Sequence Analysis of Replication Origin of Plasmid pCTP4 in γ-PGA-producing Bacillus Strain isolated from "Dan-douchi" in China

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Sequence Analysis of Replication Origin of Plasmid pCTP4 in y-PGA-producing *Bacillus* Strain isolated from "Dan-douchi" in China

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The structure of a 1.6-kb *SphI-Hin*dIII DNA sequence necessary and sufficient for the replication of a 6.3-kb plasmid pCTP4 in y-PGA-producing *Bacillus* strain isolated from "dan-douchi" in China, which is responsible for y-polyglutamate production, has been characterized by using a trimethoprim-resistance gene derived form *B. subtilis* TTK24 chromosomal DNA as a selective marker. The 1.6-kb DNA sequence contains a *rep* gene encoding the protein (333 amino acids) essential for initiation of replication and a possible origin of replication. The predicted REP protein of pCTP4 has an overall homology with the REP proteins of pUH1 (73.9% identity), pLS11 (87.4%), pBAA1 (82.0%), and pFTB14 (76. 6%) in *Bacillus* spp., pLP1 (46.5%) and pLAB1000 (32.4%) in *Lactobacillus* spp., and pUB110 (34.8%) and pC194 (21.9%) in Staphylococcus *aureus*, but has not any similarity with the REP protein of the staphylococcal plasmid pT181.

INTRODUCTION

"Douchi" is a native fermented product made from whole soybeans in China. There are two kinds of "douchi" as a raw fermented product : without or with salt, *i.* e., "dan-douchi" and "xian-douchi." "Xian-douchi" is made from steamed soybean and salt using the koji molt of *Aspergillus oryzae,* and it is found to greatly resemble "hamanatto" in Japan. "Dan-douchi," the non-salty aging product of steamed soybean by the biological action of "natto" Bacillus-like microorganisms, is an adhesive fermented soybean with a noticeable odor of ammonia, and is, then considered to be the same as Japanese "natto" (Nakao, 1972). After fermentation, the raw "dan -douchi" is sun-dried, and then the dark-colored product is mixed with red pepper and ginger and aged for a while before being eaten. However, there are some striking and possesses some acidity, whereas "natto" is predominantly sticky and contains no acid (Bo, 1984).

We have reported that a 5.8-kilobase pair (kb) plasmid designated pUH1, which encodes the y-polyglutamate-production stimulating factor (*psf*) gene responsible for y-polyglutamate production, is distributed widely in a number of *Bacillus subtilis* (*natto*) strains isolated from a fermented soybean food, "natto" (Hara et al., 1981 and 1983). *B. subtilis* and *B. subtilis* (*natto*) should be considered as one species, but these two bacilli are found to be classified separately on the basis of whether biotin is

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essential for growth or not (Amaha et al., 195'2 ;Kida *et al.*, 1963). y-Polyglutamate -producing *Bacillus* strain was isolated from "dan-douchi," which required biotin for growth and produced viscous substances, harbored a single plasmid pCTP4 (6.3 kb) (Hara et *al.*, 1993). Recently, electron microscopy showed that a heteroduplex molecule between pUH1 and pLS11 of *Bacillus subtilis* IFO 3022 contains 1.11-kb and 2.14-kb double-stranded termini (Hara et al., 1986). The 2.0-kb *Bst*EII DNA fragment of pUH1, including the 1.11-kb double-stranded region, contains a 999-bp open reading frame, a promoter for the open reading frame, and a possible replication origin upstream of the promoter (Hara et al., 1991).

Analysis of the organization of various plasmids isolated from Gram-positive bacteria, such as pT181 (Projan and Novick, 1988), pC194 (Alonso and Tailor, 1987), pE194 (Dempsey and Dubnau, 1989), pFTB14 (Murai et al., 1987), pLP1 (Perez-Martin *et al.*, 1988), pUB110 (Maciag *et al.*, 1988), pBAA1 (Devine et al., 1989), and pLAB1000 (Josson et al., 1990), has shown that all of the information necessary for replication is located on fragments of about 1.5 kb in length. These fragments harbor a *rep* **gene,** encoding a protein essential for the initiation of replication (REP) and its corresponding target site.

