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Designed horizontal transfer of stable giant DNA released from *Escherichia coli*

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DNA in the environment is a source to mediate horizontal gene transfer (HGT). Present molecular cloning methods are based on this HGT principle. However, DNA in the extracellular environment, particularly with high molecular-weight, is thought to be prone to shearing or digestion by nucleases. Here we discovered that extracellular plasmid DNA released from lysed *Escherichia coli* remained intact and stable. Furthermore, it was demonstrated that plasmids up to 100 kb in size were taken up by co-present competent *Bacillus subtilis* cells. The detailed kinetics of the process together with sensitivity to added DNase I indicated that plasmid DNA released from lysed *E. coli* into the culture medium was stable enough for quantitative efficacy in the transformation of *B. subtilis*. Our results will be useful for the development of methods to transfer giant DNAs from general host *E. coli* without their biochemical purification.

Keywords: cell lysis/DNase I sensitivity/natural genetic transformation/phage induction/plasmid transfer.

Abbreviations: Ap, ampicillin; ApR, ampicillin resistance; *bla*, β-lactamase gene; *cat*, chloramphenicol acetyl transferase gene; Cm, chloramphenicol; CmR, chloramphenicol resistance; HGT, horizontal gene transfer; *kam*, kanamycin resistance gene; Km, kanamycin; KmR, kanamycin resistance; SpcR, spectinomycin resistance; Tc, tetracycline; TcR, tetracycline resistance; *tet*, tetracycline resistance determinant gene.

DNA transfer is conducted by three major molecular mechanisms: natural transformation (*1*), conjugation (*2*) and transduction (*3*). Only natural transformation, where naked extracellular DNA plays as a mobile DNA, takes place independent of the donor and recipient genus. Therefore, it may apply to a wider host range than transduction and conjugation where DNA is protected by specific phage coat or conjugation-channel proteins. Extracellular DNA is thought to be less stable than intracellular DNA. It is vulnerable to physical and chemical damage and non-selective digestion by nucleases. Our survey of extracellular DNA from bacteria fortuitously detected stable plasmid DNA released in culture from *Escherichia coli* K-12 on lysogenic lambda induction. This finding prompted us to investigate the direct transfer of the stable extracellular plasmid DNA to co-present competent *Bacillus subtilis* 168 without biochemical plasmid purification procedures. In our standard protocol, namely simple mixing of two cultures, the DNase I-sensitive extracellular plasmids as large as 100 kb were transferred to *B. subtilis* with no structural alterations. We call the DNA transfer from *E. coli* to *B. subtilis* horizontal gene transfer (HGT) hereafter.

Materials and Methods

Bacterial strains

The bacterial strains and plasmids we used are listed in Table I. λgt11 (*c*I857) and Type II restriction enzymes were purchased from TaKaRa (Kyoto, Japan) and Toyobo (Tokyo, Japan), respectively. Molecular biological grade DNase I and RNase A were obtained from Sigma (St. Louis, MO, USA).

Construction of transferred plasmids

pGETSGFP (Fig. 3A) was derived from pGETS103 (*4*). The two open boxes represent the pBR322 region. The amp half carries *bla* for selection and an *ori* sequence for replication in *E. coli*. The region derived from the pTB522 plasmid which carries *tetL* and *repA* for selection and replication in *B. subtilis* was fused with pBR322 (*4*). The *GFPuv* gene is regulated by the λ Pr-promoter (*5*) and is repressed in the lambda lysogenic *E. coli* by binding to the Pr promoter of the CI gene product that is constitutively expressed from temperate lambda. The *GFPuv* gene is expressed in *B. subtilis* (1A1) due to the absence of a CI gene product and yields fluorescent colonies (Fig. 3B). pGETS1036 (Fig. 4A) was derived from a previously-constructed binary BAC vector pGETS118 (*6*). Open and solid arrows [L] and [R] represent the BAC vector region that carries *oriS* (the replication origin for *E. coli*), *kam* (conferring kanamycin resistance to *E. coli*), and *repE* and *parABC* for replication in *E. coli*. The 80-kb mitochondrial (mt) fragment derived from *Arabidopsis thaliana* is inserted between BAC [L] and [R] (*6*). The solid line that includes *tetL* and *repA* is similar to pGETSGFP.

