Studies on Psychrotolerant Endospore-forming Bacteria for Developing Food Preservation Methods

Kentaro Tsuda

2016

Contents

ABBREVIATIONS

BCAA	Branched-chain amino acid
BCFA	Branched-chain fatty acid
bp	Base pair
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GLC	Gas liquid chromatography
MIC	Minimum inhibitory concentration
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SFA	Saturated fatty acid
SPC	Standard Plate Count
TSB	Tryptic Soy Broth
UFA	Unsaturated fatty acid

INTRODUCTION

In order to maintain the quality of food products during storage, the growth of food spoilage microorganisms should be inhibited. In case of the food products that have been heated and then distributed under refrigeration, psychrotolerant endospore-forming bacteria are important contaminants because they can survive pasteurization and grow under low temperature. In this study, I focused on psychrotolerant endospore-forming bacteria for developing food preservation methods

Psychrotolerant endospore-forming bacteria are important contaminants in various processed foods, because the bacteria can overcome two barriers (heat-treatment and cold-storage) against food spoilage. In order to maintain the quality of the foods that have been heated and then distributed under refrigeration, it is necessary to investigate the prevalence of psychrotolerant endospore-forming bacteria in raw materials and to assess the risk of food spoilage caused by these bacteria, and then the hazard must be controlled.

Some typical examples of psychrotolerant endospore-forming bacteria isolated from foods are cited as follows: *Bacillus*, *Viridibacillus*, and *Paenibacillus* species from milk [1,2,3], *B. weihenstephanensis* from liquid egg products [4], *B. cereus*-like organisms from sausages [5], *Paenibacillus* species from zucchini purées [6], and *B. cereus*, *B. simplex*, *B. subtilis*, and *Sporosarcina aquimarina* from seafood products [7]. There are fewer cases where *Sporosarcina* species were isolated from food as psychrotolerant endospore-forming bacteria than those with *Bacillus* species.

Chapter I describes isolation of psychrotolerant endospore-forming bacteria from minced fish meat (surimi). In this chapter, I found that *Sporosarcina* species were major contaminants of surimi. Psychrotolerant endospore-forming bacteria were isolated from surimi stored at low temperatures (5°C and 10°C) after heat-treatment (80°C). The isolates were identified by partial 16S rRNA gene sequencing. I found that *Sporosarcina* species are widely distributed in surimi made from Alaska pollock, pike conger, and white croaker. The *Sporosarcina* isolates were tested for their ability to tolerate high temperatures and grow at low temperatures. Furthermore, in order to study the cold-adaptation mechanism of the isolates, the bacterial fatty acids were analyzed because the composition could change in response to growth temperature to maintain the fluidity of the cell membrane [8].

Chapter II describes mechanisms of cold tolerance in *Sporosarcina* species from the point of view of fatty acid composition. To protect surimi-based food products from bacterial contaminants, I am interested in how *Sporosarcina* strains adapt to cold environments, particularly in terms of their fatty acid composition. In this study, I investigated the growth and fatty acid compositions of several *Sporosarcina* strains at cold and moderate temperatures. Furthermore, I tested whether branched-chain amino acids affected the growth and fatty acid compositions because these compounds could be candidates for use as preservatives. I found that the contents of either *anteiso*-branched-chain or unsaturated fatty acids had crucial roles in the growth of *Sporosarcina* strains under cold conditions. The addition of branched-chain amino acids to the medium altered the fatty acid composition in a manner similar to the known mechanism. I demonstrate that the growth of *Sporosarcina* species at cold temperatures can be controlled by artificially modulating the bacterial fatty acid composition using additives. I suggest the possibility of using leucine as a food additive for preserving surimi-based products by inhibiting the growth of *Sporosarcina* strains at cold temperatures.

Chapter III describes the screening of chemicals showing antibacterial effect against *Sporosarcina* species. As mentioned in Chapter I, several *Sporosarcina* species were predominantly isolated from minced fish meat (surimi) as psychrotolerant endospore-forming bacteria, and I suggested that they should be controlled to maintain the quality of surimi-based products [44]. However, chemical compounds that exert antibacterial effect against *Sporosarcina* species are not well known. Alkyl gallates have been shown to exhibit antibacterial activities against *Salmonella choleraesuis* [57], methicillin-resistant *Staphylococcus aureus* [58], and *Bacillus subtilis* [59]. In addition, the antibacterial activities of free fatty acids have been known [60]. In this study, alkyl gallates and fatty acids-related compounds were tested for their antibacterial activities against *Sporosarcina* species.

CHAPTER I

Isolation and characterization of psychrotolerant endospore-forming *Sporosarcina* species associated with minced fish meat (surimi)

Surimi is a Japanese term referring to the minced meat of Alaska pollock (*Theragra chalcogramma*) or other fish species traditionally used in Japan as a raw material for various fish-paste products such as *kamaboko* (fish cake), *chikuwa* (fish stick), and imitation-crab stick [9]. Lately, these fish-paste products are being consumed in the US, EU, and other Asian countries as well. With global consumption of these fish-paste products, there is a shortage in the supply of fish that are used for surimi. In order to compensate, various species of fish, such as pike conger (*Muraenesox cinereus*), white croaker (*Pennahia argentata*), and Threadfin bream (*Nemipterus virgatus*) are also used as raw material of surimi [10]. Alaska pollock is mainly caught in the Bering Sea and the Sea of Okhotsk, and processed into surimi on board. On the other hand, pike conger and white croaker are mainly caught in the South China Sea and processed into surimi on land in the Southeast Asian countries (e.g., Thailand). Since surimi is made from various fish species caught in various fishing grounds, the microbiota associated with surimi is expected to be diverse. Some of the microorganisms might have a potential to cause spoilage of the fish-paste products.

Most of the Japanese fish-paste products are produced by mixing surimi with ingredients such as salt, sugar, and starch, which are heated up to about 80°C and packaged under air. Although the heating step can inactivate vegetative microorganisms present in the raw material, endospores that are resistant to high temperatures can survive [11]. The packaged fish-paste products are often kept at low temperatures (0°C to 10°C) during the shelf life. If the surviving endospore-forming bacteria are psychrotolerant, they can grow in the fish-paste product and cause food spoilage.

Psychrotolerant endospore-forming bacteria are important contaminants in various processed foods, because the bacteria can overcome two barriers (heat-treatment and cold-storage) against food spoilage. In order to maintain the quality of the foods that have been heated and then distributed under refrigeration, it is necessary to investigate the prevalence of psychrotolerant endospore-forming bacteria in raw materials and to assess the risk of food spoilage caused by these bacteria, and then the hazard must be controlled.

Some typical examples of psychrotolerant endospore-forming bacteria isolated from foods are cited as follows: *Bacillus*, *Viridibacillus*, and *Paenibacillus* species from milk [1,2,3], *B. weihenstephanensis* from liquid egg products [4], *B. cereus*-like organisms from sausages [5], *Paenibacillus* species from zucchini purées [6], and *B. cereus*, *B. simplex*, *B. subtilis*, and *Sporosarcina aquimarina* from seafood products [7]. There are fewer cases where *Sporosarcina* species were isolated from food as psychrotolerant endospore-forming bacteria than those with *Bacillus* species.

In this study, psychrotolerant endospore-forming bacteria were isolated from surimi stored at low temperatures (5°C and 10°C) after heat-treatment (80°C). The isolates were identified by partial 16S rRNA gene sequencing. I found that *Sporosarcina* species are widely distributed in surimi made from Alaska pollock, pike conger, and white croaker. The *Sporosarcina* isolates were tested for their ability to tolerate high temperatures and grow at low temperatures. Furthermore, in order to study the cold-adaptation mechanism of the isolates, the bacterial fatty acids were analyzed because the composition could change in response to growth temperature to maintain the fluidity of the cell membrane [8].

MATERIALS AND METHODS

Sample collection and preparation

Three types of frozen surimi were purchased from Matsuda Sangyo Co., Ltd. (Table 1-1). The first variety of surimi was made from minced Alaska pollock meat with sorbitol, sugar, and sodium polyphosphate to prevent freeze denaturation of fish proteins. The second and third varieties were made from pike conger and white croaker, respectively, with sugar and sodium polyphosphate. The frozen surimi samples were thawed by leaving them at 4°C for 18 h. A 100 g portion of the partially-thawed surimi was vacuum-packed at 6 mbar (microaerobic condition) in a thin bag (240 mm × 170 mm × 3 mm) in order to be heated quickly and evenly. The bag was heated by soaking in 30 l hot water at 80°C for 90 s and immediately cooled with ice water. The heat-treated surimi was stored at 5°C or 10°C for 1 or 2 weeks in the closed bag.

Enumeration and isolation of bacteria associated with surimi

To enumerate the bacteria in 10 g of surimi sample, the following method was used. A 50 g portion of surimi was homogenized in 200 g of phosphate buffered saline (PBS) for 5 min

using a homogenizer (Pro-media SH-IIM, ELMEX, Japan). Each 10 ml of the suspension was transferred to 5 sterile Petri dishes with a diameter of 150 mm mixed gently with 60 ml sterile Standard Plate Count agar (SPC agar, NISSUI, Japan), allowed to solidify, and incubated for 2 days at 30°C. The sum of colonies on the set of 5 plates was defined as the colony-forming unit (CFU) per 10 g of surimi. To simultaneously enumerate more than 100 CFU per gram of surimi, the same suspension was serially diluted tenfold with PBS, and then 1 ml of the dilution was incubated on a SPC agar in a 90 mm Petri dish. After incubation under the same conditions, the total bacterial counts (CFU/g) were calculated from the number of colonies on the plates having 30 to 300 colonies. All of the colonies on the plate with less than 30 CFU were isolated and identified.

Identification of the isolates from surimi

The isolated bacteria were identified as per the method described in The Japanese Pharmacopoeia 15th edition. Briefly, the bacteria were cultured in Tryptic Soy Broth (TSB, Becton Dickinson, USA) for 18 h at 30°C under aerobic conditions by reciprocal shaking (90 rpm). Genomic DNA was extracted from the culture with a PrepMan^{TM} Ultra Sample Preparation Reagent (Life Technologies, USA) and directly used as a PCR template to amplify the divergent region of 16S rRNA gene using 10F primer (5′-GTTTGATCCTGGCTCA-3′) and 800R primer (5′-TACCAGGGTATCTAATCC-3′). The PCR products (approximately 740 bp) were purified using a High Pure PCR Product Purification Kit (Roche applied science, Germany) for sequencing. The sequencing reaction was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) with the 10F primer. The sequencing reaction products were purified using a Sephadex G-50 Fine (GE Healthcare Life Sciences, USA) and analyzed with a 3730xl DNA analyzer (Life Technologies, USA). The obtained partial 16S rRNA gene sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed by neighbor-joining method using the ClustalW software at DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/). *Lactobacillus delbrueckii* 16S rRNA gene (AY773949) was used as outgroup of the phylogenetic tree.

Table 1-1. List of surimi tested in this study

Fish species as a raw material of surimi					
English name	Scientific name	Japanese name	Fishing ground	Manufacturer	Production year
Alaska pollock	*Theragra chalcogramma*	Sukesodara	Bering Sea	Company A in USA	2011
Pike conger	*Muraenesox cinereus*	Hamo	South China Sea	Company B in Thailand	2011
White croaker	*Pennahia argentata*	Guchi	South China Sea	Company C in Thailand	2011

Measurement of the growth rate

The isolates were cultured in TSB medium at 30°C for 18 h with reciprocal shaking (90 rpm) as primary cultures. Five microliters of the primary culture was inoculated into 5 ml of fresh TSB medium and incubated at 10°C or 30°C with seesaw shaking (20 rpm). The optical density of the cultures at 660 nm (OD_{660}) was automatically recorded every 15 min for 10 days by a Bio-photorecorder (TN-1506, ADVANTEC, Japan). The growth rate (day^{-1}) was defined as the reciprocal of the incubation time (days) when the OD_{660} was approximately 0.1.

Assessment of heat resistance of endospores

The isolated endospore-forming bacteria were incubated on SPC agar slants at 30°C for over 10 days, until at least 80% sporulation of the cells was observed using a microscope. Sporulated bacterial colonies were suspended in PBS and then centrifuged at 5,000 ×*g* for 10 min. The pellet was resuspended in PBS. The remaining vegetative cells were inactivated by heat-treatment at 80°C for 10 min. A 0.1 ml portion of the endospore suspension was taken in a 0.5-ml PCR tube and heated at 75°C, 85°C, and 95°C using a block incubator (MasterCycler, Eppendorf, Germany) in duplicate. Enumeration of the surviving endospores was carried out as described above. The decimal reduction time (*D*-value) was determined by log-linear approximation of the survival curve.

Inoculation of endospores into a fish-paste product model system

A 150 g portion of Alaska pollock surimi, 15 g potato starch, 5 g sugar, 3.5 g salt, and 75 g ice water were mixed with or without 1.5 g endospore suspension (10^4 CFU/g) by using a food processor. The mixture was stuffed into a polyvinylidene chloride casing with a diameter of 51 mm, and both ends were tightly fastened with clasps. The casing was boiled at 80°C for 15 min and rapidly cooled with ice water. After the casing was removed, the fish-paste product model system was cut into 15-mm-thick slices (approximately 20 g) and stored at 10°C under air. Enumeration of the bacteria was carried out as described above.

Analysis of fatty acid composition

The isolates were cultured in TSB medium at 28°C and 10°C with reciprocal shaking (300 rpm) until the stationary phase. The bacteria were harvested by centrifugation and dried at

120°C. The dried cells were weighed and transmethylated with 10% methanolic HCl and dichloromethane at 55°C for 2 h, using 0.5 mg n-tricosanoic acid as an internal standard. The resultant fatty acid methyl esters were extracted with n-hexane, concentrated, and analyzed by gas chromatography (GC) using a gas liquid chromatography (GLC) system (GC-2010 Plus, Shimazu, Japan) equipped with a flame ionization detector, a split injector on a TC-70 capillary column (GL-Science, Japan), and temperature programming (180°C to 260°C at 5°C/min). Fatty acid methyl ester peaks were identified and calibrated with the corresponding standard fatty acid methyl esters. The fatty acids were identified by comparison with fatty acid standards run under the same GC conditions. After conversion to pyrrolidide derivatives as described previously [12], these fatty acids were analyzed by GC-mass spectrometry (GC-MS) to determine the positions of branching and double bonds. The GC-MS analyses with an SPB-1 column (SPELCO) were carried out as described previously [13].

RESULTS

In order to investigate the bacterial diversity in surimi, three types of surimi were sampled (Table 1-1), and tested for bacterial counts at the three processing stages: raw, heat-treated, and subsequent refrigeration. The isolates from surimi were identified based on their 16S rRNA gene sequences. The total bacterial counts and other resident microbiota in Alaska pollock, pike conger, and white croaker surimi were shown in Tables 1-2, 1-3, and 1-4, respectively.

