Conversion of Temperature-sensitive to -resistant Gene Expression Due to Mutations in the Promoter Region of the Melibiose Operon in Escherichia coli

(Received for publication, February 2, 1998, and in revised form, April 21, 1998)

Eiji Tamaï†, Tadashi Shimamoto§, Masaaki Tsuda¶, Tohru Mizushima‡, and Tomofusa Tsuchiya†§

From the †Department of Microbiology, Faculty of Pharmaceutical Sciences, and §Gene Research Center, Okayama University, Tsushima, Okayama, 700-8530, Japan

The melibiose utilization system of Escherichia coli W3133, a derivative of K12, is nonfunctional between 37 and 42 °C. The reason for this temperature sensitivity was thought to be that the melibiose transporter (MelB) of W3133 cells was temperature-sensitive. A mutant W3133-2 has been isolated as a temperature-resistant strain that can utilize melibiose between 37 and 42 °C. However, we found that the melibiose transporter of the W3133-2 was still temperature-sensitive. Half-life activities of the melibiose transporter at 37 °C (or 40 °C) in both E. coli W3133 and W3133-2 were exactly the same. Furthermore, we found that the nucleotide sequence of coding region of the melB structural gene (the second gene of the melibiose operon) of W3133-2 was exactly the same as that of W3133. Activity of β-galactosidase (product of the first gene, melA, of the melibiose operon) of W3133 cells grown at 40 °C was very low, although that of W3133-2 cells grown at 40 °C was high. These observations suggested that expression of the melibiose operon in W3133 is also temperature-sensitive. In fact, we found that the expression in W3133 cells was temperature-sensitive, while that in W3133-2 cells was temperature-resistant, by analyzing mRNA levels using the Northern blot method. Furthermore, we identified mutations in the promoter region of the melibiose operon of W3133-2 that resulted in the elongation of an 18 nucleotide inverted repeat sequence to a 28-nucleotide repeat sequence present immediately upstream of the −35 region. This may stabilize a possible stem structure due to the inverted repeat at 37–42 °C.

For utilization of melibiose by Escherichia coli, both the melibiose transporter and α-galactosidase are necessary. The melibiose transporter mediates uptake of melibiose into cells. The α-galactosidase catalyzes cleavage of melibiose into galactose and glucose, which are metabolized via the glycolysis pathway. Genes encoding these two proteins, melA (α-galactosidase) and melB (melibiose transporter), constitute structural genes in the melibiose operon (1–3). The melibiose operon is inducible by either melibiose or galactitol (4), or melibitol (2). A regulatory gene for the melibiose operon, melR, is located in a region upstream of the melAB genes in the opposite orientation (5). The MelR is an activator necessary for expression of the melAB genes. An inducer such as melibiose is required to activate MelR (6).

The melibiose transporter in E. coli was first reported as the TMG1 permease II, because this transport system was found to be the second transport system for TMG (4). The first TMG transport system (TMG permease I) is known as the lactose transporter LacY (7). LacY is also able to transport melibiose (7). Therefore there are two transport systems for melibiose in E. coli cells. One of the unique properties of the melibiose transporter in E. coli K12 and its derivatives is that it is a temperature-sensitive system (4). When cells are grown at 30 °C, the melibiose transporter is active, but if cells are grown at 37 °C or above, the transporter is inactive. We have previously shown that the melibiose transporter, but not α-galactosidase, was irreversibly inactivated by incubation at 37 °C (8).

