Note

Mutational Site in Tunicamycin-resistant Mutation *tmrB8* of *Bacillus subtilis* as Revealed at the Nucleotide Sequence Level

Akihiko TANIMOTO,[†] Shigeyoshi HARADA,
Masaki MORI, Noboru YAMAJI,
Kunio YAMANE,* Kiyotaka OTOZAI,*
Koji YODA, Makari YAMASAKI
and Gakuzo TAMURA^{††}

Department of Agricultural Chemistry, The University
of Tokyo, Bunkyo-ku, Tokyo 113, Japan

*Institute of Biological Sciences, University of Tsukuba,
Sakura, Ibaraki 305, Japan

Received September 30, 1987

Gram positive bacteria, especially *Bacillus subtilis* and related species, are sensitive to tunicamycin. We isolated several tunicamycin-resistant mutants from *B. subtilis* NA64. The mutants were classified into two groups. One of the mutants, called the B7 strain, was simultaneously

endowed with hyperproductivity of α -amylase.¹⁾ The mutational site of the *tmrA7* mutant (B7 strain) was mapped upstream of *amyE*.²⁾ The mutational sites of *tmrB4*, *tmrB8* and *tmrB10* of other representative mutants without hyperproductivity of α -amylase were mapped near the *aroI* gene.²⁾ The wild type *tmrB*⁺ gene has already been cloned on the pGR71 multicopy plasmid and shown to endow host cells with tunicamycin-resistance in the multicopy state.³⁾ Recently, gene amplification of the *amyE*-*tmrB*⁺ region was found in the B7 strain. The pleiotropy of the *tmrA7* mutation, therefore, was clearly explained by the simultaneous amplification of the *amyE* and *tmrB*⁺ genes.^{4,5)}

In this study, we determined the base sequence of the *tmrB8* gene to elucidate the mechanism of tunicamycin-resistance. We found a 28 bp direct repeat insertion between the putative -10 region and the Shine-Dalgarno (SD) sequence of the *tmrB8* gene.

The bacterial strain and phage strain used were *B. subtilis* 207-21 (m₁₆₈⁻, *hsmM*, *aroI906*, *metB5*, *lys21*, *leuA8*, *amyE07*) and ρ 11d*tmrB8*-*aroI*⁺ (6), respectively. As shown in Fig. 1, ρ 11d*tmrB8*-*aroI*⁺ carries a truncated *tmrB8* gene. At present, however, this is the only available cloned gene for *tmrB8*. The 5.1 kb *EcoRI* fragment which carries the truncated *tmrB8* gene and the *aroI*⁺ region in the recombinant phage DNA was subcloned on pBR328. The truncated *tmrB8* gene was further subcloned as a 1.45 kb *HindIII*-*HincII* fragment on pUC18

FIG. 1. The Nucleotide Sequences in the 5'-Flanking Regions of the *tmrB8* and *tmrB*⁺ Genes.

The sequence for the *tmrB*⁺ gene was cited from ref. 3. The boxed base sequence represents the direct repeat. -35, -10 and SD represent the putative -35 and -10 regions, and SD sequence, respectively. The boxed ATG represents the putative initiation codon for the *tmrB*⁺ (or *tmrB8*) gene. E denotes *EcoRI* sites. J denotes the point at which an unequal illegitimate recombination between sister chromatids might have occurred as the first mutational event in the B7 strain. J also indicates the position of the junction point of the repeating unit for gene amplification found in the B7 strain. Wavy arrows above and below the genes indicate the direction of transcription.

[†] Present address: [†]Gyosei High School, Fujimi, Chiyoda-ku, Tokyo 102, Japan.

^{††} Present address: Department of Applied Biological Science, Science University of Tokyo, Noda-shi, Chiba 278, Japan.