Microbiota in raw surimi

The bacterial counts ranged from 10^4 to 10^6 CFU/g in the three types of surimi tested in the raw state. The results of the 16S rRNA gene sequencing of the isolates indicated that the microbiota of the raw surimi consisted of very diverse species. Isolates from the raw Alaska pollock surimi were related to lactic acid bacteria such as *Carnobacterium* species, actinobacteria such as *Arthrobacter* species, and gamma-proteobacteria such as *Enterobacter* species (Table 1-2). *Arthrobacter* species have been previously isolated from Alaska pollock surimi as well [14]. On the other hand, isolates from the raw pike conger and white croaker surimi were related to lactic acid bacteria such as *Carnobacterium* species, actinobacteria such as *Brevibacterium* species, alpha-proteobacteria such as *Paracoccus* species, and Bacteroidetes

Table 1-2. Bacterial counts and the microflora of Alaska pollock surimi

Surimi conditions		Total Bacterial counts	Microbiota			
Heat-treatment	Storage	(CFU/g)	Related species (Accession Number)	Identity[a]	Strain name	Population ratio[b]
None	None	75,000	*Carnobacterium maltaromaticum* (M58825)	99%	SR06	1/6
			Leuconostoc mesenteroides (CP000414)	100%	SR12	1/6
			Arthrobacter bergerei (AJ609630)	98%	SR02	1/6
			Leucobacter komagatae (D45063)	98%	SR13	1/6
			Enterobacter hormaechei (AJ508302)	98%	SR18	1/6
			Psychrobacter arenosus (AJ609273)	99%	SR03	1/6
80°C, 90 s	None	< 0.1				
80°C, 90 s	5°C, 1 week	< 0.1				
80°C, 90 s	5°C, 2 weeks	150	*Sporosarcina aquimarina* (AF202056)	97%	S42c	13/14
			Sporosarcina aquimarina (AF202056)	97%	S42d	1/14
80°C, 90 s	10°C, 1 week	1,100	*Sporosarcina aquimarina* (AF202056)	98%	S91e	9/14
			Sporosarcina aquimarina (AF202056)	98%	S91f	5/14
80°C, 90 s	10°C, 2 weeks	1,200	*Sporosarcina aquimarina* (AF202056)	99%	S92g	14/15
			Sporosarcina koreensis (DQ073393)	99%	S92h	1/15

[a] The partial 16S rRNA gene sequence (approximately 740 bp) identity with the type strain

[b] The number of identified strains per total isolates

Table 1-3. Bacterial counts and the microflora of pike conger surimi

Surimi conditions		Total Bacterial counts	Microbiota			
Heat-treatment	Storage	(CFU/g)	Related species (Accession Number)	Identity[a]	Strain name	Population ratio[b]
None	None	700,000	*Carnobacterium divergens* (M58816)	97%	HR03	1/6
			Carnobacterium maltaromaticum (M58825)	97%	HR06	1/6
			Kocuria rhizophila (Y16264)	99%	HR04	1/6
			Rothia nasimurium (AJ131121)	98%	HR10	1/6
			Paracoccus sulfuroxidans (DQ512861)	99%	HR05	1/6
			Soonwooa buanensis (FJ713810)	98%	HR01	1/6
80°C, 90 s	None	0.2	*Bacillus barbaricus* (AJ422145)	99%	H001	1/2
			Bacillus flexus (AB021185)	100%	H004	1/2
80°C, 90 s	5°C, 1 week	0.1	*Bacillus barbaricus* (AJ422145)	98%	H41v	1/1
80°C, 90 s	5°C, 2 weeks	89	*Sporosarcina aquimarina* (AF202056)	98%	H42a	15/15
80°C, 90 s	10°C, 1 week	130	*Sporosarcina aquimarina* (AF202056)	98%	H91a	8/12
			Bacillus cereus (AE016877)	99%	H91w	3/12
			Bacillus cereus (AE016877)	100%	H91x	1/12
80°C, 90 s	10°C, 2 weeks	230,000	*Sporosarcina aquimarina* (AF202056)	98%	H92b	14/14

[a] The partial 16S rRNA gene sequence (approximately 740 bp) identity with the type strain

[b] The number of identified strains per total isolates

Table 1-4. Bacterial counts and the microflora of white croaker surimi

Surimi conditions		Total Bacterial counts	Microbiota			
Heat-treatment	Storage	(CFU/g)	Related species (Accession Number)	Identity[a]	Strain name	Population ratio[b]
None	None	330,000	*Carnobacterium divergens* (M58816)	99%	GR03	1/6
			Brevibacterium epidermidis (X76565)	99%	GR22	1/6
			Nocardioides kongjuensis (DQ218275)	94%	GR06	1/6
			Psychrobacter sanguinis (HM212668)	99%	GR32	1/6
			Flavobacterium anatoliense (JF825522)	94%	GR10	1/6
			Soonwooa buanensis (FJ713810)	98%	GR08	1/6
80°C, 90 s	None	0.4	*Bacillus cereus* (AE016877)	100%	G005	1/4
			Bacillus safensis (AF234854)	100%	G006	1/4
			Bacillus vietnamensis (AB099708)	99%	G004	1/4
			Lysinibacillus fusiformis (AF169537)	99%	G002	1/4
80°C, 90 s	5°C, 1 week	0.2	*Bacillus cereus* (AE016877)	100%	G41r	2/2
80°C, 90 s	5°C, 2 weeks	12	*Sporosarcina aquimarina* (AF202056)	98%	G42a	8/12
			Sporosarcina luteola (AB473560)	99%	G42k	2/12
			Bacillus cereus (AE016877)	99%	G42p	2/12
80°C, 90 s	10°C, 1 week	480	*Sporosarcina aquimarina* (AF202056)	98%	G91a	11/20
			Paenibacillus odorifer (AJ223990)	97%	G91q	7/20
			Bacillus cereus (AE016877)	99%	G91p	2/20
80°C, 90 s	10°C, 2 weeks	6,500	*Bacillus cereus* (AE016877)	99%	G92p	12/21
			Lysinibacillus fusiformis (AF169537)	99%	G92s	9/21

[a] The partial 16S rRNA gene sequence (approximately 740 bp) identity with the type strain

[b] The number of identified strains per total isolates

such as *Flavobacterium* species (Tables 1-3 and 1-4). I noted that *Carnobacterium* species were generally isolated from all three types of surimi. *Carnobacterium divergens* and *C. maltaromaticum* have been isolated previously from various sources, such as sea water, fish, shrimp, seafood, meat, and milk [15]. *Carnobacterium* species can cause spoilage of the surimi products, as they are tolerant to freezing and capable of growth at low temperatures [15]. However, all of the identified isolates from the raw surimi were inactivated by heat-treatment as they were all non-endospore-forming bacteria [16,17,18,19,20,21,22,23,24,25,26,27,28,29].

Microbiota in surimi immediately after heat-treatment

Immediately after the raw surimi was heated at 80°C for 90 s, the bacterial counts were significantly reduced to less than 1 CFU/g, as compared to the raw state. The isolates obtained from the heated pike conger and white croaker surimi were closely related to endospore-forming bacteria such as *Bacillus* and *Lysinibacillus* species (Tables 1-3 and 1-4). These genera, defined as endospore-forming bacteria [30], are heat-resistant. These results suggest that the heat-treatment was effective in decreasing the bacterial counts in surimi, but a few endospore-forming bacteria survive in the form of dormant endospores.

Microbiota in surimi stored under refrigeration after heat-treatment

After heat-treatment, the bacterial counts increased during cold-storage depending on the temperature and time of storage. In Alaska pollock surimi, as shown in Table 1-2, the bacterial counts increased up to 150 CFU/g in 2 weeks at 5°C, and reached 1,100 CFU/g after 1 week at 10°C. The 43 isolates from the refrigerated Alaska pollock surimi were classified into six types: S42c, S42d, S91e, S91f, S92g, and S92h. Except for the isolate S92h, these isolates were closely related to *Sporosarcina aquimarina* SW28^T (Fig. 1-1 and Table 1-2). On the other hand, the isolate S92h was closely related to *Sporosarcina koreensis* F73^T (Fig. 1-1 and Table 1-2). In pike conger and white croaker surimi, as shown in Tables 1-3 and 1-4, respectively, the bacterial counts increased as well. More than half of the isolates from the refrigerated pike conger and white croaker surimi were closely related to the genus *Sporosarcina*, including *S. aquimarina* and *S. luteola*, and the others were closely related to *Bacillus cereus*, *Paenibacillus odorifer*, and *Lysinibacillus fusiformis*. As the genera *Sporosarcina* and *Paenibacillus* are defined as endospore-forming bacteria [31,32] similarly to the genera *Bacillus* and

Lysinibacillus, these isolates are heat-resistant on account of endospore formation. The phylogenetic tree of isolates from the refrigerated surimi is shown in Figure 1-1. It shows that more diverse *Sporosarcina* species were associated with the surimi samples than *Bacillus*, *Lysinibacillus*, and *Paenibacillus* species.

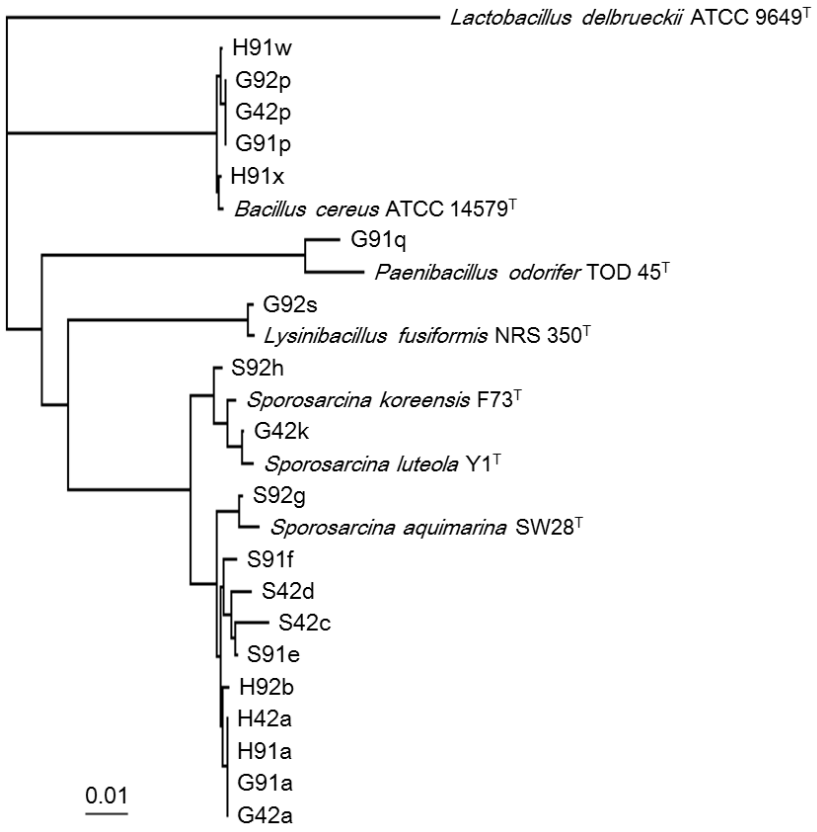

Figure 1-1. Phylogenetic tree of the isolates from the surimi that were stored under refrigeration after heat-treatment including the type strains, based on the partial 16S rRNA gene sequence (approximately 740bp). Scale bar represents 0.01 substitution per nucleotide position. The strains were named as follows: the first letter means the source of isolation (S, H, or G for Alaska pollock, pike conger, or white croaker surimi, respectively), the second letter means the storage temperature of the heated surimi (4 or 9 for 5°C or 10°C, respectively), the third letter means the storage period of the heated surimi (1 or 2 for 1 week or 2 weeks, respectively), and the last letter means the group of 16S rRNA gene sequences.

Ability of the isolates to grow at low temperatures

In order to assess the cold-adaptation of isolates from surimi stored under refrigeration after heat-treatment, the growth of isolates was monitored in TSB medium at 10°C and 30°C. The growth rates obtained are shown in Figure 1-2. The growth rates of *Sporosarcina* isolates

were 2.04 to 4.17 day^{-1} at 30°C and 0.32 to 0.63 day^{-1} at 10°C. Growth rates for *Lysinibacillus* and *Paenibacillus* isolates were 5.33 and 3.43 day^{-1} at 30°C, respectively, and 0.19 and 0.38 day^{-1} at 10°C, respectively. On the other hand, growth rates of *Bacillus* isolates were 5.65 to 19.20 day^{-1} at 30°C and less than 0.12 day^{-1} at 10°C. *Sporosarcina* isolates could proliferate more rapidly at 10°C than *Lysinibacillus*, *Paenibacillus*, and *Bacillus* isolates. These results indicate that the *Sporosarcina* isolates are more adaptive to cold environment than the other endospore-forming bacteria, such as *Bacillus*, *Lysinibacillus*, and *Paenibacillus* species.

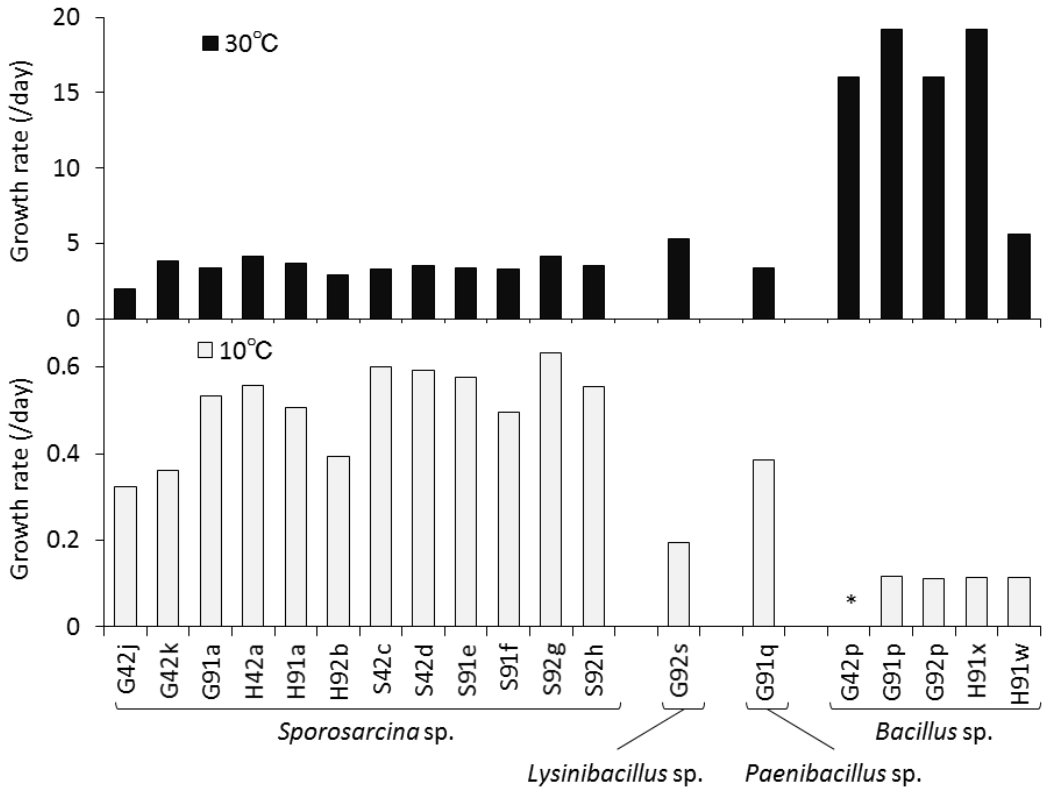

Figure 1-2. Growth rates (day-1) of the isolates from surimi at 30°C (black bar) and 10°C (gray bar) in Tryptic Soy Broth. Asterisk indicates no growth within 10 days.

Heat resistance of the endospores from the isolates

The heat resistance of the endospores of *Sporosarcina* isolates S92h and H42a was evaluated from the *D*-value. Figure 1-3 shows that the survival curves of these isolates at 75°C, 85°C, and 95°C in PBS (pH 7) were very similar to each other. I estimated that the *D*-values, which are the time required for the ten-fold reduction in endospore population, at 75°C, 85°C,

and 95°C for isolate S92h were 244.0 min, 16.0 min, and 1.9 min, while that for isolate H42a were 173.8 min, 15.2 min, and 2.7 min, respectively.

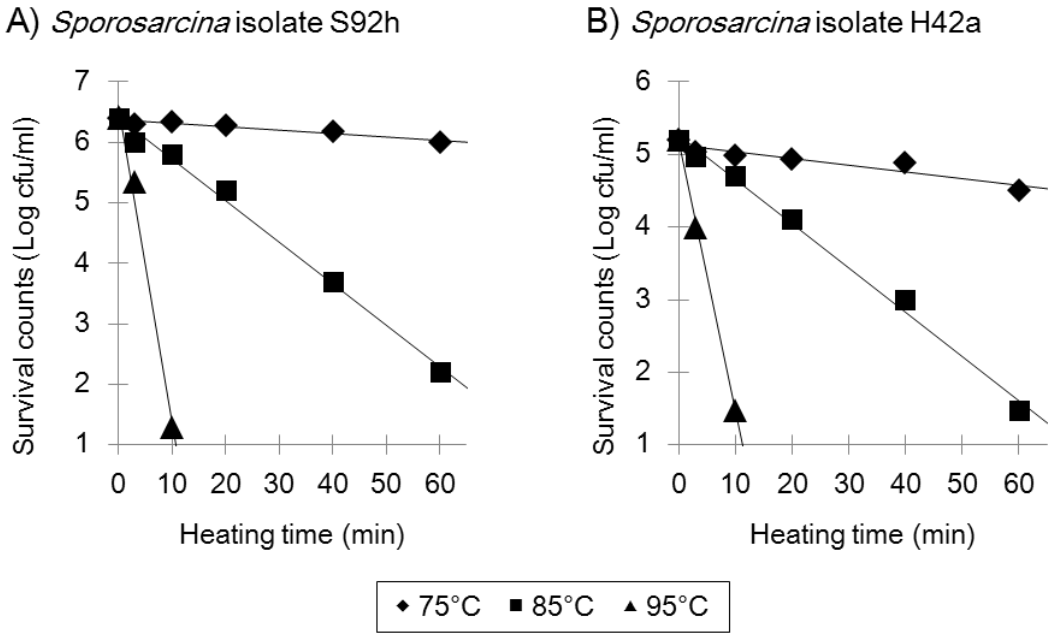

Figure 1-3. Survival curves at 75°C (diamond), 85°C (square), and 95°C (triangle) of the endospores of *Sporosarcina* isolates S92h (A) and H42a (B) in phosphate buffered saline. Each point represents the average of duplicate plate counts.