It is necessary to use a LacY-defective mutant to investigate the melibiose transporter. E. coli W3133 is a derivative of K12 and lacks the lactose transporter (9). The melibiose transporter of W3133 is temperature-sensitive (9). A mutant W3133-2 was isolated as a temperature-resistant strain derived from W3133 (9). Although cells of W3133 showed either very poor growth or no growth on melibiose as a sole source of carbon at 37 °C, cells of W3133-2 showed normal growth at this temperature (9) or even at 40 °C.² Furthermore, cells of W3133 grown and induced at 37 °C showed either little or no activity of the melibiose transporter. Cells of W3133-2 grown and induced at the same temperature showed high activity (9). Thus, it seemed very clear that the melibiose transporter of W3133 cells is temperature-sensitive and that of W3133-2 cells is temperature-resistant. We have cloned and sequenced the melB gene of E. coli. The amino acid sequence of this melibiose transporter protein has been deduced from melB (10). Thus it became possible to identify the substituted amino acid residue(s) in the melibiose transporter of the temperature-resistant W3133-2. However, we found no mutation in the nucleotide sequence of the melB gene from the mutant strain (W3133-2).³ These results suggest that the reason for the temperature-resistant growth of W3133-2 cells on melibiose is not due to changes in the primary structure of the melibiose transporter but in other factor(s) of W3133-2.

Here we report that gene expression of the melibiose operon in the parental strain is temperature-sensitive and that in the mutant strain is temperature-resistant. We found mutations in the promoter region of the melibiose operon of the mutant.

---

1 The abbreviations used are: TMG, methyl-β-D-thiogalactopyranoside; MOPS, morpholinepropanesulfonic acid.
2 E. Tamai, T. Mizushima, and T. Tsuchiya, unpublished results.
**EXPERIMENTAL PROCEDURES**

**Bacterium and Growth**—E. coli W3133, a derivative of K12, is a lactose-deleted (ΔlacZ) strain containing the wild type melibiose utilization system (9). Cells of W3133 are unable to grow on melibiose as a sole source of carbon at 37 °C or above. A mutant called W3133-2, a derivative of W3133, was isolated as a temperature-resistant strain with regard to melibiose utilization and can grow on melibiose at 37 °C (9). A chromosomal DNA fragment (ΔlacZY, amelAB) (11) was used as a host for cloning of the melibiose operon from chromosomal DNA of W3133 and W3133-2. Cells of W3133 and W3133-2 were first grown in the LB medium (12) at 37 °C, diluted 200-fold into a modified Tanaka medium (13) (Na+ salts were replaced with K+ salts) supplemented with 1% tryptone, 0.001% thiamine, and 10 mM melibiose (an inducer of the melibiose operon) and shaken at 30, 34, 37, 40, or 42 °C under aerobic conditions. Cells were harvested at logarithmic phase of growth.

For measurement of the growth on melibiose, cells were first grown in the modified Tanaka medium supplemented with 10 mM melibiose at 30 °C, diluted 200-fold into fresh medium, and shaken at various temperatures under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm.

**Measurement of Transport**—TMG uptake was measured as described previously (9) with minor modification using [methyl-3H]CTMG as a substrate. To test the effect of temperature on the transport activity, cells suspended in the modified Tanaka medium containing 10 µg/ml chloramphenicol were incubated at 30, 37, 40, or 42 °C for the indicated time. The transport assays were performed in the modified Tanaka medium containing 20 mM potassium lactate, 20 mM NaCl, 10 µg/ml chloramphenicol, cells (0.15 mg of protein/ml), and 0.1 µCi ([3H]TMG) at 25 °C.

**a-Galactosidase Assay**—a-Galactosidase activity was measured as described previously (14) using p-nitrophenyl-a-D-galactopyranoside as a substrate.

**Preparation of RNA**—Total cellular RNA was prepared from E. coli cells by the method of Alba et al. (15).

**Northern Hybridization Analysis**—RNA was denatured with formaldehyde and then electrophoresed on 1% agarose gel containing 2 M formaldehyde. Total RNA in the gel was transferred to a nylon membrane, Hybond-N (Amersham Corp.), overnight by staining with ethidium bromide. Total cellular RNA was prepared from various temperatures. Cells were harvested at logarithmic phase of growth. Initial velocity of TMG transport was measured at 25 °C.

