Application of LFH-PCR for the Disruption of *SpoIIIE* and *SpoIIIG* of *B. subtilis*

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Abstract The application of LFH-PCR (long flanking homology region-PCR) for *Bacillus subtilis* gene disruption is presented. Without plasmid- or phage-vector construction, only by PCR, based on a DNA sequence retrieved from *B. subtilis* genome data base, kanamycin resistance gene was inserted into two genes of *B. subtilis* involved in sporulation, *spoIIIE* and *spoIIIG*. The effect of gene disruption on subtilisin expression was examined and the sporulation frequency of two mutants was compared to that of the host strain. For this purpose, only 2 or 3 rounds of PCR were required with 4 primers. We first demonstrated the possibility of LFH-PCR for rapid gene disruption to characterize an unknown functional gene of *B. subtilis* or other prokaryote in the genomic era.

Keywords: *B. subtilis*, LFH-PCR, functional genomics

INTRODUCTION

With the coming of the genomic era, a huge quantity of sequence information is available, and new ORFs with unknown function are being designated. Currently the complete sequences of about 20 organisms have been published, and more organisms are being sequenced with accelarating speed. But the gap between sequence data and functional data becomes deeper. This means that the classical, one by one gene deletion methods used for functional analysis of new ORFs are no longer adequate. Some methods for the large scale functional analysis of unknown ORFs have been introduced. Serial Analysis of Gene Expression, SAGE, [1] is a smart and ingenious method but it requires complicated sample preparation and a laborious amount of sequencing work. Genetic footprinting [2] or the transposon derived method has the limitation of unequal insertion of transposable elements into the chromosome. The array based approach also has the limitation that a large number of cDNAs or PCR products must be prepared, purified and processed on the appropriate membrane or template [3-5]. Among the methods described above, the mRNA mediated method can not be applied to prokaryotic gene functional analysis due to the difficulty of specific mRNAn isolation. Results obtained with the methods mentioned above do not give as much information on the function of a gene as the classical molecular biological approach does. Therefore, it is of great concern to disrupt target ORFs to examine the specialized function of unknown genes. Antibiotic marker insertion in the middle of target ORF is one of the easiest and direct methods of target gene disruption, but the method has many limitations. Besides all the laborious molecular cloning required such as restriction enzyme digestion, ligation and sequencing woks, it needs an approapriate restriction enzyme site for molecular cloning to give enough length for homologous recombination. Other general methods [6,7] to delete or replace bacterial ORF also include classical cloning. For yeast, very simple and efficient methods have been developed [8,9] using only PCR as an experimental tool. In *S. cerevisiae*, because of the high efficiency and accuracy of mitotic recombination, even with 30-50 base pair short-flanking region, homologous recombination is possible [10]. More improved methods with long-flanking homology regions (LFH-PCR) have been devised to enhance the homologous recombination frequency [8,10]. The EUROPAN project, which involves one by one deletion of yeast unknown-function genes, is being carried out using the method. For prokaryotes, with different homologous recombination mechanisms, no such simple method [9,12] has been reported until now. Furthermore, suggested improved method used in *S. cerevisiae* [8,11] has not been adopted in prokaryotes. Methods reported in some publications [6,7] require plasmid vector construction and sequencing of the constructed region, and therefore, are inadequate for large scale and rapid gene disruption.

Bacillus subtilis is one of the most important host systems for the production of industrial enzymes due to its capability to express and secret useful proteins. To develop *B. subtilis* as a host for the production of recombinant foreign proteins, the development of strong promoters, the enhancement of plasmid stability, the improvement of protein secretion with fusion signal

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sequence, or harnessing the secretory action to the host cell, the deletion of various proteases in the host, and the prolongation of production time, by using spore negative mutants are common targets to be considered. We have been interestered in *B.subtilis* sporulation mutant as a host for foreign protein expression for some time [13-15]. In the course of several sporulation mutants constructions, we experienced difficulty because of the short ORF length and the limited number of restriction enzyme site. In this study, we report the first application of LFH-PCR for the disruption of genes involved in sporulation in *B.subtilis* and examine its effect on subtilisin expression.

