JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification and Characterization of Extracellular Cyclic Dipeptides As Quorum-Sensing Signal Molecules from *Shewanella baltica*, the Specific Spoilage Organism of *Pseudosciaena crocea* during 4 °C Storage

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ABSTRACT: Quorum-sensing (QS) signaling molecules are able to mediate specific gene expression inside spoilage bacteria in response to population density and thus are implicated in food spoilage. In the present work, a total of 102 strains of spoilage bacteria were isolated from *Pseudosciaena crocea* at 4 °C storage, and of these, 60 strains were identified as *Shewanella* spp., and 48 strains (47.1%) were identified as *S. baltica*. In addition, the spoilage capabilities of three different *S. baltica* strains (00A, 00B, and 00C) were compared by total volatile base nitrogen (TVB-N) and sensory analysis (off-odors). Furthermore, four cyclic dipeptides (diketopiperazines, DKPs) that function as QS signal molecules were isolated and characterized from the extracellular metabolites of *S. baltica* 00C which had the strongest spoilage activity based on gas chromatography mass spectrometry (GC-MS). By supplementation of four synthesized DKPs, the spoilage capability of *S. baltica* could be significantly enhanced. So far, this was the first attempt to characterize DKPs as the signaling molecules in QS of *S. baltica*. Our study may provide some evidence of the role of DKPs involved in microbial spoilage.

KEYWORDS: *Shewanella baltica, specific spoilage organisms (SSOs), diketopiperazines (DKPs), quorum sensing, Pseudosciaena crocea, gas chromatography mass spectrometry (GC-MS)*

INTRODUCTION

Microorganisms are the major cause of spoilage of most seafood products. However, only a few members of the microbial community, the specific spoilage organisms (SSOs), give rise to the offensive off-flavors and toxic compounds associated with seafood spoilage.[1] During the periods of storage, SSOs often vary according to the different conditions. Generally, *Pseudomonas* and *Shewanella* spp. are the two major SSOs of fish stored at low temperature.[2] *Shewanella* species are Gram-negative, H_2S-producing, and have motile rods and have received more attention in recent years because of their important role in spoilage of fish and fish products.[3] Several new *Shewanella* species, such as *S. baltica*, *S. glacialipiscicola*, *S. algidipiscicola*, *S. hafniensis*, *S. morhuae*, and *S. upenei*, were isolated from some kinds of marine fish.[4] Of these, *S. baltica* is considered as a main H_2S-producing organism in marine fish and can produce specific signal compounds to facilitate the potential spoilage activity.[3−5]

The response of bacteria produce and release chemical signal molecules (autoinducers) to increase in concentration as a function of cell density is called quorum sensing (QS).[6] Gram-positive and Gram-negative bacteria use QS communication circuits to regulate a diverse array of physiological activities, including symbiosis, virulence, competence, conjugation, antibiotic production, spoilage activity, motility, sporulation, and biofilm formation. One well-characterized class of signal molecules, *N*-acylhomoserine lactones (AHLs, AI-1), is used by a diverse range of Gram-negative bacteria to mediate population density-dependent gene expression. In contrast to Gram-negative bacteria, Gram-positive bacteria employ secreted peptides and peptide derivatives (AIPs) as autoinducers for QS. In addition, autoinducer-2 (AI-2), a collective term for a group of interconvertible furanones derived from dihydroxypentanedione (DPD), is known to present in both Gram-positive and Gram-negative bacteria. [7] Moreover, AI-3, an aromatic compound, is produced by some intestinal commensals and pathogens.[5,8] It has recently been proven that several new compounds, such as the cyclic dipeptides, aromatic alcohols, and multiple small RNAs, can control QS in *Pseudomonas aeruginosa*, yeast, *Vibrio* spp., and other gram-negative bacteria.[9−11]

In recent years, cyclic dipeptides (diketopiperazines, DKPs), as one of the smallest and simplest peptide compounds, have been found in many known bacterial species in nature.[12] They are isolated from many plant- and animal-derived Gram-negative bacteria, especially marine microorganisms, such as the genera *Pseudomonas*, *Ruegeria*, *Aspergillus*, *Haloterrigena*, *Burkholderia*, and *Nocardiopsis*.[9,13−18] The basic structure of diketopiperazines is a cyclic peptide derived from the folding head-to-tail of a linear dipeptide. Because of the stable framework of the six-member ring structure, two hydrogen bond donors and two hydrogen bond receptors, DKPs and their derivatives exhibit various biological and pharmacological

Received: September 3, 2013
Revised: November 7, 2013
Accepted: November 8, 2013
Published: November 8, 2013

activities.[16,19] In addition, Holden and other scientists[9] showed that several Gram-negative bacteria produced and secreted cyclic dipeptides with the ability to activate and/or antagonize other LuxR-based QS systems. It was reported that *Pseudomonas putida* WCS358 could produce and secrete four cyclic dipeptides, and some of the cyclic dipeptides interacted with the QS LuxI and LuxR homologues.[14] Hence, cyclic dipeptides are one of the important families of cell−cell signaling compounds and considered as the novel finding in Gram-negative bacteria.