**RESULTS**

**Temperature Sensitivity of Melibiose Transporter**—The melibiose transport system is the sole uptake system for melibiose (and TMG) in E. coli W3133 and W3133-2 (9). W3133 cells were unable to grow on melibiose at 37 °C or above, although cells were able to grow on other carbon sources such as glucose or amino acid mixtures at 42 °C. However, W3133-2 cells were able to grow on melibiose at 42 °C (data not shown). As reported previously (9), both types of cells grew well on melibiose at 30 °C. W3133 cells were able to grow on melibiose at 35 °C. However, 37 °C is an unreliable temperature for the measurement of growth of W3133 cells on melibiose. On some occasions the cells grew and at other occasions they did not. W3133 cells were unable to grow on melibiose at 40 °C. Thus, 40 °C instead of 37 °C is a convenient temperature for investigation of the temperature sensitivity and resistance of the melibiose utilization system of W3133 and W3133-2.

Fig. 1 shows that activity of the melibiose transporter (TMG uptake) in W3133 cells was very low when cells were grown at 37 °C or above. Although the activity was fairly high when cells were grown at 30 °C. The activity of the melibiose transporter in W3133-2 was high when cells were grown at 30–34 °C, moderate when grown at 37–40 °C, and very low when grown at 42 °C. These results suggest that the melibiose transporter in W3133-2 cells are still temperature-sensitive to a certain extent.

To compare temperature sensitivity of the melibiose transporter between W3133 cells and W3133-2 cells, we tested the effect of temperature on activity of the melibiose transporter. Fig. 2 shows decay lines of TMG transport activity when cells were incubated at 30, 37, or 40 °C. Surprisingly, W3133 cells and W3133-2 cells showed the same decay lines at 37 °C. The half-life of the melibiose transport activity in the two strains was exactly the same, 35 min. This half-life is very similar to that we reported previously with another strain (GN22) derived from K12 (8). When cells were incubated at 40 °C, again the activity decay lines were not distinguishable between W3133 and W3133-2. The half-life was 17 min. We also tested heat inactivation at 42 °C and obtained very similar decay profiles to that obtained at 40 °C in both strains (data not shown). No decrease of the transport activity was observed with both W3133 and W3133-2 when cells were incubated at 30 °C (Fig. 2). Thus, it became clear that there is no difference in the temperature sensitivity of the melibiose transport protein between W3133 cells and W3133-2 cells.

Previously we reported the nucleotide sequence of the melB gene and the deduced amino acid sequence of the melibiose transporter (10). Thus, it became possible to identify substitu-

![Fig. 1. Activity of melibiose transporter in cells grown at various temperatures. Cells of E. coli W3133 (○) or W3133-2 (●) were grown in the modified Tanaka medium supplemented with 1% tryptone and 10 mM melibiose under aerobic conditions at the indicated temperatures. Cells were harvested at the exponential phase of growth. Initial velocity of TMG transport was measured at 25 °C.](http://www.jbc.org/Downloadedfrom)
were grown in the modified Tanaka medium supplemented with 1% tryptone and 10 mM melibiose under aerobic conditions at 30 °C. Cells were harvested at the exponential phase of growth and suspended in the modified Tanaka medium containing 10 μg/ml chloramphenicol. Cells were incubated at 30 °C (circles), 37 °C (squares), or 40 °C (triangles). At the indicated time points, samples were removed and cooled in an ice bath until they were assayed. The samples were assayed for TMG transport at 25 °C. Initial values (100%) were: 35 nmol/min per mg of cell protein for W3133 and 47 nmol/min per mg of cell protein for W3133-2.

Effect of Temperature on Gene Expression—As we reported previously, the W3133-2 cells showed partially constitutive expression of the melibiose transporter, although expression in the parental W3133 cells was completely inducible (9). This indicates that there is a difference in the expression of the operon between the parent and the mutant, and suggests that the reason for temperature sensitivity is the expression of the melibiose operon. We therefore tested the effect of temperature on gene expression of the melibiose operon with the two strains. We have shown previously that α-galactosidase of E. coli was not temperature-sensitive (8). Thus, the level of α-galactosidase activity reflects the level of expression of the melibiose operon. We measured α-galactosidase activity in cells grown at various temperatures. The experiments, melibiose was added to the growth medium to fully induce the operon. The α-galactosidase activity in W3133 cells grown at 30 °C was high, moderate in cells grown at 37 °C, and very low in cells grown at 40 or 42 °C (Fig. 3). The activity in W3133-2 cells was much higher than that in W3133 cells (Fig. 3). The enzyme activity in W3133-2 cells was very high even if cells were grown at 40 or 42 °C. These results support the idea that expression of the melibiose operon in W3133 cells is temperature-sensitive while that in W3133-2 cells is not.