This communication reports that the 1.6-kb fragment of pCTP4 contains a 999-bp open reading frame, a promoter for the open reading frame, and a possible replication origin upstream of the promoter. Significant homology was observed between the amino acid sequence predicted from the 999-bp open reading frame and those of similar putative REP proteins encoded by the other well-known Gram-positive replicons.

MATERIALS AMD METHODS

Bacterial strains and plasmids. Escherichia coli JM101 and *B. subtilis MI112* (*arg* -15, *leuB8*, *thr-5*, *recE4*) were used for a cloning host. The plasmids pCTP4 (Hara *et al.*, 1993) and pATE1 (Hara et *al.*, 1991) have been described previously, and plasmid pTL12 carrying the dihydrofolate reductase gene was kindly provided by Tanaka and Kawano (1980).

Media. LB broth and Panassay broth (Difco) for both *B. subtilis* and *E. coli*, Spizizen minimal medium for *B. subtilis*, and M9 minimal medium for *E. coli* were the same as those described previously (Hara *et al.*, 1991). The cells carrying trimethoprim-resistant (Tmp^r) plasmids were grown in AA medium (Tanaka and Sakaguchi, 1978) containing 1 mg/ml of Tmp.

DNA manipulations. Plasmid DNAs from *B. subtilis* were prepared and purified as described previously (Hara et al., 1991). Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co., Ltd., and used as recommended by the manufacture. Degradation of DNA with exonuclease Ba131 (Takara Shuzo Co., Ltd.) was done by the procedure of Legerski *et al.* (1978).

Methods **for** *transformation and assessment of ori function. E. coli* was transformed by the method of Morrison (1977), and *B. subtilis* was transformed by using protoplasted cells (Chang and Cohen, 1979).

DNA sequencing. DNA fragments were subcloned into plasmids pUC18 and

pUC19, and DNA sequencing was done by the dideoxy chain termination method (Sanger et *al., 1977)* with Sequenase (United States Biochemical Corporation, Ohio, USA). Nucleotide and amino acid sequences were analyzed by the Hitachi DNASIS system.

RESULTS AND DISCUSSION

Delimitation of the replication origin

To facilitate the identification of the replication region of pCTP4, we used the trimethoprim-resistant (Tmp^r) dihydrofolate reductase gene of B. *subtilis* 168. A schematic presentation of the constructed plasmids is given in Fig. 1. The source of the dihydrofolate reductase-coding gene was a Tmp^r strain, TTK24, of B. *subtilis 168* (Tanaka and Sakaguchi, 1978) and has been cloned in the pBR322 plasmid of *E. coli*. DNAs from pTL12, carrying the Tmp^r dihydrofolate reductase gene, which was constructed by Tanaka and Kawano (1980), and pBR322 were both treated with *Eco*RI and *Hin*dIII, mixed and ligated by T4 ligase, then pATE1 was constructed. Plasmid pCTP4 was digested with *Sph*I and *Hind*III, and then the ends were filled in with the Klenow fragment to generate blunt ends. The DNA fragments were mixed and ligated to the *AatI* site of pATE1 by T4 ligase, and then added to B. *subtilis MI112* protoplasts. Several Tmp^r colonies were obtained on AA agar plates containing Tmp (1 mg/ml) and one of them was used for further study. A plasmid, pNH3, carried in such a Tmp^r colony had a molecular size of 8.2kb (Fig. 1). The physical map of pNH3 using various restriction enzymes is shown in Fig. 1.

To define the boundaries of a functional unit of pCTP4 replication, the 1.6-kb *Sph*I-*Hin*dIII fragment of pCTP4 was digested with selected restriction endonucleases to obtain a set of overlapping DNA fragments. The digests filled in with Klenow fragment were ligated with pATE1, introduced into *E. coli* by transformation, and selected for ampicillin resistance. The plasmid DNA preparations containing each generated fragment were tested for replication in B. *subtilis*. The results are summarized in Fig. 2. The constructed plasmid with the 1.4-kb *Sph*I-*Hae*III fragment (fragment 2 in Fig. 2) could replicate in the B. *subtilis* host, but the recombinant plasmid preparations containing the small fragments (fragment 3 and 4 in Fig. 2) could not replicate.