Germination and proliferation of *Sporosarcina* endospores in a fish-paste product model system

In order to confirm that the *Sporosarcina* isolates can grow in food, the endospores of the isolate S92h were inoculated into a fish-paste product model system. Bacterial counts in the model system increased to approximately 10^8 CFU/g within 8 days at 10°C (Fig. 1-4). In the absence of inocula, the bacterial counts did not increase. Morphological characteristics of the colonies isolated from the model system were indistinguishable from those of the inoculated isolate S92h (data not shown). These results indicate that the endospores of *Sporosarcina* isolate S92h can survive heat-treatment and proliferate under refrigeration in the fish-paste products.

Comparison of the bacterial fatty-acid composition at 10°C and 28°C

To study the cold-adaptation mechanism of the *Sporosarcina* isolates, fatty acid

composition of isolates S92h and H42a grown at 10°C and 28°C were analyzed (Table 1-5). At the growth temperature of 28°C, major fatty acids in the isolate S92h were *anteiso*-$C_{15:0}$ (59.0%) and *iso*-$C_{15:0}$ (23.4%), while the proportions differed slightly in *Sporosarcina koreensis* $F73^T$ as shown in Table 1-5 [33], in spite of the high degree of identity in their 16S rRNA gene sequences (99%). On the other hand, the major fatty acid in isolate H42a was *anteiso*-$C_{15:0}$ (77.2%), and the proportion was similar to *Sporosarcina aquimarina* $SW28^T$ [32] in agreement with the high degree of identity in 16S rRNA gene sequences (98%). At the growth temperature of 10°C, the sum of unsaturated fatty acids significantly increased from 2.3% to 12.5% in isolate S92h and from 3.7% to 19.9% in isolate H42a, as compared to those at 28°C. In particular, $C_{16:1}$ and *iso*-$C_{16:1}$ increased, while $C_{16:0}$ and *iso*-$C_{16:0}$ decreased, in both the strains. In addition, *anteiso*-$C_{17:1}$ also increased. The increased mono-unsaturated fatty acids have a double bond at Δ5 position. Furthermore, the sum of *anteiso* fatty acids increased and that of *iso* fatty acids decreased in both the strains.

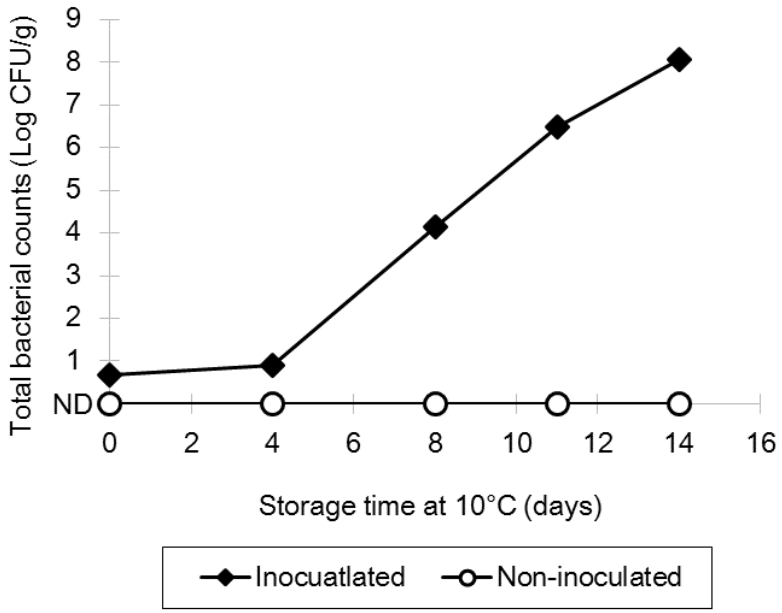

Figure 1-4. Total bacterial counts in a fish-paste product model system stored at 10°C after heat treatment at 80°C for 15 min. The raw materials were inoculated (closed diamond) or not (open circle) with endospores of *Sporosarcina* isolate S92h before the heat treatment.

Table 1-5. Fatty acid composition of *Sporosarcina* isolates from surimi and the related species

	Strain H42a		Strain S92h		Type culture	
Fatty acid	28°C	10°C	28°C	10°C	Ref. 1 [a]	Ref. 2 [b]
Fatty acid composition						
$C_{14:0}$	–	–	0.73 %	0.34 %	1.9 %	0.8 %
$C_{15:0}$	–	–	1.02 %	0.35 %	–	–
$C_{16:0}$	1.17 %	–	0.76 %	–	3.8 %	1.1 %
$C_{16:1}$ Δ5	–	1.61 %	0.38 %	1.56 %	0.6 %	0.7 %
iso-$C_{14:0}$	2.31 %	1.59 %	4.46 %	1.94 %	3.6 %	6.5 %
iso-$C_{15:0}$	8.71 %	3.62 %	23.36 %	16.11 %	5.4 %	44.4 %
iso-$C_{16:0}$	1.44 %	–	3.22 %	1.33 %	1.9 %	3.4 %
iso-$C_{16:1}$ Δ5	–	2.90 %	0.47 %	2.89 %	–	–
iso-$C_{17:0}$	–	–	0.75 %	–	–	0.7 %
anteiso-$C_{15:0}$	77.22 %	71.80 %	59.00 %	60.48 %	77.3 %	38.1 %
anteiso-$C_{17:0}$	5.51 %	3.12 %	4.44 %	6.93 %	4.1 %	1.9 %
anteiso-$C_{17:1}$ Δ5	3.65 %	15.36 %	1.43 %	8.08 %	–	–
Others[c]	–	–	–	–	1.4 %	1.2 %
Degree of unsaturation						
Sum of saturated fatty acids	96.35 %	80.13 %	97.72 %	87.47 %		
Sum of unsaturated fatty acids	3.65 %	19.87 %	2.28 %	12.53 %		
Branching pattern						
Sum of *anteiso* fatty acids	86.37 %	90.28 %	64.86 %	75.49 %		
Sum of *iso* fatty acids	12.46 %	8.11 %	32.25 %	22.26 %		

[a] *Sporosarcina aquimarina* $SW28^T$ (Yoon et al., 2001 [32])

[b] *Sporosarcina koreensis* $F73^T$ (Kwon et al., 2007 [33])

[c] Others include $C_{16:1}$ Δ9, $C_{16:1}$ Δ9 alcohol, $C_{16:1}$ Δ5 alcohol, $C_{18:0}$ and *anteiso*-$C_{13:0}$.

DISCUSSION

Surimi is cooked by steaming, boiling, and deep frying for sterilization and gelation [34]. Yokoseki (1958) studied the relationship between the heat-treatment temperature and the surviving microbiota during the production of *kamaboko* (fish cake) from white croaker surimi [11]. He found that cocci and spore-forming rods became predominant when the *kamaboko* was cooked at temperatures lower than 70°C and higher than 75°C, respectively, while several types of cocci and non-endospore-forming and endospore-forming rods were found in the raw materials. Similarly in this study, various non-endospore-forming bacteria were predominantly isolated from the raw surimi (Tables 1-2, 1-3 and 1-4). These bacteria, however, were eradicated by heat-treatment at 80°C for 90 s. Instead, endospore-forming bacteria belonging to the genera *Bacillus* and *Lysinibacillus* became predominant in the heated pike conger and white croaker surimi, although bacterial counts were very low (less than 1 CFU/g). No bacterial count was observed in Alaska pollock surimi immediately after the heat-treatment; however, some endospore-forming bacteria, including *Sporosarcina* species, may have survived, because they were detected from the subsequently refrigerated surimi (Table 1-2).

These results showed another change in microbiota during the cold-storage step. Notably, *Sporosarcina* species were predominantly isolated from 50 g of the heated surimi after 2 weeks at 5°C and 1 week at 10°C, even if they were not found in the initial stages. As the surimi samples were kept unopened during the cold-storage, *Sporosarcina* isolates were assumed to exist in the heated surimi as dormant endospores at levels below the detection limit (less than 1 CFU per 10 g). From these results, it seems that endospores of *Sporosarcina* species exist in the range of 1 CFU per 10 to 50 g of the surimi sample.

It has been reported that *Bacillus simplex*, *B. subtilis*, *S. aquimarina*, and other endospore-forming bacteria could become predominant in various kinds of surimi-based products stored at 4°C [7]. *Sporosarcina* species might have an advantage over *Bacillus*, *Lysinibacillus*, and *Paenibacillus* species to grow at low temperatures. Indeed, all of the 12 *Sporosarcina* isolates grew at 10°C faster than the *Bacillus*, *Lysinibacillus*, and *Paenibacillus* isolates (Fig. 1-2). Though growth conditions in TSB medium were not identical to those within surimi, the higher growth rates may be a reason why *Sporosarcina* species became predominant in the refrigerated surimi.

The fatty acid composition of the *Sporosarcina* isolate S92h was slightly different

from that of *S. koreensis* F73^T (Table 1-5). It has been reported that the type strain of *S. koreensis* could not grow at 10°C [33]. In contrast, I found that isolate S92h successfully grew at that temperature (Fig. 1-2). Besides, isolate S92h exhibited several other biological and chemical characters different from those of *S. koreensis*. For example, isolate S92h could not grow at 40°C, and it hydrolyzed casein but not gelatin and reduced a nitrate (data not shown). These results may indicate the existence of intra-species variation in *S. koreensis*.

The *anteiso* fatty acids within the cell membrane play a crucial role in the growth of several bacteria at low temperatures. *Listeria monocytogenes*, which is a psychrotolerant bacterium and a food-borne pathogen, can grow at 5°C by increasing *anteiso*-$C_{15:0}$ as a result of shortening fatty acid chain length and alteration of branching from *iso* to *anteiso* [35]. As the melting point of *anteiso* fatty acids appear to be lower than their analogous *iso* and straight chain forms, increase in the *anteiso* fatty acid proportion confers fluidity on cell membrane under low temperature conditions [36]. The *Sporosarcina* isolates S92h and H42a contained *anteiso*-$C_{15:0}$ as major fatty acids at 28°C. This fatty acid was also predominant at 10°C, with a slight increase in the case of isolate S92h (59% to 60%) and a slight decrease in isolate H42a (77% to 72%). The sum of *anteiso* fatty acids increased at 10°C in both the strains, and conversely, that of *iso* fatty acids decreased. The dominant proportion of *anteiso*-$C_{15:0}$ could be a reason for the faster growth at low temperatures. Besides the slight change of *anteiso*-$C_{15:0}$ level, the fatty acid chain length did not seem to be shortened when the strains were cultured at 10°C as compared to 28°C. Thus, it is implicated that the adaptive strategy of *Sporosarcina* species for low temperatures is different from that of *L. monocytogenes*. It is also notable that the *Sporosarcina* isolates contained Δ5 unsaturated fatty acids. The Δ5 desaturation could contribute to maintaining the membrane fluidity at low temperatures. It is known that *Bacillus subtilis* converts saturated to unsaturated fatty acids via Δ5 desaturase, when transferred to a lower temperature [37]. This enzyme is not detectable at 37°C but is induced transiently when the temperature is lowered [38]. In the *Sporosarcina* isolates, the proportion of unsaturated to saturated fatty acids at 10°C was higher than that at 28°C. It is therefore suggested that the membrane fluidity could be regulated by Δ5 desaturase as well as the dominant accumulation of *anteiso* fatty acids in response to low temperature.

Endospores of the *Sporosarcina* isolates S92h and H42a were resistant to heat treatment at around 75°C (Fig. 1-3). The *D*-values for *Bacillus cereus* and *Paenibacillus*

polymyxa endospores have been estimated previously as 29.5 min at 95°C and 38.1 min at 85°C, respectively [39,40]. Thus, *Sporosarcina* endospores are less heat-resistant than *Bacillus* and *Paenibacillus* endospores. As a typical fish-paste product is heated until a core temperature of 80°C is reached, *Sporosarcina* endospores could survive heat processing of the food.

The resident microbiota in surimi capable to survive through heat-treatment and cold-storage under microaerobic condition would not be always agree with those bacterial colonies emerged on SPC agar after incubation at 30°C under aerobic condition. However, in this study, *Sporosarcina* species were isolated from Alaska pollock, pike conger, and white croaker surimi by the method. Thus, *Sporosarcina* species are likely to be widely distributed in separate sea areas including Bering Sea and South China Sea. It has been reported that *Sporosarcina aquimarina*, *S. koreensis*, and *S. luteola* have been isolated from seawater [32], soil in Korea [33], and soy sauce production equipment in Japan [41], respectively. Other species belonging to the genus *Sporosarcina* have been isolated from various habitats [42,43]. These reports support the idea that the genus *Sporosarcina* is widely distributed in nature. Therefore, *Sporosarcina* species could be recognized as an important contaminant in various surimi products.

The *Sporosarcina* isolates are regarded as psychrotolerant endospore-forming bacteria. There is the possibility that fish-paste products could be spoilt by the *Sporosarcina* species, although foodborne illness outbreaks by these bacteria have not yet been reported. The isolates that were closely related to *Bacillus cereus*, well known as a foodborne pathogen, were indeed found in pike conger and white croaker surimi (Tables 1-3 and 1-4). As described, these experiments however suggested that *Sporosarcina* species could result in more severe damage of surimi products even if those bacteria were not pathogenic. Thus, they should be controlled in order to maintain the quality of surimi product by a method other than heat-treatment and cold-storage. Further studies are required to develop such a method.

SUMMARY

I studied the changes of resident microbiota in surimi—minced fish meat—during heat-treatment and subsequent cold-storage via the sequencing of partial 16S rRNA gene. Raw surimi made from Alaska pollock, pike conger, and white croaker was contaminated with 10^4 to 10^6 CFU/g of various non-endospore-forming bacteria. Immediately after heat-treatment, the

bacterial counts were significantly reduced to less than 1 CFU/g, and only endospore-forming bacteria, identified as *Bacillus* species were retrieved. Subsequently, the bacterial counts increased up to 10 to 10^5 CFU/g in the heated surimi after refrigerated storage at 5°C for 2 weeks or at 10°C for 1 week. Most of the isolates from the refrigerated surimi were identified as *Sporosarcina* species. The *Sporosarcina* isolates have an increased ability to grow at 10°C than the isolates related to the other endospore-forming bacteria, such as *Bacillus*, *Lysinibacillus*, and *Paenibacillus* species. Endospores of the *Sporosarcina* isolates were able to germinate and proliferate in a fish-paste product model system stored at 10°C within 8 days. In order to study the cold-adaptation mechanism of *Sporosarcina* species, the fatty acid composition of the isolates was analyzed. At the growth temperature of 10°C, the proportions of unsaturated to saturated fatty acids and *anteiso* to *iso* fatty acids were higher than those at 28°C. The alteration of the fatty acid composition suggests that *Sporosarcina* species adapt to cold by maintaining the fluidity of the cell membrane because unsaturated and *anteiso* fatty acids have lower melting points than saturated and *iso* fatty acids, respectively. I concluded that the endospores of *Sporosarcina* species are widely distributed in surimi, and that they can survive heat-treatment and proliferate during cold-storage in fish-paste products. Controlling *Sporosarcina* species would contribute to improving the quality of surimi product.

CHAPTER II

Modulation of fatty acid composition and growth in *Sporosarcina* species in response to temperatures and exogenous branched-chain amino acids

Psychrotolerant endospore-forming bacteria are important contaminants in various processed foods because they can survive pasteurization and grow subsequently during refrigeration. As mentioned in Chapter I, several *Sporosarcina* species were predominantly isolated from minced fish meat (surimi) as psychrotolerant endospore-forming bacteria, and I suggested that they should be controlled to maintain the quality of surimi-based products [44]. The *Sporosarcina* isolates had a higher capacity to grow at 10°C in tryptic soy broth (TSB) than other isolates from the genera *Bacillus*, *Lysinibacillus*, and *Paenibacillus*. However, the mechanisms of cold tolerance in *Sporosarcina* species remain poorly understood. Thus, a deeper understanding of these mechanisms may facilitate the development of a novel strategy to control the growth of *Sporosarcina* species in surimi-based products throughout their shelf life.