Effective methods for detection of signaling compounds are important in the identification of QS capabilities in bacteria. Recently, several methods have been developed for the direct analysis of *N*-acyl-homoserine lactone signal molecules based on chromatography coupled to bioassay, mass spectrometry or electrophoresis such as gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography mass spectrometry (HPLC-MS).[20,21] In order to detect AHLs, a convenient and sensitive bioassay was developed based on the production of pigments by *Escherichia coli* pSB403 (LuxR), *Agrobacterium tumefaciens* A136 (TraR), and *Chromobacterium violaceum* CV026 (CviR).[22] The separation by TLC coupled with biodetection gives poor resolution and works only when QS molecules are abundant, although it could give a direct and visual catalog of the acyl-HSL signal molecules.[18,23] Thus, the TLC assay is unsuited to detect the kinds of AHLs, and it cannot be used for an accurate quantitative analysis. However, GC-MS and HPLC-MS were reported for quantifying AHLs and presented as sensitive, selective, rapid, and straightforward methods to indicate the occurrence of AHLs in real samples without using chemical derivatization.[24] So far, some studies have already detected the cyclic dipeptides by GC-MS as one of the families of cell−cell signaling compounds.[13,14,16]

Large yellow croaker, *Pseudosciaena crocea*, is commercially important due to its good taste and high nutrition and is cultivated in marine net-cages in southeastern China with large yields.[25] Therefore, the aim of this work was to characterize *S. baltica* from *P. crocea* as the SSOs at low-temperature (4 °C) storage and evaluate the spoilage capabilities of individual *S. baltica* strains. Furthermore, a couple of extracellular cyclic dipeptides, the QS signal molecules, were identified from one of the *S. baltica* strains with strongest spoilage activity using the GC-MS method. In addition, four synthetic DKPs supplemented into the extracellular products of *S. baltica* strain were inoculated into sterile fish juice to evaluate their effects on spoilage capability by measuring total volatile base nitrogen (TVB-N). Our results may have important implications for understanding the ability of cyclic dipeptides to active and/or antagonize QS systems associated with spoilage potential in *S. baltica*.

MATERIALS AND METHODS

Sample Preparation. Fresh *P. crocea* were harvested from a local farm (Ningde Guanjingyang large yellow croaker breeding Co. Ltd., Ningde, Fujian province, China). The fish were kept in oxygen at a local fishery shop until they were transported to the laboratory. Then, the fish were stored in individual sterile plastic bags at 4 °C after they were kept in ice within 30 min. During the periods of storage, the growth and activities of all spoilage microorganisms were monitored at appropriate time intervals.

A 25 g dorsal fish was transferred in a sterile plastic bag, and 225 mL phosphate buffer was added by homogenizing for 1 min.[4] Samples (1 mL) from ten times serial dilutions were added in Petri dishes, then 15−20 mL of molten (46 °C ± 1 °C) Iron Agar (IA) media (Qingdao High-tech Industrial Park Haibo Biotechnology Co., Ltd., Qingdao, Shandong province, China) were added immediately. Later, the dishes were incubated at 25 °C for 72 h ± 3 h. All dilution treatments were enumerated in three replicates.

Isolation and Identification of the Dominant Bacteria. An average of 30−50 colonies were selected from each iron agar Petri dish, according to their morphological characteristics, with a total number of 102 colonies, which were purified in nutrient agar plates (Qingdao High-tech Industrial Park Haibo Biotechnology Co., Ltd., Qingdao, Shandong province, China) at 25 °C for 72 ± 3 h by streaking. All the strains were tested at 25 °C by selected physiological characteristics, such as oxidase reaction, catalase formation, fermentation of glucose and lactose, ornithine and lysine decarboxylase, and Methyl Red Voges Proskauer (MR-VP).

Bacteria genomic DNA was extracted by Biospin Bacteria Genomic DNA Extraction kit. 16S rDNA genes were amplified by PCR using primers B27F (5′AGA GTT TGATCC TGG CTC AG 3′) and B1512R (5′AAG GAG GTG ATC CAG CCG CA 3′). The PCR conditions were as follows: denaturation for 1 min at 95 °C, annealing for 2 min at 55 °C, and elongation for 3 min at 72 °C for 35 cycles. [3]

The PCR products were sequenced by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Subsequently, the sequences of the 16S rDNA gene were aligned with GenBank by BLAST.

Preparation of Spoilage Bacterial Suspension. Spoilage bacterial strains, *S. baltica* 00A, *S. baltica* 00B, *S. baltica* 00C, were purified by streaking into nutrient agar plates incubated at 25 °C for 24−48 h. Then, a colony was transferred to 100 mL of nutrient broth (in 250 mL conical flask) and incubated for 12−18 h at 25 °C, whereas bacteria liquid concentration was 10^8 cfu/mL. Bacteria were collected aseptically by centrifugation at 4 °C for 10 min at 14000*g* and resuspended on 100 mL of sterile phosphate buffer. Finally, the bacterial suspension was diluted to 1.6×10^8 cfu/mL with sterile phosphate buffer.