We tested this possibility by Northern blot analysis. The probe used was a BamHI fragment derived from the melB gene, which is located downstream from the melA gene (3, 10). Therefore, the mRNA detected in our assay is melAB mRNA (19). The mRNA level in W3133-2 cells induced with melibiose was about two times higher than that in W3133 cells when grown at 30 °C (Fig. 4). The ratio of the mRNA level in W3133-2 cells to that of W3133 cells grown at 30 °C was comparable to the ratio of the α-galactosidase activity of these cells (Table I). The levels of mRNA in both W3133-2 and W3133 increased when cells were grown at 37 °C, and the level in W3133-2 was 2.7 times higher than that in W3133. As expected, we detected a faint band of the melAB mRNA with W3133 cells grown at 40 °C, although a dense band was detected with W3133-2 cells (Fig. 4). The mRNA level in W3133-2 cells was 7.9 times higher than that in W3133 cells. A very low level of melAB mRNA was detected in W3133 cells grown at 42 °C, but a considerable level of the mRNA was detected in W3133-2 cells. The ratio of the mRNA level in W3133-2 cells to that of W3133 cells is comparable to the ratio of the α-galactosidase activity in these cells for all temperatures tested except at 42 °C (Table I). The experiments were repeated four times and very similar results were obtained. Thus, levels of α-galactosidase activity accurately reflect the levels of melAB mRNA at 30–40 °C. The reason why the mRNA level is very low at 42 °C irrespective of the relatively high α-galactosidase activity is not yet clear. In any case, our results indicate that expression of the melibiose operon in W3133 cells is temperature-sensitive while that in W3133-2 cells is fairly temperature-resistant.

Identification of a Promoter Mutation—The temperature-sensitive expression of the melibiose operon in W3133 cells and
Temperature Sensitivity in Melibiose Operon of E. coli

The temperature-resistant expression in W3133-2 cells suggested that mutation(s) may be present in the regulatory gene (melR) for the operon or in the promoter region of the melAB genes. We cloned the entire melibiose operon from both W3133 and W3133-2 cells. Plasmids carrying the melibiose operon were introduced into cells of E. coli DW1, which contains a deletion through the melA and melB genes (11). The melAB mRNA was not detected with uninduced cells of DW1 harboring the plasmid pBM3133 carrying the melibiose operon from W3133 (Fig. 5). However, we detected dense band of melAB mRNA with uninduced DW1 cells harboring a plasmid pBM3133-2 carrying the melibiose operon from W3133-2 (Fig. 5). A faint mRNA band was detected in DW1/pBM3133 cells induced with melibiose at 40 °C and a more dense band was detected with melibiose-induced cells of DW1/pBM3133-2 (Fig. 5). Thus, mutation(s) responsible for the temperature-resistant expression of the melibiose operon in W3133-2 must be present in the cloned region. Again, the melibiose operon derived from W3133-2 was partially constitutive. Therefore mutation(s) responsible for the partial constitutive phenotype must be also in the cloned region.

We sequenced the entire melibiose region. We found no difference in the nucleotide sequence of the melR coding region, in the promoter region for the melR gene, and in the melA to melB region. Instead, we found a replacement of 5 consecutive nucleotides in the promoter region for the melAB genes (Fig. 6). The transcription initiation site for the melAB genes in Fig. 6 has been reported (5). The translation initiation site for the melA gene was confirmed by amino acid sequencing of the NH₂ terminus of purified α-galactosidase (20). The sequence of the melAB promoter region from W3133 was identical with that from E. coli CS520 (3, 21), a derivative of K12, of which we were the first to report the promoter region sequence (22). There is one long inverted repeat and several short inverted repeats in the promoter region (22). The replacement is immediately downstream from the long inverted repeat which has been reported as one of the binding sites for MelR (23, 24), and lies between the long inverted repeat and the short inverted repeat (Fig. 6).

