Enhanced Recombinant Protein Productivity by Genome Reduction in Bacillus subtilis

Takuya MORIMOTO1,2,†, Ryosuke KADOTA1,2,†, Keiji ENDO1,‡, Masatoshi TOHATA1, Kazuhisa SAWADA1, Shengao LUI1, Tadahiro OZAWA1, Takeko KODAMA1,3, Hiroshi KAKESHITA1,4, Yasushi KACEYAMA1, Kenji MANABE1, Shigehiko KANAYA2, Katsutoshi ARA1, Katsuya OZAKI1, and Naotake OGASAWARA2,*

Biological Science Laboratories, Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-3497, Japan1; Graduate School of Information Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan2; Department of Bioscience and Textile Technology, Interdisciplinary Graduate School of Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan3 and Graduate School of Life and Environmental Sciences, Tsukuba University, 1-1-1 Ten-noudai, Tsukuba, Ibaraki 305-8572, Japan4

(Received 24 December 2007; accepted on 17 January 2008; published online 11 March 2008)

Abstract

The emerging field of synthetic genomics is expected to facilitate the generation of microorganisms with the potential to achieve a sustainable society. One approach towards this goal is the reduction of microbial genomes by rationally designed deletions to create simplified cells with predictable behavior that act as a platform to build in various genetic systems for specific purposes. We report a novel Bacillus subtilis strain, MBG874, depleted of 874 kb (20%) of the genomic sequence. When compared with wild-type cells, the regulatory network of gene expression of the mutant strain is reorganized after entry into the transition state due to the synergistic effect of multiple deletions, and productivity of extracellular cellulase and protease from transformed plasmids harboring the corresponding genes is remarkably enhanced. To our knowledge, this is the first report demonstrating that genome reduction actually contributes to the creation of bacterial cells with a practical application in industry. Further systematic analysis of changes in the transcriptional regulatory network of MBG874 cells in relation to protein productivity should facilitate the generation of improved B. subtilis cells as hosts of industrial protein production.

Key words: Bacillus subtilis; genome size reduction; recombinant protein productivity

1. Introduction

The emerging field of synthetic genomics is expected to facilitate the generation of microorganisms with the potential to achieve a sustainable society.1–3 One approach towards this goal is the reduction of microbial genomes by rationally designed deletions to create simplified cells with predictable behavior that act as a platform to build in various genetic systems for specific purposes. It is expected that metabolic waste in these cells will be decreased, as fewer dispensable proteins are synthesized. Challenges to reduce the genome size by the stepwise introduction of large-scale deletions have been undertaken for Escherichia coli4,5 and Bacillus subtilis.6,7

In a previous study, Posfai et al.4 reported an E. coli strain, MDS42, in which ~15% of the genome sequence (0.71 Mb) was removed by planned and
sequential deletions to eliminate all mobile DNA and cryptic virulence genes. The resultant strain displayed normal cell growth and protein expression comparable with that of the parental strain, MG1655. Interestingly, genome reduction gave rise to unexpected beneficial properties. MDS42 cells exhibit high electroporation efficiency and allow the accurate propagation of plasmids that are unstable in other strains. Another *E. coli* strain, MGF-01, with a 22% genome size reduction (1.03 Mb) was created in Japan using a similar strategy. MGF-01 cells also displayed an unexpected phenotype in that growth was as rapid as the parental W3110 strain in M9 minimal medium during the exponential phase, but continued after the wild-type strain had progressed to the stationary phase. In addition, MGF-01 secreted twice the amount of threonine as the wild-type strain.

*B. subtilis*, one of the most extensively studied model microorganisms, displays superior ability to produce various secretary enzymes. This ability has been widely applied to produce various useful enzymes in the industrial field. The 4.2 Mb *B. subtilis* genome contains 10 horizontally acquired prophage (SPβ and PBSX) and prophage-like (pro1-7 and skin) sequences. In addition, 2.8% of the genome encompasses two large operons that produce secondary metabolites (*pkb* and *pps*). Systematic inactivation of the protein-coding regions revealed that only 271 genes are indispensable for growth in rich medium (LB) at 37°C, as growth is impossible in the absence of these genes. Westers et al. reported a *B. subtilis* Δ6 mutant strain with a 7.7% reduction of the genome (0.53 Mb) produced by deleting two prophage (SPβ, PBSX), three prophage-like sequences (pro1, pro6, skin), and the *plc* operon. However, phenotypic characterization of the Δ6 cells disclosed no unique properties, including secretion of AmyQ protein, relative to wild-type 168 cells. Our group generated a strain, MGB469, in which all prophage and prophage-like sequences, except pro7, as well as *plc* and *pps* operons, were deleted. In this case, cell growth was normal, but no beneficial properties were apparent, including exogenous protein production from the plasmid harboring the corresponding genes. Moreover, we constructed the MG1M strain containing an additional six deletions in the MGB469 genome, resulting in a 0.99 Mb reduction in genome size. However, the MG1M strain displayed unstable phenotypes with regard to growth rate, cell morphology, and recombinant protein productivity after successive culture, making it inappropriate for further studies.

