

Adenosine monophosphate degradation and inosinic acid accumulation in the shrimp *Penaeus japonicus*

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Abstract Shrimp muscle contains taste component compounds, such as glutamic acid and inosinic acid (IMP). When both are present, taste intensity increases by fourfold to eightfold compared to that when only glutamic acid is present. Inosinic acid is generated via adenosine triphosphate degradation in two metabolic pathways in which adenosine monophosphate (AMP) is generated and then degraded to either IMP or adenosine (AdR). We investigated post-mortem AMP degradation pathways in the shrimp *Penaeus japonicus* by measuring the activity of enzymes extracted from shrimp muscle and by isolating bacteria from shrimp muscle and examining their role in AMP degradation. The enzyme extract degraded AMP to IMP through high AMP deaminase activity, and in addition, we identified *Shewanella* sp. and *Exiguobacterium* sp. as mediating AMP degradation to AdR. Therefore, preventing an increase in bacterial numbers during storage is important for preventing AMP from degrading to AdR.

Keywords Adenosine · Adenosine monophosphate · Bacteria · Enzyme · Inosinic acid · Shrimp

Introduction

The shrimp *Penaeus japonicus*, an economically important crustacean, inhabits the Indian Ocean, western Pacific Ocean, and Mediterranean Sea (Motoo 1990). Shrimp has substantial quantities of free amino acids (Yamanaka 2008), an appealing flavor, and high market value (Hamano 2015). The most common free amino acids in shrimp are glycine and alanine, which provide a sweet taste, and glutamic acid, which is an important taste component (Matsumoto and Yamanaka 1990; Matsumoto et al. 1991). Because flavor is improved by the simultaneous presence of glutamic acid and inosinic acid (IMP) (Zhang et al. 2008; Kobayashi et al. 2012), IMP is also important for shrimp flavor.

IMP is generated through adenosine triphosphate (ATP) degradation after an organism dies. ATP degradation produces a series of compounds: ATP → adenosine diphosphate (ADP) → adenosine monophosphate (AMP) → IMP → inosine (HxR) → hypoxanthine (Hx). ATP is generally degraded to IMP and accumulates relatively quickly in fish muscle (Srirangsan et al. 2010). AMP accumulates in substantial quantities in shrimp,

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and it degrades to either IMP or adenosine (AdR) (Fujisawa and Yoshino 1985; Matsumoto 1996; Koseki et al. 2006; Koyama et al. 2008). AdR does not appear to affect flavor, whereas the degradation of AMP in shrimp muscle to IMP increases flavor.

ATP-related compounds are generally degraded by muscle enzymes, but IMP is degraded by bacteria (Matsumoto and Yamanaka 1991; Yokoyama et al. 1996; Hayashi and Nakata 2003; Seki and Hamada-Sato 2014). Other ATP-related compounds might be degraded not only by muscle enzymes but also by bacteria. No detailed reports on AMP degradation in shrimp or on AMP degradation by muscle or bacterial enzymes have appeared in the literature. In this study, we investigated AMP degradation in shrimp to ascertain which compounds are produced in the largest quantities. We prepared an enzyme solution from shrimp and examined its effect on AMP degradation, and we isolated and identified the bacteria that degrade AMP in shrimp. In addition, we measured the amounts of ATP-related compounds and obtained an estimate of bacterial numbers in shrimp over a few days of storage. We suggest an optimal approach to the maintenance of flavor components in stored shrimp.

Materials and methods

Sample preparation

Live shrimp (*P. japonicus*, length approximately 17 cm; weight approximately 33 g) from aquacultures in Ōita Prefecture, Japan, were transported to the laboratory, where they were stunned in sterilized ice water and killed quickly by severing the first ganglia. Killing involved either immediate loss of sensibility or induction of insensibility without discomfort or pain, followed by death, while the animal was insensible.

Shrimp muscle enzyme degradation of AMP

AMP-degrading enzyme was extracted as part of an enzyme solution. Five grams of muscle was collected from each shrimp and crushed in three times its weight of ultrapure water. The mixture was dialyzed against water for 2 days, and then, the dialysate was filtered through a No. 1 filter (Advantec Co., Ltd.; Tokyo, Japan) and then a 0.20 μm filter (Toyo Roshi Kaisha, Ltd.; Tokyo, Japan) at 10 °C to remove all bacteria from the enzyme solution. Forty samples of a 3 mL reaction mixture consisting of 0.5 mL AMP (25 mM), 2 mL buffer (50 mM maleic acid/Tris/NaOH, pH 7.5), and 0.5 mL enzyme solution were incubated at 0, 5, 10, 20, 30, 40, 50, or 60 °C for 5 days, and the reaction was stopped every day in three samples for each temperature by the addition of 2 mL 10% perchloric acid. The mixture was neutralized using 10% KOH, and the resulting precipitate was removed by centrifugation (13,697 $\times g$, 5 min, 5 °C). The supernatant was then transferred to a 15 mL centrifuge tube, diluted with sterilized water to obtain a final volume of 10 mL, and filtered through a 0.20 μm filter (Millex-LG, Millipore, Merck). AMP degradation compounds were measured by high-performance liquid chromatography (HPLC) with the following equipment and parameters: pump, L2130 (Hitachi, Tokyo, Japan); column, Shodex Asahipak GS-320 HQ (Showa Denko K.K., Tokyo, Japan); mobile phase, 200 mM NaH_2PO_4 ; flow rate, 0.6 mL/min; temperature, 30 °C; detector, Hitachi L7420; and wavelength, 260 nm. Four standard curves were established, each with 0.025, 0.050, 0.125, or 0.250 mmol/L of each compound (AMP, IMP, AdR, HxR, and Hx), respectively.

Bacterial degradation of AMP

Shrimp muscle (10 g) was homogenized with 90 mL sterilized 0.9% NaCl solution in a sterilized bag (PYXON-30, ELMEX Ltd., Tokyo, Japan) using an Exnizer-400 Stomacher (ORGANO Co., Ltd., Tokyo, Japan) (30 s, 260 rpm). A 1 mL aliquot of the supernatant was diluted with 10 mL sterilized 0.9% NaCl solution. The diluted supernatant (100 μL) was smeared on Plate Count Agar (PCA) and incubated at 35 °C for 48 h to stimulate bacterial growth. In addition, the supernatant was smeared onto PCA containing 1% NaCl and incubated at 20 °C for 168 h. Previous reports (Fujii 1985; Fukuda et al. 2012) had indicated that bacteria from seawater on fresh fish grew better on PCA containing 1% NaCl and incubated at 20 °C than on PCA without added NaCl at 35 °C. After incubation, six types of bacteria that varied in color and form and had that



many colonies were selected. Each bacterial type was added to 1 mL of sterilized 0.9% NaCl solution, and the suspensions were diluted 10-, 100-, and 1000-fold; 100 μ L of each diluted supernatant was then smeared on PCA containing 1% NaCl and incubated at 20 °C for 48 h to produce a pure culture. Colonies were extracted from these cultures and suspended in 1 mL of sterilized 0.9% NaCl solution. The suspensions were then diluted fivefold with 0.9% NaCl solution and used in the analyses.

AMP degradation was measured using 3.5 mL of a reaction mixture consisting of 0.5 mL AMP (Oriental Yeast Co., LTD., Tokyo, Japan) (25 mM), 2 mL buffer (50 mM maleic acid/Tris/NaOH, pH 7.5), 0.5 mL autoclaved enzyme solution, and 0.5 mL bacterial suspension. Ninety of the reaction mixtures and 30 of the control reaction mixtures (with autoclaved enzyme solution and without bacterial suspension, and without autoclaved enzyme solution or bacterial suspension) were incubated at 20 °C for 5 days, and the reaction was stopped every day in three samples of each bacterial suspension by the addition of 1.5 mL 10% perchloric acid. AMP degradation compounds were measured by HPLC using the same procedure previously outlined. To determine the bacterial count in the reaction mixture, 0.1 mL of the reaction mixture, before the addition of perchloric acid, was diluted by a factor of five with a sterilized solution of 0.9% NaCl. The diluted supernatant (100 μ L) was then smeared on PCA plus 1% NaCl and incubated at 20 °C for 48 h. Bacterial counts were made daily.