Sterile Fish Muscle Juice. The preparation method of sterile fish muscle juice was performed as described by Dalgaard.[26] Fillets of newly killed *P. crocea* were cut into pieces, 500 mL of tap water per kg of fish was added, and the mixture was homogenized and boiled for 5 min. Then the juice was collected by filtering the mixture through gauze, and it was supplemented with trimetlylamine oxide (TMAO) (1.6 g/L), L-cysteine (40 mg/L) and L-methionine (40 mg/L). Finally, the juice was sterilized at 121 °C for 15 min for further experiment.

Measurement of Total Volatile Base Nitrogen. The prepared bacterial samples (200 μL) were inoculated into 50 mL of sterile fish muscle juice and then stored in individual sterile plastic bags at 4 °C to evaluate the spoilage capabilities through the changes of total volatile base nitrogen (TVB-N). During the storage time, the samples (15 mL) were monitored by the FOSS Kjeltec 8400 Automatic Kjeldahl Nitrogen Determination apparatus (Guangzhou Service Co., Ltd., China) in triplicates at appropriate time intervals.

Sensory Evaluation. The sensory evaluation was determined by seven member trained panelists from the local community. Panelists scored for appearance, flavor, and general acceptability, using a nine-point hedonic scale. A sensory score of five was taken as the borderline of acceptability.[27]

Isolation of Extracellular Cyclic Dipeptides. The growth rate of *S. baltica* 00C was measured by the Infinite 200 Multifunctional microplate reader (Tecan, Shanghai, China) based on the values of OD_{600} obtained during the period of 24 h growth. Cell suspensions of *S. baltica* 00C at the end of the exponential growth phase were centrifuged at 15000*g* for 10 min, and 40 mL of supernatants was extracted using the procedure described previously.[18,24,28] Briefly, 40 mL of the cell-free supernatant was extracted three times with an equal amount of chloroform. After being washed by water, the combined organic phases were taken to dryness by rotary evaporation and resuspended into 1 mL of chloroform. Samples were filtered through a 0.45 μm Millipore filter and stored at −20 °C until GC-MS analysis.

GC−MS Conditions. Experiments were conducted with a Trace 2000 GC-DSQ MS instrument (Thermofisher, USA) with the NIST (V2.0) MS library. A solution of the sample in CH_2Cl_2 was injected

directly into the GC inlet for analysis. The isomers were separated on a 30 m × 0.25 mm × 0.25 μm HP-5 ms capillary column (Agilent). Helium (>99.999%) was used as carrier gas at a constant flow of 1.0 mL min^{-1}. The column temperature was maintained at 50 °C for 2 min and then programmed at 15 °C min^{-1} to 260 °C, which was maintained for 8 min. The GC inlet temperature was 260 °C, and the transfer line temperature was 260 °C. Mass spectra were acquired by electron ionization (EI) under normal conditions: the ion-source temperature at 220 °C, the electron energy of 70 eV, the scan rate on 2 scans s^{-1}, and the mass range at 35−600 amu. GC-MS conditions, data acquisition, and processing were controlled by Xcalibur software (1.4 Version).

Chemical Synthesis of DKPs. Cyclic dipeptides were synthesized by fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS). The first step in the process of SPPS was the attachment of the first N^{α}-protected amino acid (AA1 = Pro, Leu) with resin linker. Second, in solid phase synthesis, removal of the N-Fmoc group was achieved by treatment with 20% (v/v) piperidine in dimethyl formamide (DMF). Third, the second amino acid (AA2 = Gly, Leu, Phe) was coupled and activated by a mixture of 1,3-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt), and the side chains were deprotected with trifluoroacetic acid (TFA, 95%). Finally, pure diketopiperazines were cleaved and deprotected from the resin according to the procedure of Chan and White.[29] The yield/purity of the crude products were >98.30% detected by the 2695 Quattro Micro high-performance liquid chromatography−mass spectrophotometer (Waters, USA) and stored at −20 °C in dryness for further use.

Spoilage Capability by Supplementation of DKPs. *S. baltica* 00C was plated on Luria−Bertani agar medium and incubated at 25 °C for 2 days. A colony was picked and transferred to 50 mL of liquid Luria−Bertani medium and incubated at 25 °C with continuous shaking until it reached the exponential growth phase. The bacterial suspensions (3.0 mL) were transferred to 150 mL of liquid Luria−Bertani medium and incubated at the same condition. Then, 0.5 mL DKPs solution (2 mg DKPs in 0.5 mL methanol) was added into 150 mL of liquid Luria−Bertani medium and continued to incubate. In addition, 0.5 mL of methanol was added as the control group. At the stationary phase, cell suspensions were extracted and resuspended into 1 mL of chloroform using the procedure described above. Furthermore, the prepared bacterial samples were inoculated into sterile fish muscle juice to measure the values of TVB-N as mentioned above.

RESULTS

Identification of SSOs. A number of black (H_2S-producing) and white colonies were observed from Iron Agar (25 °C, 3 days) from *Pseudosciaena crocea* stored at 4 °C. A total of 102 strains were isolated, of which 74 of them (72.5%) were H_2S-producing bacteria. The 74 H_2S-producing bacteria strains were further analyzed by conventional phenotypic method (Table 1) according to the two typical strains (*S. putrefaciens* ATCC 8071 and *S. baltica* LMG 2250). Subsequently, molecular identification by PCR amplification of bacterial 16s rDNA combined with sequencing showed that 60 of the 74 strains (81.1%) were *Shewanella* spp., and 48 of the 74 strains (64.9%) were *S. baltica*. A small portion of the 14 remaining strains were assigned to *Aeromonas* and *Enterobacteriaceae* (data not shown).