Here, we report the generation of another multiple-deletion series of mutants, from MGB469 as the starting strain through to MGB874 with a total 0.87 Mb genome deletion. When compared with wild-type cells, the regulatory network of gene expression of the mutant strain is reorganized after entry into the transition state due to the synergistic effect of multiple deletions, and productivity of extracellular cellulase and protease from transformed plasmids harboring the corresponding genes is remarkably enhanced. Our results demonstrate the effectiveness of synthetic genomics in creating novel and useful bacteria for industrial use.

2. Materials and Methods

2.1. Culture media and measurement of enzyme activity

Spizizen’s minimal medium (SMM) comprising 0.2% (NH₄)₂SO₄, 1.4% K₂HPO₄, 0.6% KH₂PO₄, 0.1% Na₃C₆H₅O₇·2H₂O, and 0.02% MgSO₄·7H₂O was supplemented with 0.5% glucose and trace elements. LB contained 1% tryptone peptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. The 2xL-Mal medium contained 2% tryptone peptone (Difco), 1% yeast extract (Difco), 1% NaCl, 7.5% maltose hydrate, and 7.5 μg/mL MnSO₄. Protease and cellulase activities in culture medium were determined as described previously.

2.2 High-resolution transcriptome analysis

The custom Affymetrix tiling chip used contains 55,430 25-mer probes for the coding strand of protein coding regions at 25–30 bp intervals and 72,218 probes for both strands of intergenic regions at 2–3 bp intervals. Cultures (200 mL) of wild-type 168 and MGB874 cells (OD₆₀₀ = 0.6) in 2xL-Mal at 30°C were inoculated into 15 L of 2xL-Mal medium within a 30 L jar fermenter. Total RNA was extracted from *B. subtilis* cells (10 OD₆₀₀), as described previously. Synthesis of cDNA, terminal labeling, and oligonucleotide chip hybridization were performed following the Affymetrix instruction manual. Briefly, cDNA was synthesized from 10 μg total RNA using random primers and reverse transcriptase (Superscript III, Invitrogen), followed by purification using QiaQuick columns (Qiagen) and digestion with DNase I (GE Healthcare Bioscience). Next, cDNA fragments were terminally labeled with biotin-ddUTP using an ENZO BioArray Terminal Labeling Kit (Enzo Life Sciences). Hybridization with the oligonucleotide chip was performed for 16 h at 42°C, followed by washing, staining, and scanning using the GeneChip Instrument System, according to the manufacturer's instructions (Affymetrix). Transcriptional signals were analyzed and visualized along the genome coordinate with the In Silico Molecular Cloning program, Array Edition (In Silico Biology). To compensate for the differences in hybridization efficiency of each 25-mer probe on the chip, we divided the hybridization intensities of cDNA
synthesized from total RNA by those of total genome DNA. The signal intensities of each experiment were adjusted to confer a signal average of 500 and normalized by MA plot analysis for comparison of MGB874 and wild-type 168 data.\textsuperscript{17,18} The average signal intensities of probes in each coding sequence were calculated after removal of the lowest and highest intensities.

3. Results and Discussion

3.1. Multiple-deletion series mutants design

To construct the multiple-deletion series mutants, we initially identified contiguous genome sequences >10 kb that did not code for RNA or essential proteins. In addition, we excluded all known and possible genes involved in primary metabolism to maintain growth in minimum medium, as well as those related to DNA metabolism to avoid genome instability. In total, 74 regions, including prophage, prophage-like, and secondary metabolite-producing sequences, were selected and individually replaced with the tetracycline-resistant gene (\textit{tet}) by selection on LB plates. As a result, we obtained deletion mutants for 63 regions, totaling up to \(~2\) Mb (Fig. 1A and Supplementary Table S1). However, a number of the deletion mutants did not grow in SMM, and others showed impaired growth, even in liquid LB medium (Supplementary Table S1).