Identification of AMP-degrading bacteria

Bacteria that showed AMP-degrading activity were identified by sequencing the 16S rRNA gene. Sequencing was performed by TechnoSuruga Laboratory Co., Ltd. (16S r-DNA-500 service; Shizuoka, Japan). Cells were lysed with achromopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain a DNA template. PCR was conducted in a 25 μ L reaction with 7.0 μ L buffer (buffer 5.0 μ L + dNTPs mixture 2.0 μ L), 0.50 μ L each of forward and reverse primers, 0.25 μ L PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan), and 0.50 μ L template DNA. The primer set was 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1406R (5'-CGGGCGGTGTGTAC-3') (Nakagawa et al. 2002). The thermal cycle program consisted of 30 cycles of 98 °C for 10 s, 57 °C for 5 s, and 72 °C for 90 s. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for the sequencing reactions. The primer set was the 9F from PCR and 536R (5'-GTATTACCGCGGCTGCTG-3'), and the thermal cycle program was 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 96 °C for 1 min, followed by a final extension of 60 °C for 4 min. An ABI PRISM 3130xl Genetic Analyzer System (Applied Biosystems) was used with the DB-BA11.0 database (Techno Suruga Laboratory, Shizuoka, Japan) and ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, Australia). Species were identified with the bacterial identification software system DB-BA11.0 (Techno Suruga Laboratory Co., Ltd.) and international nucleotide sequence databases (GenBank/DDBJ/EMBL) using BLAST (Altschul et al. 1997). CLUSTALW (Thompson et al. 1994) was used to align sequences. Distances were calculated using the Kimura 2-parameter model (Kimura 1980), and trees were constructed with neighbor-joining (Saitou and Nei 1987) using CLUSTALW (Thompson et al. 1994). The bootstrap method (Felsenstein 1985) was used with 1000 replicates to evaluate support for the trees.

Changes in levels of ATP-related compounds and bacterial counts

Shrimp were stored at 4 °C for 7 days. Three were selected each day, and ATP-related compounds were extracted by placing 2 g of muscle from one shrimp in 5 mL of 10% perchloric acid; the mixture was shaken vigorously. The muscle was pelleted by centrifugation (11,509 \times g, 10 min, 5 °C), the supernatant was transferred to a 50 mL centrifuge tube, and 10% perchloric acid was added to obtain a volume of 25 mL. The mixture was neutralized using KOH, and the precipitate was removed by centrifugation (13,697 \times g, 5 min, 5 °C). The supernatant was then transferred to a 15 mL centrifuge tube and diluted with sterilized water to obtain a final volume of 10 mL. The solution was filtered (0.20 μ m filter, Millex-LG), and ATP-related compounds were measured by HPLC using the same method outlined above. Each extract was measured daily for 7 days; extracts were stored at 4 °C over this period.

A freshly prepared bacterial suspension was used for counting bacteria in shrimp muscle. Extraction and incubation were carried out, as described above in “Bacterial degradation of AMP.” For bacterial isolation and counting, 100 μ L of the prepared suspension was spread on PCA and incubated at 35 °C for 48 h. In



addition, the suspension was spread onto PCA containing 1% NaCl and incubated at 20 °C for 168 h. The number of bacterial colonies was counted on both plates every other day for 7 days.

Statistical analysis

Data were subjected to a one-way analysis of variance using the least significant difference method. *T* tests were used for pairwise comparisons; $P < 0.05$ was considered statistically significant. Analyses were conducted with Microsoft Excel, and figures were prepared using Microsoft Excel.

Results

Shrimp muscle enzyme degradation of AMP

Table 1 shows how 2.5 mM AMP was degraded by shrimp muscle enzyme extracts after 24 h. At 0 °C, AMP was still detected at a 2.2 mM concentration after the incubation period. In contrast, at 30 °C, the AMP concentration was 0.9 mM. The amount of remaining AMP decreased as the temperature changed from 0 to 30 °C, but it increased between 30 and 60 °C ($P < 0.05$). At 0 °C, 0.4 mM IMP was produced, whereas at 30 °C, 2.1 mM was produced. The amount of IMP increased from 0 to 30 °C and decreased from 30 to 60 °C ($P < 0.05$). AMP-degrading enzymes in shrimp muscle showed the highest activity at 30 °C (Table 1). No AdR was produced, indicating that over a 24-h period, shrimp muscle enzymes degraded AMP to IMP but did not degrade AMP to AdR. We investigated AMP-degrading enzyme activity over a period of 5 days at 20, 30, and 40 °C (Fig. 1); the temperatures with the highest levels of enzyme activity. At all temperatures, AMP was degraded to IMP within 24 h, and AMP concentration on day 0 was significantly different from that on day 1 ($P < 0.05$). AMP concentration at 20 °C was 1.1 mM on day 1 and decreased to 0.59 mM on day 5 ($P < 0.05$). AMP concentrations at 30 °C were 0.88 mM on day 1 and decreased to 0.61 mM on day 3 ($P < 0.05$), remained steady between days 3 and 4, ($P > 0.05$), and decreased to 0.51 mM on day 5 ($P < 0.05$). AMP concentration at 40 °C was between 1.1 and 1.0 mM from days 1–4 ($P > 0.05$), and it decreased 0.83 mM on day 5 ($P < 0.05$). AMP concentrations at 30 °C were significantly lower than those at 20 and 40 °C between days 1 and 5 ($P < 0.05$). IMP concentrations at 20 °C increased to 2.1 mM on day 2 ($P < 0.05$), remained steady between days 3 and 4 ($P > 0.05$), and decreased to 1.8 mM on day 5 ($P < 0.05$). IMP concentrations at 30 °C increased to 2.3 mM on day 2 ($P < 0.05$), remained steady between days 3 and 4 ($P > 0.05$), and decreased to 1.8 mM on day 5 ($P < 0.05$). IMP concentrations at 40 °C were between 1.9 and 2.0 mM from days 1–4 ($P > 0.05$) and decreased to 1.6 mM on day 5 ($P < 0.05$). IMP concentrations at 30 °C were significantly higher than those at 20 and 40 °C between days 1 and 5 ($P < 0.05$). We also found that the level of HxR increased after 2 days. Over the 5-day period, we confirmed IMP production but found no evidence of AdR production.

Table 1 Degradation of AMP by enzymes extracted from shrimp muscle

Temperature (°C)	AMP (mM)	IMP (mM)	AdR (mM)	HxR (mM)	Hx (mM)
0	2.2 ± 0.02	0.4 ± 0.01	0.1 ^a	0.0 ^a	0.0 ^a
5	2.0 ± 0.04	0.8 ± 0.01	0.1	0.0	0.0
10	1.5 ± 0.03	1.4 ± 0.02	0.1	0.0	0.0
20	1.1 ± 0.01	1.9 ± 0.03	0.1	0.0	0.0
30	0.9 ± 0.01	2.1 ± 0.01	0.0	0.1	0.0
40	1.1 ± 0.01	1.9 ± 0.02	0.1	0.0	0.0
50	1.8 ± 0.03	1.1 ± 0.03	0.1	0.0	0.0
60	1.9 ± 0.04	0.9 ± 0.02	0.1	0.0	0.0

Data are presented as the mean ± SD of three replicates. The initial concentration of AMP was 2.5 mM. Concentrations are shown after 24 h of enzyme reaction