### Table I

| Temperatures (°C) | Ratios (melAB/melA) | Rationa (W3133-2/W3133) |
|------------------|--------------------|------------------------|
| 30               | 2.0                | 2.0                    |
| 37               | 2.6                | 2.7                    |
| 40               | 8.0                | 7.9                    |
| 42               | 8.6                | 4.6                    |

* Ratios were calculated from data from Figs. 3 and 4.

DISCUSSION

The cause of the temperature sensitivity of the melibiose utilization system in E. coli K12 and its derivatives has long been thought to be that the melibiose transporter is temperature-sensitive (4, 8, 9). We found that expression of the melibiose operon, in addition to the melibiose transporter, in E. coli W3133 (a derivative of K12) was temperature-sensitive. We identified a replacement of 5 consecutive nucleotides in the promoter region of the melibiose operon from the mutant strain. The 5 replaced nucleotides were located just between a long inverted repeat (18 nucleotides) and the following a short inverted repeat (5 nucleotides). In short, the replacement resulted in appearance of a huge inverted repeat (28 nucleotides). It has been reported that the original long inverted repeat is the site for binding of MelR (23, 24). Thus, it is likely that complex of MelR and melibiose (an inducer) can bind to the huge inverted repeat region even if the temperature is 40–42 °C. On the other hand, perhaps the MelR-inducer complex is unable to bind to the original inverted repeat (18 nucleotides repeat) at such high temperatures, although the complex can bind at lower temperature such as 30 °C. Although it is not clear whether the stem-loop structure of DNA is really formed in vivo, a stem structure formed with a 28-nucleotide repeat would be more stable than that formed with a 18-nucleotide repeat at 40 or 42 °C. Also it may be possible that the 18-nucleotide inverted repeat forms a stem structure at 30 °C but not at 40 °C. Although we do not have direct evidence for in vivo formation of the stem-loop structure, our results are consistent with the idea that MelR binds to the hypothetical stem

![Fig. 5. Northern analysis of melAB mRNA expressed from cloned melibiose operon.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

![Fig. 6. Mutations in the promoter region of the melibiose operon.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

*Norwesque*
structure and formation of the stem structure is temperature-sensitive. In any case, the temperature sensitivity and resistance of gene expression due to the alteration of the structure (sequence) in the promoter region of the melibiose operon of E. coli (but not due to the regulatory protein) is a very unique case. Usually protein is responsible for temperature-sensitive cellular processes (25).

The melibiose operon in the parental W3133 cells or other K12-derived cells is inducible. An inducer such as melibiose is necessary for this induction (4). The melibiose operon in the mutant W3133-2 cells, however, is partially constitutive (9). Thus, it seems that the MelR can bind to the 28-nucleotide inverted repeat structure, but not to the 18-nucleotide inverted repeat structure, to some extent without the inducer. In the presence of inducer, perhaps the MelR-inducer complex can bind to the longer inverted repeat easily in W3133-2 cells and perhaps transcription increases even at 30 °C compared with W3133 cells. Therefore, it seems likely that the induction level in W3133-2 cells is higher than that in W3133 cells.

The melibiose transport proteins in both W3133 and W3133-2 cells are equally temperature-sensitive (equally inactivated at 40 °C). Nevertheless, cells of W3133-2 are able to grow on melibiose at 40 °C and cells of W3133 are not. One possible explanation for this is as follows. Perhaps the rate of production of the melibiose transporter exceeds the rate of inactivation of the protein in W3133-2 cells at 40 °C. The efficient production of the melibiose transporter protein would be due to elevated mRNA synthesis in this strain. On the other hand, the rate of the protein inactivation exceeds the rate of the protein production in W3133 cells at 40 °C. The half-lives of the melibiose transporter inactivation rates were 35 min at 37 °C and 17 min at 40 °C. Thus, the production rate of the melibiose transport protein must exceed the inactivation rate for cells to grow on melibiose at such temperatures.