Nucleotide sequence of the replication region

The nucleotides of the 1.6-kb *Sph*I-*Hin*dIII fragment were sequenced by the method of Sanger (1977). Though the strategy is not shown, the nucleotides of both strands were sequenced using numerous restriction fragments to give enough overlapping regions (Fig. 3). Looking for possible open reading frames (ORFs), we found only one large frame (Fig. 2) designated rep, which consisted of 999 bp and encoded a protein molecule with 333 amino acids with a M_r of 38,582. Several conserved regulatory sequences similar to the *E. coli* and *Bacillus* consensus promoter sequences (McLaughlin *et al.*, 1981) were observed 5' upstream to the *rep* ORF.

Plasmid pNH3 was digested with *Bgl*II and then treated for 20 min with exonuclease Ba131 under the conditions in which about 50 bp per min were removed from

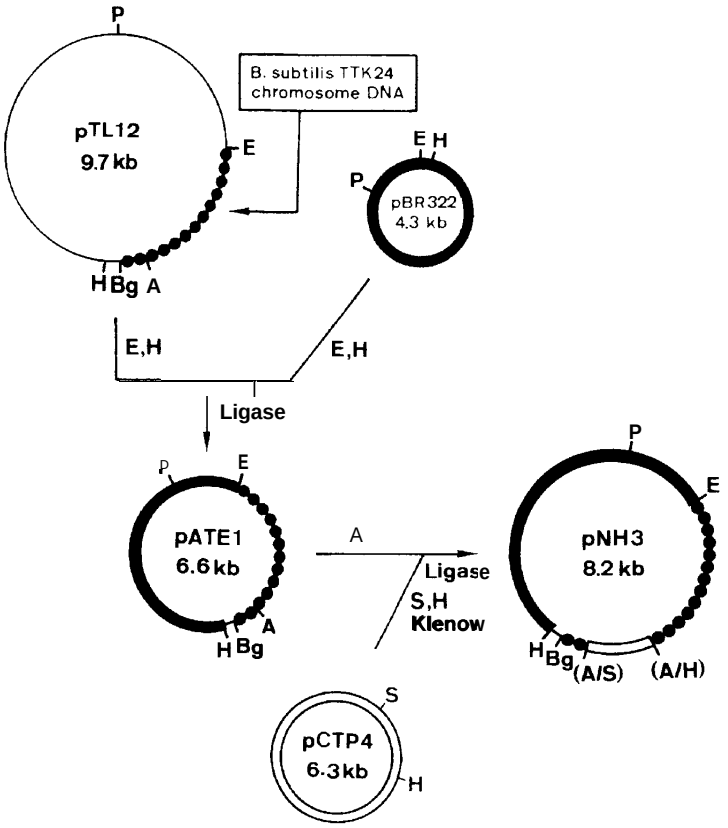

Fig. 1. Derivation of plasmids used in this study.

The chain of solid circles in the diagram indicates the DNA segment containing the Tmp' gene of *B. subtilis* TTK24. Heavy and thin lines represent the regions of pBR322 and pTL12, respectively. Double lines represent the DNA fragment of pCTP4. A, *Aat*I ; Bg, *Bgl*II ; E, *Eco*RI ; H, *Hin*dIII ; P, *Pst*I ; S, *Sph*I.

each end of the DNA molecule. After ligation by T4 ligase, the DNA was transformed into E. *coli* and subsequently introduced into *B. subtilis* by protoplast transformation. Tmp^r transformants, which contain 1.4-kb fragment 5 in Fig. 2, were obtained at high efficiencies with a 168-bp deletion plasmid generated with Bal31 (fragment 5 in Fig. 2), while no transformants were obtained with a similar 255-bp deletion plasmid (fragment 6 in Fig. 2). It suggests that the putative replication origin of pCTP4 is between position 168 and 255. To discover the sequences essential for replication, a homology search was done to find whether there are sequences within this region conserved in the registered Gram-positive replicons. A 34-bp sequence was conserved in four plasmids, pCTP4, pLS11, pUH1, pBAA1 and pFTB14 in *Bacillus* spp. (Fig. 4), and the 14-bp sequence within this region was conserved in all eight plasmids in Gram-positive bacteria including *Lactobacillus* spp. and Staphylococcus spp. This conserved 14-bp sequence is found in the 55-bp region of pC194 shown by Gros *et al.* (1987) to

