

**Note**

## **Spoilage Ability of Psychrotrophic *Paenibacillus* spp. Isolated from Cooked Food Products**

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**Five spore-forming bacterial strains were isolated as the most dominant microorganisms from five products of different types of cooked food. Three strains in particular were isolated as spoilage organisms causing acidity. These belonged to the genus *Paenibacillus*, and one of them was identified as *Paenibacillus odorifer*. All five strains grew at 10°C in brain heart infusion broth; furthermore, four strains grew at 4°C also. These strains grew optimally at 30 to 35°C. Psychrotrophic *Paenibacillus* spp. can easily be mistaken for lactic acid bacteria in routine analysis because of their colony characteristics. Because the measures that must be taken for bacterial control are different in the case of contamination by these two bacterial groups, psychrotrophic *Paenibacillus* spp. contamination must be distinguished from that caused by lactic acid bacteria.**

Key words : Psychrotrophic *Paenibacillus* spp./Spoiling ability/Lactic acid bacteria.

One of the theories in the food industry is that cooked food products can be preserved by distributing and selling them at low temperatures. By this method, most vegetative cells are destroyed by the cooking process, and the surviving spore-forming microorganisms are inhibited from growing by the cool temperatures. Therefore, with hygienic handling of the cooked products for avoidance of cross-contamination, these products have a long shelf-life.

However, this treatment typically raises opportunities for the growth of psychrotrophic spore-forming bacteria like *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides* (Francis et al., 1998) and non-proteolytic *Clostridium botulinum* (Juneja et al., 1995), which may survive the heat treatment and grow at low temperatures. Because it is difficult to control these bacteria, contamination by them is a serious problem in the food industry. The behavior of psychrotrophic *Bacillus* spp. and *Clostridium* spp. in food spoilage have been studied thoroughly (Jaquette and Beuchat, 1998; Kalinowski and

Tompkin, 1999), but there is little known about the behavior of other psychrotrophic spore-forming bacteria.

*Paenibacillus* spp. are gram-positive and spore-forming rods belonging to a class of bacilli of the phylum *Firmicutes*, formerly belonging to the genus *Bacillus* (Ash et al., 1993; Boone and Castenholtz, 2001). These bacteria have been isolated from a variety of sources, including soil, water and plants, and often contaminate foods (Bosshard et al., 2002). However, little is known about the spoilage behavior of *Paenibacillus* spp. In this study, we investigated the spoilage ability of *Paenibacillus* spp. in cooked food products at low temperatures.

Five samples of commercially available food products were obtained; two scotch eggs and two omelets individually packed by modified-atmosphere packaging, and a milk tea dessert containing tapioca pearls in a plastic cup. These samples were diluted 10-fold with a sterile 0.85% NaCl solution and treated with a stomacher (Pro-media SH-001; Elmex Ltd., Tokyo, Japan) for 1 min, and serial dilutions were then prepared. Bacterial counts were determined by the standard plate count (SPC) technique.

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Colonies from the SPC plates were selected at random and cultured on Brain Heart Infusion (BHI) agar (Oxoid Ltd., Basingstoke, Hampshire, UK) for 48 h at 30°C. BHI agar plates with the pure culture were used for Gram staining, catalase testing, oxidase testing, and morphology testing. Each pure culture was further characterized by 16S rDNA analysis and biochemical tests. A 500-bp fragment of the 16S rRNA gene was amplified from genomic DNA from each isolate by PCR, and sequenced using the reagents and protocol supplied in the MicroSeq 500 bacterial sequencing kit (Applied Biosystems, Foster City, CA, USA). The BLAST program was used to compare the present 16S rDNA sequences with sequences deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/>) and DDBJ (<http://www.ddbj.nig.ac.jp/>) databases. For phylogenetic analysis, the sequences were aligned, neighbour-joining trees were constructed using ClustalX (version 1.81) (Thompson et al., 1997), and then the tree was edited using the TreeView program (version 1.6.6) (Page, 1996). The DDBJ accession numbers for the 16S rDNA sequences of strain MCRI 9, 11, 90, 122, and 337 are AB210069, AB210070, AB210071, AB210072 and AB210073, respectively. Biochemical tests were performed by using the API 20E and API 50CHB systems (bioMerieux, Marcy-l'Etoile, France).

Growth curves at low temperatures were based on cell numbers in the BHI broth. Precultures of the strains were prepared by incubation in the BHI broth (Oxoid) for 24 h at 30°C. The cells were washed with sterile 0.85% NaCl solution and diluted to approximately  $10^4$  CFU/ml. 100  $\mu$ l of the cell suspensions were added into 10 ml of pre-cooled BHI broth and incubated at 10 or 4°C. The cell numbers were determined by plate counting with BHI agar. Growth curves at mesophilic temperatures were based on optical density in the BHI broth. Precultures (1 ml) were added into the BHI broth (100 ml) and incubated at  $25 \pm 0.2$ ,  $30 \pm 0.2$ ,  $35 \pm 0.2$ ,  $37 \pm 0.2$ ,  $39 \pm 0.2$ , or  $40 \pm 0.2$ °C in a water bath (Personal-11; TAITEC Co., Ltd., Saitama, Japan). Optical density was measured with absorbance at 660 nm.