Purification of plasmids released from lysates

The plasmid was purified from a density gradient formed with cesium chloride through ultracentrifugation in the presence of ethidium bromide using the standard procedure (*7*). Samples were prepared from 10-ml supernatant aliquots after 15-min centrifugation at 4000×*g*; this was followed by suspension in solution A [25% sucrose, 100 mM Tris–HCl (pH 7.5), 10 mM NaCl and 1 mM EDTA•Na (pH 8.0)] with 335 μg/ml RNase A for purification.

Table I. Bacterial strains.

Bacterial strain	Genotype or insert	Antibiotic selection[a]	Reference or source
E. coli			
LE392	F$^-$ *supE44 supF58 trpR55 galK2 galT22 metB1 lacY1 or (lacIZY) 6 hsdR574*(rK$^-$ mK$^+$)		(*19*)
MIC128	lysogenic LE392 by λgt11		This study
MEC5778[b]	pGETSGFP	ApR	This study
MEC5770[b]	pGETS1036	KmR	This study
B. subtilis			
1A1 (= 168*trpC2*)[c]	*trpC2*		BGSC[d]
BEST310[e]	RM125 plus *proB*::pBR[BAC, *c*I-*spc*] Pr-*neo*	SpcR	(*6*), (*20*)
BEST6606[c]	RM125 plus *proB*::pBR[BAC, *c*I-*spc*] Pr-*neo*, *leuB*::*cat*	CmR, SpcR	This study

[a]ApR; ampicillin resistance, KmR; kanamycin resistance, SpcR; spectinomycin resistance, CmR; chroramphenicol resistance.
[b]Donor strain, MEC5778 and MEC5770 are derived from MIC128.
[c]Recipient strain, 1A1 for pGETSGFP and BEST6606 for pGETS1036. BEST6606 is derived from BEST310.
[d]*Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH).
[e]Pr-*neo* gene (λ Pr-promoter fused to the neomycin resistance gene) is inserted between *Not*I sites of *yvfC* and *yveP*.

DNA transfer from E. coli to B. subtilis in mixed culture

DNA transfer protocol was shown in Fig. 2. Pre-culture of donor *E. coli* lysogen, MEC5778 or MEC5770, was prepared in LB medium supplemented with ampicillin (Ap) or kanamycin (Km) by shaking for 24 h at 30°C. This culture was diluted to 1 : 200 in pre-warmed LB (20 ml) supplemented with Ap or Km and shaken at 30°C as shown in Fig. 2. The recipient *B. subtilis*-competent cell culture was prepared as follows: *B. subtilis* (1A1 or BEST6606) pre-culture in LB medium shaken for 17 h at 37°C was diluted to 1:200 in pre-warmed competent developing medium, designated TFI [1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% Na-citrate, 0.5% glucose, 0.02% $MgSO_4{\cdot}7H_2O$, 0.05 mg/ml Trp, 0.05 mg/ml Arg, 0.05 mg/ml Leu, 0.05 mg/ml Thr: w/v] with 2% casamino acid (v/v). The *B. subtilis* TFI culture prepared after 6-h incubation was directly mixed with *E. coli* lysates at 37°C and continuously shaked for 1 h. Two *E. coli* lysates, 1 and 2 h (two arrows in shaded and open boxes in Fig. 2) after a temperature upshift were examined with various mixing ratios (parentheses in Fig. 3C) of *E. coli* and *B. subtilis*. Freshly prepared DNase I, if necessary, was added to the mixture at a final concentration of 3.4 μg/ml. Normally, 200 μl each of the culture was spread on a plate supplemented with appropriate antibiotics for the selection of *B. subtilis* transformants.