^a SD of 0



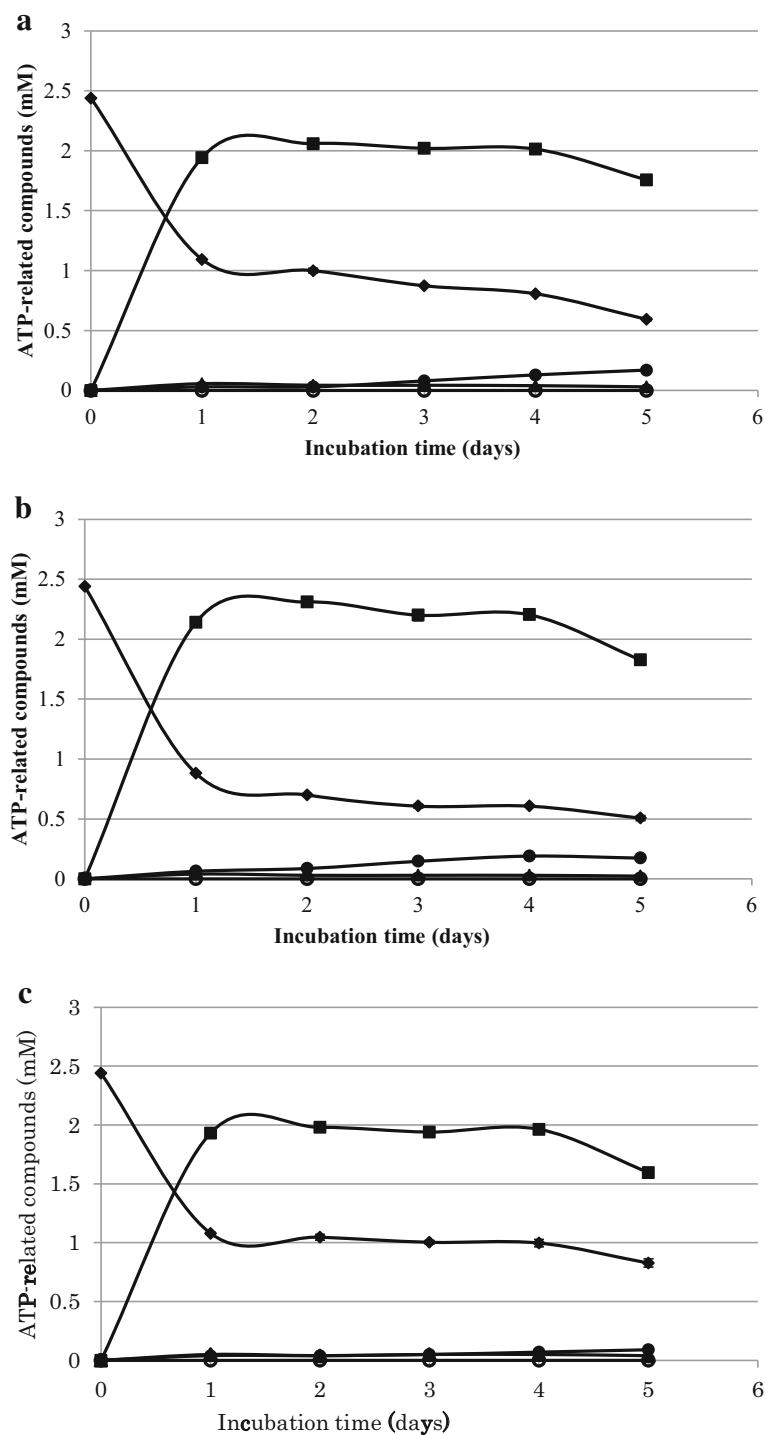


Fig. 1 Concentrations of AMP, IMP, AdR, HxR, and Hx from day 0 to day 5 during enzymatic degradation at 20 °C (a), 30 °C (b), and 40 °C (c) at pH 7.5. Filled diamonds AMP, filled squares IMP, filled triangles AdR, filled circles HxR, open circles Hx

Bacterial degradation of AMP

Few colonies grew on pure PCA, so we investigated AMP degradation using six strains (Table 2) isolated on PCA containing 1% NaCl. After 48 h of incubation, AMP, IMP, and AdR were not detected, but HxR and Hx were present in cultures with bacterial isolates 1, 5, and 6. Bacterial isolate 2 produced AdR and Hx but no



Table 2 Degradation of AMP by bacteria isolated from shrimp muscle

Bacterial isolates	AMP (mM)	IMP (mM)	AdR (mM)	HxR (mM)	Hx (mM)
No. 1	0	0	0	0.92	0.98
No. 2	0.42	0	1.40	0	0.04
No. 3	2.30	0	0	0	0
No. 4	2.30	0	0	0	0
No. 5	0	0	0	1.60	0.61
No. 6	0	0	0	0.88	1.10

The initial concentration of AMP was 2.5 mM. The concentrations after 48 h of bacterial degradation at pH 7.5 are shown. AMP was not degraded in the controls (one containing autoclaved enzyme solution without bacterial suspension, and the other without both autoclaved enzyme solution and bacterial suspension)

IMP or HxR. Our analysis indicated that bacterial isolates 1, 2, 5, and 6 could degrade AMP. In contrast, bacterial isolates 3 and 4 did not degrade AMP. Bacterial isolate 2 degraded AMP to AdR. Bacterial isolates 1, 5, and 6 degraded AMP to HxR and Hx, but it was not clear whether IMP or AdR was an intermediate. Although AdR was detected in cultures with bacterial isolate 2, the possibility that AMP was degraded to IMP could not be discounted, so the AMP degradation pathway in bacterial isolates 1, 2, 5, and 6 was investigated further. In addition, it was confirmed that AMP was not degraded in the control. We also assessed the number of bacteria in the reaction cultures over the 5-day period. In this experiment, no medium was used, but the autoclaved enzyme solution from shrimp muscle served as the nutrient source.

Bacterial isolate 1 almost completely degraded AMP by day 2. No IMP was detected, but AdR was present. The level of AdR increased to 0.74 mM at day 2, but decreased at day 3. HxR increased to 1.3 mM at day 3, but then decreased by day 5. Hx increased after day 2 and reached 2.1 mM by day 5 (Fig. 2a). Bacterial isolate 2 slowly degraded AMP to day 4. IMP was not detected, but AdR was present. The level of AdR increased to 2.1 mM at day 4 and then decreased at day 5. The level of Hx increased after day 2 to a maximum of 0.80 mM at days 3 and 4, and then decreased at day 5. HxR was not detected (Fig. 2b). Bacterial isolate 5 slowly degraded AMP to a minimum of 1.1 mM on day 5. IMP was not detected, but AdR was present at 0.19 mM on day 3. The level of HxR increased after day 4 to a maximum of 0.82 mM on day 5. Hx increased after day 2 to a maximum of 0.64 mM on day 3, then decreased slowly to day 5 (Fig. 2c). Bacterial isolate 6 almost completely degraded AMP by day 2. IMP was not detected. AdR was detected and increased after day 1 to a maximum of 0.52 mM on day 2, but decreased after day 3. HxR increased after day 1 to a maximum of 0.90 mM on day 3 and then decreased to day 5. Hx increased after day 2 to a maximum of 3.2 mM on day 4 and then decreased on day 5 (Fig. 2d). These results indicated that the bacteria degraded AMP to AdR.

Initial bacterial concentrations and growth during the experiment varied by isolate. The initial counts of isolates 1, 2, 5, and 6 were 3.3, 6.0, 1.7, and 4.2 log CFU/mL, respectively. By day 4, the isolates had counts of 11, 10, 5.3, and 7.1 log CFU/mL, respectively (Fig. 3).

Identification of AMP-degrading bacteria

Genetic analysis suggested that isolates 1 and 6 were probably *Shewanella baltica* NCTC 10735^T (98.6 and 98.4% homology, respectively), while isolate 5 was most similar to *Shewanella xiamenensis* S4^T (97.6% homology), and isolate 2 was very similar to *Exiguobacterium oxidotolerans* T-2-2^T (99.8% homology, Table 3). For each phylogenetic analysis, the top ten matches were included. Outgroup taxa included species of *Aeromonas* for isolates 1, 5, and 6 and *Salirhabdus* for isolate 5. These bacteria were the closest match from a different bacterial genus. Isolates 1 and 6 had lower homology rates (Table 3) and bootstrap support (74 and 65%, respectively; Fig. 4) with *S. baltica* than isolate 2 did with *E. oxidotolerans* (100% bootstrap support). Isolate 5 was very weakly supported (40%) as sister to a clade including *S. xiamenensis*, which was its closest match in the nucleotide databases. The 16S sequences for isolates 1, 2, 5, and 6 were submitted to the DNA Data Bank of Japan (Table 3).