Large numbers of bacteria were detected in the five food samples (Table 1). In particular, the two scotch

**TABLE 1.** Bacterial counts of products

Products	Period preserved at 10°C (days)	SPC (CFU/g)
Scotch egg A	14	$>10^7$
Scotch egg B	14	$>10^7$
Omelet A	17	$1.4 \times 10^5$
Omelet B	15	$9.6 \times 10^5$
Milk tea dessert	35	$>10^7$

eggs and milk tea dessert had soured, and contained bacteria of over  $10^7$  CFU/g. The scotch eggs and milk tea dessert had been kept for 14 and 35 days, respectively, at 10°C. In the two omelets, spoilage did not occur, but  $10^5$  CFU/g levels of bacteria were detected. Omelets A and B had been kept for 17 and 15 days, respectively, at 10°C. Several dominant organisms were isolated from each plate with the highest dilution, and five strains, MCRI 9 (culture collection of Marudai Central Research Institute), 11, 90, 122 and 337 were isolated as the most dominant organisms from the Scotch eggs A and B, Omelets A and B, and the milk tea dessert, respectively.

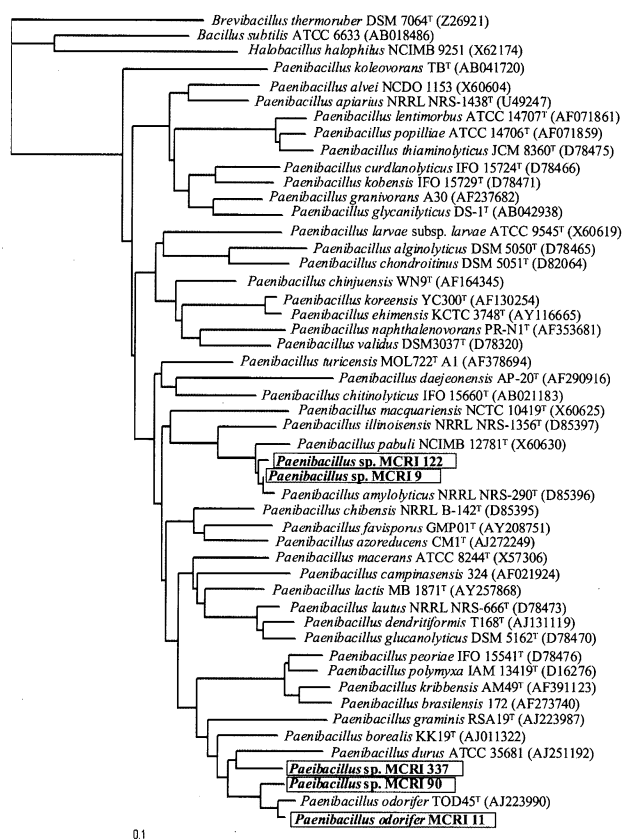
All isolates were gram positive, catalase positive,

**TABLE 2.** Phenotypic characteristics of isolates from products, and those of *Paenibacillus amylolyticus* and *Paenibacillus odorifer*.

	1	2	3	4	5	6	7
Anaerobic growth	+	+	+	+	+	+	+
Oxidase	-	-	-	+	-	-	-
Gelatin hydrolysis	+	-	-	+	-	+	NR
Nitrate reduction	+	+	-	-	-	+	+
Acid from:							
Glycerol	+	+	+	+	-	+	v
D-Arabinose	-	-	-	-	-	NR	-
L-Arabinose	+	+	+	+	+	+	+
Ribose	+	+	+	+	-	+	+
D-Xylose	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-
$\beta$ Methyl D-xyloside	+	+	+	+	+	NR	+
Galactose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Mannose	+	-	+	+	+	+	v
Sorbosose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	+	-
Mannitol	+	-	-	+	+	+	-
Sorbitol	-	-	-	-	-	-	-
$\alpha$ -Methyl-D glucoside	-	+	+	+	+	NR	+
Salicin	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+
Melibiose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Inulin	-	+	+	-	-	+	+
Melezitose	-	-	+	-	+	NR	-
Raffinose	+	+	+	+	+	-	+
Starch	+	+	+	+	-	+	+

Strains: 1, *Paenibacillus* sp. MCRI 9; 2, *P. odorifer* MCRI 11; 3, *Paenibacillus* sp. MCRI 90; 4, *Paenibacillus* sp. MCRI 122; 5, *Paenibacillus* sp. MCRI 337; 6, *P. amylolyticus* (Shida et al., 1997); 7, *P. odorifer* (Berge et al., 2002). +, positive reaction; -, negative reaction; v, variable reaction; NR, not reported.