Results

Stability of extracellular plasmid DNA released into lysates

Escherichia coli lambda lysogen was used to generate stable extracellular DNA. As shown in Fig. 1 (bottom left), lambda lysogenic *E. coli* grows normally in LB medium at 30°C. Due to a temperature-sensitive *c*I857 repressor mutation of the temperate lambda, lambda phage can be induced by a temperature shift from 30 to 37°C (Fig. 1, top and bottom left). The phage maturation accompanies cell lysis (*8*) and cellular materials are released into the environment. After 2 h at 37°C, *E. coli* was almost completely lysed. To our surprise, the normal plasmid isolation protocol produced a high yield of intact plasmid from the *E. coli* lysate (Fig. 1, bottom right).

Efficient transfer of small plasmids carrying the GFPuv gene

To verify the quality of the plasmid in the *E. coli* lysate we added separately-prepared *B. subtilis* culture

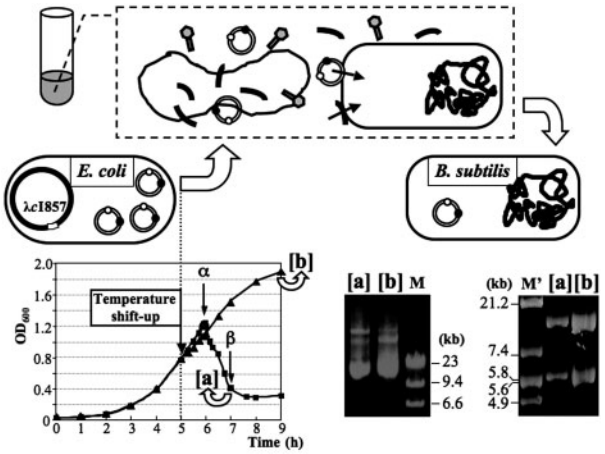

Fig. 1 Plasmid DNA transfer across genera in a test tube. *Top*: Scheme of transferring DNA released from lysed *E. coli* to *B. subtilis*. The *E. coli* genome (solid circle) is shortened in the lysate, however, the plasmid remains intact (double circle with two origins of replication, closed and open circles). The uptake of naked DNA on the cell surface of co-present *B. subtilis* is indicated by an arrow. Matured lambda particles are shaded. *Bottom left*: Growth of the lambda lysogen of *E. coli*, MEC5778, at an incubation temperature of 30°C (triangles) and after an upshift to 37°C at 5 h (squares). The colony forming units (cfu) at (α 5.26×10^8 cells/ml dropped dramatically to (β) 2.90×10^6 cells/ml. *Bottom right*: Biochemically purified pGETSGFP, ~6 μg from 10-ml lysates [a] and ~3 μg from 10 ml of uninduced lysogen [b] are indistinguishably resolved by 1% agarose gel electrophoresis; undigested (left) and digested with *Eco*RI (right). Lanes M and M′ have size markers, λ DNA digested with *Hin*dIII and *Eco*RI, respectively.

medium (Fig. 2). Naked DNA in the environment was actively and non-selectively taken up by *B. subtilis,* a Gram-positive bacterium, and incorporated into its cells (Fig. 1 top) (*9*). As the plasmid can also replicate in *B. subtilis*, we selected transformed cells by a plasmid-associated marker. We used a small (pGETSGFP, 17.1 kb in Fig. 3A) and a large plasmid (pGETS1036, 95 kb in Fig. 4A) for transformation-mediated HGT in a test tube. The small plasmid was efficiently transferred to *B. subtilis*, resulting in formation of colonies selected by tetracycline (Tc) (Fig. 3B and C). All Tc-resistant (TcR) colonies emitted green luminescence

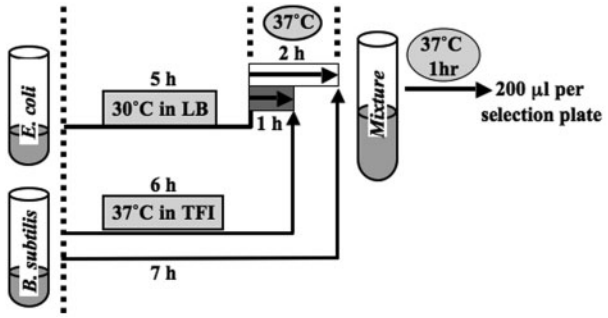

Fig. 2 Protocol for DNA transfer from *E. coli* to *B. subtilis* in mixed culture. The donor *E. coli* lysogen, MEC5778 or MEC5770, was cultured in LB medium supplemented with Ap or Km for 5 h at 30°C. The recipient *B. subtilis*-competent cell, 1A1 or BEST6606 was cultured in TFI medium until mixing with donor *E. coli* at 37°C. Details of mixing with the donor *E. coli* and the recipient *B. subtilis* is described in Materials and Methods section.