Psychrotolerant bacteria can grow under cold conditions, such as 10°C–15°C, whereas mesophilic bacteria cannot. An important explanation for their growth in cold environments is the modulation of the fatty acid composition of the cellular membrane in response to cold temperatures [45]. The modulation of the fatty acid composition is considered to be necessary to maintain the fluidity of the membrane [8].

The pattern of changes in the fatty acid composition differs among bacterial species [46]. *Listeria monocytogenes*, which is a psychrotolerant bacterium and a food-borne pathogen, can adapt to cold temperatures mainly by increasing a branched-chain fatty acid (BCFA), *anteiso*-$C_{15:0}$ [35]. *Anteiso*-fatty acids are more effective in maintaining membrane fluidity under cold conditions than straight-chain fatty acids and *iso*-fatty acids with the same carbon number because of their lower melting temperatures [47]. In addition to this mechanism, it is known that *Bacillus subtilis* converts saturated fatty acids (SFAs) into unsaturated fatty acids (UFAs) via a cold-induced Δ5-desaturase when the growth temperature decreases from 37°C to 20°C [48]. This desaturase is thought to play a role in the maintenance of membrane fluidity at low temperatures by increasing the proportion of UFAs in the cellular membrane [49].

BCFAs are major components of lipids in bacteria, such as *Bacillus* [47] and *Listeria* species [50]. In these bacteria, the BCFAs are produced from the corresponding branched-chain

amino acids (BCAAs), including valine, leucine, and isoleucine, via transamination and decarboxylation reactions and by the type II fatty acid synthase (FAS II) cycle [46], as illustrated in Fig. 2-1. Each FAS II cycle extends the alkyl chain of fatty acids by a two-carbon unit. Therefore, valine, leucine, and isoleucine can be converted into even-numbered *iso*-fatty acids (*iso*-C_{2n}), odd-numbered *iso*-fatty acids (*iso*-C_{2n+1}), and odd-numbered *anteiso*-fatty acids (*anteiso*-C_{2n+1}), respectively. Indeed, the addition of BCAA to a medium can increase the proportion of the corresponding BCFA in *L. monocytogenes* [51] and *B. subtilis* [52].

To protect surimi-based food products from bacterial contaminants, I am interested in how *Sporosarcina* strains adapt to cold environments, particularly in terms of their fatty acid composition. In this study, I investigated the growth and fatty acid compositions of several *Sporosarcina* strains at cold and moderate temperatures. Furthermore, I tested whether BCAAs affected the growth and fatty acid compositions because these compounds could be candidates for use as preservatives. I found that the contents of both *anteiso*-C_{2n+1} and UFAs had crucial roles in the growth of *Sporosarcina* strains under cold conditions. The addition of BCAAs to the medium altered the fatty acid composition in a manner similar to the known mechanism. I suggest the possibility of using leucine as a food additive for preserving surimi-based products by inhibiting the growth of *Sporosarcina* strains at cold temperatures.

MATERIALS AND METHODS

Bacterial strains and chemical reagents

Sporosarcina sp. S92h is a strain that was previously isolated from surimi [44], which was deposited at NITE Biological Resource Center under accession number NBRC 111462. *S. aquimarina* JCM 10887^T and *S. koreensis* JCM 16400^T were purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center.

A phylogenetic tree based on the partial 16S rRNA gene sequences, as described in Chapter I, is shown in Fig. 2-2 to clarify the genetic relationships among these *Sporosarcina* strains as well as those with *Bacillus cereus* ATCC 14579^T, *B. subtilis* DSM 10^T, *Lactobacillus delbrueckii* BCRC 12195^T, *L. monocytogenes* NCTC 10357^T, and *L. innocua* ATCC 33090^T. The accession numbers of these 16S rRNA genes are also shown in Fig. 2-2.

L-Valine, L-leucine, and L-isoleucine were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

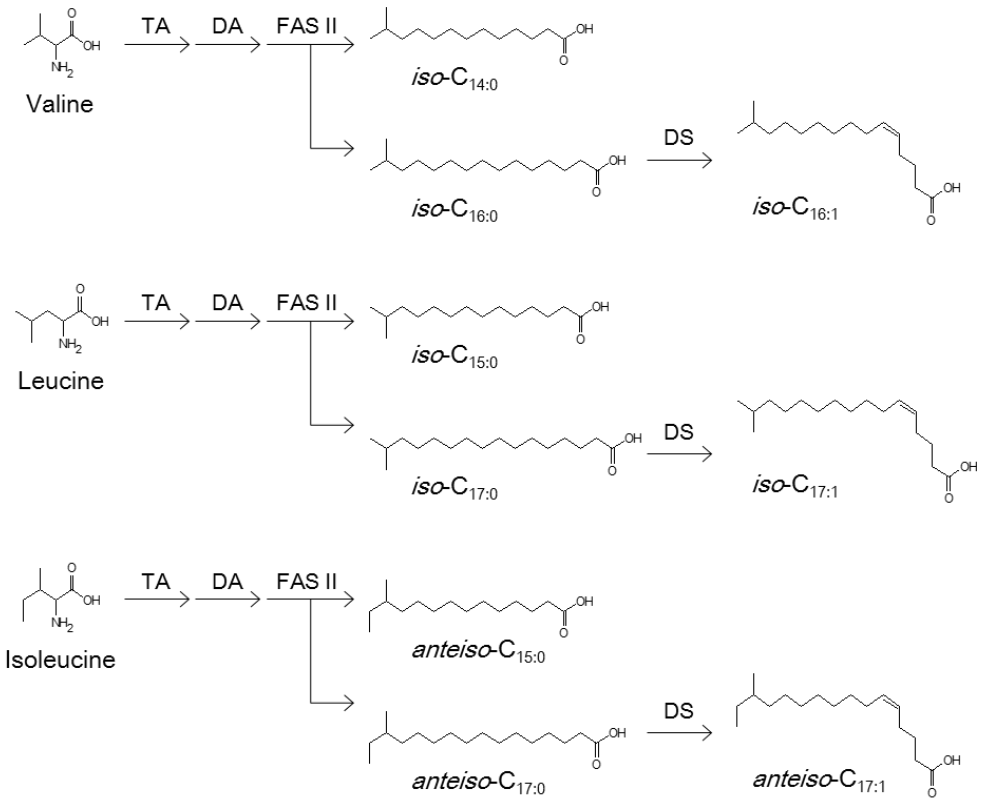

Figure 2-1. Schematic representation of the proposed pathway for the biosynthesis of branched-chain and unsaturated fatty acids from branched-chain amino acids. TA, DC, FAS II, and DS are abbreviations for transamination, decarboxylation, type II fatty acid synthase system, and desaturation, respectively.

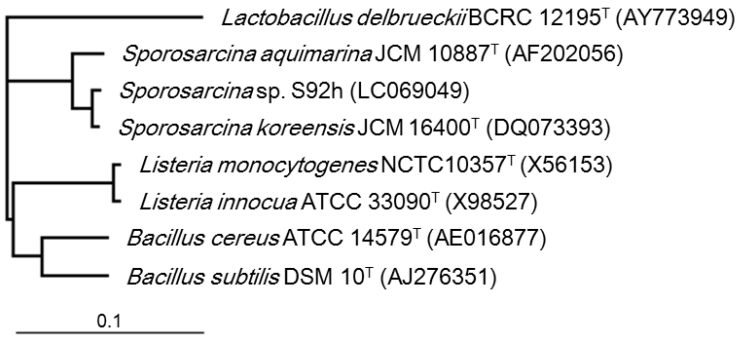

Figure 2-2. Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationships among *Sporosarcina* sp. S92h and other related species in the genera *Sporosarcina*, *Bacillus*, and *Listeria*. The tree was constructed using the neighbor-joining algorithm. *Lactobacillus delbrueckii* was used as an outgroup during tree construction. GenBank accession numbers are shown in brackets. Scale bar represents 0.1 substitution per nucleotide position.

Bacterial growth conditions and growth rate definition

A twice-concentrated BCAA solution was sterilized by filtration through a 0.2 μm membrane filter (DISMIC-25S, Toyo Roshi Kaisha Ltd, Tokyo, Japan). Twice-concentrated TSB medium (Becton, Dickinson and Company, Le Pont De Claix, France) was autoclaved at 121°C for 15 min, cooled to room temperature, then mixed with an equivalent volume of the BCAA solution to obtain TSB medium containing BCAA. As primary cultures, bacterial strains were cultured in 5 ml of TSB medium without supplementation at 30°C for 18 h with reciprocal shaking at 90 rpm. A 5 μl aliquot of the primary culture was inoculated into 5 ml of the TSB medium with or without BCAA supplementation. The inoculated medium was incubated with seesaw shaking (20 rpm) at the appropriate temperatures. The optical density of the culture at 660 nm (OD_{660}) was recorded automatically every 15 min for 10 days using a Bio-photorecorder (TN-1506, Advantec Toyo Co. Ltd, Tokyo, Japan). The growth rate (day^{-1}) was defined as the reciprocal of the incubation time (days) required for the OD_{660} value to reach 1.

Analysis of the bacterial fatty acid composition.

The bacteria were cultured in TSB medium until the late exponential phase. Next, the cells were harvested by centrifugation after washing once with phosphate-buffered saline and dried at 120°C. The dried cells were then weighed. Fatty acid methyl esters were prepared from the dried cells and analyzed by gas chromatography and gas chromatography-mass systems, as described in Chapter I.

RESULTS

Growth rates of *Sporosarcina* strains at cold and moderate temperatures.

At cold temperatures (7°C–13°C), *Sporosarcina* sp. S92h was able to grow faster than *S. koreensis* and *S. aquimarina* in TSB medium (Figs. 2-3A, 2-4A, and 2-5A). It was notable that *S. koreensis*, the species related most strongly to the strain S92h (Fig. 2-2), did not grow at 10°C within 10 days (Fig. 2-4A). Therefore, 13°C was selected as a cold growth temperature for use in the following experiments with that strain. The growth rates of strain S92h, *S. koreensis*, and *S. aquimarina* were 0.216 day^{-1} (at 7°C), 0.159 day^{-1} (at 13°C), and 0.161 day^{-1} (at 7°C), respectively. *S. aquimarina* could grow at 7°C, but it had a longer lag phase during growth. The growth rate of *S. aquimarina* was evaluated as 0.229 day^{-1} at 10°C. Therefore, 10°C was

selected as the cold growth temperature for use in the following experiments with this strain, where the growth rate was similar to that of strain S92h at 7°C.

At moderate temperatures (30°C–35°C), strain S92h grew at a similar rate as other *Sporosarcina* strains (Figs. 2-3A, 2-4A, and 2-5A). The growth rates of strain S92h, *S. koreensis*, and *S. aquimarina* were evaluated as 1.13 day^{-1} (at 35°C), 1.50 day^{-1} (at 30°C), and 1.96 day^{-1} (at 30°C), respectively. The growth rate of strain S92h at 30°C was similar to that at 35°C (data not shown), where its growth rate at 30°C was evaluated as 1.01 day^{-1}. This was not higher than the growth rates of other *Sporosarcina* strains at the same temperature.

Changes in the cellular fatty acid compositions of *Sporosarcina* strains in response to different growth temperatures.

In order to determine the mechanism that allows *Sporosarcina* strains to grow in moderate and cold temperatures, I investigated the fatty acid compositions of bacterial cells at both moderate and cold temperatures.

The fatty acid compositions of *Sporosarcina* sp. S92h at different temperatures are shown in Table 2-1. When strain S92h was grown at 35°C, the major fatty acids in the cells were *anteiso*-$C_{15:0}$ (57.6%) and *iso*-$C_{15:0}$ (20.1%), whereas straight-chain fatty acids (SCFAs), including $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, and $C_{16:1}$, were minor components. The proportion of *anteiso*-$C_{15:0}$ (68.7%) was higher in the cells grown at 7°C than at 35°C. In addition, the proportion of UFAs, including $C_{16:1}$, *iso*-$C_{16:1}$, *iso*-$C_{17:1}$, and *anteiso*-$C_{17:1}$, increased considerably from 6.8% at 35°C to 17.2% at 7°C.

The fatty acid compositions of *S. koreensis* at different temperatures are shown in Table 2-2. When *S. koreensis* was grown at 30°C, the major fatty acids in the cells were *anteiso*-$C_{15:0}$ (35.6%) and *iso*-$C_{15:0}$ (32.0%), whereas the SCFAs were minor components in a similar manner to strain S92h. The proportion of *anteiso*-$C_{15:0}$ (50.6%) was higher in the cells grown at 13°C than at 30°C. In addition, the proportion of UFAs increased from 1.2% at 30°C to 12.4% at 13°C.

The fatty acid compositions of *S. aquimarina* at different temperatures are shown in Table 2-3. When *S. aquimarina* was grown at 30°C, the proportion of *anteiso*-$C_{15:0}$ (78.8%) was relatively high but that of *iso*-$C_{15:0}$ (7.0%) was relatively low (Table 2-3) compared with those in strain S92h and *S. koreensis*. The proportion of *anteiso*-$C_{15:0}$ (83.6%) was slightly higher in the

cells grown at 7°C than those at 30°C. In addition, the proportion of UFAs increased from 2.5% at 30°C to 7.3% at 10°C.

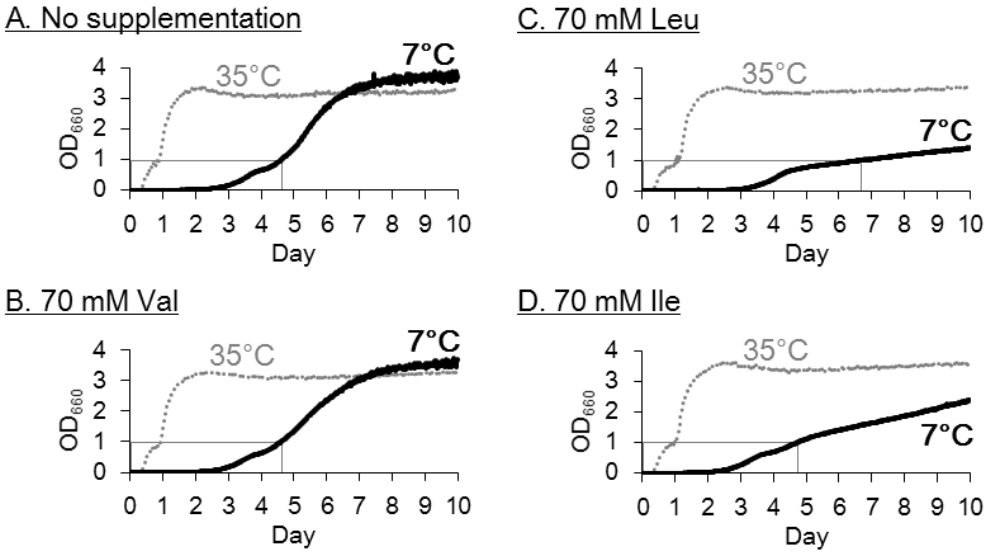

Figure 2-3. Growth rate of *Sporosarcina* sp. S92h in TSB medium at moderate (35°C, gray dotted curve) and cold (7°C, black solid curve) temperatures. The media were supplemented with: (A) no supplementation, (B) 70 mM valine, (C) 70 mM leucine, and (D) 70 mM isoleucine. The gray horizontal and vertical lines in the graphs indicate the time required to reach an OD_{660} of 1 at 7°C.