MATERIALS AND METHODS

Strain and Culture Condition

DB104::pMK101 [13-15] which has about 5 copies of subtilisin reporter gene on the chromosome, was used as a host strain for gene disruption. Schaeffers' sporulation media containing 5 ppm of chloramphenicol was used for subtilisin expression. Chromogenic substrate, n-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was used for the analysis of subtilisin activity [13-15]. Sporulation frequency was measured by heat treatment at 80°C for 15 min using samples cultured in Schaeffers' media. After transformation of the PCR product into *B.subtilis* DB104::pMK101, sporulation mutants were selected on a TBAB plate containing 10 mg/L of kanamycin and 5 mg/L of chloramphenicol .

DNA Manipulations

Basic molecular biology methodology used in this study, such as, pUB110 plasmid preparation, *B. subtilis* chromosome preparation, and two-step method for the transformation of PCR product into *B. subtilis* host DB104::pMK101 were as previously described [16].

Amplification of Target Gene and Purification of PCR Product

Expand™ Long Template PCR System (Boehringer Manheim) was used for PCR. Gene sequences for *spoIIIE* and *spoIIIG* were retrieved from *B. subtilis* genome site (http://www.pasteur.fr/Bio/subtiList.html), and the sequence for *Km^r* was obtained from japan bacillus genome data base (http://www.genome.ad.jp). Oligonucleotides were synthesized at Bioneer (Taejeon, Korea). The sequences of primers used in this study are shown in Table 1. GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) was employed for thermal cycling. PCR products were purified with the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA) using a 0.8% low melting agarose gel, if necessary.

a) step 1 : Mega primer synthesis
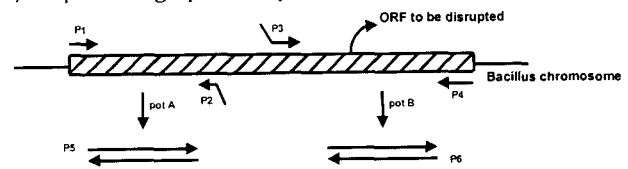
b) step 2 : Final product synthesis
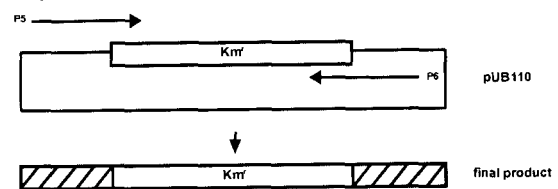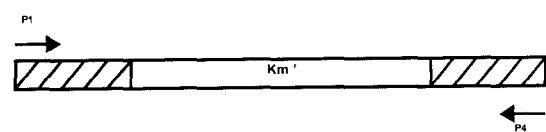

c) step 3 : Harvest of final product

Fig. 1. Strategy for the synthesis of the PCR product needed for target gene disruption; (a) synthesis of the two megaprimer P5 and P6 (step 1) : the dashed portion is ORF, which is disrupted by antibiotic marker insertion. DNA was amplified by PCR in a total volume of 50 μL. P5 and P6 were separately amplified in a different PCR tube containing 1.7 units of DNA polymerase, 50 ng of *B.subtilis* chromosome as template, 2 μM of each primer and 200 μM of each dNTP. An initial denaturation step of 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and elongation at 68°C for 1 min; (b) synthesis of the final product (step 2) : two megaprimer, P5 and P6 were sliced and purified in a single Wizard purification column with 40 μL of D.D.W. to prevent dilution. 3.5 units of DNA polymerase was used. 20 ng of pUB110 were used as template, dNTP concentration was the same as in step 1 and the reaction volume was adjusted to 50 μL. Denaturation and annealing conditions were the same as step 1. Initial 10 cycles was performed with 2 min of elongation and during the following 25 cycles, the elongation time was increased in every cycle by 10 seconds; (c) harvesting of the deactivated fragment (step 3) : To produce larger quantitties of product, it may be further amplified using primer 1, primer 4 and product from step 2 as template. For 50 μL of reaction mixture, primer concentration, dNTP concentration and amount of DNA polymerase were the same as in step 1. 1 μL of PCR product of step 2 was used as template. Thermal cycling conditions were as used in step 2.