Comparison of Spoilage Capability. Spoilage capabilities of the different strains were detected by TVB-N (Figure 1A) and sensory evaluation (off-odors) (Figure 1B). The TVB-N values of *S. baltica* 00A and *S. baltica* 00C groups were significantly higher than that of *S. baltica* 00B group ($p < 0.001$) after 5 days of storage. In addition, the sensory scores (off-odors) of fish juice inoculated with *S. baltica* 00A and *S. baltica* 00C, respectively, were both significantly lower than that inoculated with *S. baltica* 00B ($p < 0.001$) after 5 days of storage. All the above results showed that the spoilage potential of *S.baltica* 00A and *S. baltica* 00C were much higher than that of *S. baltica* 00B, indicating the same *S. baltica* strains may exhibit individual spoilage capabilities during the storage time of *P. crocea*.

Table 1. Biochemical Characteristics of the Isolated Strains from *Pseudosciaena crocea* at 4 °C Storage[a]

characteristics	typical strains: *S. baltica* LMG 2250	typical strains: *S. putrefaciens* ATCC 8071	no. of isolated strains: +	no. of isolated strains: −
oxidase reaction	+	+	74	0
catalase formation	+	+	74	0
ornithine decarboxylase	−	−	4	70
lysine decarboxylase	−	−	5	69
MR-VP	−	−	4	70
fermentation of glucose	+	−	48	26
fermentation of lactose	−	−	0	74

[a]−, Negative reaction. +, positive reaction.

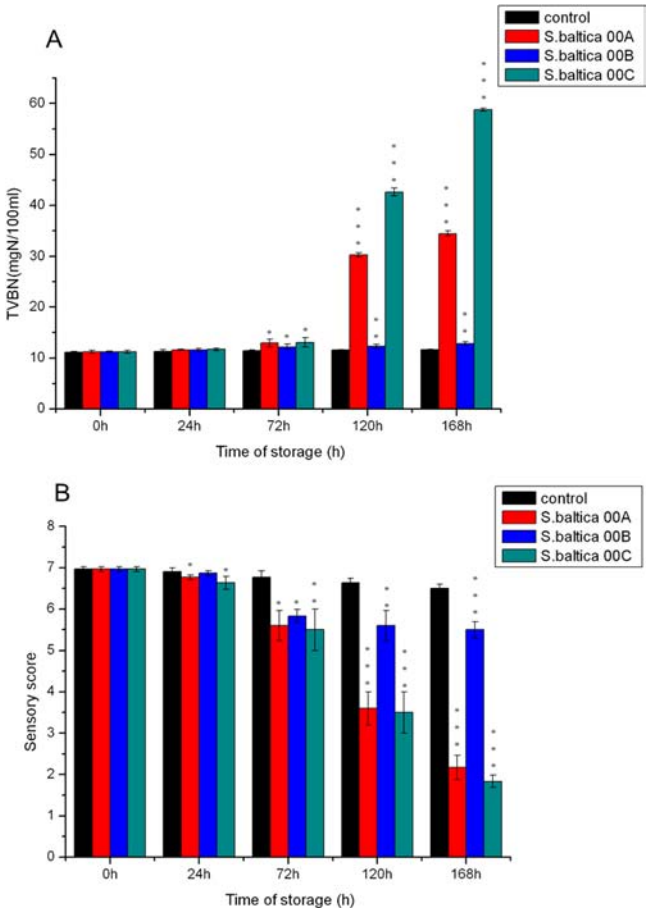

Figure 1. Comparison of spoilage capabilities of sterile fish juice inoculated with different *S. baltica* strains (00A, 00B, and 00C) during the storage time of 0, 24, 72, 120, and 168 h. (A) TVB-N values, (B) sensory scores. The error bars indicate standard deviation (±SD). Statistical significance was marked by the asterisk (* represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$).

