

Comparative phylogeny of the *Bacillaceae* species related to shrimp paste products

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Abstract

Shrimp paste is one of the traditional seafood products widely consumed in several Asian countries. Although the microbial diversity of shrimp paste remains largely undescribed to date, it has been suggested that the bacterial members of the order *Bacillales* are predominant. This study was therefore conducted to investigate the bacterial diversity especially for those of the family *Bacillaceae*, isolated from the shrimp paste products. For this 24 *Bacillaceae* strains related to the shrimp paste, as well as their nucleotide sequences of the 16S rRNA gene were retrieved from the GenBank database. To elucidate the phylogeny of these *Bacillaceae* isolates, their 16S rRNA genes were aligned and used to construct the dendrogram. Based on the analysis, these 23 *Bacillaceae* strains were categorized into three major clades: (i) *Bacillus subtilis/amyloliquefaciens* complex; (ii) *Bacillus cereus* group; and (iii) non-*Bacillus* species.

Key words: *Bacillaceae*, *Bacillus*, fermented seafood, shrimp paste.

Introduction

Fermented fishery products are widely consumed in many Asian countries, and can be generally classified based on the raw material used (i.e., fish, shrimp, etc.). Shrimp paste is one of these products typically prepared from the planktonous shrimp (*Acetes vulgaris*) or krill (*Mesopodopsis orientalis*) (Phithakpol 1993). This product is popular and generally used as a side dish or condiment/seasoning in several Asian foods. There are a variety of the shrimp paste products which depend on the raw material and the production process; these include *Belacan* (Brunei and Malaysia), *Kapi* (Thailand), *Ngapi* (Myanmar), *Saeujot* (Korea), and *Terasi* (Indonesia) (Kim et al. 2014).

Traditional production of shrimp paste consists of four major steps: substrate preparation, salt addition, sun drying, and fermentation. In brief, the shrimp (i.e., *Acetes* species) is washed thoroughly, shortly sun-dried, and mixed with salt in a ratio by weight of 3 to 1. The mixture is then pounded and spread on the clean ground under sunlight. Once drained (1 to 2 days), the paste is packed and allowed to ferment under ambient condition for at least 1 month (or longer). When ripening (observed by colour and smell), the product is ready for sale and can be stored for several months (Pongsetkul et al. 2014).

Since traditional production of shrimp paste is processed without sterilisation, it is therefore expected that there would be many microbes involved in the fermentation. In addition, the microbial community may vary considered from the raw material used, the production process and the environmental condition. It is therefore

not a surprise that knowledge of microbial diversity of the shrimp paste is scarce. For example, the microflora of *Terasi* has been previously studied and this reveals a diverse group of bacteria including those in the genera *Bacillus*, *Kurthia*, *Micrococcus*, *Pseudomonas*, *Sporolactobacillus*, and *Tetragenococcus* (Suroño, Hosono 1994a,b; Kobayashi et al. 2003).

The pioneering research work of the shrimp paste's microbiota probably comes from Korean *Saeujot* in which its microbial population was determined using the molecular approach (Han et al. 2014; Lee et al. 2014). This research showed that a diverse group of bacteria is present including those in the phyla *Proteobacteria* and *Firmicutes*. However, the *Proteobacteria* members are found at the beginning and replaced by those of the *Firmicutes* during the fermentation (Lee et al. 2014). Han et al. (2014) also pointed out that the bacterial members of the order *Bacillales* are predominant during *Saeujot*'s fermentation. The incidence of this particular bacterial group is expected considering from the selective condition provided for the shrimp paste fermentation.

At least three factors can cause such a selective condition including a protein-rich shrimp substrate, high salt concentration, and anaerobic condition. These shrimp paste products are therefore a target for screening and isolation of some proteolytic and halophilic bacteria (Setyorini et al. 2006; Pakdeeto et al. 2007; Namwong et al. 2009). Considering the potential role of this specific bacterial group, this study was performed to determine their phylogenetic relationship based on the 16S rRNA gene.

Materials and methods

A total of 23 bacterial isolates in the family *Bacillaceae* reported to be isolated from shrimp paste were initially retrieved from the GenBank database (Benson et al. 2013). These bacterial samples were obtained under the nucleotide database search using the term 'shrimp paste', 'Terasi', 'Kapi', etc. Detailed information including their identity, strain code, and accession numbers of the 16S rRNA genes was retrieved (Table 1). For comparative analysis, the bacterial type strains used were *Bacillus subtilis* DSM10 (AL009126.3), *Bacillus licheniformis* DSM13 (NC006322.1), *Bacillus amyloliquefaciens* DSM7 (FN597644), *Bacillus mojavensis* DSM9205 (AB363735), *Bacillus cereus* ATCC14579 (BC0007), *Lentibacillus kapialis* TISTR1551 (AB231905), and *Klebsiella pneumoniae* DSM30104 (X87276). These sequencing data were analysed using BLAST (Altschul et al. 1990). Sequence alignment and phylogenetic analysis were then carried out using Phylogeny.fr software (Dereeper et al. 2010).