RESULTS AND DISCUSSION

Description of the LFH-PCR Method

Target gene disruption by LFH-PCR involved two or three rounds of PCR. PCR products obtained from the first round of PCR were used as mega-primers in second

Table 1. Primer sequences used for gene disruption

SpoIIIE
Primer 1 : 5'-GCAGAACAGAACCAAGAGAC-3',
Primer 2 : 5'-<u>TCATCGGCTTTTTCGTCATCATCTG</u>-TTCCTGATCACCTGTCTCCGGTTGG-3'
primer 3 : 5'-<u>GTCTATCCCGGCATTGCCAGTCGGG</u>-GGCGTAAAGGCGAAAGTCACACAGG-3'
primer 4 : 5'-CTGCCTTCATACGGTCCGAC-3'

SpoIIIG
Primer 1 : 5'-AATTTTATGGTTAGAACCCC-3'
Primer 2 : 5'-<u>TCATCGGCTTTTTCGTCATCATCTG</u>-CCGCTCTCTTACCTGAAGCGCCTT-3'
Primer 3 : 5'-<u>CTATCCCGGCATTGCCAGTCGGGGA</u>-TAATCAGTGAGACAAGCAAGGAGC-3'
Primer 4 : 5'-CGTGGTCCTCCTCTTTATTG-3'

round PCR. Four primers were needed, primer 1 and primer 4 were normal at corresponded to each side of target gene. Primers 2 and 3 were made of two parts, one for the target gene and the other for antibiotic marker. The method is illustrated in Fig. 1.

Disruption of S*poIIIE* of *B. subtilis*

SpoIIIE of *B. subtilis* was selected as target gene for the application of LFH-PCR for the gene disruption. *SpoIIIE* participates in chromosome segregation during the initial process of spore formation, and its disruption can be easily confirmed by asporegeneous phenotype. Furthermore, its ORF (2316 base pairs) is long enough to provide sufficient length for homologous recombination at each side of the inserted antibiotic marker. Km^r gene in pUB110 was used for the inserting marker. Four primers were designed to give sufficient length for homologous recombination, considering GC contents. In primer 2 and primer 3, the underlined parts are for Km^r gene (Table 1). The synthesized mega-primers, P5 and P6 are shown (Fig. 2(a)). After step 2, very faint PCR product corresponding to exact size (3829 base pairs) were observed (data not shown). After step 3, sufficient quantity of PCR product was obtained (Fig. 2(a)). Without further purification, the total PCR product (50 μL) was used directly for transformation into *B. subtilis* DB104::pMK101(Cm'). After overnight incubation, a few colonies appeared on a TBAB plate containing 10 mg/L of kanamycin; three colonies were analyzed by PCR. Among them, the *spoIIIE* gene of two cononies was correctly disrupted by the Km^r gene (Fig. 2 (b)), and designated as DB104::ΔspoIIIE(Km')::pMK101. DB104::ΔspoIIIE(Km')::pMK101 showed a normal growth rate and did not form spores. Its sporulation frequency was lower than 10^{-6}% compared to the 1-3% of the wild type at T^7 (Table 2). Its specific subtilisin expression (units/cell O.D.) was almost the same as the host strain's (Fig. 3), DB104::pMK101. Though we tried to know the effect of *spoIIIE* disruption on subtilisin expression, this result is somewhat expected. AprE, which encodes subtilisin of *B.subtilis*, is induced and tightly regulated by the sporulation process, especially
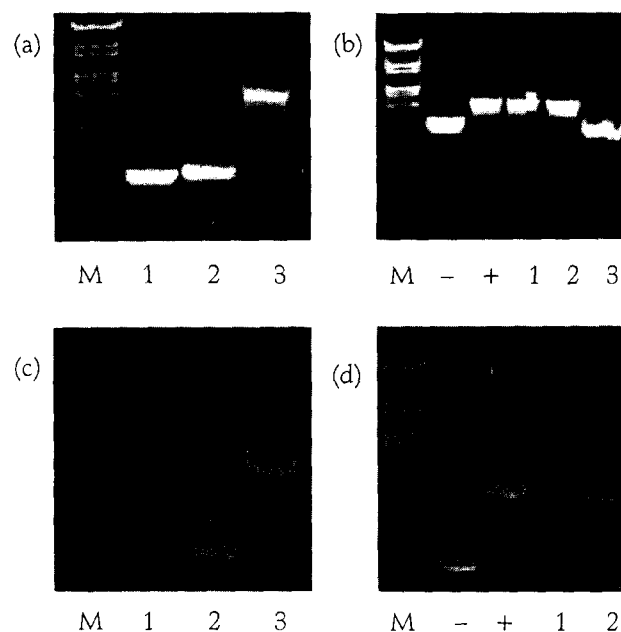