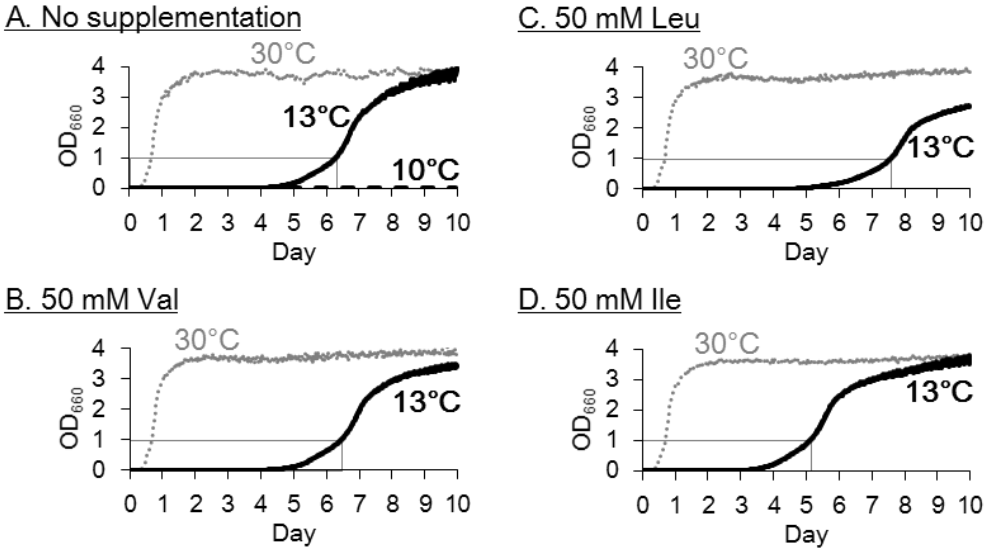

Figure 2-4. Growth rate of *Sporosarcina koreensis* in TSB medium at moderate (30°C, gray dotted curve) and cold (10°C, black dashed curve; and 13°C, black solid curve) temperatures. The media were supplemented with: (A) no supplementation, (B) 50 mM valine, (C) 50 mM leucine, and (D) 50 mM isoleucine. The gray horizontal and vertical lines in the graphs indicate the time required to reach an OD_{660} of 1 at 13°C.

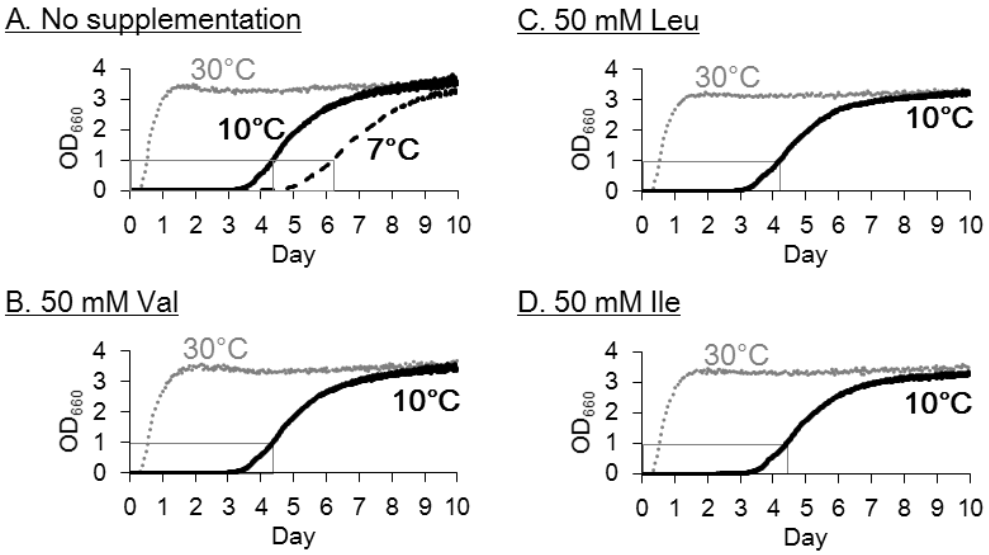

Figure 2-5. Growth rate of *Sporosarcina aquimarina* in TSB medium at moderate (30°C, gray dotted curve) and cold (7°C, black dashed curve; and 10°C, black solid curve) temperatures. The media were supplemented with: (A) no supplementation, (B) 50 mM valine, (C) 50 mM leucine, and (D) 50 mM isoleucine. The gray horizontal and vertical lines in the graphs indicate the time required to reach an OD_{660} of 1 at 7°C and 10°C.

Effects of BCAA supplementation on the growth of *Sporosarcina* strains.

The growth rates of several bacteria are affected by supplementing the growth medium with BCAAs due to changes in the cellular fatty acid composition [53,54]. Some of these changes are known to be attributable to the synthesis of BCFAs from BCAAs (Fig. 2-1). Thus, I investigated whether supplementation of the medium with valine, leucine, and isoleucine might also affect the growth rates of strain S92h, *S. koreensis*, and *S. aquimarina* at moderate and cold temperatures.

The growth rate of strain S92h at a moderate temperature was not affected by supplementation with valine, leucine, or isoleucine at concentrations of 70 mM (Figs. 2-3B, 2-3C, and 2-3D, respectively) compared with no supplementation (Fig. 2-3A). By contrast, supplementation with 70 mM leucine inhibited the growth of strain S92h at the cold temperature during the exponential phase but not in the lag phase (Figs. 2-3A and 2-3C). The growth rate of strain S92h at 7°C was evaluated as 0.150 day^{-1} in the medium supplemented with 70 mM leucine, but 0.216 day^{-1} in the non-supplemented medium. Similarly, the growth rate of *S. koreensis* at 13°C was reduced to 0.132 day^{-1} by supplementation with 50 mM leucine

compared with a rate of 0.159 day^{-1} in the absence of any BCAA (Figs. 2-4A and 2-4C). However, there was no significant effect of leucine supplementation on the growth of *S. aquimarina* (Figs. 2-5A and 2-5C).

Supplementation with 70 mM isoleucine did not affect the growth rate of strain S92h in the cold temperature (0.211 day^{-1}, evaluated from the data shown in Fig. 2-3D), but the OD_{660} value of the bacterial culture on the tenth day was relatively low compared with that under no supplementation (Figs. 2-3A and 2-3D). The addition of isoleucine appeared to affect growth after the early exponential phase (Fig. 2-3D). By contrast, supplementation with 50 mM isoleucine shortened the lag phase in *S. koreensis* when grown in the cold temperature and the growth rate of this bacterium at 13°C increased from 0.159 day^{-1} in the medium with no supplementation to 0.196 day^{-1} under isoleucine supplementation (Figs. 2-4A and 2-4D). These effects of isoleucine supplementation were not observed in the growth of *S. aquimarina* (Figs. 2-5A and 2-5D).

Valine supplementation had no significant effects on the growth of the *Sporosarcina* strains when tested in the moderate and cold temperatures (Figs. 2-3B, 2-4B, and 2-5B).

Effects of supplementation with BCAAs on the fatty acid compositions of *Sporosarcina* strains.

I analyzed the fatty acid compositions of *Sporosarcina* sp. S92h, *S. koreensis*, and *S. aquimarina* when grown to the late exponential phase in TSB medium supplemented with BCAAs at moderate and cold temperatures (Tables 2-1, 2-2, and 2-3, respectively). In summary, supplementation with valine, leucine, and isoleucine clearly increased the proportions of *iso*-C_{2n}, *iso*-C_{2n+1}, and *anteiso*-C_{2n+1}, respectively.

In addition to the increases in the BCFAs, I noted that the proportions of UFAs were influenced by supplementation with BCAAs. For instance, supplementation with leucine caused increases in the UFAs in strain S92h at both cold and moderate temperatures (Table 2-1). However, the UFAs did not increase significantly in *S. koreensis* and *S. aquimarina* (Tables 2-2 and 2-3). In addition, supplementation with valine caused the higher proportion of *iso*-$C_{16:1}$ at the cold temperatures than the moderate temperatures (Tables 2-1, 2-2, and 2-3). Under isoleucine supplementation at cold temperatures, there were no significant increase in the UFA proportions of these three strains (Tables 2-1, 2-2, and 2-3).

Table 2-1. Fatty acid composition (%) of *Sporosarcina* sp. S92h grown in TSB medium supplemented with branched-chain amino acids at 35°C and 7°C

	35°C				7°C			
	No supplement	70 mM Val	70 mM Leu	70 mM Ile	No supplement	70 mM Val	70 mM Leu	70 mM Ile
Component								
$C_{14:0}$	1.0	1.2	2.3	1.3	0.1	0.2	0.9	0.6
$C_{15:0}$	1.2	0.5	0.4	0.5	0.0	0.0	0.0	0.0
$C_{16:0}$	1.5	1.4	2.7	1.6	0.1	0.1	1.6	0.3
$C_{16:1}$	0.2	0.2	1.3	0.2	0.7	0.7	5.7	1.0
iso-$C_{14:0}$	4.1	**18.5**	1.8	0.3	1.3	**9.4**	1.2	0.2
iso-$C_{16:0}$	3.3	**12.1**	1.0	0.3	0.4	**3.7**	0.1	0.0
iso-$C_{16:1}$	0.1	**0.6**	0.6	0.0	2.4	**11.5**	1.5	0.1
iso-$C_{15:0}$	20.1	11.7	**38.4**	2.4	11.7	7.0	**30.9**	0.5
iso-$C_{17:0}$	0.3	0.2	**0.6**	0.1	0.1	0.0	**0.2**	0.0
iso-$C_{17:1}$	0.0	0.0	**0.0**	0.0	2.7	4.0	**1.6**	2.4
anteiso-$C_{15:0}$	57.6	44.5	27.9	**75.7**	68.7	57.1	27.4	**82.8**
anteiso-$C_{17:0}$	4.3	3.1	1.7	**6.2**	0.0	0.0	0.0	**0.0**
anteiso-$C_{17:1}$	6.6	5.6	19.8	**10.0**	11.4	5.6	25.0	**11.0**
Others[a]	0.0	0.4	1.6	1.6	0.4	0.7	3.9	1.1
Sum								
iso-C_{2n}	7.4	**31.1**	3.3	0.6	4.1	**24.6**	2.8	0.3
iso-C_{2n+1}	20.4	12.0	**39.0**	2.4	14.5	11.0	**32.7**	2.9
anteiso-C_{2n+1}	68.4	53.2	49.4	**91.8**	80.1	62.7	52.4	**93.8**
UFAs[b]	6.8	6.4	21.6	10.1	17.2	21.8	33.8	14.5

Fatty acids corresponding to the supplemented branched-chain amino acids are highlighted in bold.

[a]Others include unidentified minor fatty acids.

[b]Unsaturated fatty acids include $C_{16:1}$, *iso*-$C_{16:1}$, *iso*-$C_{17:1}$, and *anteiso*-$C_{17:1}$.

Table 2-2. Fatty acid composition (%) of *Sporosarcina koreensis* JCM 16400^T grown in TSB medium supplemented with branched-chain amino acids at 30°C and 13°C

	30°C				13°C			
	No supplement	50 mM Val	50 mM Leu	50 mM Ile	No supplement	50 mM Val	50 mM Leu	50 mM Ile
Component								
$C_{14:0}$	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2
$C_{15:0}$	0.3	0.3	0.1	0.4	0.3	0.5	0.0	0.2
$C_{16:0}$	0.5	0.6	0.3	0.8	0.3	0.4	0.2	0.4
$C_{16:1}$	0.3	0.4	0.4	0.1	0.9	0.9	0.6	0.8
iso-$C_{14:0}$	3.0	**12.5**	1.3	0.6	5.2	**17.5**	3.7	0.9
iso-$C_{16:0}$	2.5	**9.2**	0.3	0.7	1.2	**8.5**	0.2	0.3
iso-$C_{16:1}$	0.5	**1.5**	0.5	0.0	6.8	**14.7**	4.3	1.4
iso-$C_{15:0}$	32.0	26.2	**60.3**	7.7	20.7	13.6	**45.4**	4.7
iso-$C_{17:0}$	0.7	0.5	**0.8**	0.2	0.1	0.1	**0.1**	0.0
iso-$C_{17:1}$	0.0	0.0	**0.6**	0.0	0.1	0.0	**1.6**	0.0
anteiso-$C_{15:0}$	35.6	25.7	15.0	**63.8**	50.6	27.4	29.7	**68.4**
anteiso-$C_{17:0}$	3.0	2.0	0.5	**6.5**	2.3	2.1	0.3	**3.7**
anteiso-$C_{17:1}$	0.4	0.0	0.0	**0.0**	4.6	1.7	0.0	**0.0**
Others[a]	20.9	20.8	19.7	18.9	6.8	12.5	13.8	19.0
Sum								
iso-C_{2n}	6.1	**23.2**	2.1	1.4	13.1	**40.6**	8.2	2.5
iso-C_{2n+1}	32.7	26.8	**61.7**	8.0	20.9	13.7	**47.1**	4.7
anteiso-C_{2n+1}	39.0	27.7	15.5	**70.3**	57.5	31.1	30.1	**72.1**
UFAs[b]	1.2	1.9	1.5	0.1	12.4	17.3	6.5	2.2

Fatty acids corresponding to the supplemented branched-chain amino acids are highlighted in bold.

[a]Others include unidentified minor fatty acids.

[b]Unsaturated fatty acids include $C_{16:1}$, *iso*-$C_{16:1}$, *iso*-$C_{17:1}$, and *anteiso*-$C_{17:1}$.

Table 2-3. Fatty acid composition (%) of *Sporosarcina aquimarina* JCM 10887^T grown in TSB medium supplemented with branched-chain amino acids at 30°C and 10°C

	30°C				10°C			
	No supplement	50 mM Val	50 mM Leu	50 mM Ile	No supplement	50 mM Val	50 mM Leu	50 mM Ile
Component								
$C_{14:0}$	1.4	1.2	2.2	1.1	0.4	0.5	0.6	0.4
$C_{15:0}$	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0
$C_{16:0}$	2.1	3.3	2.7	1.6	0.5	0.6	0.7	0.5
$C_{16:1}$	0.4	0.7	0.8	0.3	1.0	1.3	1.1	0.9
iso-$C_{14:0}$	2.0	**5.8**	1.9	0.2	2.3	**9.5**	2.0	0.2
iso-$C_{16:0}$	0.9	**6.0**	0.8	0.1	0.2	**2.3**	0.1	0.0
iso-$C_{16:1}$	0.3	**1.2**	0.4	0.0	1.8	**6.3**	1.5	0.2
iso-$C_{15:0}$	7.0	6.6	**20.4**	0.8	3.8	3.8	**12.2**	0.4
iso-$C_{17:0}$	0.1	0.2	**0.3**	0.1	0.0	0.0	**0.0**	0.0
iso-$C_{17:1}$	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.3**	0.0
anteiso-$C_{15:0}$	78.8	69.1	66.3	**90.1**	83.6	69.3	76.7	**90.0**
anteiso-$C_{17:0}$	3.1	4.5	2.6	**4.0**	1.1	2.0	0.3	**0.7**
anteiso-$C_{17:1}$	1.8	0.5	0.4	**0.4**	4.5	3.6	3.4	**5.7**
Others[a]	1.9	0.9	1.2	1.2	0.6	0.5	1.1	0.8
Sum								
iso-C_{2n}	3.3	**13.0**	3.1	0.3	4.3	**18.2**	3.5	0.4
iso-C_{2n+1}	7.1	6.8	**20.6**	0.9	3.9	3.9	**12.5**	0.4
anteiso-C_{2n+1}	83.8	74.1	69.3	**94.6**	89.2	75.0	80.4	**96.5**
UFAs[b]	2.5	2.3	1.6	0.8	7.3	11.3	6.3	6.8

Fatty acids corresponding to the supplemented branched chain amino acids are highlighted in bold.

[a]Others include unidentified minor fatty acids.

[b]Unsaturated fatty acids include $C_{16:1}$, *iso*-$C_{16:1}$, *iso*-$C_{17:1}$, and *anteiso*-$C_{17:1}$.

DISCUSSION

The results of this study indicate that *anteiso*-$C_{15:0}$ and UFAs both play important roles in cold tolerance by members of the genus *Sporosarcina*, where these bacteria modulate their fatty acid compositions in response to the growth temperature and supplementation with BCAAs. Thus, it is possible that exploiting the bacterial response to BCAAs could facilitate the control of bacterial growth at cold temperatures.

Sporosarcina sp. S92h is closely related to *S. koreensis*, although several of its biological and chemical characteristics differ from those of *S. koreensis*, as described in Chapter I. Among these characteristics, the difference in the cold tolerance of the two strains was particularly notable (Figs. 2-3A and 2-4A). Thus, I analyzed their cellular fatty acid compositions as a possible cause of this difference. The fatty acid composition of strain S92h was not identical to those of *S. koreensis* and *S. aquimarina* but it shared greater similarity with *S. koreensis* than *S. aquimarina* (Tables 2-1, 2-2, and 2-3), thereby agreeing with the phylogenetic relationship based on partial 16S rRNA gene sequences (Fig. 2-2).

