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Quorum sensing system-regulated genes affect the spoilage potential of *Shewanella baltica*

Linglin Fu[a], Chong Wang[a], Nana Liu[a], Aijin Ma[b,*], Yanbo Wang[a,*]

[a] Key Laboratory for Food Microbial Technology of Zhejiang Province, School of Food Science and Biotechnology, Zhejiang Gongshang University, 18 Xue Zheng Street, Hangzhou 310018, China

[b] China National Institute of Standardization, No. 4 Zhichun Road, Haidian District, Beijing 100191, China

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ABSTRACT

Large yellow croaker (*Pseudosciaena crocea*) is a popular and nutritious but also highly perishable fish species, with *Shewanella baltica* being the primary spoilage bacteria during low-temperature storage. Clarifying the factors promoting spoilage will facilitate efforts to predict and control the shelf life of foods. This study focused on spoilage-related genes in two *Shewanella baltica* strains with different spoilage potentials. Using whole genome sequencing and alignment, three distinguishing genes (*torT*, *cysM* and *trxB*) were identified. Further protein sequence comparison and protein structure modeling revealed possible motifs responsible for the spoilage activity. Moreover, diketopiperazine (DKP) *quorum sensing* (QS) signaling molecules regulated biofilm formation and spoilage gene expression, indicating a relationship between the QS system, biofilm formation and spoilage potential. Our results suggest that DKPs and spoilage genes are potential targets for developing novel food antiseptics, as well as new markers for fish product spoilage.

1. Introduction

Fish and fish products are consumed as foods all over the world. Fish products are nutrient-rich foods containing high-quality proteins that include all of the essential amino acids for human health, making them complete protein sources (Zhao, Li, Wang, & Lv, 2012). However, fish products are highly perishable, which renders them unfit for human consumption upon spoilage and results in significant economic losses. Thus, it is imperative to clarify the mechanisms of fish product spoilage to enable shelf life to be predicted and controlled (Bruhn et al., 2004).

Studies have observed that microorganisms are the most common causes of food spoilage. Even with the development of modern preservation techniques, excessive amounts of foods are lost due to microbial spoilage (Bai & Rai, 2011). Due to the different abilities of microorganisms to adapt to different environments, the rate and extent of microbial food spoilage changes with different storage conditions. For instance, fermentative Gram-negative bacteria, such as the *Vibrionaceae* family, are the cause of fish spoilage in the absence of effective preservation conditions (Ghaly, Dave, Budge, & Brooks, 2010), whereas psychrotolerant Gram-negative bacteria *Pseudomonas* spp. and *Shewanella* spp. cause chilled fish spoilage (Gram & Huss, 2000). Under vacuum-pack conditions, lactic acid bacteria, such as *Lactobacillus* and *Carnobacterium*, psychtrotrophic Enterobacteriaceae and Gram-negative fermentative bacteria, such as *Photobacterium phosphoreum*, are typical microbiota (Hansen, Gill, & Hussa, 1995; Jorgensen, Huss, & Dalgaard, 2000; Leroi, Joffraud, Chevalier, & Cardinal, 1998). Thus, not all bacteria cause food spoilage, and the primary inducers for food spoilage are usually referred to as specific spoilage organisms (SSOs), although they are typically present in very low numbers and comprise a low percentage of the microbiota present on fresh food (Gram & Dalgaard, 2002). SSOs decompose nitrogenous substances, such as amino acids and proteins, into amines (trimethylamine), sulfides, alcohols, ketones, aldehydes and organic acids via their metabolism (Gram et al., 2002; Gram & Dalgaard, 2002), producing unacceptable off odors and unpleasant off flavors (Dainty & Mackey, 1992; Jaffrès et al., 2011).

