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短報（Note）

Strain Typing of *Bacillus subtilis* (*natto*) Using a Modified Randomly Amplified Polymorphic DNA Method

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Abstract

In the process of fermentation of the soybean food natto, particular *Bacillus subtilis* strains, termed *B. subtilis* (*natto*) or *B. subtilis* var. *natto*, are selectively used. *B. subtilis* (*natto*) strains produce viscous substances to a greater extent than do other common *B. subtilis* strains. Randomly Amplified Polymorphic DNA (RAPD) strain typing of *B. subtilis* (*natto*) is difficult, although discrimination between *B. subtilis* (*natto*) and other *B. subtilis* strains is easier. In this study, a modified RAPD analysis method for strain typing of *B. subtilis* (*natto*) has been developed. To discriminate among strains, PCR was performed for each genomic DNA template using a selected 21-mer primer specific for the insertion sequence IS*4Bsu1*, which differs in frequency and position in almost all *B. subtilis* (*natto*) strains, and 11 RAPD primers. Thus, the 11 PCR products of each strain may be compared. Applying this method, several *B. subtilis* (*natto*) and *B. subtilis* strains were discriminated, regardless of their retention of IS*4Bsu1*.

Keywords: *Bacillus subtilis* (*natto*), RAPD, strain typing, insertion sequence IS*4Bsu1*

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Natto is a traditional fermented soybean food in Japan, and is recognized as a probiotic (Hosoi and Kiuchi, 2008). For natto production, cooked soybeans are fermented with particular *Bacillus subtilis* strains, termed *B. subtilis* (*natto*) or *B. subtilis* var. *natto*. Although *B. subtilis* (*natto*) and other *B. subtilis* strains possess similar sequences of the 16S rRNA gene, *B. subtilis* (*natto*) strains have a tendency to produce a viscous substance (a mixture of polyglutamic acid and fructan) to an extent greater than shown by other common *B. subtilis* strains, including *B. subtilis* Marburg 168 and the type strain of *B. subtilis*. Between-strain differences in genome structure are known (Itaya and Matsui, 1999; Qiu et al., 2004), and the ease of formation of competent cells differs between *B. subtilis* (*natto*) and other *B. subtilis* strains (Ashikaga et al., 2000). In addition, certain *B. subtilis* (*natto*) strains have been reported to retain the insertion sequences IS*4Bsu1* and IS*256* in genomic DNA, but other *B. subtilis* strains do not (Nagai et al., 2000; Qiu et al., 2004; Kimura and Ito, 2007). The frequencies and positions of IS*4Bsu1* in genomic DNA differ depending on the *B. subtilis* (*natto*) strain examined (Nagai et al., 2000; Qiu et al., 2004). When IS*4Bsu1* transposes into the *comP* gene controlling synthesis of polyglutamic acid, production of the viscous substance is reduced.

For strain typing of microbes, Randomly Amplified

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Table 1. Primer list used in this study.

Name	Target	Sequence	References
RAPD 1	-	5'-AGTCAGCCAC-3'	Lett. Appl. Microbiol., 43, 237 (2006)
RAPD 2	-	5'-CCGAGTCCA-3'	Lett. Appl. Microbiol., 27, 168 (1998)
RAPD 3	-	5'-GTTTCGCTCC-3'	Appl. Environ. Microbiol., 67, 1035 (2001)
RAPD 4	-	5'-AAGAGCCCGT-3'	Appl. Environ. Microbiol., 67, 1035 (2001)
RAPD 5	-	5'-CCGCAGCCAA-3'	Lett. Appl. Microbiol., 32, 139 (2001)
RAPD 6	-	5'-TGCCGAGCTG-3'	Appl. Environ. Microbiol., 65, 5182 (1999)
RAPD 7	-	5'-AATCGGGCTG-3'	Appl. Environ. Microbiol., 65, 5182 (1999)
RAPD 8	-	5'-CAATCGCCGT-3'	Appl. Environ. Microbiol., 65, 5182 (1999)
RAPD 9	-	5'-GGTGATCAGG-3'	Plant Cell Rep., 21, 814 (2003)
RAPD 10	-	5'-CCGGCGGCG-3'	Int. J. Syst. Evol. Microbiol., 52, 101 (2002)
RAPD 11	-	5'-AGTCGGGTGG-3'	J. Clin. Microbiol., 35, 2573 (1997)
RAPD 12	-	5'-AGGGGGTTCC-3'	J. Clin. Microbiol., 35, 2573 (1997)
IS*4Bsu1* 124F	IS*4Bsu1*	5'-AAGGACAATAAGCATGGATAAG-3'	J. Bacteriol., 182, 2387 (2000)
IS*4Bsu1* 635F	IS*4Bsu1*	5'-GATAAAGCGGTGCTGACAAACG-3'	in this study
IS*4Bsu1* 1280F	IS*4Bsu1*	5'-TGCACTCGTCAAAGATTATAG-3'	in this study
IS*4Bsu1* 145R	IS*4Bsu1*	5'-CTTATCCATGCTTATTGTCC-3'	in this study
IS*4Bsu1* 1235R	IS*4Bsu1*	5'-TTCGTAGCCATAAATGAGCGGA-3'	in this study
IS*4Bsu1* 1301R	IS*4Bsu1*	5'-ACTATAATCTTTGACGAGTGCA-3'	J. Bacteriol., 182, 2387 (2000)