(Fig. 1). The base sequence was determined by the dideoxy chain termination method⁷⁾ and partly by the method of Maxam and Gilbert.⁸⁾

We have already determined the base sequence of the *tmrB*⁺ gene with its 5'-flanking region.³⁾ A base sequence difference between the *tmrB*⁺ gene and the truncated *tmrB8* gene was found only in the 5'-flanking region. As shown in Fig. 1, a 28 bp direct repeat insertion was found in that region of the *tmrB8* gene. We have not determined the mRNA start point. However, the insertion point is located between the putative -10 region and the SD sequence. The indicated -10 and -35 regions coincide partly with the consensus sequence for the σ^{43} promoter.⁹⁾ As a result of the 28 bp insertion, the *tmrB8* gene has an additional -10 region. If this sequence also functions as the binding site of the σ^{43} type RNA polymerase, it may contribute to the higher expression of the *tmrB8* gene than the wild *tmrB*⁺ gene. No other promoter-like sequence was found in the 5'-flanking region. Judging from the fact that the *tmrB*⁺ gene endows host cells with tunicamycin-resistance only in the multicopy state,³⁾ an increase in the gene product seems to be necessary for the phenotypic expression of tunicamycin-resistance. So far as known there are few examples of such a short distance between a -10 region and a SD sequence, as postulated for the *tmrB*⁺ gene. Several ten bp sequences are present between the -10 region and the SD sequence in most cloned *B. subtilis* genes. The insertion of 28 bp between the putative -10 region and the SD sequence could otherwise function as a favorable spacer or a mRNA stabilizing sequence for better translation efficiency.

A striking feature of the insertion point is its exact coincidence with the junction point of the repeating unit for gene amplification found in the B7 strain (*tmrA7* mutant).^{5,10)} The first mutational event in the B7 strain is supposed to be an unequal illegitimate recombination between sister chromatids at the very insertion point. This point, therefore, seems to be a hot spot for DNA rearrangement. We have not determined the 3'-end about 200 bp of the *tmrB8* gene because this portion was deleted during the cloning procedure.⁶⁾ We cannot eliminate the possibility of a further base sequence difference between the *tmrB8* and *tmrB*⁺ genes in the deleted portion.

In conclusion, we found a 28 bp direct repeat insertion

between the putative -10 region and the SD sequence. The insertion may enhance the transcription and/or translation efficiency of the *tmrB*⁺ gene resulting in the expression of tunicamycin-resistance.

Acknowledgment. This work was supported by a Grant-in-Aid for Special Distinguished Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) T. Sasaki, M. Yamasaki, B. Maruo, Y. Yoneda, K. Yamane, A. Takatsuki and G. Tamura, *Biochem. Biophys. Res. Commun.*, **70**, 125 (1976).
- 2) S. Nomura, K. Yamane, T. Sasaki, M. Yamasaki, G. Tamura and B. Maruo, *J. Bacteriol.*, **136**, 818 (1978).
- 3) S. Harada, K. Yoda, A. Tanimoto, M. Mori, K. Yamane, T. Furusato, A. Takatsuki, M. Yamasaki and G. Tamura, *Agric. Biol. Chem.*, submitted.
- 4) K. Hashiguchi, A. Tanimoto, S. Nomura, K. Yamane, K. Yoda, S. Harada, M. Mori, T. Furusato, A. Takatsuki, M. Yamasaki and G. Tamura, *Agric. Biol. Chem.*, **49**, 545 (1985).
- 5) K. Hashiguchi, A. Tanimoto, S. Nomura, K. Yamane, K. Yoda, S. Harada, M. Mori, T. Furusato, A. Takatsuki, M. Yamasaki and G. Tamura, *Mol. Gen. Genet.*, **204**, 16 (1986).
- 6) K. Otozai, Y. Takeishi, A. Nakayama, K. Yamane, A. Tanimoto, M. Yamasaki, G. Tamura, S. Nomura, F. Kawamura and H. Saito, *J. Gen. Appl. Microbiol.*, **30**, 15 (1984).
- 7) F. Sanger, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci., U.S.A.*, **74**, 5463 (1977).
- 8) A. M. Maxam and W. Gilbert, "Methods in Enzymology," Vol. LXV, ed. by L. Grossman and K. Moldave, Academic Press, New York 1979, p. 499.
- 9) C. P. Moran, Jr., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero and R. Losick, *Mol. Gen. Genet.*, **186**, 339 (1982).
- 10) M. Mori, A. Tanimoto, K. Yoda, S. Harada, N. Koyama, K. Hashiguchi, M. Obinata, M. Yamasaki and G. Tamura, *J. Bacteriol.*, **166**, 787 (1986).