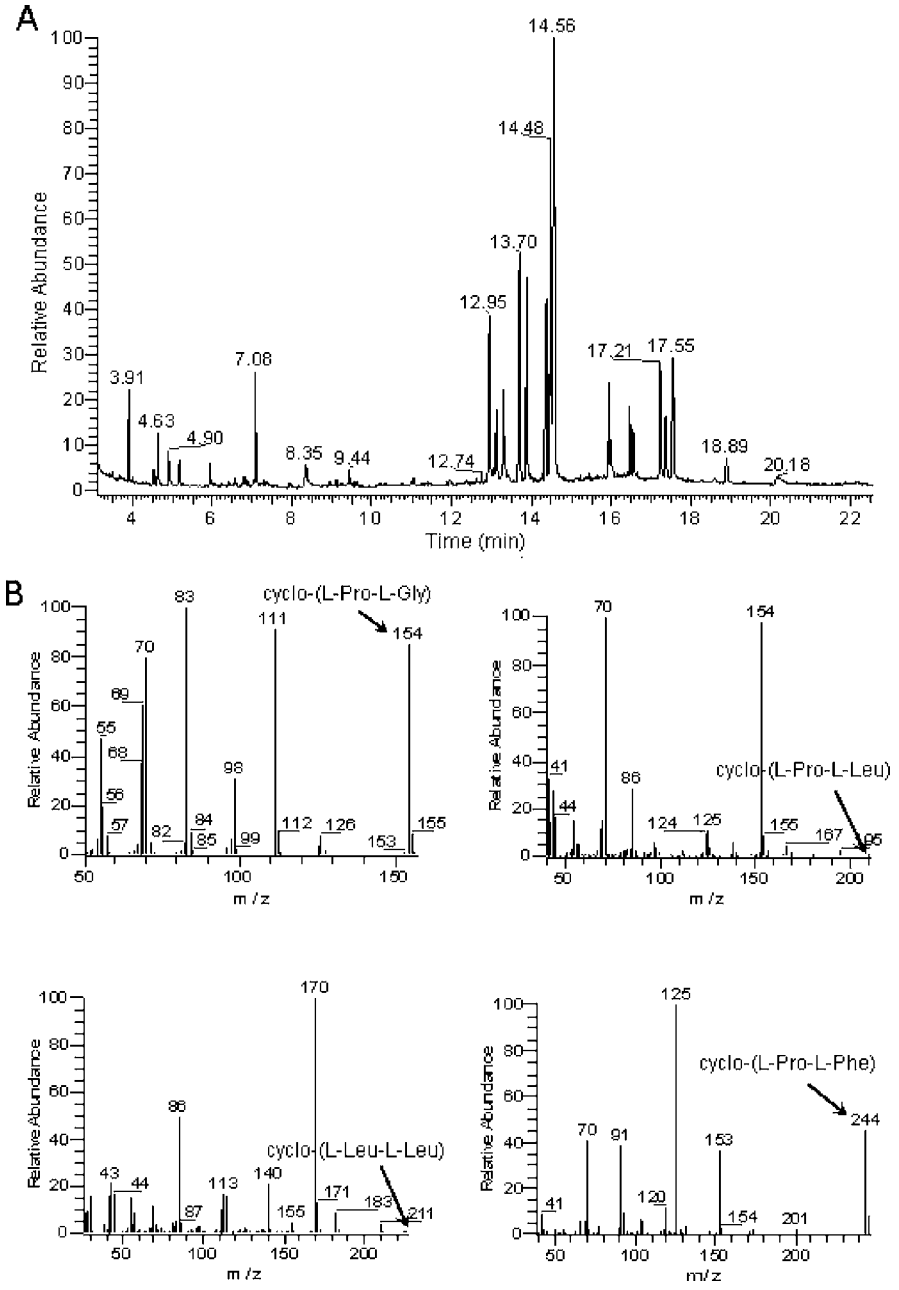

Figure 2. Gas chromatography (GC) chromatogram (A) and electron ionization mass spectrometry (EI-MS) spectra (B) of *S. baltica* supernatants recorded for cyclic dipeptides.

Characterization of Extracellular Cyclic Dipeptides. Figure 2 presented the GC/EI-MS chromatogram of *S. baltica* 00C supernatant recorded for cyclic dipeptides. All peaks were detected and identified from the respective mass spectra (data not shown). Of all the peaks, four peaks with retention times of 13.30, 14.48, 15.98, and 17.21 min, those possibly representative of DKPs, were selected for further characterization (Table 2). Total ion current chromatogram (TIC) profile from a typical analysis of supernatant of the *S. baltica* 00C is shown in Figure 2A, and spectra of four DKPs obtained by EI-MS are shown in Figure 2B. In addition, the structures of these four DKPs were identified as cyclo-(L-Pro-L-Gly) (m/z 154), cyclo-(L-Pro-L-Leu) (m/z 210), cyclo-(L-Leu-L-Leu) (m/z 226), and cyclo-(L-Pro-L-Phe) (m/z 244) (Figure 3).

In order to clarify the role of these signal molecules in spoilage capability of the *S. baltica* 00C, four cyclic dipeptides were chemical synthesized using Fmoc SPPS and detected by HPLC/ESI-MS/MS. The yield/purity of the four chemical synthetic cyclic dipeptides were >98.30% according to the peak area of HPLC chromatogram (Figure 4A and Table 3). Figure 4B presents the ESI-MS ($[M + H]^+$ and $[M - H]^-$) spectra recorded for the four chemical synthetic cyclic dipeptides,

Table 2. Elemental Composition and Mass Fraction of Cyclic Dipeptides

compound	retention time (min)	name	molecular formula	formula weight
1	13.30	cyclo-(L-Pro-L-Gly)	$C_7H_{10}N_2O_2$	154
2	14.48	cyclo-(L-Pro-L-Leu)	$C_{11}H_{18}N_2O_2$	210
3	15.98	cyclo-(L-Leu-L-Leu)	$C_{12}H_{22}N_2O_2$	226
4	17.21	cyclo-(L-Pro-L-Phe)	$C_{14}H_{16}N_2O_2$	244