All three *Sporosarcina* strains possessed *anteiso*-$C_{15:0}$ and *iso*-$C_{15:0}$ as the major fatty acid components. The melting point of *anteiso*-$C_{15:0}$ is 23.0°C, which is much lower than those of *iso*-$C_{15:0}$ and *n*-$C_{15:0}$ (51.7°C and 52.5°C, respectively) [46]. The proportion of *anteiso*-$C_{15:0}$ in the cellular fatty acid composition is considered to be an important factor for bacteria that contain BCFAs because it maintains the fluidity of the cell membrane under cold conditions [45]. The proportions of *anteiso*-$C_{15:0}$ in the *Sporosarcina* strains increased at cold temperatures compared with those at moderate temperatures, whereas those of *iso*-$C_{15:0}$ decreased (Tables 2-1, 2-2, and 2-3). Therefore, it is likely that the *Sporosarcina* strains respond to cold temperatures by modulating the proportion of *anteiso*-$C_{15:0}$ in their fatty acid compositions.

S. koreensis had a relatively high proportion of *iso*-$C_{15:0}$ but a low proportion of *anteiso*-$C_{15:0}$ in the cold temperature compared with strain S92h and *S. aquimarina* (Tables 2-1, 2-2, and 2-3). By contrast, most of the fatty acids in *S. aquimarina* comprised *anteiso*-$C_{15:0}$ (Table 2-3). The difference in the fatty acid compositions of these strains probably explain the variations in their capacity to grow at 10°C. In strain S92h, the proportion of *anteiso*-$C_{15:0}$ was 57.6% in the moderate temperature and 68.7% in the cold temperature (Table 2-1). These proportions were not as high as those in *S. aquimarina* but they were higher than those in *S. koreensis*. In addition, strain S92h possessed *iso*-$C_{15:0}$ as a relatively major fatty acid component.

Therefore, the fast growth of this strain was not fully explained by the proportion of *anteiso*-$C_{15:0}$. However, these results suggest that more than 50% of *anteiso*-$C_{15:0}$ may be required for the growth of *Sporosarcina* strains below 10°C.

UFAs play a role in microbial cold adaptation [55], where their lower melting points compared with those of SFAs containing an equal number of carbon atoms are crucial for maintaining the fluidity of the cellular membrane. Indeed, *Bacillus cereus* ATCC 14579 grows at 15°C with a very high proportion of UFAs (43.06%) and a very low ratio of *anteiso*-$C_{15:0}$ (5.06%) [56]. Similarly, *anteiso*-$C_{15:0}$ as well as UFAs may be considered important factors for growth under cold conditions by *Sporosarcina* strains. Strain S92h had a higher proportion of UFAs than *S. aquimarina* (Tables 2-1 and 2-3), so it is likely that the higher proportion of UFAs allowed strain S92h to grow faster in the cold temperature, although this strain had a lower proportion of *anteiso*-$C_{15:0}$ than *S. aquimarina*.

The fatty acid compositions of the *Sporosarcina* species were modified successfully by supplementation with BCAAs (Tables 2-1, 2-2, and 2-3), where the changes in the fatty acid compositions agreed with those predicted from the known pathway for BCFA biosynthesis (Fig. 2-1). Thus, these results indicate that *Sporosarcina* strains may possess a BCFA biosynthesis pathway similar to other bacteria.

Additionally, supplementation with valine and leucine altered the proportions of UFAs and BCFAs. Supplementation with valine led to increases in *iso*-$C_{16:1,}$ as well as *iso*-$C_{16:0,}$ in all of the *Sporosarcina* strains. The increases in *iso*-$C_{16:1}$ were more prominent in the fatty acids from the cold-cultured cells. These results indicate that *Sporosarcina* strains possess a desaturase activity that increases at cold temperatures. The double bond in *iso*-$C_{16:1}$ is positioned at Δ5 [44], so it is possible that a cold-induced desaturase gene is involved in the increased desaturation of *iso*-$C_{16:0}$ due to supplementation with valine. A similar cold-induced Δ5-desaturase is known in *Bacillus subtilis* [48]. In addition, supplementation with leucine increased the UFAs at both the moderate and cold temperatures, specifically in strain S92h. This strain-specific increase in UFAs due to the addition of leucine indicates the presence of a specific fatty acid desaturase activity in strain S92h, which might have contributed to increased UFAs and the superior growth capacity of strain S92h at cold temperatures.

Supplementation with leucine affected the growth of strain S92h and *S. koreensis* at cold temperatures, but not that of *S. aquimarina* (Figs. 2-3, 2-4, and 2-5). Leucine is a precursor

of *iso*-C_{2n+1} and thus *iso*-$C_{15:0}$ increased in the cells grown in the medium supplied with leucine (Tables 2-1, 2-2, and 2-3). As a result, the proportion of *anteiso*-$C_{15:0}$ decreased to less than 30% in strain S92h and *S. koreensis*, which was lower than that of *iso*-$C_{15:0}$. However, the proportions of *anteiso*-$C_{15:0}$ and *iso*-$C_{15:0}$ in *S. aquimarina* at 10°C were 76.7% and 12.2%, respectively. This strain contained a much higher proportion of *anteiso*-$C_{15:0}$ than the other strains, so it is possible that the higher proportion of *anteiso*-$C_{15:0}$ might have attenuated the growth inhibition effect of leucine on *S. aquimarina* at 10°C. In addition to leucine, supplementation with valine caused decreases in the proportion of *anteiso*-$C_{15:0}$ by increasing the proportions of *iso*-C_{2n} and unsaturated *iso*-$C_{16:1}$ (Tables 2-1, 2-2, and 2-3). Despite the decrease in *anteiso*-$C_{15:0}$, the growth rates of the *Sporosarcina* strains were relatively unaffected in cold temperatures (Figs. 2-3, 2-4, and 2-5). Supplementation with valine also caused increases in the UFAs, as described above. These UFAs might be able to compensate for the loss of membrane fluidity caused by the decrease in *anteiso*-$C_{15:0}$. Moreover, supplementation with isoleucine caused increases in *anteiso*-$C_{15:0}$ (Tables 2-1, 2-2, and 2-3) and it affected the growth rate at cold temperatures negatively, positively, and negligibly in strain S92h, *S. koreensis*, and *S. aquimarina*, respectively (Figs. 2-3, 2-4, and 2-5). *S. aquimarina* contained sufficient *anteiso*-$C_{15:0}$ without isoleucine supplementation, so its growth may have been affected little as a consequence. By contrast, the increase in *anteiso*-$C_{15:0}$ might have allowed *S. koreensis* to grow slightly faster at 13°C. There is no obvious explanation for the inhibitory effect of isoleucine on the growth of strain S92h at 7°C, but it is known that bacteria can adapt to cold temperatures via various mechanisms in addition to modulating their membrane fatty acid composition [45].

These results help to elucidate the cold adaptation mechanism of *Sporosarcina*, but further studies are still required. However, I found that supplementation with leucine had an inhibitory effect on the growth of some *Sporosarcina* strains. Thus, these results demonstrate that the growth of bacteria at cold temperatures can be controlled by artificially modulating the bacterial fatty acid composition using additives. I hope that my findings contribute to quality maintenance in refrigerated surimi-based foods.

SUMMARY

Psychrotolerant endospore-forming *Sporosarcina* species have been predominantly isolated from minced fish meat (surimi), which is stored under refrigeration after heat treatment. To develop a better method for preserving surimi-based food products, I studied the growth and fatty acid compositions of the isolated strain S92h as well as *Sporosarcina koreensis* and *S. aquimarina* at cold and moderate temperatures. The growth rates of strain S92h and *S. koreensis* were the fastest and slowest at cold temperatures, respectively, although these strains grew at a similar rate at moderate temperatures. In all three strains, the proportions of *anteiso*-$C_{15:0}$ and unsaturated fatty acids (UFAs) were significantly higher at cold temperatures than at moderate temperatures. Furthermore, supplementation with valine, leucine, and isoleucine resulted in proportional increases in *iso*-$C_{16:0}$, *iso*-$C_{15:0}$, and *anteiso*-$C_{15:0}$, respectively, among the fatty acid compositions of these strains. The proportions of the UFAs were also altered by the supplementation. At cold temperatures, the growth rates of strain S92h and *S. koreensis*, but not of *S. aquimarina*, were affected by supplementation with leucine. Supplementation with isoleucine enhanced the growth of *S. koreensis* at cold temperatures but not that of the other strains. Valine did not affect the growth of any strain. These results indicate that *anteiso*-$C_{15:0}$ and UFAs both play important roles in the cold tolerance of the genus *Sporosarcina* and that these bacteria modulate their fatty acid compositions in response to the growth environment.

CHAPTER III

Effects of alkyl gallates and fatty acid-related compounds on growth of *Sporosarcina* species

As mentioned in Chapter I, several *Sporosarcina* species were predominantly isolated from minced fish meat (surimi) as psychrotolerant endospore-forming bacteria, and I suggested that they should be controlled to maintain the quality of surimi-based products [44]. As mentioned in Chapter II, *Sporosarcina* species modulate their fatty acid compositions in response to the growth temperature to maintain the fluidity of the cell membrane under cold conditions. In addition, I demonstrate that the growth of *Sporosarcina* species at cold temperatures can be controlled by artificially modulating the bacterial fatty acid composition using additives. Here, I examined the effects of hydrophobic compounds that might interact with the cell membrane on the growth of *Sporosarcina* species.

Alkyl gallates have been shown to exhibit antibacterial activities against *Salmonella choleraesuis* [57], methicillin-resistant *Staphylococcus aureus* [58], and *Bacillus subtilis* [59]. In addition, the antibacterial activities of free fatty acids have been known [60]. In this study, alkyl gallates and fatty acids-related compounds, which can be mainly used as food additives, were tested for their antibacterial activities against *Sporosarcina* species. The compounds were further categorized based on their activities and physicochemical characteristics to reveal the required properties as the antibacterial compounds. I studied the relationship between the hydrophobicities, the molecular weights and the antibacterial effects of the tested compounds to develop a model to predict efficacious compounds in the preservation of surimi-based products.

MATERIALS AND METHODS

Bacterial strains and chemical regents

Sporosarcina sp. S92h is a strain from surimi and is deposited at NITE Biological Resource Center under accession number NBRC 111462. Alkyl gallates, fatty acids, monoacylglycerols, and triacylglycerols were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Calculation of ACD/LogP values

The ACD/LogP values of the tested compounds were calculated using a software ACD/ChemSketch 14.01 (Advanced Chemistry Development, Inc., Toronto, Canada).

Bacterial growth conditions

Methyl, ethyl, propyl, butyl, isoamyl gallates were dissolved in water at a concentration of 20 mM, respectively. Octyl, lauryl, and stearyl gallates were dissolved in acetone at a concentration of 20 mM, respectively. A 50 μl aliquot of the alkyl gallate solution was added to 4.95 ml of tryptic soy broth (TSB; Becton, Dickinson and Company, Le Pont De Claix, France) medium. Stearic, oleic, linoleic, α-linolenic acids were dissolved in ethanol at a concentration of 40 mM, respectively. A 25 μl aliquot of the fatty acid solution was added to 4.975 ml of TSB medium. As primary cultures, *Sporosarcina* sp. S92h was cultured in 5 ml of TSB medium at 30°C for 18 h with reciprocal shaking at 90 rpm. A 5 μl aliquot of the primary culture was inoculated into 5 ml of the TSB medium. The inoculated medium was incubated with seesaw shaking (20 rpm) at 30°C. The optical density of the culture at 660 nm (OD_{660}) was recorded automatically every 15 min for 10 days using a Bio-photorecorder (TN-1506, Advantec Toyo Co. Ltd, Tokyo, Japan).

Determination of minimum inhibitory concentrations

Two-fold serial dilutions of the test compounds were prepared in DMSO, and 1 μl of each dilution was added to 100 μl of Mueller-Hinton broth. *Sporosarcina* sp. S92h was cultured for 48 h at 28°C on Standard Plate Count agar (SPC agar, NISSUI, Japan). The colonies were suspended in 0.9% (w/v) saline to achieve an absorbance of 0.132 at 600 nm (0.5 McFarland Standard). A 1 μl aliquot of the bacterial suspension was inoculated into the broth containing the test compound. After incubation of the cultures at 28°C for two days under static condition, the minimum inhibitory concentration (MIC) was regarded as the lowest concentration of the test compound showing no visible growth.

RESULTS and DISCUSSION

Effects of alkyl gallates on growth of *Sporosarcina* sp. S92h

Alkyl gallates were added to TSB medium at a concentration of 200 μM, and the

growths of *Sporosarcina* sp. S92h in those media were evaluated by comparing with the growth in normal TSB medium. Presence of methyl, ethyl, propyl, butyl, and isoamyl gallates in the medium did not show a significant effect on the growth at 30°C (Figure 3-1A), but resulted in the growth inhibition at 10°C (Figure 3-1B). In particular, isoamyl gallate completely inhibited the growth of *Sporosarcina* sp. S92h at 10°C for ten days (Figure 3-1B).

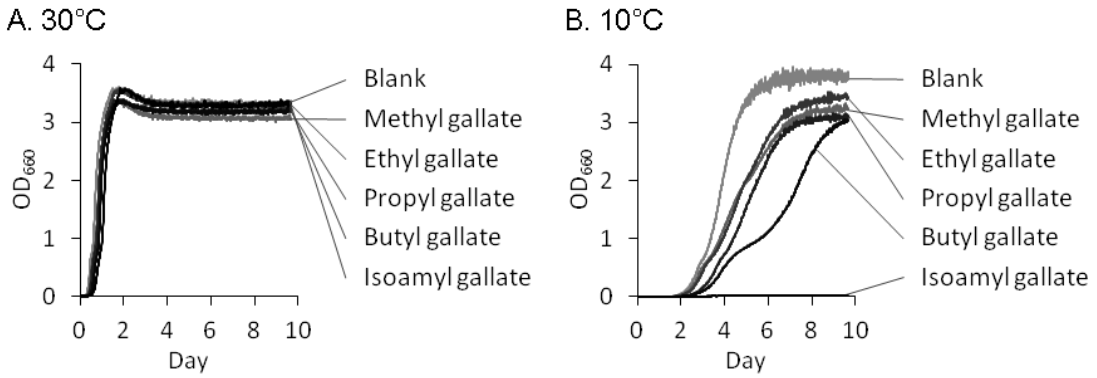

Figure 3-1. Growth of *Sporosarcina* sp. S92h at 30°C (A) and 10°C (B) in TSB medium with or without 200 μM methyl, ethyl, propyl, butyl, and isoamyl gallates. 'Blank', indicating 'without alkyl gallate'.

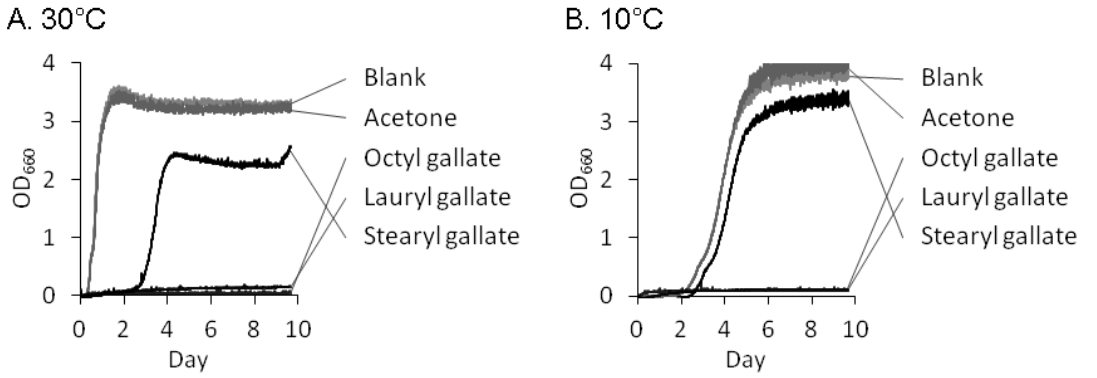

Figure 3-2. Growth of *Sporosarcina* sp. S92h at 30°C (A) and 10°C (B) in TSB medium with or without 200 μM octyl, lauryl, and stearyl gallates. The growth in a medium with 1% (v/v) acetone used as a solvent for them were also shown. 'Blank', indicating 'without alkyl gallate'.

On the other hand, the effects of octyl, lauryl, and stearyl gallates were similarly examined using acetone as a solvent as these compounds have low solubility in water. Octyl and lauryl gallates completely inhibited the growth of *Sporosarcina* sp. S92h at 30°C and at 10°C for ten days (Figure 3-2A and 3-2B, respectively). Stearyl gallate delayed the growth at 30°C, but such an inhibitory effect was attenuated at 10°C (Figure 3-2A and 3-2B). Acetone used as a solvent had negligible impacts on the growth at 30°C and at 10°C (Figure 3-2A and 3-2B, respectively).

