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# Inhibition of biofilm development and spoilage potential of *Shewanella baltica* by quorum sensing signal in cell-free supernatant from *Pseudomonas fluorescens*

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ABSTRACT

The objective of this study was to in vitro evaluate the effect of a cell-free supernatant (CFS) containing quorum sensing (QS) signal of *Pseudomonas fluorescens* on the growth, biofilm development and spoilage potential of *Shewanella baltica*, and preliminarily assess the interactive influences of various chemically synthesized autoinducers on spoilage phenotypes of *S. baltica*. PF01 strain isolated from spoiled *Pseudosciaen crocea* was identified *P. fluorescens*. The addition of 25% and 50% CFS to *S. baltica* culture had no effect on the growth rate during the lag and exponential phase, however, caused cell decline during the stationary phase. The presence of CFS from *P. fluorescens* significantly inhibited biofilm development, and greatly decreased the production of trimethylamine (TMA) and biogenic amino in *S. baltica*. Various signal molecules of QS in the CFS of *P. fluorescens* culture were detected, including seven *N*-acyl-L-homoserine lactones (AHLs), autoinducer-2 (AI-2) and two diketopiperazines (DKPs). Exogenous supplement of synthesized seven AHLs containing in the CFS decreased biofilm formation and TMA production in *S. baltica*, while exposure to exogenous *cyclo*-(L-Pro-L-Leu) was showed to promote spoilage potential, which revealed that *S. baltica* also sense the two QS molecules. Furthermore, the stimulating effect of *cyclo*-(L-Pro-L-Leu) was affected when AHL was simultaneously added, suggesting that the inhibitory activity of spoilage phenotypes in *S. baltica* might be attributed to a competitive effect of these QS compounds in the CFS of *P. fluorescens*. The present studies provide a good basis for future research on the role of QS in the regulation of spoilage microbial flora.



## 1. Introduction

Fresh fish and lightly preserved fish are highly perishable due to enzymatic autolysis, chemical oxidations and microbial growth, and microbial activity is by far the most important cause affecting the quality of fresh fish (Gram and Huss, 1996). Bacterial spoilage in chilled fresh fish under aerobic storage conditions is mainly caused by Gram-negative psychrotrophic organisms such as *Shewanella* spp, *Pseudomonas* spp and genera of the *Enterobacteriaceae* family (Gram et al., 2002; Skandamis and Nychas, 2012). *Shewenalla* spp. and *Pseudomonas* spp. are largely responsible for the specific spoilers in iced marine fish and freshwater fish, respectively (Gram et al., 2002). The two bacteria were also linked to the spoilage of large yellow croaker (*Pseudosciaena crocea*) stored aerobically under refrigeration (Zhu et al., 2016). Additionally, *Shewanella baltica* was identified to be the dominant species producing spoilage in iced stored marine fish caught in the Danish Baltic Sea (Vogel et al., 2005) and refrigerated *P. crocea* (Gu et al., 2013; Zhu et al., 2016). Furthermore, various spoilage related bacteria have been confirmed to form biofilm on various food surfaces, such as *Shewanella*, *Pseudomonas*, and *Lactobacillus* (Bagge et al., 2001; Fazli et al., 2014; Fernández Ramírez et al., 2015), resulting in a continuous source of contamination.

Quorum sensing (QS) have been found in a diverse group of bacteria which communicate with each other and govern their behaviour using signal molecules in food ecosystems. In recent years, the role of cell-to-cell communication in food microbial ecology has been explored. Various QS signaling compounds, including acylated homoserine lactones (AHLs), autoinducer-2 (AI-2) and diketopiperazines (DKPs), have been reported to present or increase their concentration in spoiled milk, meat, vegetables and aquatic product (Lu et al., 2004; Rasch et al., 2005; Blana and Nychas, 2014; Zhu et al., 2015, 2016). The increasing evidence implicated that QS could be involved in bacterial food spoilage (Skandamis and Nychas, 2012; Blana and Nychas, 2014), however, QS exact role has not been completely elucidated. Additionally, relatively limited attention has paid to how both intra- and inter-species communication modulate bacterial growth responses and biofilm formation by adding exogenous QS signaling compounds and potential signalings existing in cell-free supernatant (CFS) from food spoilage or pathogenic bacteria (Dourou et al., 2011; Nychas et al., 2009;

