Preliminary Communication

Conversion of Bacillus subtilis 168: Natto Producing Bacillus subtilis with Mosaic Genomes

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Sequential replacement of sequences in the Bacillus subtilis 168 genome with DNA from Bacillus subtilis (natto) conferred the trait of the ability to ferment soybeans to B. subtilis 168 as its genome became mosaic. All mosaic strains retained competence, an intrinsic polygenic trait of the recipient B. subtilis 168.

Key words: Bacillus subtilis; Bacillus subtilis (natto); mosaic genome; horizontal transfer; transformation

Bacillus subtilis and Bacillus subtilis (natto) are endospore-forming Gram-positive bacteria. B. subtilis (natto) ferments unsalted boiled soybeans for production of the traditional Japanese food natto. This fermentation is complicated and polygenic, that is, regulated by a number of genes. Proteases secreted by B. subtilis (natto) growing on soybean surfaces degrade soy proteins to oligopeptides and amino acids. These products are converted to poly(γ-glutamic acid), part of the mucous material on the soybean surface characteristic of natto as shown in Fig. 1, and to other metabolites that confer the characteristic fragrance and tastiness, that is termed umami in Japanese.

B. subtilis does not ferment beans but can take up foreign DNA with sequences similar to its own and integrate it into its own genome by recombination. The trait is regulated by at least 39 genes in the 4,215-kb genome. DNAs longer than 50 kb can be transferred to the B. subtilis 168 genome. Substitutions at least two regions take place randomly along the recipient genome. This promiscuous integration is useful when we want to assemble natto genes in the B. subtilis 168 genome. B. subtilis (natto) BEST195, used as the DNA donor strain, was from our laboratory collection. It has no indigenous plasmids. None of the BEST195 genome has been sequenced, but its SfiI restriction map (unpublished results) is similar to that of B. subtilis 168. The number and location of natto genes in the B. subtilis (natto) genome are not known, but natto traits were not conferred on B. subtilis 168 in an experiment with only one transfer (unpublished results). Perhaps certain parts of the B. subtilis (natto) genome need to be assembled first, or some kind of physiological adaptation is needed. Here, we repeated DNA transfer a total of seven times in both selective and nonselective ways.

In BEST3055, a starter recipient strain of B. subtilis 168, twelve SfiI sites have been eliminated. BEST3055 has three antibiotic resistance markers in three of the 12 lost sites: neo (neomycin resistance) between the ywnE and mta genes, bsr (blasticidin S resistance) between the yapA and yxyF genes, and cat (chloramphenicol resistance) between the yvdC and yvdB genes. The absence of SfiI allowed us to detect substitutions by the SfiI site from the B. subtilis (natto) genome.

Bacteria were grown at 37°C in all media except at 42°C in natto production. Genomic DNA in liquid form prepared by the method of Saito and Miura (1999) was used for the transfer experiments. Endonuclease SfiI was obtained from New England Biolabs. (Beverly, Mass., USA). Protocols for the preparation and transformation of competent B. subtilis 168 cells were as described elsewhere, as were the preparation and digestion of genome DNA for gel electrophoresis on a contour-clamped homogeneous electric field.

In the first four transfers ([1] to [4] in Fig. 1) replacements at the seven SfiI loci of the recipient genome were made.

The tetracycline resistance marker leuB::tet was introduced into BEST3055 with plasmid pBMAP105TT, giving rise to BEST3095. The leuB::tet marker (blue letters "tet" in Fig. 2) was used repeatedly as a catalyst marker. In the first transfer [1], B. subtilis (natto) genomic DNA was added to competent BEST3095 cells, of which 67 leu+ transformants were then selected. This change from leuB::tet to leuB occurred because of substitution by the corresponding leuB region of the B. subtilis (natto) genome. The concentration of B. subtilis (natto) DNA used was at least 3 μg/ml to allow substitutions at other loci than leuB. Screening of the leu+ transformants for recombinants that had lost antibiotic sensitivity gave BEST3096, which had lost both bsr and neo as indicated by [1] in Fig. 2. cat marker had not been replaced in BEST3096. From new SfiI fragments identified by pulsed-field gel electrophoresis, the replaced loci were located with reference to the original physical map. One additional region was replaced as indicated by [1] in Fig. 2.