Effects of free fatty acids on the growth of *Sporosarcina* sp.S92h

I also examined the effects of 200 μM stearic, oleic, linoleic, and α-linolenic acids on the growth of *Sporosarcina* sp. S92h in TSB medium. As described in Materials and Methods section, ethanol was used as a primary solvent. Stearic acid did not show a significant effect on the growth of *Sporosarcina* sp. S92h either at 30°C or at 10°C (Figure 3-3A and 3-3B), but oleic, linoleic, and α-linolenic acids completely inhibited the growth at 30°C and at 10°C for ten days (Figure 3-3A and 3-3B, respectively). No significant influence of temperature on the effects of those free fatty acids was observed, unlike the alkyl gallates described. Ethanol used as a solvent had negligible impacts on the growth at 30°C and at 10°C (Figure 3-3A and 3-3B, respectively).

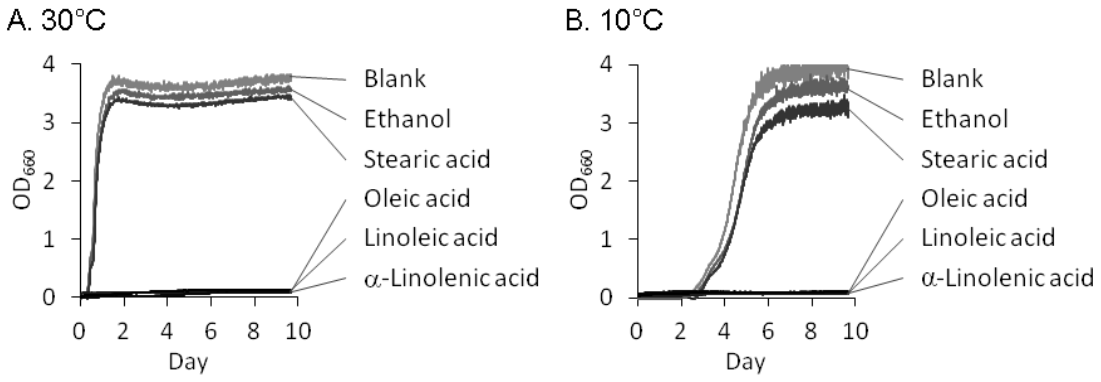

Figure 3-3. Growth of *Sporosarcina* sp. S92h at 30°C (A) and 10°C (B) in TSB medium with or without 200 μM stearic, oleic, linoleic, and α-linolenic acids. The growth in a medium with 0.5% (v/v) ethanol used as a solvent for them were also shown. 'Blank', indicating 'without acyl gallate'.

Table 3-1. Physicochemical properties and growth-inhibitory effects of the tested alkyl gallates and fatty acids.

			Tested concentration		Growth inhibition[b]	
Compound	MW[a]	ACD/LogP	(μM)	(μg/ml)	30°C	10°C
Alkyl gallates						
Methyl gallate (C1)	184.2	1.54	200	36.8	−	−
Ethyl gallate (C2)	198.2	2.07	200	39.6	−	−
n-Propyl gallate (C3)	212.2	2.60	200	42.4	−	+
n-Butyl gallate (C4)	226.2	3.13	200	45.2	−	+
Isoamyl gallate (C5)	240.3	3.48	200	48.1	−	++
n-Octyl gallate (C8)	282.3	5.26	200	56.5	++	++
n-Lauryl gallate (C12)	338.4	7.38	200	67.7	++	++
n-Stearyl gallate (C18)	422.6	10.57	200	84.5	+	−
Free fatty acids						
Stearic acid (C18:0)	284.5	8.22	200	56.9	−	−
Oleic acid (C18:1)	282.5	7.70	200	56.5	++	++
Linoleic acid (C18:2)	280.5	7.18	200	56.1	++	++
α-Linolenic acid (C18:3)	278.4	6.50	200	55.7	++	++

[a] Molecular weight

[b] The effect to delay the growth of *Sporosarcina* sp. S92h in TSB medium for less than one day (−), more than one day (+), or more than ten days (++).

Effects of fatty acid-related compounds on the growth of *Sporosarcina* sp. S92h

The growth-inhibitory effect of the fatty acid-related compounds, monoacylglycerols and triacylglycerols, was further investigated. To research more quickly and deeply, the growth-inhibitory effects of those compounds were evaluated by determining the minimum inhibitory concentrations (MICs). The MICs of monoacylglycerols and triacylglycerols against *Sporosarcina* sp. S92h were determined at 28°C for two days under static condition and shown in Table 3-2. Among monoacylglycerols tested, monomyristin exhibited the most effective

MIC of 15.6 mg/L (51.6 μM), followed by monolaurin and monocaprin with MICs of 31.3 mg/L (114.1 μM) and 125 mg/L (507.5 μM), respectively. Monopalmitin, monostearin, and monoarachidin did not show the growth-inhibitory effect at concentrations less than 2000 mg/L. On the other hand, among triacylglycerols tested, tricaproin was the most effective with a MIC of 500 mg/L (1293.7 μM) and the only triacylglycerol exhibiting the MIC less than 2000 mg/L. However, the MIC of tricaproin was much higher than those of monomyristin and monolaurin.

Relationship between the physicochemical properties of the tested compounds and their antibacterial activities against *Sporosarcina* sp. S92h

The logarithm of *n*-octanol/water partition coefficient (log *P*) has been widely used as a hydrophobic parameter of organic compounds. The log *P* values of the tested compounds (alkyl gallates and fatty acids-related compounds) were predicted by using a well-known software package ACD/ChemSketch based on a 'structure-fragment' approach [61]. The molecular weights and ACD/LogP values of the tested compounds were shown in Tables 3-1 and 3-2. Those were plotted in Figure 3-4 with closed and open symbols for compounds with and without the growth-inhibitory effects at a concentration of 200 μM, respectively. The physicochemical molecular weights of the compounds with the growth-inhibitory effects were around 300 and the ACD/LogP values of them ranged from 4.04 to 7.70. Either the ACD/LogP value or molecular weight of the other compounds without growth-inhibitory effects was out of the range. Therefore, estimating these parameters could be useful for predicting if a hydrophobic compound were able to inhibit the growth of *Sporosarcina* sp. S92h. Although further studies are necessary to generalize this finding to other compounds and bacteria containing *Sporosarcina* species, I hope that this finding would contribute to searching a novel compound useful to prevent the damage of surimi-based products by bacterial contamination.

Table 3-2. Minimum inhibitory concentrations of the fatty acid-related compounds tested against *Sporosarcina* sp. S92h.

Compound	MW[a]	ACD/LogP	MIC[b]
Monoacylglycerol			
Monocaprin (C10)	246.3	2.98	125 mg/L (507.5 μM)
Monolaurin (C12)	274.4	4.04	31.3 mg/L (114.1 μM)
Monomyristin (C14)	302.4	5.10	15.6 mg/L (51.6 μM)
Monopalmitin (C16)	330.5	6.17	2000 mg/L (6051.4 μM)
Monostearin (C18)	358.6	7.23	> 2000 mg/L
Monoarachidin (C20)	386.6	8.29	> 2000 mg/L
Triacylglycerol			
Tributyrin (C4)	302.4	2.95	> 2000 mg/L
Trivalerin (C5)	344.4	4.54	> 2000 mg/L
Tricaproin (C6)	386.5	6.14	500 mg/L (1293.7 μM)
Trienanthin (C7)	428.6	7.73	> 2000 mg/L
Tricaprylin (C8)	470.7	9.33	> 2000 mg/L
Tricaprin (C10)	554.8	12.5	> 2000 mg/L
Trilaurin (C12)	639.0	15.7	> 2000 mg/L

[a] Molecular weight

[b] Minimum inhibitory concentration at 28°C for two days in Mueller-Hinton broth under static condition

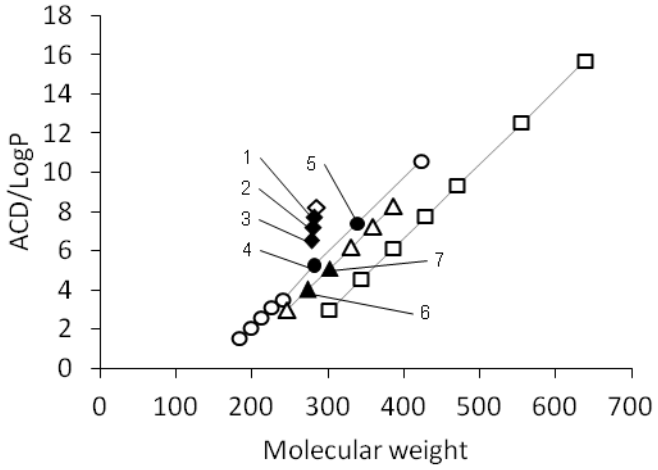

Figure 3-4. Relationship between the physicochemical properties of the tested compounds and their antibacterial activities against *Sporosarcina* sp. S92h. The molecular weights and ACD/LogP values of alkyl gallates (circle), free fatty acids (diamond), monoacylglycerols (triangle), and triacylglycerols (square) are indicated. The tested compounds with and without the growth-inhibitory effects at a concentration of 200 μM are indicated with closed and open symbols, respectively. 1, Oleic acid; 2, linoleic acid; 3, α-linolenic acid; 4, octyl gallate; 5, lauryl gallate; 6, monolaurin; 7, monomyristin.

SUMMARY

As mentioned in Chapter I, several *Sporosarcina* species were predominantly isolated from minced fish meat (surimi) as psychrotolerant endospore-forming bacteria, and I suggested that they should be controlled to maintain the quality of surimi-based products. As mentioned in Chapter II, *Sporosarcina* species modulate their fatty acid compositions in response to the growth temperature to maintain the fluidity of the cell membrane under cold conditions. In addition, I demonstrate that the growth of *Sporosarcina* species at cold temperatures can be controlled by artificially modulating the bacterial fatty acid composition using additives. Here, I examined the effects of hydrophobic compounds (alkyl gallates and fatty acids-related compounds) that might interact with the cell membrane on the growth of *Sporosarcina* species. The compounds were further categorized based on their activities and physicochemical characteristics to reveal the required properties as the antibacterial compounds. I studied the relationship between the hydrophobicities, the molecular weights, and the antibacterial effects of the tested compounds to develop a model to predict efficacious

compounds in the preservation of surimi-based products. As a result, I found that 200 μM octyl gallate, lauryl gallate, oleic acid, linoleic acid, and α-linolenic acid showed significant effects to inhibit the growth of *Sporosarcina* sp. S92h in TSB medium at 30°C for ten days, and monolaurin and monomyristin also showed significant effects to inhibit the growth in Mueller-Hinton broth at 28°C for two days at a concentration of less than 200 μM. Their physicochemical properties tend to converge on around 300 in molecular weight and a range from 4.04 to 7.70 in the ACD/LogP value. This information will be useful for future investigation of antibacterial compounds against food contaminations by *Sporosarcina* species.

CONCLUSIONS

The results described in each chapter are summarized as follows:

CHAPTER I

This chapter described isolation and characterization of psychrotolerant endospore-forming *Sporosarcina* species associated with minced fish meat (surimi). I studied the changes of resident microbiota in surimi during heat-treatment and subsequent cold-storage via the sequencing of partial 16S rRNA gene. Raw surimi made from Alaska pollock, pike conger, and white croaker was contaminated with 10^4 to 10^6 CFU/g of various non-endospore-forming bacteria. Immediately after heat-treatment, the bacterial counts were significantly reduced to less than 1 CFU/g, and only endospore-forming bacteria, identified as *Bacillus* species were retrieved. Subsequently, the bacterial counts increased up to 10 to 10^5 CFU/g in the heated surimi after refrigerated storage at 5°C for 2 weeks or at 10°C for 1 week. Most of the isolates from the refrigerated surimi were identified as *Sporosarcina* species. The *Sporosarcina* isolates have an increased ability to grow at 10°C than the isolates related to the other endospore-forming bacteria, such as *Bacillus*, *Lysinibacillus*, and *Paenibacillus* species. Endospores of the *Sporosarcina* isolates were able to germinate and proliferate in a fish-paste product model system stored at 10°C within 8 days. In order to study the cold-adaptation mechanism of *Sporosarcina* species, the fatty acid composition of the isolates was analyzed. At the growth temperature of 10°C, the proportions of unsaturated to saturated fatty acids and *anteiso* to *iso* fatty acids were higher than those at 28°C. The alteration of the fatty acid composition suggests that *Sporosarcina* species adapt to cold by maintaining the fluidity of the cell membrane because unsaturated and *anteiso* fatty acids have lower melting points than saturated and *iso* fatty acids, respectively. I concluded that the endospores of *Sporosarcina* species are widely distributed in surimi, and that they can survive heat-treatment and proliferate during cold-storage in fish-paste products. Controlling *Sporosarcina* species would contribute to improving the quality of surimi product.

CHAPTER II

This chapter described modulation of fatty acid composition and growth in *Sporosarcina* species in response to temperatures and exogenous branched-chain amino acids.

Psychrotolerant endospore-forming *Sporosarcina* species have been predominantly isolated from minced fish meat (surimi), which is stored under refrigeration after heat treatment. To develop a better method for preserving surimi-based food products, I studied the growth and fatty acid compositions of the isolated strain S92h as well as *Sporosarcina koreensis* and *S. aquimarina* at cold and moderate temperatures. The growth rates of strain S92h and *S. koreensis* were the fastest and slowest at cold temperatures, respectively, although these strains grew at a similar rate at moderate temperatures. In all three strains, the proportions of *anteiso*-$C_{15:0}$ and unsaturated fatty acids (UFAs) were significantly higher at cold temperatures than at moderate temperatures. Furthermore, supplementation with valine, leucine, and isoleucine resulted in proportional increases in *iso*-$C_{16:0}$, *iso*-$C_{15:0}$, and *anteiso*-$C_{15:0}$, respectively, among the fatty acid compositions of these strains. The proportions of the UFAs were also altered by the supplementation. At cold temperatures, the growth rates of strain S92h and *S. koreensis*, but not of *S. aquimarina*, were affected by supplementation with leucine. Supplementation with isoleucine enhanced the growth of *S. koreensis* at cold temperatures but not that of the other strains. Valine did not affect the growth of any strain. These results indicate that *anteiso*-$C_{15:0}$ and UFAs both play important roles in the cold tolerance of the genus *Sporosarcina* and that these bacteria modulate their fatty acid compositions in response to the growth environment.

CHAPTER III

This chapter described effects of alkyl gallates and fatty acid-related compounds on growth of *Sporosarcina* species. As mentioned in Chapter I, several *Sporosarcina* species were predominantly isolated from minced fish meat (surimi) as psychrotolerant endospore-forming bacteria, and I suggested that they should be controlled to maintain the quality of surimi-based products. As mentioned in Chapter II, *Sporosarcina* species modulate their fatty acid compositions in response to the growth temperature to maintain the fluidity of the cell membrane under cold conditions. In addition, I demonstrate that the growth of *Sporosarcina* species at cold temperatures can be controlled by artificially modulating the bacterial fatty acid composition using additives. Here, I examined the effects of hydrophobic compounds (alkyl gallates and fatty acids-related compounds) that might interact with the cell membrane on the growth of *Sporosarcina* species. The compounds were further categorized based on

their activities and physicochemical characteristics to reveal the required properties as the antibacterial compounds. I studied the relationship between the hydrophobicities, the molecular weights, and the antibacterial effects of the tested compounds to develop a model to predict efficacious compounds in the preservation of surimi-based products. As a result, I found that 200 μM octyl gallate, lauryl gallate, oleic acid, linoleic acid, and α-linolenic acid showed significant effects to inhibit the growth of *Sporosarcina* sp. S92h in TSB medium at 30°C for ten days, and monolaurin and monomyristin also showed significant effects to inhibit the growth in Mueller-Hinton broth at 28°C for two days at a concentration of less than 200 μM. Their physicochemical properties tend to converge on around 300 in molecular weight and a range from 4.04 to 7.70 in the ACD/LogP value. This information will be useful for future investigation of antibacterial compounds against food contaminations by *Sporosarcina* species.

