Among 45 Bacillus subtilis strains isolated from non-salted types of fermented soybean products produced in several Southeast Asian countries, 20 had the insertion sequence IS4Bsu1 in the chromosome. In contrast, none of 49 B. subtilis strains of non-food origin contained IS4Bsu1. Frequent occurrence of this mobile DNA element in the soybean-fermenting B. subtilis would reflect the fact that few strains flourish on soybeans and thereby contribute to soybean fermentation.

Key words: Bacillus subtilis; insertion sequence; soybean fermentation; Southern blotting

A variety of fermented soybean foods are produced in Southeast and East Asia. Unlike natto (a Japanese non-salted type of soybean food) fermented by a pure Bacillus subtilis starter, naturally occurring microorganisms or seeds (a portion of the product) are used to produce other non-salted types of Asian fermented soybeans.1–4) B. subtilis is a major microorganism in Asian fermented soybeans,1–5) so this bacterium likely has a key role in the fermentation processes as do the natto starter B. subtilis strains. However, little is known whether specific B. subtilis strains prevail in fermenting soybeans or diverse B. subtilis strains can ferment soybeans. Assuming only a few B. subtilis strains can grow readily on soybeans, they would have much chance to acquire common transferable genetic elements such as plasmids and insertion sequences (ISs), small mobile DNA elements capable of horizontal transmision via plasmids, bacteriophages, or genetic transformation. Because of their transmissible nature, IS elements would be distributed widely among bacteria sharing the same niche where they can easily make contact for conjugation or they may become infected by the same phage and receive DNA from neighboring cells.6–9) Early studies1–3,10) reported frequent distribution of mobilizable plasmid pUH1 (=pTA1015) among B. subtilis strains isolated from Asian fermented soybeans, implying that certain B. subtilis strains would frequently occur in the fermenting soybeans, although plasmids appear not to contribute to soybean fermentation.11) IS4Bsu1 is the first IS discovered in B. subtilis as a genetic element that causes genetic instability of the poly-γ-glutamate (γ-PGA) production by translocating at high frequency into the comP gene encoding a sensor-kinase of the ComQXPA quorum sensing system essential for γ-PGA synthesis.12,13) IS elements have been used as markers in diagnosis and epidemic studies.14,15) This study examined frequency of IS4Bsu1 among B. subtilis strains isolated from the Asian fermented soybean foods.

A total of 36 B. subtilis strains were isolated from fermented soybeans, including doucha from China and Myanmar, mac thua nai from Laos, thua nai from Thailand, and chine peboke from Myanmar and identified according to Bergey’s manual.16) Only two strains were selected from each product to minimize isogenic strains. Nine B. subtilis strains isolated previously from different Nepalese kinema products5) were obtained from the MAFF Gene Bank (http://www.gene.afrc.go.jp/index_j.html). Chromosomal DNA extracted from 45 isolates was digested with the restriction endonuclease EcoRV, which does not cut IS4Bsu1, resolved by agarose gel electrophoresis and blotted onto nylon membranes.12) Fragments of DNA carrying the IS were detected with use of the PCR-amplified IS4Bsu1 sequence (from nucleotides [nt] 124 to 1301) as the probe.12) Among the 45 strains, 20 of them (44%) had an IS element (Fig. 1). These consisted of 2 of 4 isolates from Chinese doucha, 9 of 10 from Thai thua nai, 5 of 9 from Nepalese kinema, 1 of 6 from mac thua nai of Laos and 3 of 12 from chine peboke of Myanmar. None of the 4 strains from doucha of Myanmar contained an IS element. Except that the two isolates from Chinese doucha showed the same Southern blot profile of a single band, only one of the pairs had the...
IS or the pairs showed different IS profiles. Some strains from *thau nao* of Cheng Mai and Cheng Rai (Thailand) had very similar, but distinct Southern profiles, whereas others had quite different profiles. Likewise, *B. subtilis* strains having identical or very similar IS profiles also occur in independent products of Nepalese *kinema*: the presence of the two bands common to all isolates from *kinema* suggests that these strains share the same origin. These results indicate that *IS4Bsu1* appears to be distributed frequently among the *B. subtilis* strains in the Asian fermented soybeans. We wondered whether or not the frequency of *IS4Bsu1* is also high in phage-typed *B. subtilis* strains of non-food origins. We accordingly analyzed a total of 49 strains obtained from H. W. Ackermann at Laval University, Quebec.\(^{(17)}\) Again, only one or two strains from each of 29 phage-types strains\(^{(17)}\) were examined to avoid duplication of the same strains. All of these strains had the IS (data not shown), ruling out the notion that *IS4Bsu1* is ubiquitous in *B. subtilis* strains.