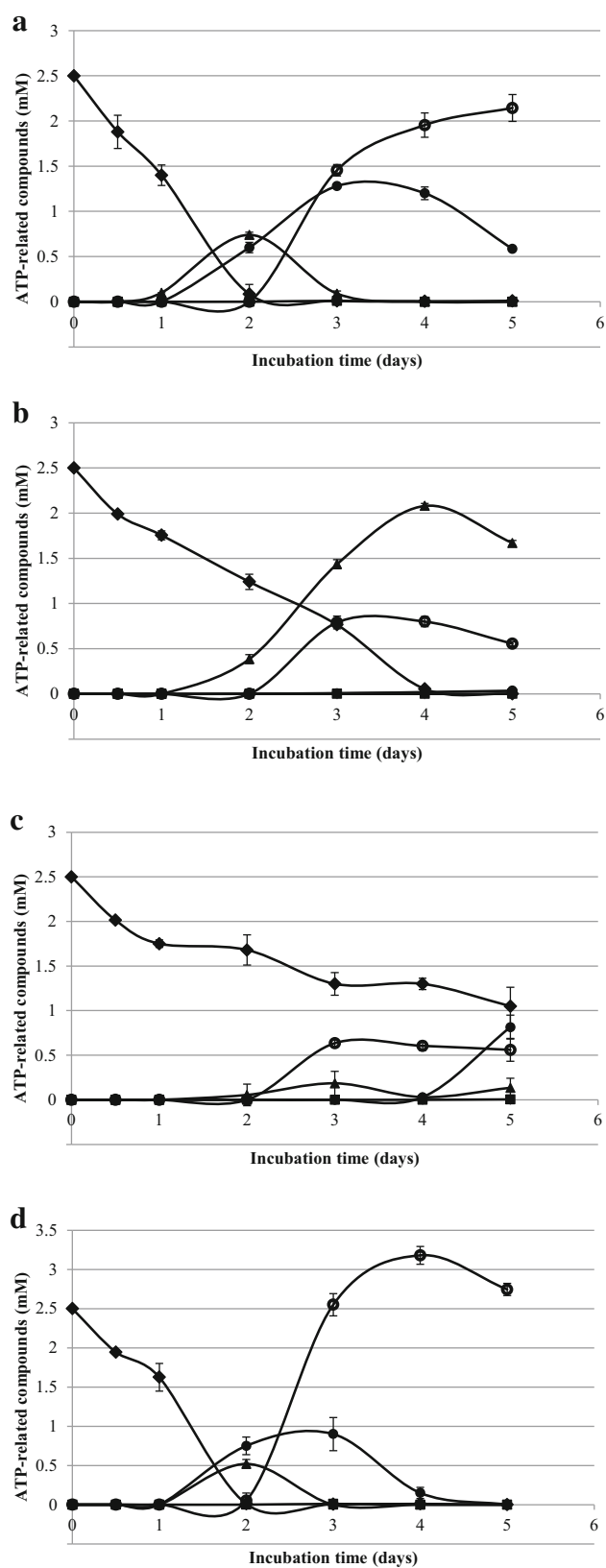


Fig. 2 Concentrations of AMP, IMP, AdR, HxR, and Hx from days 0 to 5 during bacterial degradation at 20 °C, pH 7.5. Bacterial isolate 1 (**a**), 2 (**b**), 5 (**c**), and 6 (**d**). Filled diamonds AMP, filled squares IMP, filled triangles AdR, filled circles HxR, open circles Hx



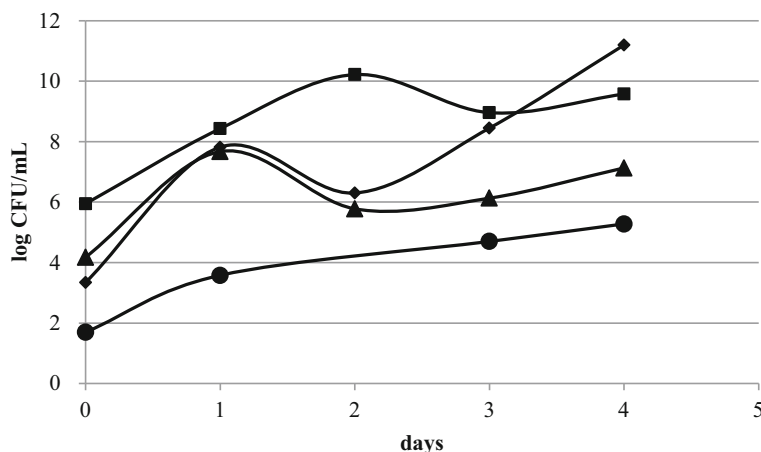


Fig. 3 Counts of AMP-degrading bacteria on Plate Count Agar containing 1% NaCl and incubated at 20 °C. Filled diamonds LC160132 (isolate 1), filled squares LC160133 (isolate 2), filled circles LC160134 (isolate 5), filled triangles LC160135 (isolate 6)

Changes in levels of ATP-related compounds and bacterial counts

Changes in the levels of ATP-related compounds in shrimp extracts were monitored for 7 days at 4 °C (Fig. 5). The initial level of ATP (4.3 $\mu\text{mol/g}$) approached zero (0.26 $\mu\text{mol/g}$) by day 2. The initial amount of ADP (6.6 $\mu\text{mol/g}$) decreased to 1.1 $\mu\text{mol/g}$ on day 2. Thereafter, the level of ADP decreased slowly to 0.58 $\mu\text{mol/g}$ on day 7. The initial amount of AMP was 1.2 $\mu\text{mol/g}$, which increased to 4.4 $\mu\text{mol/g}$ on day 2 and then decreased to 1.5 $\mu\text{mol/g}$ on day 7. The initial amount of IMP was 0.11 $\mu\text{mol/g}$, which increased to 7.1 $\mu\text{mol/g}$ on day 4 and then decreased. IMP was the most abundant ATP-related compound after day 3. AdR was not detected during the 7-day experiment. HxR was not detected initially, but was present on day 2 and rose to a maximum level of 2.0 $\mu\text{mol/g}$ on day 6. Hx was not detected initially, but was present on day 2 and reached a maximum level of 1.7 $\mu\text{mol/g}$ on day 7. Overall, ATP degradation in shrimp increased with time, and degradation to AdR was not observed; all the AMP degraded to IMP.

Changes in bacterial numbers in shrimp extracts over the 7-day period are shown in Fig. 6. A bacterial count of <2.0 log CFU/g was found from samples incubated at 35 °C on day 7. In contrast, the bacterial count for samples incubated at 20 °C was initially 3.5 log CFU/g, and after day 2, the bacterial count increased and reached 7.2 log CFU/g by day 7. It was found that there were increases in the numbers of bacteria that can grow in the presence of NaCl.

Discussion

Shrimp muscle enzyme degradation of AMP

Shrimp muscle extracts demonstrated enzymatic activity that degraded AMP to IMP (Table 1; Fig. 1); however, AdR production from AMP degradation was detected only at a low level and was significantly less than IMP production from AMP degradation ($P < 0.05$). AMP degradation to IMP is catalyzed by AMP deaminase through excision of the amino group. When AMP is degraded to AdR, AMP-5'-nucleotidase excises the phosphate group. IMP accumulates in fish owing to an inherently high AMP deaminase activity (Yokoyama and Sakaguchi 1998). AMP is reported to degrade to both IMP and AdR in shrimp (Koseki et al. 2006; Koyama et al. 2008), presumably through both AMP deaminase and AMP-5'-nucleotidase activities. Both IMP and AdR have been detected in the pink shrimp *Pandalus hypsinotus*, and it was reported that the primary degradation pathway in this species is AMP to AdR mediated by AMP-5'-nucleotidase (Arai and Terasaki 1966). Therefore, the activity of AMP-5'-nucleotidase is higher than that of AMP deaminase. In the Japanese spiny lobster *Panulirus japonicus*, AMP was degraded to both IMP and AdR, although IMP



Table 3 Sequences used in genetic analyses to identify bacterial isolates from shrimp

Species name	Homology rate (%) ^a	Accession number ^b
Isolate 1	–	LC160132
<i>Shewanella baltica</i> NCTC 10735 ^T	98.6	AJ000214
<i>Shewanella aestuarii</i> SC18 ^T	98.2	JF751004
<i>Shewanella pacifica</i> KMM3597 ^T	97.1	AF500075
<i>Shewanella japonica</i> KMM3299 ^T	96.8	AF145921
<i>Shewanella gaetbuli</i> TF-27 ^T	95.5	AY190533
<i>Shewanella putrefaciens</i> ATCC8071 ^T	96.0	X82133
<i>Shewanella frigidimarina</i> ACAM591 ^T	95.1	U85903
<i>Shewanella vesiculosa</i> M7 ^T	94.9	AM980877
<i>Shewanella livingstonensis</i> LMG19866 ^T	94.9	AJ300834
<i>Aeromonas sobria</i> ATCC 43979 ^{T,c}	94.9	X74683
Isolate 2	–	LC160133
<i>Exiguobacterium oxidotolerans</i> T-2-2 ^T	99.8	AB105164
<i>Exiguobacterium artemiae</i> 9AN ^T	96.9	AM072763
<i>Exiguobacterium acetylicum</i> DSM20416 ^T	96.9	DQ019167
<i>Exiguobacterium indicum</i> HHS_31 ^T	96.7	AJ846291
<i>Exiguobacterium soli</i> DVS3y ^T	96.3	AY864633
<i>Exiguobacterium antarcticum</i> DMS14480 ^T	96.3	DQ019164
<i>Exiguobacterium undae</i> L2 ^T	95.9	AJ344151
<i>Exiguobacterium sibiricum</i> 255-15 ^T	96.7	CP001022
<i>Exiguobacterium aurantiacum</i> DSM6208 ^T	96.9	DQ019166
<i>Salirhabdus euzebyi</i> CVS-14 ^{T,c}	88.8	AM292417
Isolate 5	–	LC160134
<i>Shewanella xiamenensis</i> S4 ^T	97.6	FJ589031
<i>Shewanella hafniensis</i> P010 ^T	97.0	AB205566
<i>Shewanella putrefaciens</i> ATCC8071 ^T	96.3	X82133
<i>Shewanella oneidensis</i> MR-1 ^T	96.1	AE014299
<i>Shewanella decolorationis</i> CCTCC_M_203093 ^T	95.0	AJ609571
<i>Shewanella vesiculosa</i> M7 ^T	96.0	AM980877
<i>Shewanella livingstonensis</i> LMG19866 ^T	95.7	AJ300834
<i>Shewanella frigidimarina</i> ACAM591 ^T	94.5	U85903
<i>Shewanella donghaensis</i> LT17 ^T	94.7	AY326275
<i>Shewanella sairae</i> SM2-1 ^T	95.2	AB081762
<i>Shewanella marinintestina</i> IK-1 ^T	95.2	AB081757
<i>Shewanella schlegeliana</i> HRKA1 ^T	94.9	AB081760
<i>Shewanella gaetbuli</i> TF-27 ^T	94.9	AY190533
<i>Shewanella aestuarii</i> SC18 ^T	94.5	JF751004
<i>Aeromonas molluscorum</i> 848 ^{T,c}	93.7	AY532690
Isolate 6	–	LC160135
<i>Shewanella baltica</i> NCTC 10735 ^T	98.4	AJ000214
<i>Shewanella aestuarii</i> SC18 ^T	98.0	JF751004
<i>Shewanella pacifica</i> KMM3597 ^T	96.9	AF500075
<i>Shewanella vesiculosa</i> M7 ^T	94.7	AM980877
<i>Shewanella livingstonensis</i> LMG19866 ^T	94.7	AJ300834
<i>Shewanella frigidimarina</i> ACAM591 ^T	94.9	U85903
<i>Shewanella putrefaciens</i> ATCC8071 ^T	95.7	X82133