Although SSOs change dramatically based on the storage methods and fish species (Alfaro & Hernandez, 2013; Mace et al., 2012; Rudi, Maugesten, Hannevik, & Nissen, 2004), many studies have shown that *Shewanella putrefaciens* is the primary spoilage bacterium of low-temperature stored marine foods (Fonnesbech Vogel, Venkateswaran, Satomi, & Gram, 2005), and *Shewanella baltica* is the primary spoilage bacteria of the large yellow croaker and Danish marine fish during low-temperature storage (Fonnesbech Vogel et al., 2005; Gu, Fu, Wang, & Lin, 2013). *Shewanella* spp. are motile Gram-negative bacteria with the

Abbreviations: DKP, diketopiperazine; QS, *quorum sensing*; TMAO, trimethyl-amine-N-oxide; TVB-N, total volatile basic nitrogen

* Corresponding authors.
E-mail addresses: maaj@cnis.gov.cn (A. Ma), wangyb@mail.zjgsu.edu.cn (Y. Wang).

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capability of anaerobic respiration using electron acceptors other than oxygen, such as trimethyl-amine-N-oxide (TMAO) (Brettar, Christen, & Hofle, 2002; Venkateswaran et al., 1999). Most *Shewanella* spp. are able to decompose TMAO into trimethylamine (TMA), dimethylamine (DMA) and formaldehyde (FA), thereby producing a strong fishy smell. However, the regulation of the relevant spoilage genes in *Shewanella* spp. has not been revealed.

Bacteria communicate with one another using chemical signal molecules called autoinducers. This process, termed *quorum sensing* (QS) (Fuqua, Winans, & Greenberg, 1994; Hammer & Bassler, 2003; Pinto, Pappas, & Winans, 2012), allows bacteria to monitor cell density and regulates a diverse array of physiological activities, including symbiosis, virulence, competence, conjugation, antibiotic production, spoilage activity, motility, sporulation, and biofilm formation (Jiang & Li, 2013; Mangwani, Dash, Chauhan, & Das, 2012; Perez-Velazquez, Golgeli, & Garcia-Contreras, 2016). The most widely used autoinducers are N-acylhomoserine lactones (AHLs) and autoinducing peptides (AIPs) in Gram-negative and Gram-positive bacteria, respectively (Miller & Bassler, 2001). Additionally, diketopiperazines (DKPs) have been suggested to be another type of autoinducer involved in the QS system of Gram-negative bacteria, such as *Pseudomonas putida* and *Shewanella baltica*, and the role of DKPs in inducing fish spoilage has also been revealed (Degrassi et al., 2002; Gu et al., 2013). In fact, DKPs but not AHLs can be detected in *Shewanella baltica* (Zhu, Zhao, Feng, & Gao, 2016). However, the detailed mechanism of the DKPs-mediated QS system has not been thoroughly elucidated to date, and the role and regulatory system of DKP-induced fish spoilage is also undefined.

The large yellow croaker (*Pseudosciaena crocea*), a commonly cultivated fish species, has become one of the most produced and consumed fish in China due to its significant nutritional value and great taste (Duan, Mai, Zhong, Si, & Wang, 2001). However, due to its high nutritional value and water content, the large yellow croaker is susceptible to decomposition, leading to considerable economic losses every year (Li, Li, Hu, Chen, & Li, 2014; Zhu et al., 2016). Achieving the ability to control large yellow croaker spoilage by understanding the spoilage mechanism is urgently important.

Overall, this work focused on the spoilage of the large yellow croaker by *Shewanella baltica* and identifying the mechanism of bacterial fish spoilage at the gene level in particular. To this end, the whole genomes of two *Shewanella baltica* strains with major differences in spoilage activity were sequenced and compared to reveal genetic differences between these two strains. In addition, variations in gene expression and the regulatory role of the DKP-based QS system were determined using real-time PCR. Additionally, the relevance of biofilm formation in this process was investigated. Our results have important implications for understanding the mechanism of fish product spoilage and developing new approaches for spoilage detection and prevention.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Different *Shewanella* strains were previously isolated from fresh large yellow croaker and stored at 4 °C (Gu et al., 2013). Two strains, termed *Shewanella baltica* 025 and *Shewanella baltica* 003 (later identified as *Shewanella baltica* OS185 and *Shewanella baltica* OS223, respectively), were streaked onto nutrient agar plates and incubated at 25 °C for 24 h. Next, single colonies were transferred to 50 mL of nutrient broth (in 100 mL conical flasks) and incubated for 15 h at 25 °C for further studies.