Fig. 2. Generation of PCR product and confirmation of antibiotic marker insertion into *spoIIIE* and *spoIIIG* of *B. subtilis*. M is denoted as molecular DNA marker λ/BstEII; (a) Synthesis of P5, P6 and final products for s*poIIIE* disruption : Lanes 1 and 2 are megaprimers P5 and P6 generated from step 1 with lengths of 1219 and 1281 base pairs each. Lane 3 is the final PCR product for gene disruption (3829 base pairs) from step 3; (b) Confirmation of antibiotic marker insertion into *spoIIIE* : Primers 1 and 4 were used for this experiment. Thermal cycling condition and concentration of components were the same as used in step 2. Templates were prepared as follows-a tooth-picked single colony was suspended in an eppendorf tube containing 100 μL of D.D.W. and boiled for 2 min. 1 μL of the suspension was used as template. '–' denotes negative control from wild type DB104::pMK101. '+' denotes positive control obtained from step 2 (lane 3 of (A)). Lane 1, 2 and 3 are PCR products of three different colonies obtained after transformation; (c) Synthesis of P5, P6 and final products for s*poIIIG* disruption : Lanes 1 and 2 are megaprimers P5 and P6 each generated from step 1 with lengths of 558 and 557 base pairs each. Lane 3 is the final PCR product for gene disruption (2334 base pairs) from step 3; (d) Confirmation of antibiotic marker insertion into *spoIIIG* : Experimental procedure is the same as legend of (b). Lane 1 and 2 PCR products of two colonies obtained after transformation.

at the stage of sporulation initiation. Subtilisin is transiently produced only from stage 0 to stage II of sporulation. Gene disruption, which can arrest spore formation at spoII stage, such as *spoIIG*, was reported as an effective way of enhancing the expression of the subtilisin promoter of *B. subtilis* [17], and the importance of the sporulation mutant was extensively investigated in fed-batch fermentation [14]. The effect of stage 0 sporulation mutants on subtilisin expression has also been studied [18], and stage 0 sporulation genes was

Table 2. Comparison of sporulation frequencies for different sporulation mutants

Strain	sporulation frequency (%)
DB104 ::pMK101	2.3
DB104 ΔspoIIIE::Km^r::pMK101	<1.0 E-6 [a]
DB104 ΔspoIIIG::Km^r::pMK101	<1.0 E-6 [a]

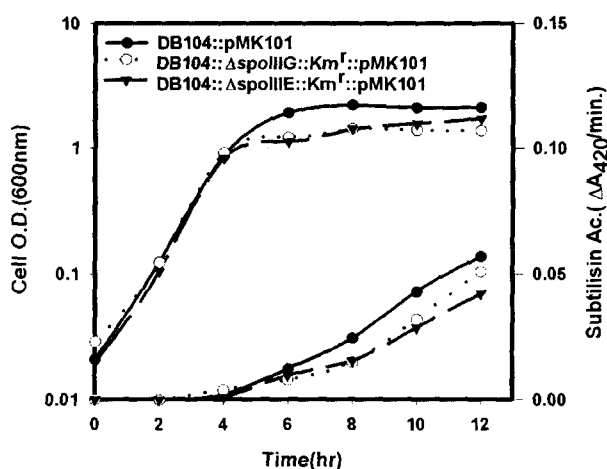[a] This value of sporulation frequency means that no spore was formed at T$_7$

Fig. 3. Comparison of subtilisin activities of wild type and sporulation mutants in flask culture

found to have a negative effect upon *aprE* promoter expression. *SpoIIIE* can be considered as another sporulation gene, which has little effect on subtilisin expression.