3.2. Step-by-step \textit{B. subtilis} genome reduction

Next, we attempted to construct a reduced genome \textit{B. subtilis} strain by step-by-step deletions in regions where single deletions did not affect cell growth, applying the \textit{upp} (encoding uracil-phosphoribosyltransferase) cassette and 5-fluorouracil (5-FU) selection to remove the drug-resistant markers used to introduce primary deletions (Fig. 2). Accordingly, sequential deletions in 11 non-essential gene clusters were introduced in the MGB469 strain to generate MGB874 depleted of 874 kb (20.7\%) of the sequence spanning 865 genes from the original \textit{B. subtilis} 168 genome (Fig. 1A and Supplementary Table S2). Although the growth rate of MGB874 cells was reduced (30\% in LB and 50\% in SMM) compared with the wild-type 168 strain (Fig. 1B), cell

![Figure 1](image-url)

Figure 1. Design and basal phenotypic analysis of MGB874. (A) Outer concentric ring: genome coordinate (bases) of the \textit{B. subtilis} 168 genome. Ring 2 (green): positions of deleted sequences in MGB874, including prophages and prophage-like regions (SP\textsubscript{b}, PBSX, skin, pro1-7) and polyketide and plipastatin synthesis operons (pks, pps). Ring 3 (dark blue): regions of single deletion. Rings 4 and 5 (light blue): protein coding regions in clockwise (Ring 4) and counterclockwise (Ring 5) orientations. Ring 6 (red): rRNA and tRNA genes. (B) Growth profiles of MGB874 (red squares) and wild-type 168 (blue diamonds) cells in LB and SMM medium. The doubling time of growth is specified. (C) Cell morphology, chromosome distribution, and mean values of cell lengths of wild-type 168 and MGB874 cells. MGB874 and 168 cells were cultured at 37°C in LB or SMM medium, and images were obtained during the exponential growth phase after staining with 4,6-diamindino-2-phenylindole (DAPI). The average cell length is indicated (~200 cells analyzed).
morphology and chromosome distribution were normal (Fig. 1C). The reduced growth rate indicates that certain non-annotated and deleted genes contribute to the metabolic capacity of *B. subtilis* cells under normal growth conditions. It is also possible that this phenotype is caused by unexpected synergetic effects of the deletions of annotated genes. Moreover, MGB874 cells did not form spores (data not shown), owing to the deletion of genes essential for spore formation, including *spoIVCB* and *spoIIIC* that encode the N- and C-terminal regions of sporulation-specific sigma factor-K, respectively.

### 3.3. Exogenous protein productivity in the *B. subtilis* genome reduction mutants

To assess the productivity of MGB874 cells in terms of exogenous protein secretion, we examined the production of thermostable alkaline cellulase, Egl237, and alkaline protease (M-protease) from a multi-copy plasmid, pHY300PLK (~50 copies per cell) harboring the respective genes under the control of a constitutive SigA-dependent promoter of Egl237. We introduced the pHYS237 (for cellulase production) or pHP237-K16 (for protease production) plasmids into the multiple-deletion series strains generated (MGB469 to MGB874) and measured the protease and cellulase activities after 75 h culture in 2xL-Mal, a model medium for industrial protein production (Fig. 3A). Unexpectedly, the production of both enzymes increased in proportion to genome deletion lengths, with maximum levels estimated in the MGB874 strain. The activities of cellulase and protease in the culture medium of MGB874 cells were about 1.7- and 2.5-fold higher than those from wild-type cells, respectively. Increase in cell mass was similar for MGB874 and wild-type cells (Fig. 3B), and cellulase production was arrested in wild-type
168 cells from 28 h upwards (Fig. 3C). In contrast, the cellulase level in MGB874 cells continued to increase throughout the culture period to about twice that obtained from wild-type 168. The production period for protease was similarly elongated in the mutant strain (data not shown). Furthermore, maltose consumption in the culture medium was enhanced in MGB874 cells (Fig. 3D), indicating that the efficiency of carbon source utilization also is improved as a result of genome reduction.

3.4. Reprogramming of the transcriptional regulatory network in MGB874 cells

To assess the molecular events underlying these unexpected phenomena, we compared the transcriptome profiles of MGB874 and wild-type 168 cells during growth in 2xL-Mal medium using a custom Affymetrix tiling chip, as described previously,15 and expression levels of each genes in MGB874 and 168 cells were determined as described in Materials and Methods (Supplementary Table S3). Transcriptome analysis revealed that the expression of a limited number of genes was disrupted in MGB874 cells at the early exponential growth phase. The correlation coefficient of the expression levels of each gene between the two strains was 0.94 (Fig. 4A), but decreased as a function of culture time to 0.71 after 60 h (Fig. 4B–F).