2.2. Whole genome sequencing and analysis

Bacterial genomic DNA was extracted according to the instruction of the Biospin Bacteria Genomic DNA Extraction kit (BioFlux, Alameda, CA, USA). The genomic DNA was qualified using a Nanodrop (Thermo Fisher Scientific, San Jose, CA, USA) with a guaranteed OD_{260}/OD_{280} of between 1.7 and 2.0. Whole genome sequencing was done by Bio-x Research Institute, Shanghai Jiaotong University using an Ion PGM Sequencer (Life Technologies, Grand Island, NY, USA). Protein sequences were translated from whole genome sequencing data using NCBI ORF Finder. Spoilage proteins, including those involved in the Tor respiratory system, biogenic amine production, sulfide production and biofilm formation, were selected and the sequences of the same proteins from different strains were aligned using NCBI BLAST.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from the cultured cells using RNAiso Plus (Total RNA extraction reagent, Takara Shuzo, Ghiga, Japan) by following the instructions for Gram-negative bacteria. The total RNA was qualified using a Nanodrop, ensuring an OD_{260}/OD_{280} between 1.7 and 2.1. Next, the RNA was reverse-transcribed using the GoScript Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions with random primers. The product was incubated at 70 °C for 15 min to inactivate reverse transcriptase and stored at −20 °C for further real-time PCR analysis.

2.4. Real-time PCR

Real-time PCR was carried out with a LightCycler Nano System (Roche Applied Science, Indianapolis, IN, USA). Each 20 μL reaction contained 2 μL of cDNA, 10 μL of SYBR green PCR master mix, 0.4 μL of each primer (20 μmol/L), and 7.2 μL of double-distilled water. Also, 16S rRNA was used as the reference gene, and specific primers were designed using the Primer 5.0 software and synthesized by Invitrogen (Invitrogen, Shanghai, China). All primers for real-time PCR are shown in Table 1.

Real-time PCR was run using the following conditions: hold at 95 °C for 600 s; 40 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 20 s, the fluorescent signal was collected at this step; and melting from 60 to 95 °C at 0.1 °C/s. Relative quantitation was used to evaluate the expression level of the spoilage genes (Ginzinger, 2002).

Three independent experiments were performed using triplicate samples. The statistical analysis was performed using Origin 8.0. Error bars represent the SD from the mean of the measurements.

2.5. DKP treatment

Four DKPs (cyclo-(L-Pro-L-Gly), cyclo-(L-Pro-L-Leu), cyclo-(L-Leu-L-Leu), and cyclo-(L-Pro-L-Phe)), were synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China) and dissolved in methanol as 4 mg/mL stocks. DKPs were added into the bacteria culture medium with a final concentration of 40 ng/mL, and equal volume of methanol was

Table 1
Real-time PCR primers used in this study.

Gene name	Forward primer (5′-3′)	Reverse Primer (5′-3′)
16 s rRNA	ACTCCTACGGGAGGCAGCAG	GTATTACCGCGGCTGCTGG
torT (*S. baltica* OS185)	TTACTTGCCCAATTTACC	TGCGGATGTTGTGATGTT
torT (*S. baltica* OS223)	CTGACTAAACCCGTGCTA	GTTGCGGATGTTGTGATG
cysM (*S. baltica* OS185)	TGTAATCCTGACATCACCA	CTTGCTCTTCAACATCCAT
cysM (*S. baltica* OS223)	GGACGACAGGCACCATAA	GAAAATCCCCGGCAAGTA
trxB (*S. baltica* OS185)	CGACCTGTGATGGTTTCT	GTGAAGGATGATGTTGCC
trxB (*S. baltica* OS223)	CTGTGCGACCTGTGATGG	TTTCTGAGCGGAAGCTGT
torCAD operon	ACTCGAAGCCACCAATACCG	CGCGAACGCCAGAGTGATTC