Table 3 continued

Species name	Homology rate (%) ^a	Accession number ^b
<i>Shewanella gaetbuli</i> TF-27 ^T	95.3	AY190533
<i>Aeromonas sobria</i> ATCC 43979 ^{T,c}	94.7	X74683

^T Type strain

^a Similarity determined by BLAST search

^b Accession number in GenBank

^c Outgroup taxon used for phylogenetic reconstruction

accumulated to a greater extent, indicating that AMP deaminase activity was higher than AMP-5'-nucleotidase activity (Shirai et al. 1996). In the shovel-nosed lobster *Ibacus ciliatus*, a higher level of AdR than IMP accumulated, indicating higher AMP-5'-nucleotidase activity than AMP deaminase activity (Shirai et al. 1996). In *Pandalus borealis*, AMP deaminase activity was high, while AMP-5'-nucleotidase activity was minimal (Fijisawa and Yoshino 1987). These reports indicate that the relative activities of AMP deaminase and AMP-5'-nucleotidase vary by species. In the present study, AdR production was significantly lower than IMP production ($P < 0.05$; Fig. 1). This indicates that AMP deaminase activity was higher than AMP-5'-nucleotidase activity.

The pathway and rate of AMP degradation depend on temperature (Koyama et al. 2008). In the vannamei shrimp *Litopenaeus vannamei* (syn. *Penaeus vannamei*), HxR and Hx accumulated at 0–5 °C, but IMP accumulated at 20–40 °C, indicating that different AMP-degrading enzymes are active at these temperature ranges (Koyama et al. 2008). Therefore, in this study, the amount of IMP and AdR that were generated by shrimp enzymes was measured at different temperatures on day 5 to investigate temperature dependency of AMP deaminase and AMP-5'-nucleotidase activity in shrimp (Fig. 7). From Fig. 7, it is clear that AMP deaminase activity was lowest at 0 °C and highest at 30 °C. Activity decreased when the temperature increased above 30 °C. In contrast, AMP-5'-nucleotidase activity was the highest at 0 °C and decreased to 30 °C, before increasing again above 30 °C. These results are similar to those reported for vannamei shrimp, in which AMP deaminase and AMP-5'-nucleotidase activities were temperature dependent (Koyama et al. 2008). AMP deaminase and AMP-5'-nucleotidase are AMP-degrading enzymes, and it is likely that AMP is degraded to IMP by AMP deaminase and further to AdR by AMP-5'-nucleotidase. Figure 7a shows AMP deaminase activity, and Fig. 7b shows AMP-5'-nucleotidase activity. As both enzymes are different, the substrates are different as well. Overall, we concluded that AMP deaminase and AMP-5'-nucleotidase are active in shrimp at certain temperatures. In addition, as AMP deaminase activity is higher than AMP-5'-nucleotidase activity, almost all AMP is degraded to IMP in shrimp.

Bacterial degradation of AMP

We demonstrated that *S. baltica*, *E. oxidotolerans*, and *S. xiamenensis* closely matched the isolated bacteria that degraded AMP in shrimp. *Shewanella baltica* has been isolated from the oyster *Crassostrea gigas* and from seawater in Delaware Bay (Richards et al. 2008), plaice *Pleuronectes platessa*, cod *Gadus morhua*, and flounder *Platichthys flesus* from the Baltic Sea (Vogel et al. 2005), and tuna *Thunnini* sp., swordfish *Xiphias gladius* (Serio et al. 2014), and vannamei shrimp *Penaeus vannamei* (Mace et al. 2014) from markets. *Exiguobacterium oxidotolerans* has been isolated from a drain in a fish processing plant in Hokkaido (Yumoto et al. 2004) and from sea mud (Chen et al. 2010). *Shewanella xiamenensis* has been isolated from sea floor sediment (Ng et al. 2014; Huang et al. 2010). These three species of bacteria are present on the sea floor where shrimp live, so it was not surprising to encounter them in our samples.

In this study, we investigated whether the bacteria from shrimp degrade AMP. We showed that bacterial isolates 1, 2, and 6 from shrimp could degrade AMP to AdR in muscle (Table 2; Fig. 2). Isolates 1, 2, and 6 degraded AMP not to IMP but to AdR. On the other hand, the amount of AdR in isolate 5 increased slightly at day 3, after which IMP was no longer detected. The amount of Hx in isolate 5 increased after day 2, indicating that isolate 5 degrades AMP to Hx immediately and has an enzyme that shows very strong activity with AMP. The bacterial counts in the reaction mixture increased with time (Fig. 3), which indicated that AMP



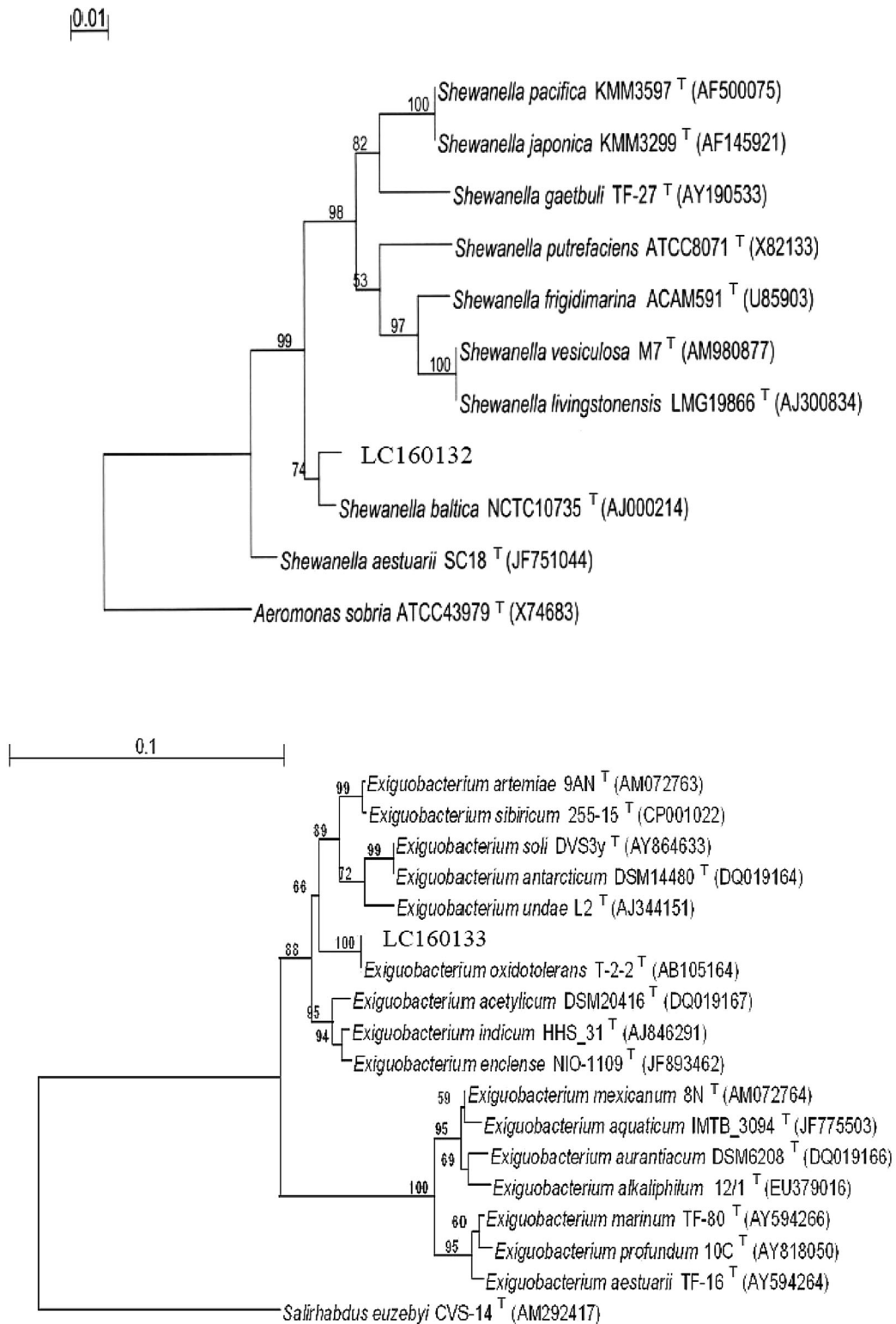


Fig. 4 Phylogenetic trees based on DNA sequences of the closest matches to bacterial isolates LC160132 (isolate 1), LC160133 (isolate 2), LC160134 (isolate 5), and LC160135 (isolate 6). Bootstrap values (%) are shown on the internal branches. ^T indicates type strain