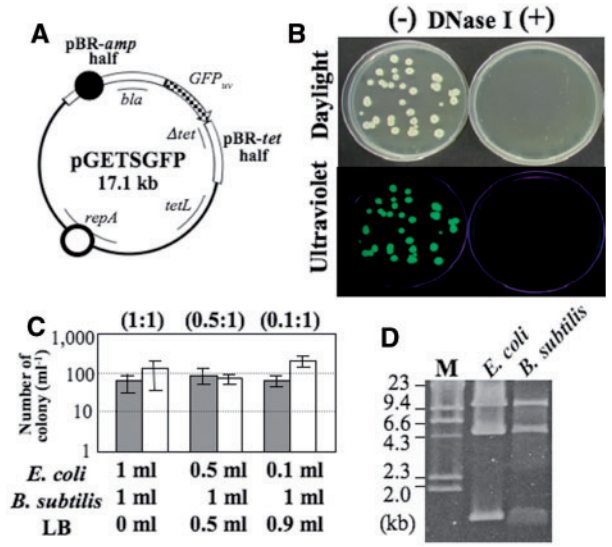

Fig. 3 Transfer kinetics of a 17-kb plasmid from *E. coli* to *B. subtilis*. (A) Structure of the 17.1-kb pGETSGFP. Small circles on the circular plasmid represent the origin of DNA replication for *E. coli* (closed) and *B. subtilis* (open). Other details are described in Materials and Methods. (B) *B. subtilis* colonies selected upon HGT. All colonies fluoresced because the *GFPuv* gene was carried by the plasmid. DNase I treatment completely suppressed HGT. (C) Number of transformants resulting from HGT per ml of mixed cultures. Each mixing ratios (parentheses) and actual volumes (bottom) are shown. The number of *B. subtilis* transformants remains constant during different periods of *E. coli* lysing (1 h: shaded, 2 h: open) and at different mixing volumes of *E. coli* lysate/*B. subtilis* culture. (D) The resolution in 1% agarose gel electrophoresis for *Eco*RV digests of pGETSGFP recovered from the *E. coli* donor MEC5778, and the *B. subtilis* transformant. Lane M: *Hin*dIII digests of λ DNA, fragment sizes are indicated at the left.

under UV light at 365 nm (Fig. 3B), confirming the functioning of the monitor *GFPuv* gene in the plasmid. Plasmids, from all *B. subtilis* transformants examined, showed an *Eco*RV digestion pattern identical to that of pGETSGFP (Fig. 3D). The presence of commercial DNase I (3.4 µg/ml) in the mixed culture completely abolished HGT. These results proved the occurrence of plasmid transfer across genera from *E. coli* to *B. subtilis* via stable naked DNA in the medium. There were no significant changes in the number of TcR transformants at various ratios of *E. coli* and