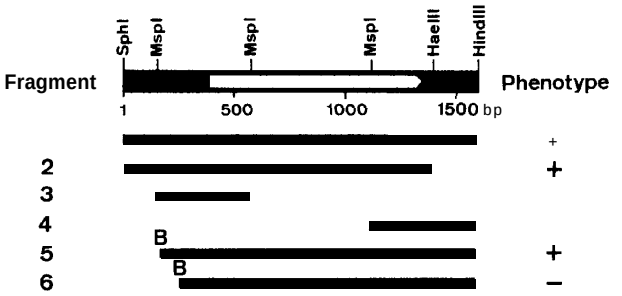

Fig. 2. Structure and replication activity of the derived fragments of the 1.6-kb *ori* fragment.

The open arrow in the restriction map indicates an open reading frame found in the sequence data (see Fig. 3). + and – indicate, respectively, ability and inability to replicate in the *B. subtilis* host. Derivatives were made by deletion with the indicated restriction enzyme or by Ba131 digestion (B) from the *Bgl*II site of pNH3.

have origin activity. Within this 14-bp sequence, in addition, the sequence CTTGATA is the sequence at which nicking of the plus-strand occurs in the initiation of replication of the coliphage ϕX174 (Shlomai and Kornberg, 1980), and this conserved sequence was found in a hairpin region of ϕX174.

Amino acid sequence homology of pCTP4 REP protein with different Gram-positive replication proteins

The amino acid sequence of the REP protein coding region of pCTP4 was compared with a number of amino acid sequence of proteins registered in GenBank by the homology search system GENAS (Kuhara *et al.*, 1984). Homologies between the predicted amino acid sequence of the REP protein of pCTP4 and those of the REP protein of pUH1, pLS11, pBAA1, pFTB14, pLP1, pLAB1000, pUB110 and pC194 are illustrated in Fig. 5. The REP protein encoded on pCTP4 showed a substantial degree of homology to three REP proteins in *Bacillus* plasmids: 87.4% identity with REPpLS11, 82.0% with REPpBAA1, 76.6% with REPpFTB14, and 73.9% with REPpUH1. The REPpCTP4 is similar to REPpLP1 (46.5% identity) and REPpAB1000 (32.4%) from lactic acid bacteria but also to REPpUB110 (34.8%) and REPpC194 (21.9%) from S. *aureus*. The REP protein of pFTB14 stretches for 1,017 bp, a promoter region for rep expression, and a possible replication origin for rep expression, which is upstream of the promoter. The rep product is trans-active and essential for plasmid replication (Murai *et al.*, 1987). The tyrosine, which acts as an active site of REP in the rolling circle mechanism (Van Mansfeld *et al.*, 1986), was conserved in all of the Gram-positive initiation proteins (Fig. 5). Khan *et al.* (1982) identified the start site of pT181 DNA synthesis within a 127-bp segment and showed that a 168 bp segment containing the replication start site is enough to initiate unidirectional replication. Furthermore, like REP protein of pT181 (Projan and Novick, 1988), the protein of the *E. coli* plasmid R6K (Stalker *et al.*, 1982 ; Germino and Bastia, 1982) does not

Fig. 3. Nucleotide sequence of the 1.6-kb *Sph*I–*Hind*III DNA fragment.

Nucleotide residues are numbered in the 5'-to-3' direction, beginning with the 5'-end residue originated from the *Sph*I site. The deduced amino acid sequence is given below the nucleotide sequence. Putative promoter elements (-35, -10 and the ribosome binding site) are underlined.

