Characterization of pTZ12, a Chloramphenicol-resistance Plasmid in *Bacillus subtilis*

Megumi KONO, Takashi AOKI, Masanori SASATSU,
Norihisa NOGUCHI and Koji O'HARA

Department of Microbiology, Tokyo College of Pharmacy,
1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

Received November 12, 1984

The chloramphenicol-resistance (CP^r) plasmid pTZ12 (2.55 kb) in *Bacillus subtilis* was genetically analyzed in detail, and the CP^r determinant and the functional unit of replication were mapped. The plasmids pTZ12 and pBR322 were digested with suitable restriction endonucleases and ligated with T4 ligase. The ligated DNAs were introduced into *E. coli* by transformation and CP-resistant transformants were selected. In conclusion, the CP^r determinant was mapped between a *TaqI* site and a *BclI* site (about 900 base pairs) on pTZ12. A set of pTZ12-pBR322 recombinant plasmids isolated from *E. coli* was introduced into *B. subtilis* by transformation to test for ability to replicate in *B. subtilis*. From the results, the region of the functional unit of pTZ12 replication was mapped. It was also proved that the gene product of this CP^r determinant was chloramphenicol acetyltransferase (CAT) and the native CAT in the cells carrying pTZ12 was a dimeric protein with two identical subunits having a molecular weight of approximately 24,000 (24 K).

It has been reported that several antibiotic resistance plasmids originally detected in Staphylococci and Streptococci can be introduced into *B. subtilis* and are stably maintained and expressed in the new host.^{1~5)} These plasmids would become very useful cloning vectors in *B. subtilis*.

This laboratory has described a number of plasmid cloning vectors from *S. aureus*.^{2,6,7)} In a previous paper,⁸⁾ we reported CP^r plasmid pTZ11 and its derivatives from *Corynebacterium xerosis*. Among these plasmids, CP^r plasmid pTZ12 had a small molecular size of 2.55 kilobases (kb), and produced chloramphenicol acetyltransferase (CAT) extracellularly. This paper deals with the genome characterization of the pTZ12, the mapping of the CP^r determinant and the functional unit of the replication, and with the gene products of the CP^r determinant, or CAT.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used as

recipients in transformation were *B. subtilis* RM125 (*argA15*, *leuA8*, *hsrM*, *hsmM*)⁹⁾ and *E. coli* ML1410, which is a nalidixic acid-resistant mutant of *E. coli* K12. Strain *B. subtilis* BKS 2-2 is a derivative of *B. subtilis* RM125 that carries the CP^r plasmid pTZ12.⁸⁾ In our reports, "pTP" has been used as a plasmid name, but we took the advice of Dr. Lederberg of the Plasmid Reference Center and exchanged this plasmid name for "pTZ." The plasmids pTP11 and pTP12 in a previous paper⁸⁾ are named pTZ11 and pTZ12 in this paper.

Media and chemicals. For preparing plasmids from *B. subtilis* and *E. coli*, cells were grown in Trypto-soy broth (Eiken, Tokyo) or in L broth.¹⁰⁾ For other experiments, Penassay broth (Difco) and brain heart infusion (BHI) broth (Eiken, Tokyo) were used routinely for bacterial growth in liquid media. Heart infusion (HI) agar (Eiken, Tokyo) was used for agar plates. All antibiotics were purchased from the Sigma Chemical Company.

Plasmid DNA isolation. The procedures for the preparation of plasmid DNA from *B. subtilis* and *E. coli* were described by Tanaka *et al.*¹¹⁾ and Birnboim *et al.*,¹²⁾ respectively.

Transformation procedure. Protoplast cells of *B. subtilis* RM125 were transformed by the method of Chang and Cohen.¹³⁾ Competent cells of *E. coli* ML1410 were made according to the method of Mandel and Higa.¹⁴⁾

FIG. 1. Restriction Endonuclease Cleavage Map of pTZ12.

The unique *DdeI* site is the point of reference. Distance from the *DdeI* site is shown in kb.

Transformants were selected using HI agar plates or DM3 plates containing 10 μ g of CP per ml.