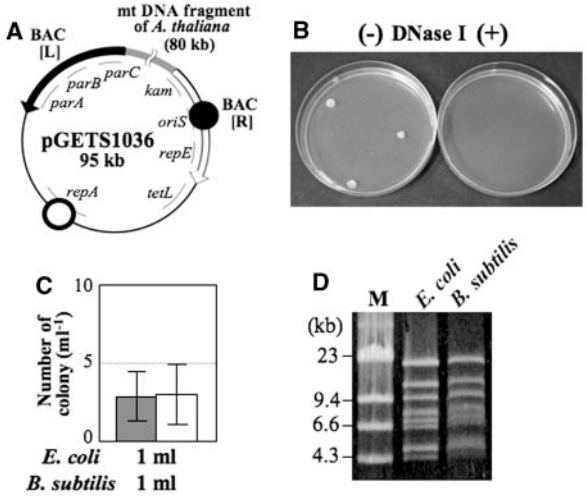

Fig. 4 Transfer of a 95-kb plasmid from *E. coli* to *B. subtilis*. (A) Structure of the 95-kb pGETS1036. Small circles on the circular plasmid represent the origin of DNA replication for *E. coli* (closed) and *B. subtilis* (open). Other details are found in Materials and Methods. (B) *B. subtilis* colonies selected upon HGT. (C) Number of transformants resulting from HGT per milli liter of mixed cultures. The number of *B. subtilis* transformants was unaffected during different periods of *E. coli* lysing (1 h: shaded, 2 h: open) in equal mixture volumes (1 ml) of *E. coli* lysate/*B. subtilis* culture. (D) The resolution in 1% agarose gel electrophoresis for *Eco*RV digests of pGET1036 recovered from the *E. coli* donor MEC5770 and the *B. subtilis* transformant. Lane M: *Hin*dIII digests of λDNA; fragment sizes are indicated at the left.

B. subtilis, suggesting that practical plasmid concentrations were constant under our experimental conditions in which many debris including the genomes from lysing *E. coli* seem to compete with plasmids.

Transfer of large-sized shuttle plasmids

Since *B. subtilis* efficiently takes up naked DNA exceeding 100 kb (*6*, *10–12*), we extended HGT study to larger plasmid. The 95-kb pGETS1036 plasmid depicted in Fig. 4A was prepared previously from a bacterial artificial chromosome (BAC) vector that contained an extra origin for DNA replication in *B. subtilis* (*6*). The pGETS1036 carriers for *E. coli* and *B. subtilis* were selected by kanamycin (Km) and Tc, respectively. We adopted a restriction-modification mutant of *B. subtilis* as a recipient to avoid possible restriction problems (Table I). In a protocol similar to that used for pGETSGFP (Fig. 2), TcR colonies of *B. subtilis* transformants were obtained, however, their number was smaller (Fig. 4B and C). The addition of DNase I completely abolished transformation. Several transformants we investigated contained the 95-kb pGETS1036 as demonstrated by *Eco*RV digestion (Fig. 4D). The reduced efficiency compared to the 17-kb plasmid may be attributable to (i) a lower copy number of the 95-kb plasmid (one or two copies per *E. coli* cell compared to 50 copies for the 17-kb plasmid) and (ii) reduced transformation efficiency for large DNA in the recipient *B. subtilis* (*10*, *13*).

Discussion

We reported our novel experimental findings that plasmid DNA, up to, and possibly longer than 100 kb, can

be transferred from *E. coli* to *B. subtilis* upon simple mixing the culture of both bacteria (the specific protocol is described in Materials and Methods and Fig. 2). In the two cases, with pGETSGFP (17 kb) and pGETS1036 (95 kb), the number of *B. subtilis* transformants remains constant during different periods of *E. coli* lysing (1 h: shaded, 2 h: open in Figs. 3C and 4C, respectively) and at different mixing volumes of *E. coli* lysate/*B. subtilis* culture (Fig. 3C). We posit that this is primarily attributable to competition with the simultaneous uptake of *E. coli* genomic DNA as depicted in Fig. 1 (top) (*14*). Unidentified interactions with other cellular materials present in the mixed culture medium may have adverse effects during HGT.

The damage-free handling of DNA larger than 100 kb in test tubes is increasingly difficult. This has been a bottleneck in its regular use (*6*) and even in synthetic biology (*15*). Our findings may facilitate a more rapid and convenient massive DNA transfer to *B. subtilis* without the manual preparation of damage-free naked DNA. Although *E. coli* is the fundamental host cell for the construction of plasmid-based engineered DNA, the possibility of using other bacterial species as recipients for naked DNA transformation should be investigated. Our finding that intact DNA remains stable for some period and that it is sensitive to added DNase I is an encouragement for identifying other methods to lyse *E. coli*. In additon experimental information yielded by HGT and sequence information on bacterial genomes will shed light on the role of extracellular DNA in the natural environment (*16–18*). Mimicking transformation-mediated DNA transfer should not only illuminate our current understanding of HGT but also advance the manipulation of beneficial bacteria.

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Conflict of interest
None declared.