When nutrients in the culture medium were exhausted, B. subtilis cells entered the transition state, accompanied by various phenomena, such as
competence for transformation, secretion of degradative enzymes, and induction of motility through a complex network of global regulators, including ComPA and DegSU two-component systems, the AbrB transition state regulator, and the alternative sigma factor, SigD. Finally, phosphorylation of a two-component regulator, Spo0A, triggers a regulatory program for spore formation involving the activation of an alternative sigma factor, Spo0H, and sporulation-specific sigma factors, SigF and SigG, in prespores and SigE and SigK in mother cells. A closer examination of the changes in transcriptome profiles associated with entry into the stationary phase, and activation of the sporulation program in MGB874 and wild-type cells disclosed remarkable differences in these processes between the two strains. The ComA regulator that induces competence was activated earlier in MGB874 cells, observed as the expression of the srf operon and pel gene that are directly stimulated by ComA. Moreover, competence genes that are indirectly activated by ComA via the master regulator, ComK, were induced earlier in MGB874 cells, compared with the wild-type strain (Fig. 5A). The degU regulator participating in competence activation in the non-phosphorylated form and protein production in the highly phosphorylated form was induced earlier and maintained at high levels throughout the culture period in MGB874 cells (Fig. 5B). This altered feature may also contribute to the earlier competence development in MGB874 cells. However, degradative protein production, possibly dependent on DegU, was similar in both MGB874 and wild-type cells, except for major intracellular serine protease (IspA) and bacillopeptidase F (Bpr), which were strongly induced in MGB874 cells at the late growth phase (Fig. 5B). In contrast, inhibition of the expression of the transition state regulator, AbrB, which represses transition-state genes in the exponential growth phase and is suppressed by phospho-Spo0A, was delayed in MGB874 cells (Fig. 5C). Consistent with this observation, MGB874 cells displayed disrupted expression of SigH, which is repressed by AbrB and activated by phospho-Spo0A. In addition, activation of sporulation-specific sigma factors was delayed in MGB874 cells, as evident from the delay in the

Figure 4. Comparison of gene expression in wild-type 168 and MGB874 cells. Total RNA was extracted from both strains grown in 2xL-Mal medium for 1 (A), 7.5 (B), 13 (C), 26 (D), 40 (E), and 60 (F) hours (Fig. 3B) and used for tiling chip analysis. The average signal intensities of probes in each coding sequence were calculated after removal of the lowest and highest intensities. Scatter plots of expression in wild-type 168 and MGB874 cells are presented. Genes deleted in MGB874 were excluded from the analysis. The correlation coefficient is indicated.
expression patterns of genes under their control (except those directed by SigK, which is deleted in MGB874) (Fig. 5D). These findings suggest that the transition state is extended in MGB874 cells, compared with the wild-type strain. Furthermore, expression levels of the ctaABCDEFG operon encoding cytochrome c oxidase and the atpABEFHAGDC operon encoding FoF1 ATP synthase in MGB874 cells were maintained throughout the culture period (Fig. 5E). Interestingly, appDFABC and dppBCDE encoding ABC
transporters for oligopeptide and dipeptide, respectively, malP representing maltose-specific PTS enzyme, and bioWFADB coding for biotin biosynthesis enzymes were active throughout the culture period (Fig. 5E). Thus, it appears that metabolic activities are maintained in the extended transition state of MGB874 cells. Global regulators for the transition state influence each other in terms of expression, forming a complex regulatory network. Moreover, activities of ComA, DegU, and SpoOF (phosphorylates Spo0A) are regulated by Rap family proteins to control the precise activation timing. Six rap genes among the 11 encoded in the B. subtilis genome were deleted in the MGB874 strain, which may attribute to the disruption of transition state timing and gene expression during sporulation. A significant increase of protein productivity occurred in the deletion process from MGB625 to MGB723 (Fig. 3A). The rapG gene and its regulator phrG were deleted in this process, suggesting that the deletion of RapG had a major impact on protein productivity among deletions of six rap genes.