Construction and analysis of plasmids. Restriction endonucleases and T4 DNA ligase, purchased from New England Biolabs and Bethesda Research Laboratories, were used according to the manufacturer's instructions. Agarose gel electrophoresis was done to analyze the plasmids as described in our previous paper.⁸⁾

Assay of chloramphenicol acetyltransferase. Chloramphenicol acetyltransferase (CAT) activity was assayed by spectrophotometry.¹⁵⁾

Preparation of crude extract. An overnight culture of bacteria was inoculated into fresh BHI broth and shaken for about 3 hr at 37°C. The culture in exponential growth phase was centrifuged at 10,200 $\times g$ for 20 min. The broken cells obtained by sonic oscillation were centrifuged at 20,000 $\times g$ for 30 min, and the supernatant fluid were ultracentrifuged at 105,000 $\times g$ for 2 hr. This supernatant fluid was used as a crude extract.

Partial purification of crude extract. A Sephadex G-100 column (1.9 $\times$ 40 cm) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.8. Crude extract was put on the Sephadex column and eluted with the same buffer at a flow rate of 12 ml/hr. Fractions of 10 ml were collected and each fraction was assayed for CAT activity. Two ml of the peak fraction showing the highest CAT activity was put on the same column again and eluted with the same buffer, and 1.5 ml fractions were collected. Each fraction was assayed for the enzyme activity, and the protein concentration was estimated by ultraviolet absorption at 280 nm. To examine the elution volume of protein markers, 2 ml of 0.05 M Tris-HCl buffer, pH 7.8 containing BSA (68K), ovalbumin (44K), chymotrypsinogen (25K), and cytochrome *c* (13K) was put on the same column and eluted under the same conditions. The marker elution profiles were determined by absorbance at 280 nm.

SDS polyacrylamide gel electrophoresis. Estimates of the molecular weights of the enzyme subunits were made in

FIG. 2. Construction of pTZ12-pBR322 Recombinant Plasmids.

(A) pTZ121, obtained by cloning the full-length pTZ12 digested with *BclI* into the unique *BamHI* site of pBR322; (B) pTZ122, obtained by cloning pTZ12 *MboI* fragment A into the unique *BamHI* site of pBR322; (C) pTZ123, obtained by cloning pTZ12 *HpaII* fragment A into the unique *ClaI* site of pBR322; (D) pTZ124, obtained by cloning pTZ12 *TaqI* fragment A into the unique *ClaI* site of pBR322.

the presence of 0.1% SDS in 14% polyacrylamide gel using the protein markers described above. Proteins were detected by staining with a silver stain kit (Ag-STAIN "DAIICHI," Daiichi Pure Chemicals Co., Ltd., Tokyo).

RESULTS

Restriction endonuclease map of pTZ12

A restriction endonuclease map of CP^r plasmid pTZ12 was constructed (Fig. 1). The relative positions of restriction sites of the plasmid were determined from the molecular weights of DNA fragments produced by digestion with appropriate combinations of restriction endonucleases.

FIG. 3. Schematic Diagram Summarizing Properties of pTZ12-pBR322 Recombinant Plasmids.

Recombinant plasmids (pTZ121, pTZ122, pTZ123, and pTZ124) constructed by cloning pTZ12 DNA fragments into pBR322 expressed CP resistance in *E. coli* and these recombinant plasmids were tested for ability to replicate in *B. subtilis*.

Map region of CP^r determinant on pTZ12

To determine the map region of the CP^r determinant, recombinant plasmids constructed by insertion of the various pTZ12 DNA fragments into pBR322 were tested for ability to express CP resistance in *E. coli*. Plasmids pTZ12 and pBR322 were digested with *Bcl*I and *Bam*HI, respectively, mixed, and ligated with T4 ligase. The recombinant plasmids thus obtained were used in a transformation experiment with *E. coli* ML1410 as the recipient. Transformants resistant to CP were selected and screened for resistance to ampicillin (ABPC). Plasmid DNA was isolated from one of the transformants resistant to CP and ABPC, and characterized by restriction endonuclease digestion. This recombinant plasmid (pTZ121) contained full-length pTZ12 inserted into the unique *Bam*HI site of pBR322 (Fig. 2A). Thus, it was proved that the CP^r determinant on pTZ12 had the ability to express CP resistance in *E. coli*. Plasmids pTZ12 and pBR322 were then digested with *Mbo*I and *Bam*HI, respectively, mixed, and ligated. The ligated DNA mixture was used for a transformation experiment with *E. coli* ML1410 as the recipient. CP- and ABPC-resistant transformants were selected. Plasmid DNAs were isolated from these transformants and characterized by restriction endonuclease digestion. One recombinant plasmid (pTZ122) contained the larger *Mbo*I fragment (A, 2.0 kb) from pTZ12 (Fig. 2B). The recombinant plas-