Results and discussion

Fermented shrimp paste is traditionally produced; without prior sterilisation of raw materials, it is expected that, during the fermentation, this would lead to the growth of various microbes. Unfortunately, information regarding

microbial diversity of the shrimp paste products is scarce compared to that of other products (i.e., fermented dairy and soybean products). Recently, Han et al. (2014) described the microbial dynamics and succession during the fermentation of *Jeotgal*, a Korean fermented shrimp. Using the denaturing gradient gel electrophoresis technique, they found that a diverse group of bacteria was present in which the members of the order *Bacillales* were predominant (Han et al. 2014).

In this study, an initial search using the terms 'shrimp paste' and other local names of the related products (i.e., *Kapi* and *Terasi*) revealed approximately 60 literature sources. These studies included the primer set and the 16S rRNA gene sequences of various bacteria isolated from the products. It should be noted that 27 isolates were in the phyla Proteobacteria, consisting of two genera, *Methylobacterium* and *Pseudomonas* (data not shown). These strains were isolated from *Bulacan*, the Philippine shrimp paste. Two strains were lactic acid bacteria (*Tetragenococcus halophilus* and *Tetragenococcus muriaticus*) (data not shown). The remaining isolates identified as the members of the family *Bacillaceae* were then selected for this study and listed in Table 1.

Their 16S rRNA gene sequences were compiled and analysed for their phylogenetic relationship. Based on the evolutionary tree constructed, Fig. 1 showed that there were at least three major clades. Group 1 consisted of five strains

Table 1. List of the *Bacillaceae* bacteria isolated from the shrimp paste and related products used in this study

Source	Species	Strain	Accession No.	Remarks
<i>Belachan</i>	<i>Bacillus cereus</i>	RE01-BS05	KJ742939	Unpublished (Malaysia)
Fermented shrimp	<i>B. mojavensis</i>	F412	LC020019	Unpublished (Japan)
	<i>Bacillus</i> sp.	AUSH4	EU878032	Unpublished (Korea)
<i>Kapi</i>	<i>Bacillus</i> sp.	K-A7	HQ222303	Unpublished (Thailand)
	<i>Bacillus</i> sp.	C5	JQ814721	Unpublished (Thailand)
	<i>Bacillus</i> sp.	KPa7	JQ814720	Unpublished (Thailand)
	<i>Lentibacillus</i>	PN7-6	NR041319	Pakdeeto et al. 2007
	<i>Oceanobacillus</i>	SSK2-2	NR112740	Namwong et al. 2009
	<i>Virgibacillus</i>	KN3-8-4	LC041942	Unpublished (Thailand)
	<i>Virgibacillus</i>	TKNR13-3	HM364289	Unpublished (Thailand)
<i>Ngapi</i>	<i>B. subtilis</i>	NP1-1	AB168129	Aung et al. 2004
	<i>Bacillus</i> sp.	NP3-2	AB168130	Aung et al. 2004
	<i>B. licheniformis</i>	1LB5	JQ266341	Unpublished (India)
<i>Terasi</i>	<i>Bacillus</i> sp.	S2-3	FJ217159	Unpublished (Thailand)
	<i>Bacillus</i> sp.	S4-5	FJ217160	Unpublished (Thailand)
	<i>Bacillus</i> sp.	S1-13	JX441363	Unpublished (Thailand)
<i>Saeujot</i>	<i>Alkalibacillus</i>	BH163	AY671976	Jeon et al. 2005
	<i>B. cereus</i>	SCH-1	KC878876	Han et al. 2014
	<i>Paenibacillus</i>	SCH-2	KC878877	Han et al. 2014
	<i>Virgibacillus</i>	YIM-kkny16	AY121439	Unpublished (China)
Shrimp paste	<i>Allobacillus</i>	B3A	NR116607	Sheu et al. 2011
	<i>Bacillus</i>	NSPM	FJ347756	Unpublished (Taiwan)
Shrimp sauce	<i>B. subtilis</i>	SK11004	FJ437210	Unpublished (China)