*B. subtilis* (*natto*) carries the conjugative plasmid pLS20 of 55 kb and the small mobilizable plasmid pTA1015 of 5.8 kb.\(^{(18,19)}\) These and related plasmids have been identified in *B. subtilis* (*natto*) strains.\(^{(11,20,21)}\) However, neither pLS20 nor pTA1015 has *IS4Bsu1*.\(^{(12,19)}\) Furthermore, because no bands emitted intense signals due to a copy number effect of a plasmid and the IS are present as multiple copies in most strains, the investigated strains may have the IS in the chromosome. Irrespective of the mechanism involved in horizontal transfer of the IS, the sequences adjacent to the IS might have a clue to the transfer mechanism. We therefore analyzed the flanking sequences of the IS in the isolate (strain NFRI83552) from Chinese *doucha*, which has a single copy of *IS4Bsu1*. The chromosomal DNA of this strain was digested with the restriction endonuclease EcoRV and circularized using a DNA ligation kit (Takara Shuzo). The flanking regions were then amplified by inverse PCR with the ligated DNA as the template and the oligonucleotide primers, 5’-*CTC*\(^{\text{GC}}\)\(^{\text{AGTTTGTGGAAGAAATCGG}}*-3’ and 5’*-GGA*\(^{\text{AAATCCCGCTATTATG}}\text{GCTACG-3’ which correspond to nt 182–206 (complementary) and 1209–1233 of *IS4Bsu1* and* nucleotide sequences adjacent to the IS were determined. The flanking sequences of 350 bp analyzed showed that this sequence corresponds (97% identity) to positions 2,723,972 to 2,724,312 of the *B. subtilis* 168 genome,\(^{(22)}\) and that the IS is located at 2,724140 in the intergenic region between the *cycD* and *yrdN* genes encoding a putative membrane protein (290 amino acids) and a protein (129 amino acid) of no suggested function\(^{(22)}\) respectively (Fig. 2). These flanking genes are also present in *B. subtilis* 168 and have no homology to known plasmid and phage genes, so the transmission mechanism of *IS4Bsu1* remains unclear.

An important notion of this study is that *IS4Bsu1*, like plasmid pUH1, tends to occur frequently among *B. subtilis* strains in fermented soybeans, such as *thau nao* from Thailand and in *kinema* from Nepal. *B. subtilis* strains may not occur at random in these fermenting soybeans, rather, particular strains or groups of strains of *B. subtilis* likely arise and become involved in the fermentation process. Although a genetic mechanism underlying transmission of the IS element is unknown, the transmission frequency may not be high. Therefore, there must be many strains without the IS even if they share the same niche with donor strains.

**Fig. 1.** Southern Blots of *IS4Bsu1* in *B. subtilis* Strains Isolated from Fermented Asian Soybeans. Only one is presented when isolates from the same source showed the same fragment profile of *IS4Bsu1*. Brackets, a pair of strains from the same product; circles, two isolates with the same profile; triangles, only one strain had an IS. Fermented soybean products and their countries are abbreviated as follows: C, chine peboke; D, doucha; K, kinema; T, *thau nao*; M, *mac thau nau*; N, the *natto* starter *B. subtilis* (natto) Miura\(^{(19)}\); Ch, China; Jp, Japan; La, Laos; My, Myanmar; Np, Nepal; Th, Thailand.

**Fig. 2.** Location of *IS4Bsu1* on the Chromosome of Strain NFRI83552. Numbers refer to nucleotide positions of the *B. subtilis* 168 genome and gene annotation is according to Kunst *et al.*\(^{(20)}\) Flanking inverted repeats, IRL and IRR, of *IS4Bsu1*\(^{(13)}\) are not drawn to scale.
Acknowledgment

We thank H. W. Ackermann and the MAFF Gene Bank for the phage-typed \textit{B. subtilis} strains and the typing phages and for the \textit{B. subtilis} strains from \textit{kinema}, respectively.

References