mids pTZ123 and pTZ124 were also obtained by a similar experimental procedure. Plasmid pTZ123 was obtained by cloning the larger *Hpa*II fragment (A, 1.85 kb) of pTZ12 (Fig. 2C). Plasmid pTZ124 was obtained by cloning the largest *Taq*I fragment (A, 1.65 kb) of pTZ12 (Fig. 2D). In *E. coli*, these recombinant plasmids specified resistance to at least 25 µg of CP per ml. Plasmid pTZ12 DNA portions of each recombinant plasmid are shown in Fig. 3. From the data, it was proved that the CP^r determinant could be mapped between the *Taq*I site and the *Bcl*I site (about 900 base pairs) on pTZ12 (Fig. 3).

Map region of replication function of pTZ12

To define the functional unit of pTZ12 replication, we tried to introduce the recombinant plasmids (pTZ122, pTZ123 and pTZ124) into *B. subtilis* by transformation. The results are summarized in Fig. 3. Neither plasmid pTZ123 nor pTZ124 could replicate in *B. subtilis*, whereas it was found that pTZ122, which contains pTZ12 *Mbo*I fragment A, could replicate. This shows that the functional unit of pTZ12 replication must be on the *Mbo*I fragment A of pTZ12 and that the *Taq*I site and the *Hpa*II site must be in this region (Fig. 3).

Molecular nature of chloramphenicol acetyl-transferase

Figure 4 summarizes the results obtained by

FIG. 4. Second Gel Filtration of the Cell Extracts from *B. subtilis* BKS2-2 on Sephadex G-100 Column.

Each 1.5 ml fraction was assayed for CAT activity (○) and protein (●). Protein markers A; BSA (68K), B; ovalbumin (44K), C; chymotrypsinogen (25K), D; cytochrome *c* (13K) were eluted under the same conditions. From the elution patterns of protein markers, this CAT was calculated to be 52K. The peak fraction (fraction No. 19) was used as a partially-purified CAT.

FIG. 5. SDS-Polyacrylamide Gel Electrophoresis of Cell Extracts from *B. subtilis* BKS2-2.

Proteins (partially-purified enzyme prepared from CP-induced culture, lane A; crude enzyme prepared from CP-induced culture, lane B; crude enzyme prepared from non-induced culture, lane C) were prepared as described in the text and analyzed on an SDS-14% polyacrylamide gel. BSA, ovalbumin, chymotrypsinogen, and cytochrome *c* were used as molecular standards.

the second gel filtration on a Sephadex G-100 column using the partially-purified CAT which was prepared from the crude extract of CP-

induced *B. subtilis* BKS2-2, carrying pTZ12. Protein markers were put on the same column and eluted under the same conditions. The molecular weight of the native enzyme from *B. subtilis* BKS2-2 was approximately 52K by comparison of the elution pattern of protein markers. On the other hand, the crude extract was prepared from the *B. subtilis* BKS2-2 cells grown with or without 12.5 μ g of CP per ml in BHI broth. The CAT activity of the crude extract prepared from CP-induced culture was 1.61 μ mol/mg protein/min and that of the crude extract prepared from the culture without CP was 0.03 μ mol/mg protein/min. Each two μ g of proteins, partially-purified enzyme prepared from the CP-induced culture, crude enzyme from CP-induced culture, and crude enzyme from non-induced culture, were analyzed by SDS-polyacrylamide gel electrophoresis and by Ag staining for protein. The results are shown in Fig. 5. A major protein band of partially-purified enzyme was found at the position which is approximately 24K in molecular weight. The 24K protein band was also found in the crude enzyme from the CP-induced culture, but the band was not found in the crude enzyme from the non-induced culture. These suggested that the 24K band was a CAT subunit.