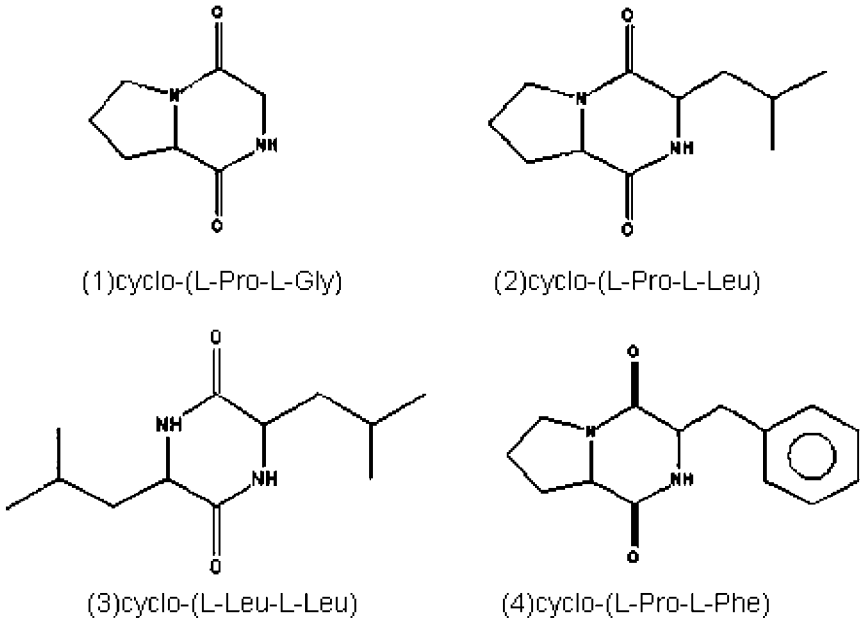

Figure 3. Chemical structures of diketopiperazines isolated from cell-free supernatants of *Shewanella baltica*.

shown as cyclo-(L-Pro-L-Gly) (m/z 154.2), cyclo-(L-Pro-L-Leu) (m/z 210.3), cyclo-(L-Leu-L-Leu) (m/z 226.3), and cyclo-(L-Pro-L-Phe) (m/z 244.3), respectively.

Effect of Synthetic DKPs Supplementation on Spoilage Capability. The TVB-N values were measured in both synthetic DKPs supplemented and nonsupplemented (control) groups after inoculation of group samples into sterile fish muscle juice (Figure 5). The TVB-N values in groups treated with synthetic DKPs (cyclo-(L-Pro-L-Gly), cyclo-(L-Pro-L-Leu), cyclo-(L-Leu-L-Leu), and cyclo-(L-Pro-L-Phe)) were all significantly higher ($p < 0.001$) than that of the control group after a 5-day storage of fish muscle juice. The activity for enhancement of spoilage capability in the cyclo-(L-Pro-L-Phe) treated group was the highest, while that in the cyclo-(L-Pro-L-Leu) treated group was the lowest. After a 24-h storage, the TVB-N value in the cyclo-(L-Pro-L-Phe) treated group was significantly higher ($p < 0.001$) than that in the other DKPs treated groups. All the above results indicated that DKPs as a kind of signaling molecule were capable of improving the spoilage capability of *S. baltica* during the storage of *P. crocea*.

DISCUSSION

As previously studied, the main spoilage bacteria of marine seafood stored at low-temperature were *Photobacterium phosphoreum*, *Shewanella putrefaciens*, *Brochothrix thermosphacta*, *Pseudomonas* spp., *Aeromonas* spp. and lactic acid bacteria.[3,4] In addition, the species of dominant bacteria of fresh *P. crocea* was identified as *Vibrionaceae* spp. However, *Enterobacteriaceae* spp. could be the main spoilage bacteria, while the marine fish were harvested from a polluted sea area.[30] In the present work, the results showed that *S. baltica* was the specific spoilage organism in *P. crocea* stored in air at 4 °C. After screening of the typical *S. baltica* strains, sterile fish muscle juice was further selected as a specific medium to determine the bacterial spoilage potential of the three different strains. Spoilage potential and activity can be assessed in several fish substrates as heat-sterilized fish juice or on sterile muscle blocks.[26,31] However, the fish muscle juice analyses can be performed much more conveniently, reliably, and effectively than using blocks by sterilizing the samples at 121 °C for 15 min. Moreover, the TVB-N value mainly composed of ammonia and amines, which results from degradation of proteins and nonprotein nitrogenous compounds and endogenous enzymes, was used as an evaluation indicator of spoilage potential in this study.[32] By comparison of spoilage potential in three typical *S. baltica* strains, *S. baltica* 00C was proven to have the highest spoilage capability indicated by the results of biochemical and sensory evaluation. The results also indicated that the same *S. baltica* species could present different spoilage capabilities in individual strains, which may be explained by the diversity of *S. baltica* isolate genomes.[4] Further study will be needed to characterize the genomic differences in these *S. baltica* isolates.