REFERENCES

1. **Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P., Heyndrickx, M.**: Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst. Appl. Microbiol.*, **31**, 126-40 (2008)

2. **Huck, J. R., Sonnen, M., Boor, K. J.**: Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *J. Dairy Sci.*, **91**, 1218-28 (2008)

3. **Ivy, R. A., Ranieri, M. L., Martin, N. H., den Bakker, H. C., Xavier, B. M., Wiedmann, M., Boor, K. J.**: Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. *Appl. Environ. Microbiol.*, **78**, 1853-64 (2012)

4. **Baron, F., Cochet, M. F., Grosset, N., Madec, M. N., Briandet, R., Dessaigne, S., Chevalier, S., Gautier, M., Jan, S.**: Isolation and characterization of a psychrotolerant toxin producer, *Bacillus weihenstephanensis*, in liquid egg products. *J. Food Prot.*, **70**, 2782-91 (2007)

5. **Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B., Wilcks, A.**: Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiol. Lett.*, **250**, 129-36 (2005)

6. **Guinebretiere, M.-H., Berge, O., Normand, P., Morris, C., Carlin, F., Nguyen-The, C.**: Identification of Bacteria in Pasteurized Zucchini Purées Stored at Different Temperatures and Comparison with Those Found in Other Pasteurized Vegetable Purées. *Appl. Environ. Microbiol.*, **67**, 4520-30 (2001)

7. **Coton, M., Denis, C., Cadot, P., Coton, E.**: Biodiversity and characterization of aerobic spore-forming bacteria in surimi seafood products. *Food Microbiol.*, **28**, 252-60 (2011)

8. **Zhang, Y. M., Rock, C. O.**: Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol.*, **6**, 222-33 (2008)

9. **Park, J. W., Nozaki, H., Suzuki, T., J., B.**: Historical review of surimi technology and market developments. *Surimi and surimi seafood*, 4-23 (2013)

10. **Guenneugues, P., Ianelli, J.**: Surimi resources and market. *Surimi and surimi seafood*, 25-54 (2013)

11. **Yokoseki, M.**: Studies on the internal spoilage of fish jelly products. I. Surviving

microorganisms in jelly fish products cooked at different temperatures. *Bull. Japan. Soc. Sci. Fish* **23**, 539-42 (1958)

12. **Ogawa, J., Matsumura, K., Kishino, S., Omura, Y., Shimizu, S.**: Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, **67**, 1246-52 (2001)

13. **Kikukawa, H., Sakuradani, E., Kishino, S., Park, S. B., Ando, A., Shima, J., Ochiai, M., Shimizu, S., Ogawa, J.**: Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system. *J. Biosci. Bioeng.*, **116**, 672-6 (2013)

14. **Himelbloom, B. H., Brown, E. K., Jong, S. L.**: Microorganisms Isolated from Surimi Processing Operations. *Journal of Food Science*, **56**, 299-301 (1991)

15. **Leisner, J. J., Laursen, B. G., Prévost, H., Drider, D., Dalgaard, P.**: *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS Microbiol. Rev.*, **31**, 592-613 (2007)

16. **Collins, M. D., Farrow, J. A. E., Goodfellow, M., Minnikin, D. E.**: *Brevibacterium casei* sp. nov. and *Brevibacterium epidermidis* sp. nov. *Syst. Appl. Microbiol.*, **4**, 388-95 (1983)

17. **Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S., Jones, D.**: Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and Some Catalase-Negative, Asporogenous, Rod-Shaped Bacteria from Poultry in a New Genus, *Carnobacterium*. *Int. J. Syst. Evol. Microbiol.*, **37**, 310-16 (1987)

18. **O'Hara, C. M., Steigerwalt, A. G., Hill, B. C., Farmer, J. J., 3rd, Fanning, G. R., Brenner, D. J.**: *Enterobacter hormaechei*, a new species of the family Enterobacteriaceae formerly known as enteric group 75. *J. Clin. Microbiol.*, **27**, 2046-9 (1989)

19. **Takeuchi, M., Weiss, N., Schumann, P., Yokota, A.**: *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall. *Int. J. Syst. Evol. Microbiol.*, **46**, 967-71 (1996)

20. **Kovacs, G., Burghardt, J., Pradella, S., Schumann, P., Stackebrandt, E., Marialigeti, K.**: *Kocuria palustris* sp. nov. and *Kocuria rhizophila* sp. nov., isolated from the rhizoplane of the narrow-leaved cattail (*Typha angustifolia*). *Int. J. Syst. Evol. Microbiol.*, **49**, 167-73 (1999)

21. **Collins, M. D., Hutson, R. A., Baverud, V., Falsen, E.**: Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of

Stomatococcus mucilaginosus as *Rothia mucilaginosa* comb. nov. *Int. J. Syst. Evol. Microbiol.*, **50**, 1247-51 (2000)

22. **Mora, D., Scarpellini, M., Franzetti, L., Colombo, S., Galli, A.**: Reclassification of *Lactobacillus maltaromicus* (Miller et al. 1974) DSM 20342(T) and DSM 20344 and *Carnobacterium piscicola* (Collins et al. 1987) DSM 20730(T) and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov. *Int. J. Syst. Evol. Microbiol.*, **53**, 675-8 (2003)

23. **Romanenko, L. A., Lysenko, A. M., Rohde, M., Mikhailov, V. V., Stackebrandt, E.**: *Psychrobacter maritimus* sp. nov. and *Psychrobacter arenosus* sp. nov., isolated from coastal sea ice and sediments of the Sea of Japan. *Int. J. Syst. Evol. Microbiol.*, **54**, 1741-5 (2004)

24. **Irlinger, F., Bimet, F., Delettre, J., Lefevre, M., Grimont, P. A.**: *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov., novel coryneform species isolated from the surfaces of cheeses. *Int. J. Syst. Evol. Microbiol.*, **55**, 457-62 (2005)

25. **Liu, X. Y., Wang, B. J., Jiang, C. Y., Liu, S. J.**: *Paracoccus sulfuroxidans* sp. nov., a sulfur oxidizer from activated sludge. *Int. J. Syst. Evol. Microbiol.*, **56**, 2693-5 (2006)

26. **Yoon, J. H., Lee, J. K., Jung, S. Y., Kim, J. A., Kim, H. K., Oh, T. K.**: *Nocardioides kongjuensis* sp. nov., an N-acylhomoserine lactone-degrading bacterium. *Int. J. Syst. Evol. Microbiol.*, **56**, 1783-7 (2006)

27. **Joung, Y., Song, J., Lee, K., Oh, H. M., Joh, K., Cho, J. C.**: *Soonwooa buanensis* gen. nov., sp. nov., a member of the family Flavobacteriaceae isolated from seawater. *Int. J. Syst. Evol. Microbiol.*, **60**, 2061-5 (2010)

28. **Wirth, S. E., Ayala-del-Río, H. L., Cole, J. A., Kohlerschmidt, D. J., Musser, K. A., Sepúlveda-Torres, L. e. C., Thompson, L. M., Wolfgang, W. J.**: *Psychrobacter sanguinis* sp. nov., recovered from four clinical specimens over a 4-year period. *Int. J. Syst. Evol. Microbiol.*, **62**, 49-54 (2012)

29. **Kacagan, M., Inan, K., Belduz, A. O., Canakci, S.**: *Flavobacterium anatoliense* sp. nov., isolated from fresh water, and emended description of *Flavobacterium ceti*. *Int. J. Syst. Evol. Microbiol.*, **63**, 2075-81 (2013)

30. **Ahmed, I., Yokota, A., Yamazoe, A., Fujiwara, T.**: Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *Int. J. Syst. Evol. Microbiol.*, **57**, 1117-25 (2007)

31. **Ash, C., Priest, F. G., Collins, M. D.**: Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. *Antonie Van Leeuwenhoek*, **64**, 253-60 (1993)

32. **Yoon, J. H., Lee, K. C., Weiss, N., Kho, Y. H., Kang, K. H., Park, Y. H.**: *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of th. *Int. J. Syst. Evol. Microbiol.*, **51**, 1079-86 (2001)

33. **Kwon, S. W., Kim, B. Y., Song, J., Weon, H. Y., Schumann, P., Tindall, B. J., Stackebrandt, E., Fritze, D.**: *Sporosarcina koreensis* sp. nov. and *Sporosarcina soli* sp. nov., isolated from soil in Korea. *Int. J. Syst. Evol. Microbiol.*, **57**, 1694-8 (2007)

34. **Okada, M., Miyauchi, D., and Kudo, G.**: "Kamaboko": The Giant Among Japanese Processed Fishery Products. *Marine Fisheries Review*, **35**, 1-6 (1973)

35. **Annous, B. A., Becker, L. A., Bayles, D. O., Labeda, D. P., Wilkinson, B. J.**: Critical role of anteiso-$C_{15:0}$ fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Appl. Environ. Microbiol.*, **63**, 3887-94 (1997)

36. **Chan, Y. C., Wiedmann, M.**: Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. *Crit Rev Food Sci Nutr*, **49**, 237-53 (2009)

37. **Fulco, A. J.**: The biosynthesis of unsaturated fatty acids by bacilli. I. Temperature induction of the desaturation reaction. *J. Biol. Chem.*, **244**, 889-95 (1969)

38. **Aguilar, P. S., Lopez, P., de Mendoza, D.**: Transcriptional control of the low-temperature-inducible des gene, encoding the delta5 desaturase of *Bacillus subtilis*. *J. Bacteriol.*, **181**, 7028-33 (1999)

39. **Casadei, M. A., Ingram, R., Skinner, R. J., Gaze, J. E.**: Heat resistance of *Paenibacillus polymyxa* in relation to pH and acidulants. *J. Appl. Microbiol.*, **89**, 801-06 (2000)

40. **Leguerinel, I., Spegagne, I., Couvert, O., Gaillard, S., Mafart, P.**: Validation of an overall model describing the effect of three environmental factors on the apparent D-value of *Bacillus cereus* spores. *Int. J. Food Microbiol.*, **100**, 223-9 (2005)

41. **Tominaga, T., An, S. Y., Oyaizu, H., Yokota, A.**: *Sporosarcina luteola* sp. nov. isolated from soy sauce production equipment in Japan. *J. Gen. Appl. Microbiol.*, **55**, 217-23 (2009)

42. **Kämpfer, P., Falsen, E., Lodders, N., Schumann, P.**: *Sporosarcina contaminans* sp. nov. and *Sporosarcina thermotolerans* sp. nov., two endospore-forming species. *Int. J. Syst. Evol. Microbiol.*, **60**, 1353-7 (2010)

43. **Wolfgang, W. J., Coorevits, A., Cole, J. A., De Vos, P., Dickinson, M. C., Hannett, G. E., Jose, R., Nazarian, E. J., Schumann, P., Van Landschoot, A., Wirth, S. E., Musser, K. A.**: *Sporosarcina newyorkensis* sp. nov. from clinical specimens and raw cow's milk. *Int. J. Syst. Evol. Microbiol.*, **62**, 322-9 (2012)

44. **Tsuda, K., Nagano, H., Ando, A., Shima, J., Ogawa, J.**: Isolation and characterization of psychrotolerant endospore-forming *Sporosarcina* species associated with minced fish meat (surimi). *Int. J. Food Microbiol.*, **199**, 15-22 (2015)

45. **Chintalapati, S., Kiran, M. D., Shivaji, S.**: Role of membrane lipid fatty acids in cold adaptation. *Cell. Mol. Biol. (Noisy-le-grand)*, **50**, 631-42 (2004)

46. **Kaneda, T.**: Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Rev.*, **55**, 288-302 (1991)

47. **Kaneda, T.**: Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev*, **41**, 391-418 (1977)

48. **Aguilar, P. S., Cronan, J. E., de Mendoza, D.**: A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J. Bacteriol.*, **180**, 2194-200 (1998)

49. **Lyons, J. M., Asmundson, C. M.**: Solidification of unsaturated/saturated fatty acid mixtures and its relationship to chilling sensitivity in plants. *J Am Oil Chem Soc*, **42**, 1056-8 (1965)

50. **Feresu, S. B., Jones, D.**: Taxonomic studies on *Brochothrix*, *Erysipelothrix*, *Listeria* and atypical lactobacilli. *J. Gen. Microbiol.*, **134**, 1165-83 (1988)

51. **Julotok, M., Singh, A. K., Gatto, C., Wilkinson, B. J.**: Influence of fatty acid precursors, including food preservatives, on the growth and fatty acid composition of *Listeria monocytogenes* at 37 and 10degreesC. *Appl. Environ. Microbiol.*, **76**, 1423-32 (2010)

52. **Cybulski, L. E., Albanesi, D., Mansilla, M. C., Altabe, S., Aguilar, P. S., de Mendoza, D.**: Mechanism of membrane fluidity optimization: isothermal control of the *Bacillus subtilis* acyl-lipid desaturase. *Mol. Microbiol.*, **45**, 1379-88 (2002)

53. **Klein, W., Weber, M. H., Marahiel, M. A.**: Cold shock response of *Bacillus subtilis*: isoleucine-dependent switch in the fatty acid branching pattern for membrane adaptation to

low temperatures. *J. Bacteriol.*, **181**, 5341-9 (1999)

54. **Zhu, K., Ding, X., Julotok, M., Wilkinson, B. J.**: Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-$C_{15:0}$ fatty acid required for low-temperature growth of *Listeria monocytogenes*. *Appl. Environ. Microbiol.*, **71**, 8002-7 (2005)

55. **Haque, M. A., Russell, N. J.**: Strains of *Bacillus cereus* vary in the phenotypic adaptation of their membrane lipid composition in response to low water activity, reduced temperature and growth in rice starch. *Microbiology*, **150**, 1397-404 (2004)

56. **de Sarrau, B., Clavel, T., Clerté, C., Carlin, F., Giniès, C., Nguyen-The, C.**: Influence of anaerobiosis and low temperature on *Bacillus cereus* growth, metabolism, and membrane properties. *Appl. Environ. Microbiol.*, **78**, 1715-23 (2012)

57. **Kubo, I., Fujita, K., Nihei, K.**: Anti-*Salmonella* activity of alkyl gallates. *J. Agric. Food Chem.*, **50**, 6692-6 (2002)

58. **Kubo, I., Xiao, P., Fujita, K.**: Anti-MRSA activity of alkyl gallates. *Bioorg. Med. Chem. Lett.*, **12**, 113-6 (2002)

59. **Kubo, I., Fujita, K., Nihei, K., Nihei, A.**: Antibacterial activity of akyl gallates against *Bacillus subtilis*. *J. Agric. Food Chem.*, **52**, 1072-6 (2004)

60. **Desbois, A. P., Smith, V. J.**: Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Appl. Microbiol. Biotechnol.*, **85**, 1629-42 (2010)

61. **Osterberg, T., Norinder, U.**: Prediction of drug transport processes using simple parameters and PLS statistics. The use of ACD/logP and ACD/ChemSketch descriptors. *Eur J Pharm Sci*, **12**, 327-37 (2001)

ACKNOWLEDGEMENTS

The present thesis is based on the studies carried out from 2013 to 2016 at Laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University; Food Development Laboratories, Nippon Shinyaku Co. Ltd.

I wish to express my sincere thanks to Professor Jun Ogawa of Kyoto University, for his kind guidance, warm understanding and encouragement throughout the course of this study.

I greatly thank Professor Jun Shima, Assistant Professor Akinori Ando, and Dr. Hideaki Nagano for their kindly instruction in experimental technologies, direction of this study, critical reading of the manuscripts, and continuous encouragements.

I am also grateful to Emeritus Professor Nobuo Kato, Emeritus Professor Sakayu Shimizu, Assistant Professor Koichi Tanaka, Dr. Toru Akita, and Mr. Masayuki Fujino for their warm supports, encouragements, and thoughtful advice during the course of this study.

I greatly appreciate to Ms. Atsuko Kitamura and all members of Laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University; Industrial Microbiology, Graduate School of Agriculture, Kyoto University; Research Unit for Physiological Chemistry, Kyoto University; Food Development Laboratories, Nippon Shinyaku Co. Ltd.

Finally, but not the least, I would like to acknowledge the strong support and affectionate encouragement of my family throughout the course of this study.

津田 謙太郎

PUBLICATIONS

Tsuda, K., Nagano, H., Ando, A., Shima, J., Ogawa, J.: Isolation and characterization of psychrotolerant endospore-forming *Sporosarcina* species associated with minced fish meat (surimi). *Int. J. Food Microbiol*., **199**, 15-22 (2015)

Tsuda, K., Nagano, H., Ando, A., Shima, J., Ogawa, J.: Modulation of Fatty Acid Composition and Growth in *Sporosarcina* Species in Response to Temperatures and Exogenous Branched-chain Amino Acids. in preparation

Tsuda, K., Nagano, H., Ando, A., Shima, J., Ogawa, J.: Effects of alkyl gallates and fatty acid-related compounds on growth of *Sporosarcina* species. in preparation