DISCUSSION

The CP^r plasmid pTZ12 of *B. subtilis* was investigated using the *in vitro* recombination technique. We found that the essential region of CP resistance and replication of pTZ12 was in the *Mbo*I fragment A of 2.0 kb (Fig. 3). From the mapping data of the CP^r determinant and the functional unit of replication, it was thought that plasmid pTZ12 was a more useful cloning vector because this plasmid was smaller, with a high copy number, 180~200 per chromosome.⁸⁾

CAT from *B. subtilis* carrying pTZ12 exists as native enzyme with the molecular weight of approximately 52K in the crude extract, but the molecular weight of this CAT was found to be approximately 24K from the result of SDS-

polyacrylamide gel electrophoresis. The CP^r determinant was mapped in the region of about 900 base pairs on pTZ12, therefore the molecular weight of this inducible CAT should theoretically be below 36K. This suggests that this CAT exists as a dimer of two identical subunits in the crude extract and that the molecular weight of this CAT monomer is approximately 24K by SDS-polyacrylamide gel electrophoresis. The CAT gene coding the molecular weight of 24K protein must contain at least 600 base pairs of DNA with another 50 to 100 base pairs allotted for the promoter-operator region, and therefore the 900 base pairs region on pTZ12 is enough to code for this CAT gene.

All CAT variants studied in detail are tetrameric proteins of four identical subunits, each subunit having a molecular weight in the range 23K~25K.^{16~18)} The CAT of pTZ12 has a molecular weight in this range, but it is noteworthy that this CAT may be composed of two identical subunits. The studies on pTZ12 will be done in more detail to extend the knowledge of the structural and functional properties of the CAT.

REFERENCES

- 1) S. D. Ehrlich, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1680 (1977).
- 2) M. Kono, M. Sasatsu and H. Hamashima, *Microbios. Lett.*, **5**, 55 (1978).
- 3) T. J. Gryczan, S. Contente and D. Dubnau, *J. Bacteriol.*, **134**, 318 (1978).
- 4) M. Kono, H. Hamashima, T. Aoki and M. Sasatsu, *Agric. Biol. Chem.*, **46**, 569 (1982).
- 5) M. Espinosa, P. Lopez, M. T. Perez-Urena and S. A. Lacks, *Molec. Gen. Genet.*, **188**, 195 (1982).
- 6) M. Kono, M. Sasatsu, H. Hamashima and T. Yamakawa, *Microbios. Lett.*, **6**, 67 (1978).
- 7) N. Noguchi, K. Shishido, T. Ando and M. Kono, *Gene*, **21**, 105 (1983).
- 8) M. Kono, M. Sasatsu, T. Aoki and N. Noguchi, *Agric. Biol. Chem.*, **48**, 821 (1984).
- 9) T. Uozumi, T. Hoshino, K. Miwa, S. Horinouchi, T. Beppu and K. Arima, *Molec. Gen. Genet.*, **152**, 65 (1977).
- 10) E. S. Lennox, *Virology*, **1**, 190 (1955).
- 11) T. Tanaka, M. Kuroda and K. Sakaguchi, *J. Bacteriol.*, **129**, 1487 (1977).
- 12) H. C. Birnboim and J. Doly, *Nucleic Acids Res.*, **7**, 1513 (1979).
- 13) S. Chang and S. N. Cohen, *Molec. Gen. Genet.*, **168**, 111 (1979).
- 14) M. Mandel and A. Higa, *J. Mol. Biol.*, **53**, 159 (1970).
- 15) S. Iyobe, M. Kono, K. Ohara, H. Hashimoto and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **5**, 68 (1974).
- 16) W. V. Shaw, *Methods Enzymol.*, **43**, 737 (1975).
- 17) Y. Zaidenzaig, J. E. Fitton, L. C. Packman and W. V. Shaw, *Eur. J. Biochem.*, **100**, 609 (1979).
- 18) W. V. Shaw, *Crit. Rev. Biochem.*, **14**, 1 (1983).