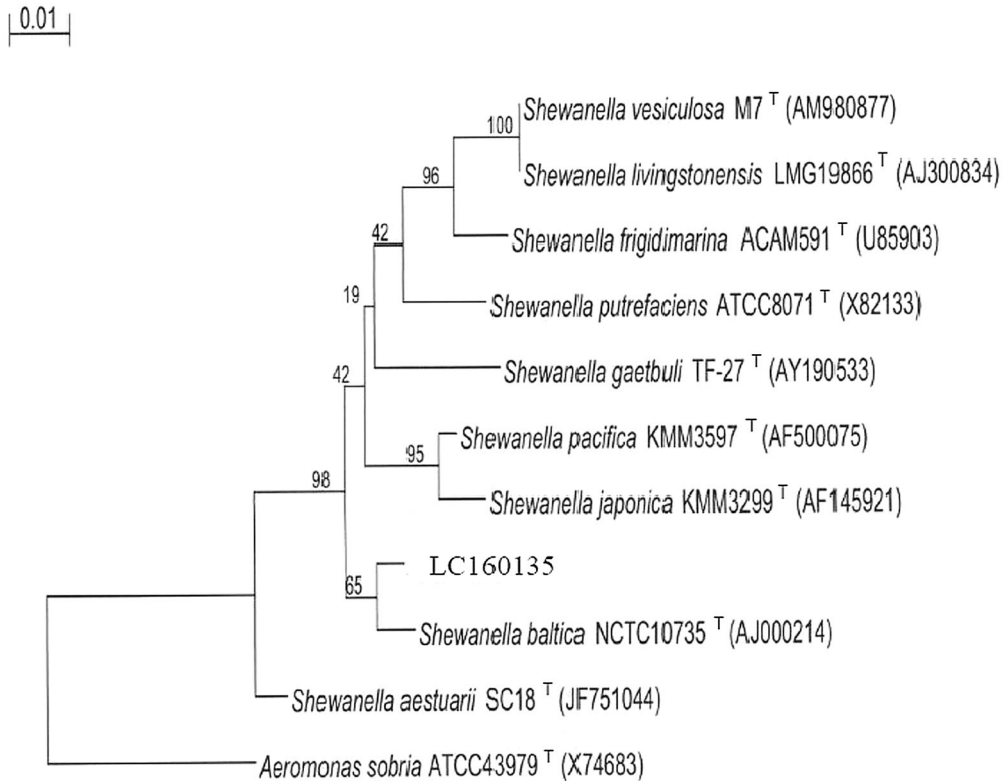
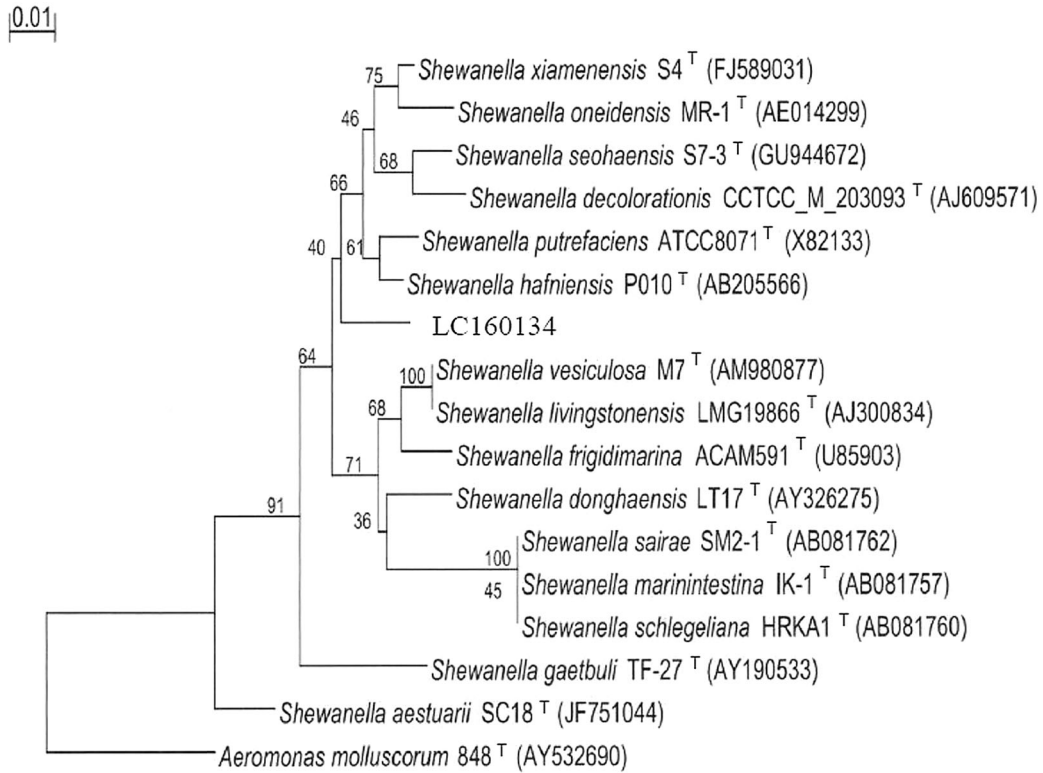


Fig. 4 continued

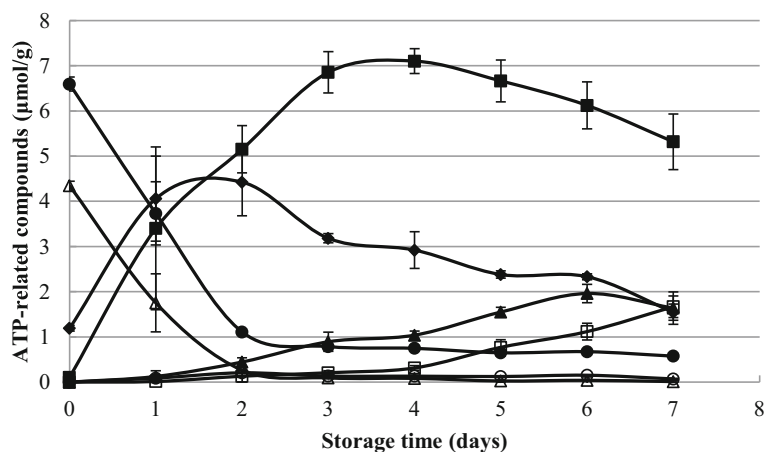


Fig. 5 Levels of ATP-related compounds at different times in shrimp stored at 4 °C. Bars denote the standard deviation ($n = 9$). Open triangles ATP, filled circles ADP, filled diamonds AMP, filled squares IMP, open circles AdR, filled triangles HxR, open squares Hx

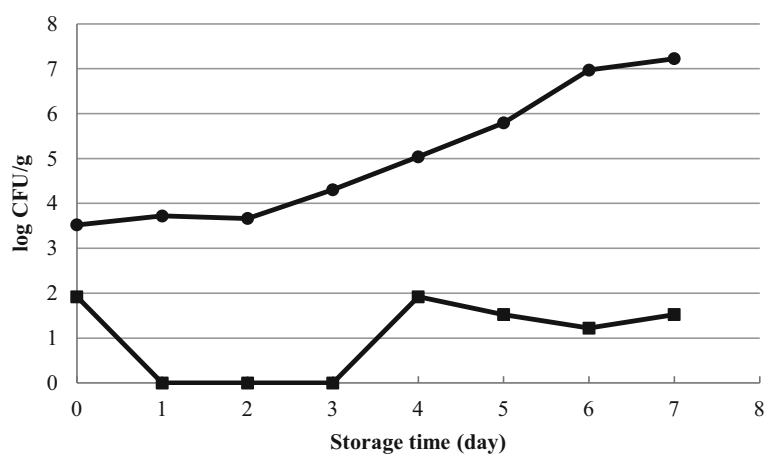


Fig. 6 Counts of bacteria on Plate Count Agar at 35 °C (filled squares) and on Plate Count Agar containing 1% NaCl at 20 °C (filled circles)

degradation was dependent on the bacteria, because only the bacteria isolated from shrimp degraded AMP. When AMP is degraded to AdR, the phosphate group is removed by a phosphatase, such as AMP-5'-nucleotidase. This enzyme removes phosphate from a nucleotide composed of a base + carbohydrate + phosphate. In this study, there is a possibility that the isolated bacteria from shrimp yielded phosphatases that removed the phosphate group from AMP.