Quorum-sensing (QS) is a process of bacterial cell-to-cell communication involving the production of signaling molecules whose circuits control a variety of physiological functions including bioluminescence, sporulation, antibiotics, spoilage potential, virulence, and biofilm formation.[4−6,33,34] It is well-known that the AI-2 group of signaling molecules are produced by both Gram-positive and Gram-negative bacteria called a "universal signal".[33] All *Shewanella* spp. species produce AI-2 synthesized from a byproduct of *S*-adenosylmethionine (SAM) metabolism by a LuxS-based quorum-sensing system.[5,35] In this work, four known cyclic dipeptides were isolated and characterized from the cell-free culture supernatants of *S. baltica* by gas chromatography coupled with EI mass spectrometry (GC/EI-MS). The identified four compounds have been isolated previously from marine sponges and microorganisms, including the genus *Haloterrigena*, *Bacillus*, and *Pseudoalteromonas*.[13,36] However, this study was the first attempt to characterize the DKPs as the signaling molecules in QS of *S. baltica*, the main spoilage bacteria of large yellow croaker.

A series of cyclic dipeptides and their derivatives were found from plants, animals, and marine microorganisms in recent years.[16,17] In addition, it is shown that their functions are not limited to antimicrobial, auxin and antioxidative activity, but they also play an important role in the regulatory mechanism of QS as signal molecules.[37] Holden et al.[9] reported that cyclo-(D-

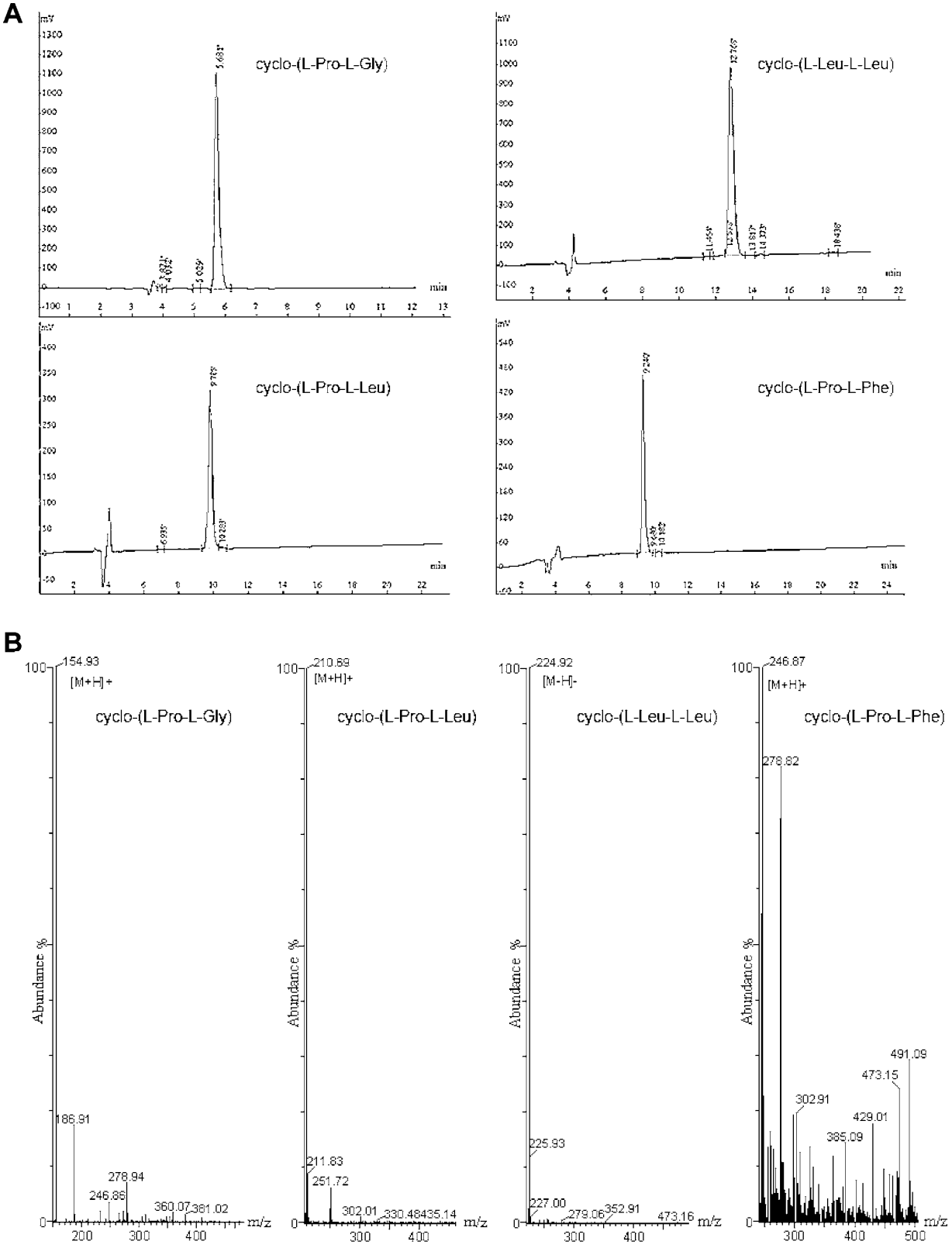

Figure 4. HPLC/ESI-MS chromatograms of chemical synthetic cyclic dipeptides. (A) HPLC on a column of GS-120-5-C18-BP. Ten microliters of sample was injected into a column (250 mm × 4.6 mm, flow rate: 1.0 mL/min), gradient eluted with Solvent A (0.1% TFA in 100% water) and Solvent B (0.1% TFA in 100% acetonitrile) and detected at a wavelength of 220 nm. The yield/purity of the products were >98.30% according to the peak area. (B) ESI-MS spectra.