and spore forming rods. In strain MCRI 11, BLAST analysis of the 16S rDNA sequence showed similarity with that of *Paenibacillus odorifer*, with identity of over 99% to *P. odorifer* TOD45<sup>T</sup> (accession number AJ223990) without single nucleotide polymorphisms. The biochemical properties of strain MCRI 11 were consistent with those of *P. odorifer* according to biochemical tests using the API systems (Table 2) (Berge et al., 2002). Therefore, strain MCRI 11 was identified as *P. odorifer*. The 16S rDNA sequences of strains MCRI 9 and 122 showed identity of over 99% to *Paenibacillus amylolyticus* NRRL NRS-290<sup>T</sup> (accession number D85396) without single nucleotide polymorphisms. However, their biochemical properties were not consistent with those of *P. amylolyticus*, because acid production from lactose and raffinose was positive and from inositol and inulin was negative (Shida et al., 1997). Thus, strains MCRI 9 and 122 were not identified as species in this study. The 16S



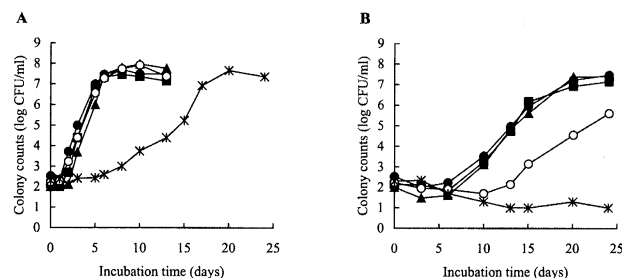
**FIG. 1.** Phylogenetic relationship of five strains, MCRI 9, 11, 90, 122 and 337, to the published *Paenibacillus* species and some other aerobic, rod-shaped, endospore-forming bacteria based on 16S rDNA sequences. The branching pattern was generated by the neighbour-joining method. A total of approximately 500 bases between positions 5 and 531 (*E. coli* numbering) were used for this analysis. Sequence accession numbers are in parentheses. Bar, 0.1 nucleotide substitutions per site.

rDNA sequences of strains MCRI 90 and 337 showed identity of under 97% to all other bacteria identified for species in databases, showing similarity with that of *Paenibacillus* spp. According to the phylogenetic analysis results, it was confirmed that strains MCRI 9, 90, 122 and 337 belong to genus *Paenibacillus* (Fig. 1).

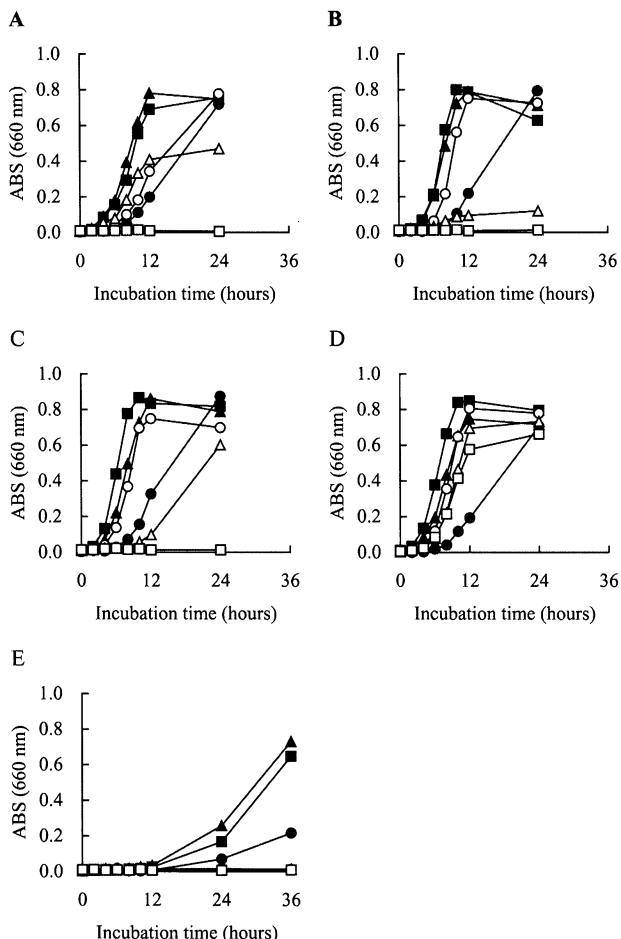
These five *Paenibacillus* strains grew at 10°C in BHI broth (Fig. 2A). Four strains in particular, MCRI 9, 11, 90 and 122, grew rapidly; the initial cell numbers of 10<sup>2</sup> CFU/ml level increased to over 10<sup>7</sup> CFU/ml in 6 days. Furthermore, these four strains grew at 4°C, while *Paenibacillus* sp. MCRI 337 did not grow (Fig. 2B). Three strains, MCRI 9, 11 and 90, tended to grow rapidly at 4°C; they increased to 10<sup>7</sup> CFU/ml over 20 days. Four strains excluding *Paenibacillus* sp. MCRI 337 grew optimally at 30 to 35°C (Fig. 3). The maximum growth temperatures of *Paenibacillus* sp. MCRI 9, 90 and *P. odorifer* MCRI 11 were 37 to 39°C (Figs. 3A-C). *Paenibacillus* sp. MCRI 122 tended to grow at a higher temperature, and its maximum temperature for growth was 40°C (Fig. 3D) because this strain did not grow at 42°C (data not shown). In the growth of *Paenibacillus* sp. MCRI 337, its optimum and maximum temperatures were approximately 30 and 35°C, respectively (Fig. 3E).