Polymorphic DNA (RAPD) and/or Pulsed Field Gel Electrophoresis (PFGE) are often used. However, strain typing of *B. subtilis* (*natto*) by RAPD analysis is difficult, possibly because only small differences in genome structure exist between *B. subtilis* (*natto*) strains (Qiu et al., 2004). Although it has been reported that RAPD analysis discriminated several *B. subtilis* strains obtained from various fermented soybean foods in Thailand, using a single RAPD primer (Table 1, denoted as 'RAPD 1') (Inatsu et al., 2006), we confirmed that the primer RAPD 1 did not permit discrimination among several *B. subtilis* (*natto*) strains used in natto production in Japan (data not shown).

We therefore developed a new method for strain typing of *B. subtilis* (*natto*) strains, by modification of the RAPD analysis protocol. We selected 11 other RAPD primers (Table 1, denoted as 'RAPD 2'-'RAPD 12'), most of which have been used for RAPD strain typing of genus *Bacillus* strains. As PCR templates, we isolated genomic DNA from 25 strains of *B. subtilis*, including *B. subtilis* (*natto*) NBRC 3013 and 13169 (NITE Biological Resource Center), *B. subtilis* TFC 4205 (Tokyo Metropolitan Food Technology Research Center), and *B. subtilis* JCM 1465^{T} (Japan Collection of Microorganisms, RIKEN BioResource Center), using a DNeasy Plant Mini Kit (Qiagen). *B. subtilis* (*natto*) NBRC 3013 and 13169 retain the insertion sequence IS*4Bsu1*, whereas *B. subtilis* TFC 4205 and *B. subtilis* JCM 1465^{T} do not possess IS*4Bsu1*, as was confirmed by PCR using two primer pairs specific for IS*4Bsu1* (Table 1, denoted as 'IS*4Bsu1* 124F'-'IS*4Bsu1* 1301R' and 'IS*4Bsu1* 635F'-' IS*4Bsu1* 1235R') (data not shown). We first confirmed that it was impossible to discriminate among the four *B. subtilis* strains by RAPD analysis using any of the 11 RAPD primers ('RAPD 2'-'RAPD 12') in the manner in which the primer 'RAPD 1' is employed (data not shown).

However, we found that strain typing could be achieved by comparing the 11 PCR products from each strain by agarose gel electrophoresis. The PCR products were obtained using a definitive 21-mer primer specific for IS*4Bsu1* (Table 1, denoted as 'IS*4Bsu1* 1280F') plus each of 11 short RAPD primers (Table 1, denoted as 'RAPD2'-'RAPD12'), employing the HotStarTaq Master Mix Kit (Qiagen). Of the six tested primers specific for IS*4Bsu1* (Table 1, denoted as 'IS*4Bsu1* 124F'-'IS*4Bsu1* 1301R'), primer IS*4Bsu1* 1280F was optimal to discriminate among strains in the modified RAPD analysis of the present study. Figure 1 shows results for