Acknowledgment—We thank Dr. Manuel F. Varela of Eastern New Mexico University for critically reading the manuscript.

REFERENCES

1. Schmitt, R. (1968) J. Bacteriol. 96, 462–471
2. Leviathan, M. (1971) J. Bacteriol. 105, 1047–1052
3. Hanatani, M., Yazyu, H., Shiota-Niiya, S., Moriyama, Y., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) J. Biol. Chem. 259, 1807–1812
4. Prestridge, L. S., and Pardee, A. B. (1965) Biochim. Biophys. Acta 100, 591–593
5. Webster, C., Kaempfeli, K., Booth, I., and Busby, S. (1987) Gene (Amst.) 58, 253–263
6. Webster, C., Gardner, L., and Busby, S. (1989) Gene (Amst.) 83, 207–213
7. Kennedy, E. P. (1970) in The Lactose Operon (Beckwith, J. R. and Zipser, D., eds) pp. 49–82, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Tsuchiya, T., Lopilato, J., and Wilson, T. H. (1978) J. Membr. Biol. 42, 45–59
9. Lopilato, J., Tsuchiya, T., and Wilson, T. H. (1978) J. Bacteriol. 134, 147–156
10. Yazyu, H., Shiota-Niiya, S., Shimamoto, T., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) J. Biol. Chem. 259, 4320–4326
11. Wilson, D. M., and Wilson, T. H. (1987) Biochim. Biophys. Acta 904, 191–200
12. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) in Molecular Cloning: A Laboratory Manual, p. 440, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Tanaka, S., Lerner, S. A., and Lin, E. C. C. (1967) J. Bacteriol. 93, 642–648
14. Burstein, C., and Kepes, A. (1971) Biochim. Biophys. Acta 230, 52–63
15. Aiba, H., Adhya, S., and de Crombrugghe, B. (1981) J. Biol. Chem. 256, 11905–11910
16. Ohtani, K., Sakurai, H., Oh, E., Iwata, E., Tsuchiya, T., and Tsuda, M. (1995) J. Neurochem. 65, 605–614
17. Berna, K. I., and Thomas, C. A., Jr. (1965) J. Mol. Biol. 11, 476–490
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
19. Shimamoto, T., Noguchi, K., Kuroda, M., Tsuda, M., and Tsuchiya, T. (1994) J. Biochem. 115, 1185–1189
20. Nagao, Y., Nakada, T., Imoto, M., Shimamoto, T., Sakai, S., Tsuda, M., and Tsuchiya, T. (1988) Biochem. Biophys. Res. Commun. 151, 236–241
21. Clarke, L., and Carbon, J. (1976) Cell 9, 91–99
22. Shimamoto, T., Yazyu, H., Futai, M., and Tsuchiya, T. (1984) Biochem. Biophys. Res. Commun. 121, 41–46
23. Williams, J., Michan, C., Webster, C., and Busby, S. (1994) Biochem. J. 300, 757–763
24. Keen, J., Williams, J., and Busby, S. (1996) Biochem. J. 318, 443–449
25. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1983) in Molecular Biology of the Cell, pp. 611–671, Garland Publishing Inc., New York
Conversion of Temperature-sensitive to -resistant Gene Expression Due to Mutations in the Promoter Region of the Melibiose Operon in Escherichia coli

Eiji Tamai, Tadashi Shimamoto, Masaaki Tsuda, Tohru Mizushima and Tomofusa Tsuchiya

J. Biol. Chem. 1998, 273:16860-16864.
doi: 10.1074/jbc.273.27.16860

Access the most updated version of this article at http://www.jbc.org/content/273/27/16860

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 10 of which can be accessed free at http://www.jbc.org/content/273/27/16860.full.html#ref-list-1