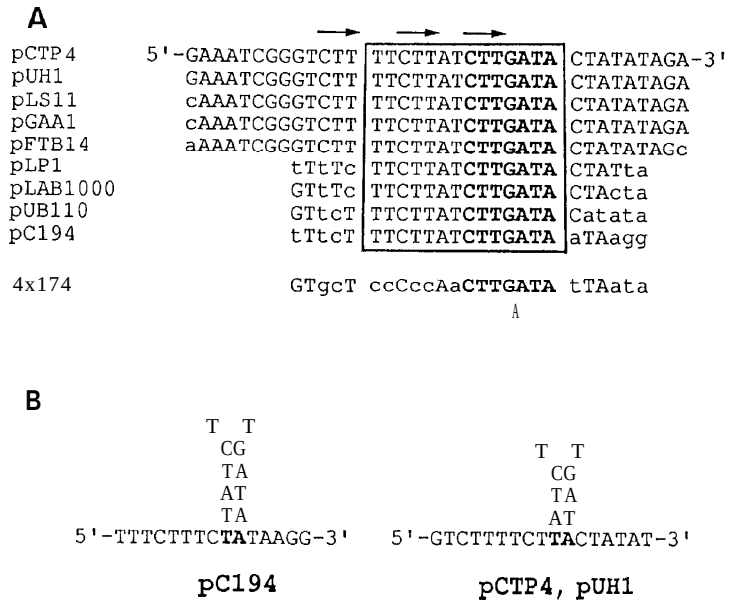

Fig. 4. (A) Homology at the origin for plus-strand synthesis among several plasmids in Gram-positive microorganisms. The nucleotides in boxes are conserved well among them. The sequence CTTGATA, which is the site at which nicking of the plus strand occurs in phage ϕX174, are boldface. The DNA bond between nucleotides G and A will be nicked by the REP protein and is indicated by an arrow. (B) Hairpin structure of the pC194 origin region proposed by S. D. Ehrlich and the predicted structure of origin region of plasmids pCTP4 and pUH1.

have any significant homology in its amino acid sequences with those of the REP proteins of pCTP4, pUH1, pLS11 and also pFTB14 deduced from the ORFs (data not shown). Comparison of dendrograms made on the basis of 16S rRNA hybridizations and the percentage of homology on REP protein are illustrated in Fig.6. The degree of homology of REPpCTP4 with the REP proteins of pLP1 and pLAB1000 in *Lactobacillus* spp. strains was almost the same as those with the REP proteins of pUB110 and pC194 in S. *aureus*. Taxonomic studies (based on the 16S rRNA similarities) showed that *Bacillus* spp. and S. *aureus* strains are more closely related to each other than *Staphylococcus* spp. and *Lactobacillus* spp. (Stackebrandt and Teuber, 1988). The plasmid homologies suggest an exchange of plasmid replicons by recent horizontal transfer through the different genera, including *B. subtilis* (*natto*).

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Fig. 5. Amino acid sequence comparison of the REPpCTP4 protein with different Gram-positive replication proteins.

The original names of these proteins have been replaced by REP, followed by a letter code indicating the plasmid from which they originate. The amino acid identities with several plasmids in Gram-positive microorganisms with REPpCTP4 are indicated (*). Amino acid numbers follow the sequence of the REP protein from the amino-terminal methionine to the carboxy terminus. Gaps have been inserted to gain maximum matching. The one-letter amino acid code has been used. The tyrosine residue (marked with arrow) probably involved in the binding of the REP protein to the DNA is indicated by an arrow.

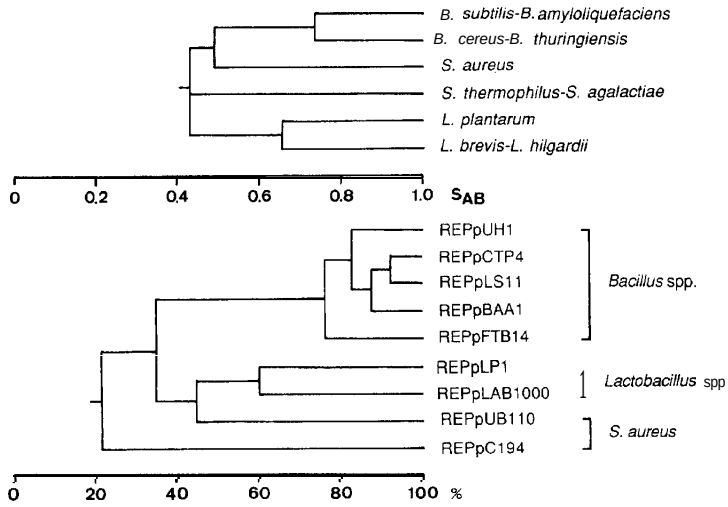

Fig. 6. Comparison of dendrograms made on the basis 16SrRNA hybridizations (A) and the percentages of homology on Rep proteins among several plasmids in Gram-positive microorganisms (B).
The microorganisms from which the plasmids originate are shown. Dendrograms were made by the unweighted average pair group method (Sneath and Sokal, 1973).

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