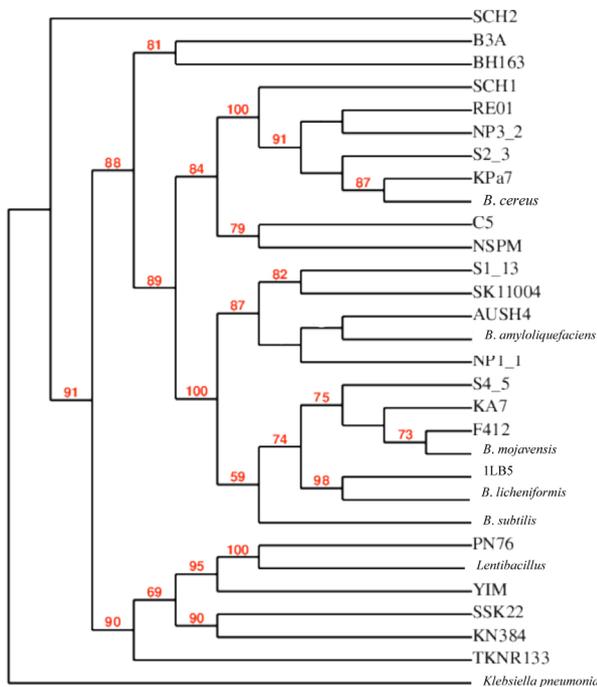


Fig. 1. Cladistic tree constructed with the 16S rRNA gene sequences of the *Bacillaceae* bacteria isolated from the shrimp paste products. Bootstrap confidence levels greater than 50% are presented at the nodes.

(SCH1, RE01, NP3-2, S2-3, and KPa7) which were clustered with a *B. cereus* type strain. There were two strains (C5 and NSPM) that formed a distinct clade related to Group 1 and initially designated as *Bacillus* spp. from the unpublished work of Thailand and Taiwan, respectively. Group 2 was comprised of eight strains (S1-13, SK11004, AUSH4, NP1-1, S4-5, KA7, F412, and 1LB5) including the type strains of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. mojavensis*. The eight remaining isolates (SCH2, B3A, BH163, PN7-6, YIM-kkny16, SSK2-2, KN3-8-4, and TKNR13-3) were classified in Group 3 and represented the non-*Bacillus* species.

In addition, as shown in Table 1, there were nine bacterial isolates in which their identity remained unknown, including the strains AUSH4, K-A7, C5, KPa7, NP3-2, S2-3, S4-5, S1-13, and NSPM (thus designated as *Bacillus* spp.). These bacterial isolates were then identified based on relatedness of the 16S rRNA gene sequence, as shown in Table 2. In general, the species identification was in agreement with the data obtained from the phylogenetic tree. For example, the unidentified strains of S2-3, NP3-2, and Kpa7 were identified as *B. cereus* (Fig. 1 and Table 2). The strain NSPM was re-identified as *B. cohnii* distantly related to the *B. subtilis* group.

The phylogenetic relationship of the *Bacillaceae* strains isolated from the shrimp paste products was determined in the present study. It should be noted, however that the sole use of the 16S rRNA gene sequence proved to be difficult for *Bacillus* species identification (especially for

Table 2. Species identification of the unidentified or wrongly identified *Bacillaceae* bacteria isolated from the shrimp paste products. Nucleotide sequence similarities were presented in the parentheses

Strain	Species identification
AUSH4	<i>B. atrophaeus</i> (86.38)
K-A7	<i>B. subtilis</i> (99.54)
C5	<i>B. infantis</i> (99.42)
KPa7	<i>B. cereus</i> (99.79)
NP3-2	<i>B. cereus</i> (99.46)
S2-3	<i>B. cereus</i> (99.86)
S4-5	<i>B. subtilis</i> (99.56)
S1-13	<i>B. subtilis</i> (99.68)
NSPM	<i>B. cohnii</i> (96.34)

B. subtilis species). There are some *Bacillus* species that are closely related to *B. subtilis*, and they are often referred to as the ‘*subtilis*-group’ or ‘*B. subtilis* species complex’ which includes *B. amyloliquefaciens*, *B. atrophaeus*, and *B. mojavensis* (Rooney et al. 2009). Additional information on such strains regarding phenotypic characteristics (i.e., morphology and biochemical profiles) as well as other biomarkers (i.e., sequencing data of the gyrase and RNA polymerase genes) is important for further analysis. Once available, these data can be expected to provide insight on the antiquity and diversity of this widely consumed food.

Conclusions

Information on the shrimp paste microbiota is presently scarce. This paper highlights the bacterial diversity especially for those of the family *Bacillaceae*. Considered that shrimp paste products are widely distributed in several Asian countries, the *Bacillaceae* members appear to be predominant. This is the first report to describe the phylogeny of the *Bacillaceae* bacteria isolated from shrimp paste products.

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