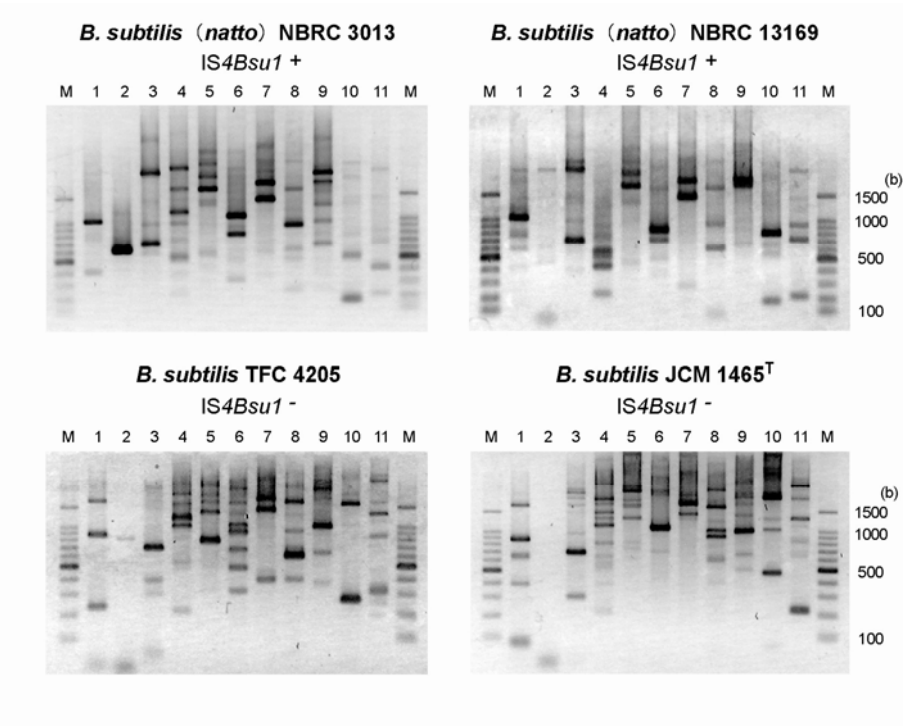

Fig. 1. Strain typing of four *B. subtilis* strains by modified RAPD analysis.

PCR was performed using the primer 'IS*4Bsu1* 1280F' shown in Table 1, plus each of 11 RAPD primers ('RAPD 2'-'RAPD 12'), with the HotStarTaq Master Mix Kit (Qiagen). After PCR, the 11 PCR products for each strain (lanes 1-11) were examined by agarose gel electrophoresis. The PCR protocol comprised an initial heat activation step at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 60 s, annealing at 36°C for 60 s, extension at 72°C for 180 s, and a final extension at 72°C for 5 min. Retention of IS*4Bsu1* in each strain was explored by PCR using two primer pairs (IS*4Bsu1* 124F-1301R and 635F-1235R). Lane M: Molecular size marker (100 bp DNA Ladder; Takara Bio).

the four *B. subtilis* strains *B. subtilis* (*natto*) NBRC 3013 and 13169, *B. subtilis* TFC 4205, and *B. subtilis* JCM 1465^T. We also confirmed that the electrophoretic banding patterns differed depending on the type of PCR enzyme kit used. We employed a HotStarTaq Master Mix Kit (Qiagen), an Amplitaq Gold PCR Master Mix (Applied Biosystems), and an RBC SensiZyme Hotstart Taq Premix (RBC Bioscience) (data not shown).

Although *B. subtilis* TFC 4205 and JCM 1465^T do not possess IS*4Bsu1*, the primer IS*4Bsu1* 1280F functioned to produce varying PCR amplicons, resulting in different electrophoretic banding patterns. We suggest that the 3' end of the primer IS*4Bsu1* 1280F anneals to certain sites in genomic DNA of *B. subtilis* strains regardless of retention of IS*4Bsu1*, and that genomic sequences near the binding sites of the primer IS*4Bsu1* 1280F are more diverse than those of the other five primers tested.

In conclusion, we have developed a modified RAPD analysis method to discriminate among *B. subtilis* (*natto*) strains, using a primer specific for IS*4Bsu1* plus 11 short RAPD primers. This method is useful to discriminate between and conduct quality-control for *B. subtilis* (*natto*) strains, which form colonies with similar morphology. To obtain higher resolution and/or to simplify the method further, modifications in primers, PCR conditions, and the PCR polymerase used, may be effective.

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改変RAPD法による納豆菌及び枯草菌の菌株識別

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摘　要

大豆発酵食品のひとつである納豆の製造には，枯草菌 *Bacillus subtilis* のうち，納豆菌 *Bacillus subtilis* (*natto*) と呼ばれる粘質物産生能の高い菌株が使用されている．しかしながら，Randomly Amplified Polymorphic DNA（RAPD）法を用いて菌株識別を実施すると，納豆菌と枯草菌間の識別は比較的容易なものの，納豆菌間の菌株識別は困難である．そこで，RAPD 法の改変による納豆菌の菌株識別法の開発を試みた．その結果，各菌株のゲノム DNA を鋳型として, 納豆菌がゲノム上に異なる様式で保有しているとされる挿入配列 IS*4Bsu1* に特異的な 21mer のプライマー1 種と RAPD プライマー11 種類を組み合わせて PCR を行い，得られる 1 菌株につき 11 種類の PCR 増幅産物をアガロースゲル電気泳動により菌株間で比較することにより，納豆菌及び枯草菌の菌株識別が可能であることが明らかとなった．また，本法は，IS*4Bsu1* を保有しない枯草菌株間の識別にも適用可能であった.

キーワード：納豆菌，枯草菌，RAPD 解析，菌株識別，挿入配列 IS*4Bsu1*

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