Application to Shorter ORF: Disruption of *SpoIIIG* of *B. Subtilis*

SpoIIIG of *B. subtilis* codes sigma factor of RNA polymerase which dominate late fore-spore specific gene expression. Its length is 780 base pairs and about 1.1 kb is available if the non-transcribed region between ORFs is included. Same procedure was followed for PCR product generation and transformation procedure as was used for *spoIIIE*. Synthesized mega-primers P5 (558 base pairs), P6 (557 base pairs) and final product (2334 base pairs) were identified (Fig. 2(c)). An initial two attempts at the transformation experiment were unsuccessful, due to insufficient length for homologous recombination. Therefore a ten times higher dosage of PCR product, prepared by the procedure described in Fig. 1, was used for transformation, because of its reduced recombination frequency resulting from a shorter ORF length. After overnight incubation, five colonies were isolated from TBAB plate. Four colonies were correctly disrupted in *spoIIIG* by Km^r inserted antibiotic marker (Fig. 2(d)). These were designated as DB104::ΔspoIIIG (Km^r)::pMK101. DB104::ΔspoIIIG(Km^r)::pMK101 showed

normal growth rate and its sporulation frequency was 10^{-6}% lower than the wild type at T^7 (Table 2) as was DB104::ΔspoIIIE(Km^r)::pMK101. Specific subtilisin expression (units/cell O.D.) was similar compared to that of the host strain (Fig. 3). This result is somewhat unexpected. From the results of *spoIIG* mutation on subtilisin production, [14,17] disruption of genes involved in stage II or III of spore formation, were expected to have positive effect on subtilisin expression, similarly genes involved in the later stages of sporulation (stage IV-V) were expected to have little effect on subtilisin expression, in view of the exprssion period of subtilisin. *SpoIIIG* is activated by *spoIIG* through precise signal transduction during *B. subtilis* spore formation. It dominates prespore gene expression, just as *spoIIG* dominates mother cell gene expression in the sporulation process. *SpoIIG* and *spoIIIG* have similar roles in spore formation and its expression period is also similar. One of the possible reasons for this result is that *aprE* is mainly expressed in the mother cell and that the deletion of mother cell sigma factor SpoIIG has a positive effect on subtilisin production; deletion of fore spore sigma factor SpoIIIG may not exert such an effect. Another possibility is that expansion of time period which is governed by *spoIIIG* occurs too late for the control of subtilisin expression, and that therefore *spoIIG* may be the last gate for the control of subtilisin expression. Four sigma factors (*spoIIAC, spoIIG, spoIIIG and spoIIIC*) are involved in *B. subtilis* spore formation. SpoIIAC and SpoIIIG dominate fore spore development and SpoIIG and SpoIIIC govern mother cell development. We will get the exact answers to the above result and assumptions, when *spoIIAC* and *spoIIIC* mutants are constructed; this work is currently underway in our laboratory.

In the field of yeast genetics, LFH-PCR is actively used for gene disruption, but, its application for other organisms has been scarcely reported. The aim of this study was to apply LFH-PCR to disrupt *B.subtilis* ORFs. The results showed that LFH-PCR, originally developed for yeast gene disruption, was effective at rapidly disrupting *B.subtilis* sporulation-associated genes. The application of this method is restricted by several factors. The rate of homologous recombination for gene disruption is roughly proportional to the available flanking region on each side of the antibiotic marker [19]. Many have reported that the average length of ORF is about 1kb. (In the case of *E.coli*, 4288 ORFs in 4,632, 221 base pairs and in the case of *B.subtilis*, 4100 ORFs in 4,214,810 base pairs). Therefore, the length of spoIIIG (780 base pairs) can be considered as an average. Though spoIIIG needed about a ten times higher dosage of the PCR product compared to *spoIIIE* disruption, it was successful. One point, which should be remembered is that many genes in prokaryote are polycistronic. If antibiotic marker is inserted into prokaryotic ORF by LFH-PCR, a polar effect in DNA transcription may occur, which is not encountered in yeast genetics. However, this problem is inherent in the genome of prokaryote and it lies beyond LFH-PCR, and antibiotic

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marker insertion is one of the most widely used method of gene disruption for the functional analysis of target gene. We expect this method can be further used not only *B. subtilis* gene disruption but also other prokaryotes such as *E. coli*.