Alkaline phosphatase has been found in marine bacteria isolated from the sea urchin *Strongylocentrotus intermedius* (Nedashkovskaya et al. 2005a), green alga *Ulva fenestrata* (Nedashkovskaya et al. 2005b), and soft coral *Paragorgia arborea* (Nedashkovskaya et al. 2005c). In addition, an alkaline phosphatase that was active at low temperatures was refined from a species of *Shewanella* (Ishida et al. 1998; Tsuruta et al. 1998). The *Shewanella* spp. identified in this study may also have alkaline phosphatase. However, the alkaline phosphatase from the *Shewanella* sp. studied by Tsuruta and Aizono (1999) has been shown to remove phosphoric acid only from phosphotyrosine, not from ATP, ADP, or AMP. The species of *Shewanella* identified in our study had different activity, but as there are many different species within the genus, multiple phosphatases are likely. At present, the type(s) of phosphatase(s) in the three species identified in this study are not known, although several studies (Ishida et al. 1998; Murakawa et al. 2002; Yasuda et al. 2002; Yamaguchi et al. 2006; Tsuruta et al. 2010) have examined phosphatase production in *Shewanella* spp. The species of bacteria identified in our study probably produce a phosphatase that degrades AMP to AdR in shrimp. A



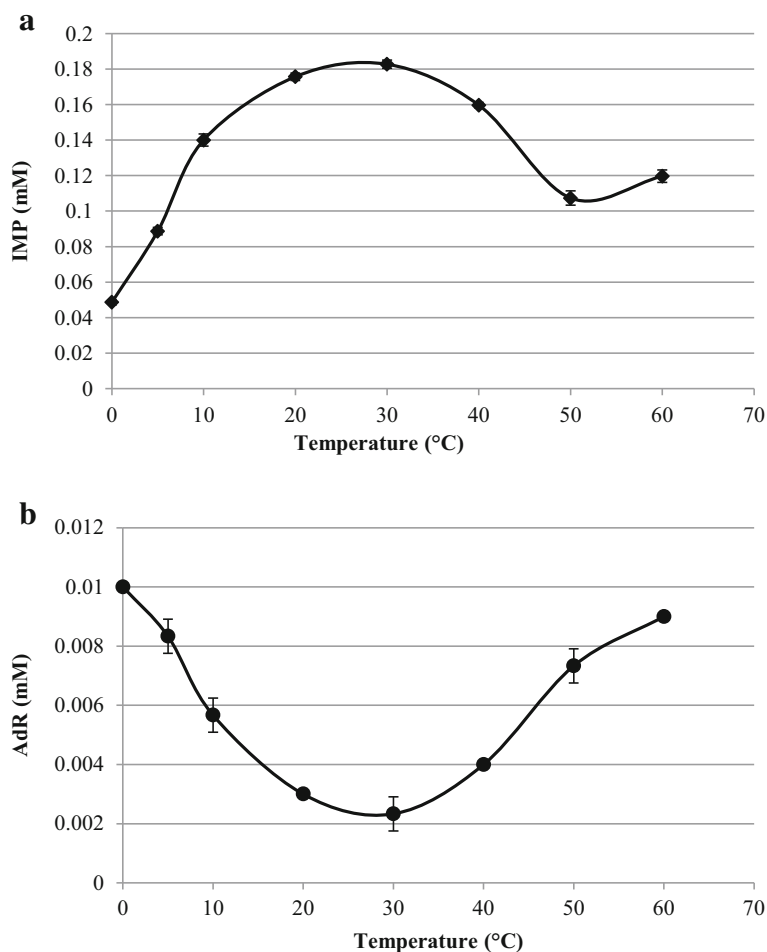


Fig. 7 Effects of temperature on the amount of IMP (**a**, filled diamonds) and AdR (**b**, filled circles) generated by shrimp enzymes from muscle by day 5 of incubation. Bars denote the standard deviation ($n = 3$). IMP is produced from degradation by AMP deaminase, and AdR is produced from degradation by AMP-5'-nucleotidase

deaminase is required to degrade AMP to IMP, and although a glucosamine-6-phosphate deaminase has been extracted from *Vibrio cholerae* non-O1 (Fujishima et al. 1996, 1997), it is unlikely that this deaminase affects AMP degradation, because IMP was not detected (Table 2; Fig. 2).

Changes in levels of ATP-related compounds and bacterial counts

We found that ATP and ADP were degraded relatively quickly and that AMP and IMP accumulated in the shrimp (Fig. 5). Shrimp and lobsters fall into two groups with respect to AMP degradation: in one group, AMP degrades to IMP, and in the other, AMP degrades to AdR (Koseki et al. 2006; Koyama et al. 2008). AdR was not detected in this study, so *P. japonicus* belongs to the first group. In the vannamei shrimp *P. vannamei*, the level of IMP accumulation depended on temperature (Koyama et al. 2008). IMP has been detected in shrimp held at -1 to 0 °C (Matsumoto and Yamanaka 1990) or at 2 °C (Nakamura and Ishikawa 1986). In this study, IMP was detected in shrimp extracts at 5 °C, indicating that AMP was degraded to IMP, because AMP deaminase activity was higher than AMP-5'-nucleotidase activity.

We found that the bacteria from the shrimp degraded AMP to AdR (Table 2; Fig. 2) and that the bacterial numbers increased after 2 days of incubation on agar plates with 1% NaCl at 20 °C (Fig. 6). However, AdR was not detected in shrimp tissue stored for up to 7 days at 4 °C (Fig. 5). These apparently contradictory results are due to the low level of enzyme produced by bacteria at 4 °C in comparison with the high level of



active enzyme in shrimp muscle at the same temperature. Alternatively, it is possible that the types of bacteria that can grow in the presence of NaCl but do not degrade AMP increased in shrimp muscle.

Our results indicate that suppressing the growth of bacteria during storage is necessary to prevent AMP degradation to AdR, which negatively affects shrimp flavor. Bacteria are not generally present in muscle; however, after death, bacteria may invade the muscle from organs and the shrimp's external environment. Therefore, it is important to remove organs and wash the surface of the shrimp as soon as possible.

Conclusions

In this study, we investigated AMP degradation in shrimp using enzymes extracted and bacteria isolated from shrimp muscle. An enzyme with high AMP deaminase activity in shrimp degraded AMP to IMP. In contrast, bacterial isolates 1, 2, and 6 degraded AMP to AdR with a phosphatase. Isolate 5 produced an enzyme with high activity that immediately degraded AMP to Hx. The amount of enzyme that was produced from bacteria was less than that in shrimp muscle, so no AdR but considerable IMP was confirmed when shrimp was stored for 7 days at 5 °C, demonstrating that refrigeration is important for preserving the taste component in shrimp and preventing bacteria from degrading IMP.