Maintenance of metabolic activities in the extended transition state of MGB874 cells would be a basis for increased enzyme productivity. In addition, the remarkable changes in gene expression observed might be related to higher protein productivity of MGB874 cells (Fig. 5F). B. subtilis SigD, activated in the transition state, is responsible for the transcription of genes for flagellar assembly, motility, chemotaxis, and autolysis. However, significantly low transcription of SigD and genes under its control was observed in MGB874 cells. The argGH and argCJBD operons for arginine biosynthesis were induced during the transition state in wild-type cells, but not in the mutant MGB874 cells. In contrast, a number of genes were strongly induced in MGB874 cells, including yakJ encoding L-malate and citrate transporter, levDEFG encoding fructose-specific PTS enzyme, and oxdC encoding cytosolic oxalate decarboxylase (Fig. 5G). We additionally observed the earlier and stronger induction of genes related to phosphate metabolism under the control of the PhoPR two-component system in MGB874 cells, including gipQ encoding glycerol-phosphoryl diester phosphodiesterase, phoD (phosphodiesterase), phoA (alkaline phosphatase A), pstSCAB (phosphate transporter), and tuaABCDEFG (teichuronic acid biosynthesis) (Fig. 5H).

These results indicate that considerable reprogramming of the transcriptional regulatory network occurs in MGB874 cells, probably due to the synergistic effect of multiple deletions, although the molecular basis for these changes and their relationship to increased protein productivity await further investigation.

4. Conclusion

To our knowledge, this is the first report demonstrating that genome reduction actually contributes to the creation of bacterial cells with a practical application in industry. It is not clear at the moment that phenomena we observed are due to a global synergistic effect of large-scale genome reduction or mainly due to the deletion of several regulators. Further systematic analysis of changes in the transcriptional regulatory network of MGB874 cells in relation to protein productivity should facilitate the generation of improved B. subtilis cells as hosts of industrial protein production. We are in the process of introducing further rational deletions on the basis of transcriptome data, gene function information, and comparative genomics, with a view to generating simple, predictable cells comprising genes with defined functions as a new platform of development of bacterial strains for industrial use.

Acknowledgements: We are grateful to Shu Ishikawa and Taku Oshima for help in transcriptome analysis and to Junichi Sekiguchi, Kouji Nakamura, and Fujio Kawamura for valuable discussions. This work is part of the subproject ‘Development of a Technology for the Creation of a Host Cell’ included within the industrial technology project ‘Development of Generic Technology for Production Process Starting Productive Function’ of the Ministry of Economy, Trade and Industry, entrusted by the New Energy and Industrial Technology Development Organization (NEDO), Japan.

Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

Funding

This work is part of the subproject ‘Development of a Technology for the Creation of a Host Cell’ included within the industrial technology project ‘Development of Generic Technology for Production Process Starting Productive Function’ of the Ministry of Economy, Trade and Industry, entrusted by the New Energy and Industrial Technology Development Organization (NEDO), Japan.

References

1. Ball, P. 2007, Synthetic biology: designs for life, Nature, 448, 32–33.
2. Drubin, D. A., Way, J. C. and Silver, P. A. 2007, Designing biological systems, Genes Develop., 21, 242–254.
3. Forster, A. C. and Church, G. M. 2007, Synthetic biology projects in vitro, Genome Res., 17, 1–6.
4. Posfai, G., Plunkett, G. III, Feher, T., Frisch, D., Keil, G. M., Umemoto, K., Kolinskychenko, V., Stahl, B., Sharma, S. S., de Arruda, M., et al. 2006, Emergent properties of reduced-genome Escherichia coli, Science, 312, 1044–1046.

5. Mizoguchi, H., Mori, H. and Fujio, T. 2007, Escherichia coli minimum genome factory, Biotechnol. Appl. Biochem., 46, 157–167.

6. Ara, K., Ozaki, K., Nakamura, K., Yamane, K., Sekiguchi, J. and Ogasawara, N. 2007, Bacillus minimum genome factory: effective utilization of microbial genome information, Biotechnol. Appl. Biochem., 46, 169–178.

7. Westers, L., Dorenbos, R., van Dijl, J. M., Kabel, J., Flanagan, T., Devine, K. M., Jude, F., Seror, S. J., Beekman, A. C., Darmon, E., et al. 2003, Genome engineering reveals large dispensable regions in Bacillus subtilis, Mol. Biol. Evol., 20, 2076–2090.

8. Westers, L., Westers, H. and Quax, W. J. 2004, Bacillus subtilis as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism, Biochim. Biophys. Acta, 1694, 299–310.

9. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., et al. 1997, The complete genome sequence of the gram-positive bacterium Bacillus subtilis, Nature, 390, 249–256.

10. Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., et al. 2003, Essential Bacillus subtilis genes, Proc. Natl. Acad. Sci. USA, 100, 4678–4683.