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Chorianopoulos et al., 2010; Zhang et al., 2014; Wang et al., 2013). Microbial cell-free meat extract containing QS compounds enhanced the growth rate of two spoilage bacterial, *Pseudomonas fluorescens* and *Serratia marcescens* (Nychas et al., 2009). The AHLs-containing culture extract could reduce growth time and inhibit biofilm formation of *Pseudomonas aeruginosa* (Zhang et al., 2014).

*Shewanella*, which are associated with marine fish spoilage, was found to have *luxS* gene and AI-2 activity (Bodor et al., 2008). DKPs and AI-2 activities, rather than AHLs, were detected in the *S. baltica*, as a specific spoilage organism (SSO) in refrigerated shrimp (*Litopenaeus vannamei*) and large yellow croaker (*P. crocea*) (Gu et al., 2013; Zhu et al., 2015). Our previous studies indicated that the spoilage potential of *S. baltica* might be regulated by DKPs-based quorum sensing (Zhu et al., 2016). So far, the most available studies on QS in *Shewanella* have focused on the detection of QS system and the possible role of autoinducers in the spoilage (Bodor et al., 2008; Gu et al., 2013; Zhu et al., 2015, 2016). Nevertheless, limited research studies have assessed the possible responses of *Shewanella* to sense signaling molecules produced by other spoilage bacterial species in microbial flora, particularly AHLs which *Shewanella* do not synthesize (Zhu et al., 2015, 2016). Considering that fish spoilage is associated with the presence of *Pseudomonas* with the capability of producing AHLs signaling, it is interesting to evaluate how interspecies communication affect the biofilm formation and spoilage potential of *S. baltica*.

Therefore, the aim of the present study was to investigate the effect of cell-free culture supernatant from *P. fluorescens* on the growth, biofilm development and spoilage phenotypes of *S. baltica*, a SSO in *P. crocea*. The QS signaling compounds produced by *P. fluorescens* were characterized, and the competitive effects of synthetic related different autoinducers on the biofilm and TMA formation in *S. baltica* were further confirmed.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*S. baltica* 02 strain originating from spoiled large yellow croaker (*P. crocea*) were identified the specific spoilage organism in our laboratory (Zhu et al., 2016). A strain PF01 without producing $H_2S$ isolated newly from spoiled *P. crocea* was also sourced from our laboratory. *S. baltica* and PF01 were routinely incubated in Luriae-Bertani (LB) broth at 25 °C and 30 °C respectively.

### 2.2. Sequencing 16S rRNA sequence of PF01

DNA was extracted from a single colony of the PF01 strain after growth in LB broth at 30 °C for 24 h using bacterial DNA isolation kit (Bioer Techology Co., Ltd. Hangzhou, China) according to the manufacturer's instructions. 16S rRNA gene analysis was performed and the PCR product was sequenced by Sangon Biotech Company (Shanghai, China). Phylogenetic analysis was carried out using MEGA 5.2 software by comparing the 16S rRNA sequences of PF01 to the closely related sequences available in GenBank database.

### 2.3. Preparation of CFS

*P. fluorescens* was cultured in 200 mL of LB broth at 30 °C for 12 h. Cultures were then centrifuged (Sigma 3–18 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 12,000 *g* for 15 min at 4 °C, followed by filtration through a 0.22 μm-pore-size filter (Whatman, Inc., Cilfton, NJ, USA) to obtain CFS of *P. fluorescens*. After heated at 100 °C for 5 min, the CFS was immediately detected for the presence of QS signal molecules as described below. The initial pH of the CFS was 7.19. The growth, biofilm, TMA and biogenic amino in the tested *S. baltica* were analyzed in the broth containing no CFS (control), 20% (v/v) CFS and 50% CFS.