Before the second transfer, leuB::tet was introduced into BEST3096 with the plasmid pBMAP105TT, giving in BEST3098. In the second transfer [2], the change from leuB::tet to leuB+ selected for and we found 228 leu+ transformants, from which BEST3102, which had lost cat, was isolated. BEST3102 had acquired one more SfiI site indicated by [2] in Fig. 2 in addition to the expected locus.

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Fig. 1. Assembling Genes for natto Production in the B. subtilis 168 Genome by Repeated Horizontal Transfer.

Left: Progressive substitution of the B. subtilis 168 genome (red circle) by B. subtilis (natto) DNA (pink arcs). Details are described in the text.

Middle: Boiled soybeans fermented by the strains indicated. Fermentation of boiled soybeans,\(^4\) for 24 hours at 42°C produces characteristic viscous materials and the natto fragrance. Fermentation process is described in detail in the text.

Right: Phenotypes 1 to 4 are categorized as absent (−) to very pronounced (+ + +). (1) Competence, measured in terms of tetracycline-resistant colonies as described elsewhere.\(^5\) (2) Growth on Spizizen plates with tryptophan, leucine, and proline.\(^6\) (3) Colonies grown on a plate that contained the soybean extract, Phyton, for 24 hours at 42°C, were wrinkled and mucous.\(^7\) (4) Protease secretion was measured in terms of the diameter of the halo that formed in the plate, which also contained casein.\(^8\)

The leuB gene of BEST3102 was reused for direction of substitution at the locus in the ndhF gene after being marked by neo using pSOFT10A\(^9\) indicated by [3] in Fig. 2. Similarly, the genomic locus [4] in the yhcV gene marked by neo (pSOFT1A\(^9\)) was used for replacement in transfer [4]. We isolated BEST3106 and BEST3109 in transfers [3] and [4], respectively. Because loci [1] to [4] do not have genes related to competence,\(^1\) BEST3109 did not affect the ability of competence (Fig. 1).

Nonregional directed substitutions were done in three consecutive screenings for four natto-related phenotypes. The traits were utilization of phyton, formation of natto-like materials on soybean surface, production of natto fragrance, and protease secretion. Three intermediate variants (BEST3125, BEST3136, BEST3145) were isolated in transfers [5], [6], and [7]. The leuB::tet catalyst marker was used as before in these transfers. The gradual acquisition of the natto-related phenotypes was in parallel with the gradual increase in the total amount of replacement. SfiI patterns of BEST3145 (not shown) did not differ from those of BEST3136, although the ability to produce natto increased. This finding indicates that the additional DNA transfer to BEST3136 in the transfer [7] occurred at loci distant from those not containing SfiI.

Possibly the natto genes that accumulated in the recipient genome changed a non-natto producer to a producer without loss of competence traits. The stable intermediate variants shown in Fig. 1 had phenotypes somewhere between the two extremes. Several groups have reported conversion of a small part of the recipient B. subtilis 168 genome,\(^11\)-(13) but our results showed that repeated transfer created greater genomic diversity.

It was hard to estimate the exact length of the substitutions because (i) the ends of the substitution were not clear and (ii) substitutions not containing SfiI sites could not be detected by this method. An estimation was made from results for a 27-kb substitution at one locus reported elsewhere.\(^14\) BEST3145 had at least 12 substituted loci. On this basis, 324 kb (= 27 kb × 12), 7.7% of the 4,215 kb B. subtilis genome, was mosaic. BEST3145 should be a useful starter strain for the investigation of natto production.

The new concept of 'laundering the genome' may be
applicable to other bacterial conjugation or transformation systems, providing experimental approaches to the design of bacterial genomes for medical or industrial uses. It may occur more often in nature than is believed now. 15

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**References**


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