bacterial strains and pre-culturing

Bacterial strains used in this study are listed in Table 1. Strains were grown in Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) at 20 °C during 24 h, for *Morganella* strains, and during 24–48 h for other strains, except for *P. phosphoreum* which was cultivated at 15 °C in BHI with 2% NaCl. The strains were stored at −80 °C in their culture medium with 10% glycerol.

2.2. DNA extraction

For bacterial cultures, DNA extraction was performed on 1.5 ml cultures that have reached a concentration of at least 8 log CFU/ml. After centrifugation during 10 min at 8500 ×*g*, the chromosomal DNA of all bacterial isolates was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, S.A., Courtaboeuf, France).

The DNA extraction on fish tissue was adapted from a protocol developed for raw salmon to quantify *P. phosphoreum* by RTi qPCR (Macé et al., 2013). Briefly, 30 g portion of seafood samples (tuna, mackerel or herring) was aseptically weighed in a sterile stomacher bag and 5-fold diluted with sterile peptone-salt water (0.1% peptone, 0.85% salt). Ten milliliters of homogenized suspension were filtered on a Nucleospin Filter L (Macherey-Nagel, Hoerdt, France). The following extraction of DNA was performed as described by Macé et al. (2013). DNA was purified using the DNeasy Blood & Tissue Kit as described in the Qiagen instruction manual.

2.3. Genomics data and primer design

Shotgun sequencing was performed in Denmark using Roche FLX 454 pyrosequencing on DNA from the *M. morganii* strain U6/1 and on DNA from the type strain of *M. psychrotolerans* U2/3^T = LMG 23374^T = DSM 17886^T (Emborg et al., 2006; Meyer et al., 2008). Sequencing was done using the FLX Titanium sequencing kit and 1 region of an XLR70 pico titre plate per strain. Contigs for each strain were assembled using the Newbler assembler software version 2.0.01.14 provided with the GS FLX instrument and annotated by using the RAST annotation server (Aziz et al., 2008). Genome sequencing and assembly of the *M. morganii* U6/1 and *M. psychrotolerans* U2/3^T resulted in 51 and 28× coverage on 3.9 Mb and 4.2 Mb size genomes, respectively. Genomes were assembled into 123 (*M. morganii* U6/1) and 292 (*M. psychrotolerans* U2/3^T) large contigs (>500 bp).

Thirty primer pairs were designed for *M. psychrotolerans* and *M. morganii* using the Geneious Software (Geneious version 6.1, Biomatters Ltd.) based on the Primer3 calculation method (Untergasser et al., 2007) and the Primer-Blast software (NCBI, UK). Primer pairs were initially evaluated *in silico* by using the nucleotide Blast program to check their specificity for *M. morganii* or *M. psychrotolerans* against all genomic data of GenBank (NCBI, UK) and tested in vitro as described in 2.4 using appropriate hybridization temperatures. The selected primers VasD-F4 (5′-AAATCGCCATCACACT CCTTG-3′) and VasD-R4 (5′-TTCAAAACGGGAGTCCTCACTG-3′) were designed on the *vasD* gene from the Type VI secretion system of *M. psychrotolerans*. This primer set matched respectively positions 146–166 and 234–255 of the *M. psychrotolerans* U2/3^T *vasD* gene (GenBank accession number KP069481). For *M. morganii*, the primers GalK-F4 (5′-ACAGTGCTTCGGCGCATCCC-3′) and GalK-R4 (5′-GCAGCC ACCACGCAGACCTT-3′) were obtained on the galactokinase gene (*galK*) and matched respectively positions 39–58 and 190–209 of the galactokinase gene of *M. morganii* U6/1 (GenBank accession number KP069480).

2.4. Real-time PCR amplification

Inclusivity and exclusivity of primers (TAG Copenhagen, Denmark or Invitrogen, Illkirch, France) designed for *M. morganii* and *M. psychrotolerans* were tested on bacterial DNA extracted from the strains listed in Table 1. Genomic DNA was measured using a Nanovalue (Applied Biosystem, Saint-Aubin, France) and diluted to 4 ng/μl. Specificity of the RTi qPCR assay was tested using 4 ng of DNA.

Real-time qPCR was conducted in a 15 μl reaction volume using the following reaction mixture: 1 U of Iq SYBR® Green Supermix (Biorad, Hercules, US), 300 nM of each VasD or GalK forward and reverse primers, nuclease free H_2O and 1 μl of DNA template. Real-time PCR cycling was performed using a CFX-96 instrument (Biorad, Marnes-la-Coquette, France) or a Mx3000P thermocycler (Stratagene, AH Dianostics, Aarhus, Denmark). The cycling parameters were as follows: 95 °C hold for 180 s for initial denaturation and activation of