### 2.4. Growth assay of S. baltica

Overnight cultures of *S. baltica* strain were inoculated into LB broth containing a final concentration of 0% (control), 25% and 50% CFS respectively, and the treated samples then were incubated for at 25 °C and at 4 °C with shaking (180 rpm). The population of *S. baltica* was examined by plating on iron agar (IA, Qindao Hope Co., Ltd. Qindao, China) at 3 h intervals up to 30 h.

### 2.5. Biofilm assay of S. baltica

Overnight cultures of *S. baltica* were diluted at 1:10,000 ratio in fresh sterile TSB medium. The dilutions were transferred into the wells of 24-well tissue culture plate supplemented with a final concentration of 0% (control), 25% and 50% CFS respectively. After incubation without shaking at 25 °C, biofilm content was determined every 24 h using a crystal violet binding assay (Djordjevic et al., 2002). After incubation, the cultures in plate wells were then aspirated, and washed thrice with sterile phosphate buffer solution (PBS) to remove loosely adherent cells. After drying, the surface-attached cells in the biofilm were stained with 1% crystal violet. The wells were then washed with sterile distilled water, and 95% ethanol was then added to re-solubilize the crystal violet-stained biofilm. The absorbance was measured at 595 nm using a microplate reader (Infinite 200, Tecan, Switzerland).

### 2.6. TMA and biogenic amino analyses in S. baltica

For TMA analyses, *S. baltica* strain was cultured in the LB broth containing 10 mM trimethylamine oxide (TMAO) and a different concentration of 0% (control), 25% and 50% CFS at 25 °C for 6 h, 12 h and 24 h with shaking (180 rpm). TMA levels in supernatant were determined spectrophotometrically by following the colorimetric formation of picric acid salt (Dyer, 1945). For biogenic amines analyses, *S. baltica* strain was cultured in LB broth with addition of 0.5% L-histidine hydrochloride, L-lysine hydro-chloride, L-ornithine hydrochloride, 0.25% L-tyrosine disodium salt supplemented with 0.0005% pyridoxal-HCl. Meanwhile a final concentration of 0% (control), 25% and 50% CFS was added to the medium respectively. After incubation at 25 °C for 24 h with shaking (180 rpm), 1 mL of bacterial culture was mixed with 4 mL 0.6 M cold (4 °C) perchloric acid soultion and centrifuged (Sigma 3–18 K) at 10,000 × *g* for 15 min. The supernatant was used to measure biogenic amines by HPLC (HPLC 1100, Agilent Technologies Inc., Santa Clara, CA, USA) according to the method of Wang et al. (2014) and Zhu et al. (2016) with little modification.

### 2.7. Screening for QS autoinducer

#### 2.7.1. AHL analyses

AHLs in CFS from *P. fluorescens* were rapidly screened using *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains in a semi-quantitative well diffusion bioassay as described by the methods of Ravn et al. (2001). Meanwhile, AHLs were further extracted trice from 50 mL CFS of *P. fluorescens* PF01 with acidified ethyl acetate (0.1% *v*/v formic acid). The solvent was removed by rotary evaporation, and the dried residue was reconstituted in 1 mL of ethyl acetate. The extract was injected for LC-MS/MS analysis (TSQ Vantage, Thermo Fisher Scientific, Waltham, MA, USA) using a accucore XL C18 column (150 mm × 4.6 mm × 4 μm) (Thermo Fisher Scientific, USA) according to the method of Ortori et al. (2011) with little modification. Ten synthetic AHLs and oxo-derivatives of known carbon chain lengths were used as standards for comparison. These synthetic AHLs, including *N*-butanoyl-L-homoserinelactone ($C_4$-HSL), *N*-hexanoyl-L-homoserine lactone ($C_6$-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (O-$C_6$-HSL), *N*-octanoyl-L-homoserine lactone ($C_8$-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone(O-$C_8$-HSL), *N*-decanoyl-L-homoserine lactone