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References

- Altschul SF, Madden TF, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Arai K, Terasaki M (1966) Nucleotides in the muscles of marine invertebrates. *Nippon Suisan Gakk* 32:174–179
- Chen S, Hong Y, Shao Z, Liu Z (2010) A cold-active β -glucosidase (Bg11C) from a sea bacteria *Exiguobacterium oxidotolerans* A011. *World J Microbiol Biotechnol* 26:1427–1435
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Fujisawa K, Yoshino M (1987) Activities of adenylate-degrading enzymes in muscles from vertebrates and invertebrates. *Comp Biochem Physiol B Biochem Mol Biol* 86:109–112
- Fujii T (1985) Comparative studies on methods for determination of bacterial counts in seafoods. I. Comparisons of media, incubation temperatures and plating methods. *Bull Tokai Reg Fish Res Lab* 118:71–79
- Fujisawa K, Yoshino M (1985) Distribution of AMP deaminase and adenosine deaminase in muscle of mammals, fish, molluscs and crustaceans. *J Jpn Soc Nutr Food Sci* 38:322–326
- Fujishima S, Yamano N, Wang J (1996) Degradation of chitin by marine bacteria *Vibrio* sp. *Chitin Chitosan Res* 2:120–121
- Fujishima S, Yamano N, Maruyama A, Higashihara T (1997) *N*-Acetylglucosamine metabolism of marine bacteria. *Chitin Chitosan Res* 3:164–165
- Fukuda T, Furushita M, Shiba T (2012) Viable counts of bacteria determined on food fishes by the standard-agar-plate method incubated at 35 °C and nutrient-agar-plate method incubated at 20 °C. *J Natl Fish Univ* 60:183–188
- Hamano K (2015) Strategies to improve aquaculture and disease controlling agent of prawn in Japan. The report of Setouchi, vol 21, pp 10–12. http://feis.fra.affrc.go.jp/event/h26seika_happyokai/houkoku/youshi3_hamano.pdf. Accessed May 2015
- Hayashi M, Nakata K (2003) Effect of contaminating bacteria on the inosinic acid content of chicken meat. *J Home Econ* 54:441–448
- Huang J, Sun B, Zhang X (2010) *Shewanella xiamenensis* sp. nov., isolated from coastal sea sediment. *Int J Syst Evol Microbiol* 60:1585–1589
- Ishida Y, Tsuruta H, Tsuneta ST, Uno T, Aizono Y, Watanabe K (1998) Characteristics of psychrophilic alkaline phosphatase. *Biosci Biotechnol Biochem* 62:2246–2250
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kobayashi H, Kadowaki M, Fujimura S (2012) Regulation of muscular free glutamate content by the nutrition of animal diet. *Proc Jpn Soc Anim Nutr Metab* 56:1–12
- Koseki S, Kitakami S, Kato N, Arai K (2006) Rigor mortis of fish and shellfish and evaluation of freshness of their muscles as K value. *J School Mar Sci Technol Tokai Uni* 4:31–46
- Koyama N, Matsukawa M, Shimada M, Sato R (2008) The change of ATP and its related compounds of the *Penaeus vannamei* muscle and effect of the sense of taste. *Nippon Suisan Gakk* 74:1068–1074



- Mace S, Cardinal M, Jaffres E, Cornet J, Lalanne V, Chevalier F, Serot T, Pilet MF, Dousset X, Joffraud JJ (2014) Evaluation of the spoilage potential of bacteria isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging. *Food Microbiol* 40:9–17
- Matsumoto M (1996) Freshness and extractive components of marine product invertebrate animals. *J Cook Sci Jpn* 29:306–313
- Matsumoto M, Yamanaka H (1990) Changes in contents of glycolytic metabolites and free amino acids in the muscle of kuruma prawn during storage. *Nippon Suisan Gakk* 56:1515–1520
- Matsumoto M, Yamanaka H (1991) Influences of antibiotics chloramphenicol on post-mortem biochemical changes in the muscle of Kuruma Prawn during storage. *Nippon Suisan Gakk* 57:2291–2297
- Matsumoto M, Yamanaka H, Hatae K (1991) Effect of “aria” treatments on the biochemical changes in the Kuruma Prawn muscle. *Nippon Suisan Gakk* 57:1383–1387
- Motoo H (1990) Resource and use of a shrimp. The biology of tiger shrimps. *J Food Sci* 145:24–30
- Murakawa T, Yamagata H, Tsuruta H, Aizono Y (2002) Cloning of cold-active alkaline phosphatase gene of a psychrophile, *Shewanella* sp., and expression of the recombinant enzyme. *Biosci Biotechnol Biochem* 66:754–761
- Nakagawa Y, Sakane T, Suzuki M, Hatano K (2002) Phylogenetic structure of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* deduced from 16S rRNA sequence analysis. *J Gen Appl Microbiol* 48:155–165
- Nakamura K, Ishikawa S (1986) Changes in freshness of kuruma prawn muscle during chill-storage. *Bull Tokai Reg Fish Res Lab* 120:69–72
- Nedashkovskaya OI, Frolova GM, Mikhailov VV, Kim SB, Bae KS, Lysenko AM, Lee DH, Kim IS (2005a) *Gramella echinicola* gen. nov., sp. nov., a novel halophilic bacterium of the family Flavobacteriaceae isolated from the sea urchin *Strongylocentrotus intermedius*. *Int J Syst Evol Microbiol* 55:391–394
- Nedashkovskaya OI, Frolova GM, Mikhailov VV, Kim SB, Bae KS, Lysenko AM (2005b) *Bizionia paragorgiae* gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the soft coral *Paragorgia arborea*. *Int J Syst Evol Microbiol* 55:375–378
- Nedashkovskaya OI, Shevchenko LS, Frolova GM, Mikhailov VV, Kim SB, Lee DH, Lee KH, Bae KS, Lysenko AM (2005c) *Roseivirga ehrenbergii* gen. nov., sp. nov., a novel marine bacterium of the phylum ‘Bacteroidetes’, isolated from the green alga *Ulva fenestrata*. *Int J Syst Evol Microbiol* 55:231–234
- Ng IS, Chen T, Lin R, Zhang X, Ni C, Sun D (2014) Decolorization of textile azo dye and Congo red by an isolated strain of the dissimilatory manganese-reducing bacterium *Shewanella xiamenensis* BC01. *Appl Microbiol Biotechnol* 98:2297–2308
- Richards GP, Watson MA, Crane EJ III, Burt IG, Bushek D (2008) *Shewanella* and *Photobacterium* spp. in oysters and seawater from the Delaware Bay. *Appl Environ Microbiol* 74:3323–3327
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Seki H, Hamada-Sato N (2014) Identification of bacteria that contribute to IMP degradation in horse mackerel. *J Food Process Technol* 5:363
- Serio A, Fusella GC, Chaves LC, Sacchetti G, Paparella A (2014) A survey on bacteria isolated as hydrogen sulfide-producers from marine fish. *Food Control* 39:111–118
- Shirai T, Hirakawa Y, Koshikawa Y, Toraishi H, Suzuki T, Hirano T, Terayama M (1996) Taste components of Japanese spiny and shovel-nosed lobsters. *Fish Sci* 62:283–287
- Srirangsan P, Hamada-Sato N, Kawai K, Watanabe M, Suzuki T (2010) Improvement of fish freshness determination method by the application of amorphous freeze-dried enzymes. *J Agric Food Chem* 58:12456–12461
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Tsuruta H, Aizono Y (1999) Enzymatical properties of psychrophilic phosphatase I. *J Biochem* 125:690–695
- Tsuruta H, Tsuneta ST, Ishida Y, Uno T, Aizono Y, Watanabe K (1998) Purification and some characteristics of phosphatase of a psychrophile. *J Biochem* 123:219–225
- Tsuruta H, Mikami B, Higashi T, Aizono Y (2010) Crystal structure of cold-active alkaline phosphatase from the psychrophile *Shewanella* sp. *Biosci Biotechnol Biochem* 74:69–74
- Vogel BF, Gram L, Venkateswaran K, Satomi M (2005) Identification of *Shewanella baltica* as the most important H₂S-producing species during iced storage of Danish marine fish. *Appl Environ Microbiol* 71:6689–6697
- Yamaguchi H, Tsuruta H, Yamagata H, Aizono Y (2006) Enzymatic characteristics of cold-active alkaline phosphatase. *Mem Grad Sch Sci Technol Kobe Univ* 24:A23–A31
- Yamanaka H (2008) Fish brands 11) live prawn. *Food Sci* 51:31–34
- Yasuda J, Nakasine K, Kato C, Horikoshi K (2002) Cloning and sequencing of the *phoA* gene encoding alkaline phosphatase from a deep-sea piezophilic bacterium, *Shewanella violacea* strain DSS12. *JAMSTEC Rep Res Dev* 45:69–74
- Yokoyama Y, Sakaguchi M (1998) ATP metabolism in muscle after death of fish/shellfish and related items. *Jpn Soc Comp Physiol Biochem* 15:193–200
- Yokoyama Y, Sakaguchi M, Azuma Y, Kawai F, Kanamori M (1996) Postmortem changes of ATP and its related compounds in oyster tissues in the presence of antibiotic chloramphenicol. *Fish Sci* 62:312–316
- Yumoto I, Hishinuma-Narisawa M, Hirota K, Shingyo T, Takebe F, Nodasaka Y, Matsuyama H, Hara I (2004) *Exiguobacterium oxidotolerans* sp. nov., a novel alkaliphile exhibiting high catalase activity. *Int J Syst Evol Microbiol* 54:2013–2017
- Zhang F, Xu H, Pronin A, Liu H, Tachdjian C, Li X, Klebansky B, Fine RM (2008) Molecular mechanism for the umami taste synergism. *Proc Natl Acad Sci USA* 105:20930–20934