REFERENCES

[1] Velculescu V. E, Zhang L., Vogelstein B., and Kinzler K. W. (1995) Serial Analysis Gene Expression. *Science* 270: 484-487.

[2] Smith V., Bostein D., and Brown P. O. (1995) Genetic footprinting: A genomic strategy for determining a gene's function given its sequence. *Proc. Natl. Acad. Sci. USA* 92: 6479-6483.

[3] Lockhart D. J., Dong H., Byrne M. C., Follettie M. T., Gallo M. V., Chee M. S., Mittmann M., Wang C., Kobayashi M., and Horton H., Brown (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnol.* 14: 1675-1680.

[4] Schena M., Shalon D., Heller R., Chai A., Brown P. O., and Davis R. W. (1996). Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. *Proc. Natl. Acad. Sci. USA* 93: 10614-10619.

[5] Shoemaker D. D., Lashkari D. A., Morris D., Mittman M., and Davis R. W. (1996) Quantitative phenotype analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nature Genet.* 14: 450-456.

[6] Link A. J., Phillips D., and Church G. M., (1997) Methods for generating precise deletion and insertion in the genome of wild-type *Escherichia coli*: Application to open reading frame characterization. *J. Bacteriol.* 179: 6228-6237.

[7] Leenhouts K., Buist G., Bolhuis A., ten Berge A., Kiel J., Mierau I., Dabrowska M., Venema G., and Kok J. (1996). A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol. Gen. Genet.* 253: 217-224.

[8] Amberg D. C., Bostein D., and Beasley E. M. (1995) Precise gene disruption in Saccharomyces cerevisiae by dou-

ble fusion polymerase chain reaction. *Yeast* 11: 1275-1280.

[9] Baudin A., Ozier-Kalogeropoulos O., Denouel A., Lacroute F., and Cullin C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae. Nucleic Acids Res.* 21: 3329-3330.

[10] Oliver S. G., Winson M. K., Kell D. B., and Baganz F. (1998) systematic functional analysis of the yeast genome. *TIBTECH* 16: 373-378.

[11] Wach A. (1996) PCR-synthesis of marker cassette with long flanking homology regions for gene disruption in *S. cerevisiae. Yeast* 12: 259-265.

[12] Lorenz M. C., Muir R. S., Lim E., McElver J., Weber S. C., and Heitman J. (1995) Gene disruption with PCR products in *Saccharomyces cerevisiae. Gene* 158: 113-117.

[13] Oh M. K., Park S. H., and Kim B. G. (1994) Development and characterization of sporulation mutants for overexpression of recombinant protein of *B. subtilis. Korean J. Biotechnol. Bioeng.* 9: 16-25.

[14] Oh M. K., Kim B. G., and Park S. H. (1995) Importance of spore mutants for fed-batch and continuous fermentation of *Bacillus subtilis. Biotechnol. Bioeng.* 47: 696-702.

[15] Oh M. K., Park S. H., and Kim B. G. (1995, b) Enhanced subtilisin production with spore mutants of *Bacillus subtilis* and their characterization. *Ann. NY Acad. Sci.* 750: 444-451.

[16] Harwood C. R. and Cutting S. M. (1990) *Molecular Biological Methods for* Bacillus. John Wiley & Sons, NY, USA.

[17] Pierce J. A., Robertson C. R., and Leighton T. J. (1992) Physiological and genetic strategies for enhanced Subtilisin production by *Bacillus subtilis. Biotechnol. Prog.* 8: 211-218.

[18] Ferrari E., Sandra M. H., Howard, and Hoch J. A. (1986) Effect of stage 0 sporulation mutations on subtilisin expression. *J. Bacteriol.* 166: 173-179.

[19] Khasanov F. K., Zvingila D. J., Zainullin A. A., Prozorov A. A., and Bashkirov V. I. (1992) Homologous recombination between plasmid and chromosomal DNA in *Bacillus subtilis* requirs approximately 70 bp of homology. *Mol. Gen. Genet.* 234: 494-497.