11. Anagnostopoulos, C. and Spizizen, J. 1961, Requirements for transformation in Bacillus subtilis, J. Bacteriol., 81, 741–746.

12. Harwood, C. R. and Archibald, A. R. 1990, Growth, maintenance and general techniques, In: Harwood, C. R. and Cutting, S. M. (eds), Molecular Biological Methods for Bacillus, Chichester, New York, Brisbane, Toronto, Singapore: John Wiley & Sons, p. 549.

13. Hakamada, Y., Hatada, Y., Koike, K., Yoshimatsu, T., Kawai, S., Kobayashi, T. and Ito, S. 2000, Deduced amino acid sequence and possible catalytic residues of a thermostable, alkaline cellulase from an Alkaliphilic bacillus strain, Biosci. Biotech. Biochem., 64, 2281–2289.

14. Kobayashi, T., Hakamada, Y., Adachi, S., Hitomi, J., Yoshimatsu, T., Koike, K., Kawai, S. and Ito, S. 1995, Purification and properties of an alkaline protease from alkalophilic Bacillus sp. KSM-K16, Appl. Microbiol. Biotechnol., 43, 473–481.

15. Ishikawa, S., Ogura, Y., Yoshimura, M., Okumura, H., Cho, E., Kawai, Y., Kurokawa, K., Oshima, T. and Ogasawara, N. 2007, Distribution of stable DNA binding sites on the Bacillus subtilis genome detected using a modified ChIP-chip method, DNA Res., 14, 155–168.

16. Igo, M. M. and Losick, R. 1986, Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in Bacillus subtilis, J. Mol. Biol., 191, 615–624.

17. Quackenbush, J. 2002, Microarray data normalization and transformation, Nature Genet., 32, Suppl., 496–501.

18. Hirai, M. Y., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D. B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., et al. 2005, Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics, J. Biol. Chem., 280, 25590–25595.

19. Itaya, M. 1992, Construction of a novel tetracycline resistance gene cassette useful as a marker on the Bacillus subtilis chromosome, Biosci. Biotech. Biochem., 56, 685–686.

20. Fabret, C., Ehrlich, S. D. and Noirot, P. A. 2002, A new mutation delivery system for genome-scale approaches in Bacillus subtilis, Mol. Microbiol., 46, 25–36.

21. Hodge, J. E. and Hofreiter, B. T. 1962, Analysis and preparation of sugars, In: Whistler, R. L. and Wolfrom, M. L. (eds), Methods in Carbohydrate Chemistry, New York, San Francisco, London: Academic Press, p. 388–389.

22. Piggot, P. J. and Hilbert, D. W. 2004, Sporulation of Bacillus subtilis, Curr. Opin. Microbiol., 7, 579–586.

23. Ogura, M., Yamaguchi, H., Yoshida, K., Fujita, Y. and Tanaka, T. 2001, DNA microarray analysis of Bacillus subtilis DegU, ComA and PhoP regulons: an approach to comprehensive analysis of B. subtilis two-component regulatory systems, Nucleic Acids Res., 29, 3804–3813.

24. Hamoen, L. W., Van Werkhoven, A. F., Venema, G. and Dubnau, D. 2000, The pleiotropic response regulator DegU functions as a priming protein in competence development in Bacillus subtilis, Proc. Natl. Acad. Sci. USA, 97, 9246–9251.

25. Olmos, J., de Anda, R., Ferrari, E., Bolivar, F. and Valle, F. 1997, Effects of the sinR and degU32 (Hy) mutations on the regulation of the aprE gene in Bacillus subtilis, Mol. Gen. Genet., 253, 562–567.

26. Mirel, D. B., Estacio, W. F., Mathieu, M., Olmsted, E., Ramirez, J. and Marquez-Magana, L. M. 2000, Environmental regulation of Bacillus subtilis sigma(D)-dependent gene expression, J. Bacteriol., 182, 3055–3062.

27. Serizawa, M., Yamamoto, H., Yamaguchi, H., Fujita, Y., Kobayashi, K., Ogasawara, N. and Sekiguchi, J. 2004, Systematic analysis of SigD-regulated genes in Bacillus subtilis by DNA microarray and northern blotting analyses, Gene, 329, 125–136.

28. Antelmann, H., Scharf, C. and Hecker, M. 2000, Phosphate starvation-inducible proteins of Bacillus subtilis: proteomics and transcriptional analysis, J. Bacteriol., 182, 4478–4490.