References

1. Lorenz, M.G., and Wackernagel, W. (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev.* **58**, 563–602
2. Ippen-Ihler, K. (1990) Bacterial conjugation in *Gene Transfer in the Environment* (Levy, S.B., and Miller, R.V., eds.), pp. 33–72, McGraw-Hill Book Co., New York
3. Kokjohn, T.A. (1989) Transduction: mechanism and potential for gene transfer in the environment *Gene Transfer in the Environment* (Levy, S.B., and Miller, R.V., eds.), pp. 73–97, McGraw-Hill Book Co., New York
4. Tsuge, K., and Itaya, M. (2001) Recombinational transfer of 100-kilobase genomic DNA to plasmid in *Bacillus subtilis* 168. *J. Bacteriol.* **183**, 5453–5458
5. Ohashi, Y., Ohshima, H., Tsuge, K., and Itaya, M. (2003) Far different levels of gene expression provided by an oriented cloning system in *Bacillus subtilis* and *Escherichia coli*. *FEMS Microbiol. Lett.* **221**, 125–130
6. Kaneko, S., Akioka, M., Tsuge, K., and Itaya, M. (2005) DNA shuttling between plasmid vectors and a genome vector: Systematic conversion and preservation of DNA libraries using the *Bacillus subtilis* genome (BGM) vector. *J. Mol. Biol.* **349**, 1036–1044
7. Sambrook, J., Fritsch, E.C., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition, pp. 1.42–1.45, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
8. Arber, W. (1983) Lambda II in *A Beginner's Guide to Lambda Biology* (Hendrix, R.W., Roberts, J.W., Stahl, F.W., and Weisberg, R.A., eds.), pp. 381–394, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Chen, I., Christie, P.J., and Dubnau, D. (2005) The ins and outs of DNA transfer in bacteria. *Science* **310**, 1456–1460
10. Itaya, M. (1999) Genetic transfer of large DNA inserts to designated loci of the *Bacillus subtilis* 168 genome. *J. Bacteriol.* **181**, 1045–1048
11. Itaya, M., Fujita, K., Kuroki, A., and Tsuge, K. (2008) Bottom-up genome assembly using the *Bacillus subtilis* genome vector. *Nat. Methods* **5**, 41–43
12. Itaya, M., Tsuge, K., Koizumi, M., and Fujita, K. (2005) Combining two genomes in one cell: Stable cloning of the Synechocystis PCC6803 genome in the *Bacillus subtilis* 168 genome. *Proc. Natl Acad. Sci. USA* **102**, 15971–15976
13. Itaya, M., Fujita, K., Koizumi, M., Ikeuchi, M., and Tsuge, K. (2003) Stable positional cloning of long continuous DNA in the *Bacillus subtilis* genome vector. *J. Biochem.* **134**, 513–519
14. Yonemura, I., Nakada, K., Sato, A., Hayashi, J., Fujita, K., Kaneko, S., and Itaya, M. (2007) Direct cloning of full-length mouse mitochondrial DNA via *Bacillus subtilis* genome vector. *Gene* **391**, 171–177
15. Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A. 3rd, and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345
16. Baur, B., Hanselman, K., Schlimme, W., and Jenni, W. (1996) Genetic transformation in fresh water: *Escherichia coli* is able to develop natural competence. *Appl. Environ. Microbiol.* **62**, 3673–3678
17. Demanèche, S., Kay, E., Gourbiére, F., and Simonet, P. (2001) Natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* in soil. *Appl. Environ. Microbiol.* **67**, 2617–2621
18. Stone, B.J., and Kwaik, Y.A. (1999) Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. *J. Bacteriol.* **181**, 1395–1402
19. Itaya, M., and Crouch, R.J. (1991) Correlation of activity with phenotypes of *Escherichia coli* partial function mutant of *rnh*, the gene encoding RNase H. *Mol. Gen. Genet.* **227**, 433–437
20. Itaya, M., and Tanaka, T. (1997) Predicted and unsuspected alteration of the genome structure of genetically defined *Bacillus subtilis* 168 strains. *Biosci. Biotech. Biochem.* **61**, 56–64