Ala-L-Val), cyclo-(L-Pro-L-Tyr), and cyclo-(L-Phe-L-Pro) could antagonize the 3-oxo-C_6-HSL-mediated induction of bioluminescence, suggesting that these DKPs might compete for the same LuxR-binding site. Tommonaro et al.[17] isolated one of the DKPs, cyclo-(L-Pro-L-Val), in an extremely halophilic archaeon and showed that the compound could activate

Table 3. The Yield/Purity of the Compounds According to the Peak Area of HPLC Chromatogram

compound	name	rank	time	conc (%)	area	height
A	cyclo-(L-Pro-L-Gly)	4	5.681	98.3316	11019669	1118630
B	cyclo-(L-Pro-L-Leu)	2	9.785	98.7063	4887382	307739
C	cyclo-(L-Leu-L-Leu)	3	12.768	98.8293	19047949	933774
D	cyclo-(L-Pro-L-Phe)	1	9.240	98.3120	4885943	431156

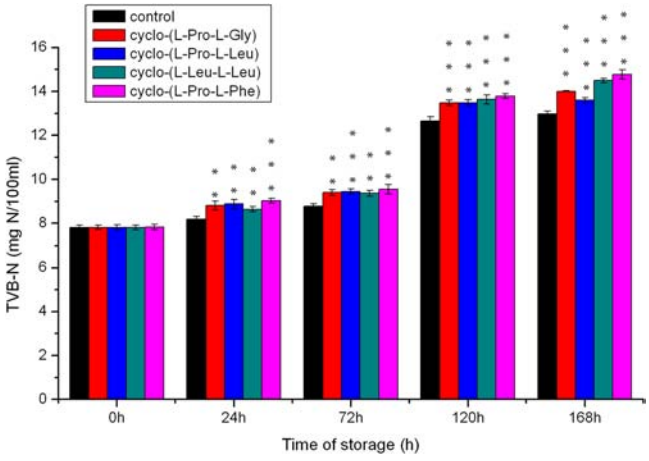

Figure 5. TVB-N values of sterile fish juice inoculated with metabolites of *S. baltica* strain 00C after adding four different synthetic DKPs during a storage time of 0, 24, 72, 120, and 168 h. The four DKPs molecules refer to cyclo-(L-Pro-L-Gly), cyclo-(L-Pro-L-Leu), cyclo-(L-Leu-L-Leu), and cyclo-(L-Pro-L-Phe). The error bars indicate standard deviation (±SD). Statistical significance was marked by the asterisk (* represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$).

AHLs. Other studies also demonstrated that several cyclic dipeptides may activate or inhibit an AHL-based biosensor in bacteria.[36,38] In order to confirm the significant role of DKPs in modulating QS systems, the cyclic dipeptides isolated and characterized from *S. baltica* were obtained by chemical synthesis using Fmoc SPPS. Metabolites of *S. baltica* cultures by adding these four DKPs were respectively inoculated into sterile fish juice to evaluate the spoilage capabilities determined by TVB-N. Our results showed that the four cyclic dipeptides have individual effects on promotion of spoilage capability of the *S. baltica* strain, which might indicat that the four DKPs regulated the expression of different target genes. Recently, numerous signaling molecule-mediated sensing and response pathways have been defined.[39] However, the molecular mechanism of these cyclic dipeptides in the metabolic and signal pathway of *S. baltica* is poorly understood. It is possible to suppose that cyclic dipeptides might play an alternative role in spoilage bacteria interactions, which can function instead of AI-2 involved in the quorum-sensing system of *Shewanella* spp.

In conclusion, this study demonstrated that the specific spoilage organism of *P. crocea* stored at 4 °C was *S. baltica*. Moreover, cyclic dipeptides could be produced and isolated from the cell-free culture supernatants of *S. baltica*, a Gram-negative spoilage-bacterium, functioning in a population density-dependent manner involved in regulation of the QS system. By comparison of the spoilage potential between control and DKPs-supplemented groups, it was found that all four synthesized DKPs could improve the spoilage capability of *S. baltica* during the storage of *P. crocea*. It was also shown that cyclo-(L-Pro-L-Phe) had the highest capability in enhancement of spoilage potential among these DKPs. So far, this was the first attempt to characterize DKPs as the signaling molecules in QS of *S. baltica*. However, further study will be needed to clarify the regulatory mechanisms and response pathways of these four DKPs in order to better understand their important roles in QS systems.

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Funding

This study was supported by the National Natural Science Foundation of China (31072221 and 31101921), Zhejiang Provincial Natural Science Foundation of China (R3110345), and Zhejiang Provincial Science and Technology Foundation of China (2012C24024).

Notes

The authors declare no competing financial interest.

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