Generally, because cooked food products are produced with good quality ingredients contaminated by a small quantity of microorganisms, the initial bacterial counts are very small, below 10 CFU/g in most cases. Nevertheless, *Paenibacillus* sp. MCRI 9, *P. odorifer* MCRI 11 and *Paenibacillus* sp. MCRI 337 were isolated as spoilage organisms causing acidity due to their growth property at low temperatures. Although *Paenibacillus* sp. MCRI 90 and 122 did not spoil the foods we tested, it is believed that they also possess spoilage ability. These bacteria are considered to mainly originate from plant materials.

*Paenibacillus* spp. formed pinpoint colonies on SPC agar or Plate Count Agar with Bromocresol



**FIG. 2.** Growth curves of *Paenibacillus* sp. MCRI 9 (●), *Paenibacillus odorifer* MCRI 11 (▲), *Paenibacillus* sp. MCRI 90 (■), *Paenibacillus* sp. MCRI 122 (○) and *Paenibacillus* sp. MCRI 337 (\*) in BHI broth at 10°C (A) and 4°C (B).



**FIG. 3.** Growth curves of *Paenibacillus* sp. MCRI 9 (A), *Paenibacillus odorifer* MCRI 11 (B), *Paenibacillus* sp. MCRI 90 (C), *Paenibacillus* sp. MCRI 122 (D) and *Paenibacillus* sp. MCRI 337 (E) in BHI broth at 25°C (●), 30°C (▲), 35°C (■), 37°C (○), 39°C (△) and 40°C (□).

Purple (PCABP) like lactic acid bacteria (data not shown). *Paenibacillus* spp. in particular form colonies accompanied by a yellow halo on PCABP, and it is therefore easy to mistake them for lactic acid bacteria in routine bacterial analysis. Lactic acid bacterial contamination in cooked foods is almost always caused by cross-contamination after cooking because lactic acid bacteria have low heat tolerance (Hamasaki et al., 2003) while *Paenibacillus* spp., originating from ingredients, survive during the cooking process and then contaminate foods. Because the measures that must be taken for bacterial control are different in the case of contamination by these two bacterial groups, contamination by *Paenibacillus* spp. must be distinguished from contamination by lactic acid bacteria. In routine analysis, catalase testing is a good method for distinguishing between these two groups; *Paenibacillus* spp. show positive results whereas lactic acid bacteria show negative results.

The heat tolerance of psychrotrophic spore-forming bacteria tends to be lower than those of other spore-forming bacteria. For example, non-proteolytic *C. botulinum* type B and psychrotrophic *B. cereus* indicate D-values of 0.8 and 2.2-9.2 min, respectively, at 90°C (Juneja et al., 1995; Dufrenne et al., 1995). Furthermore, *Paenibacillus polymyxa* indicates the D-value of 1.3-7.7 min at 90°C in milk (Casadei et al., 2000). It is expected that retort sterilization is unnecessary to sterilize psychrotrophic spore-forming bacteria, but at least several minutes of heating at approximately 90°C are needed for destruction of these bacteria. In recent years, manufacturers have tended to avoid excessive heating during the cooking process, so as to preserve taste. Therefore, foods are being cooked for shorter amounts of time or at lower temperatures, and psychrotrophic spore-forming bacteria can easily survive in these cooked foods. If there is a risk of such bacterial contamination, manufacturers need to cook foods more thoroughly.

In conclusion, psychrotrophic *Paenibacillus* spp. were newly isolated as spore-forming bacteria which survive the cooking process, grow at low temperatures and then cause food spoilage. To preserve food, these must be controlled similarly to psychrotrophic *Bacillus* spp. and *Clostridium* spp. Because psychrotrophic *Paenibacillus* spp. can easily be mistaken for lactic acid bacteria in routine analysis, the person in charge of bacterial analysis should give proper attention to the presence of these bacteria.

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