## Molecular Structure of the Replication Origin of a Bacillus subtilis (natto) Plasmid, pUH1

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The structure of a 2.0-kb BstEII DNA sequence necessary and sufficient for the replication of a 5.7-kb Natto plasmid, pUH1, which is responsible for  $\gamma$ -polyglutamate production by Bacillus subtilis (natto), has been characterized by using a trimethoprim resistance gene derived from B. subtilis chromosomal DNA as a selective marker. The 2.0-kb DNA sequence contains an open reading frame, rep, stretching for 999 bp; a promotor region for rep expression; and a possible replication origin for the plasmid upstream of the promotor. The predicted Rep protein has highly homologous amino acid sequences with rep<sub>14</sub> of pFTB14 in B. amyloliquefaciens, RepB of pUB110, and protein A, which is necessary for pC194 replication in staphylococci throughout the protein molecule, but is not homologous with RepC of staphylococcal plasmid pT181.

In a previous paper (5), we reported that a 5.7-kb plasmid designated pUH1, which encodes the  $\gamma$ -glutamyltranspeptidase gene responsible for  $\gamma$ -polyglutamate production, was detected in a *Bacillus subtilis* (*natto*) strain isolated from a



FIG. 1. Derivation of plasmids used in the present study. The chain of solid circles in the diagram indicates the DNA segment containing the Tmp<sup>r</sup> gene of *B. subtilis* TTK24. Heavy and thin lines represent the regions of pBR322 and pTL12, respectively. Double lines represent the DNA fragment of pUH1. A, *Aat*I; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hind*III; P, *Pst*I.

fermented soybean food, natto. Recently, electron microscope studies of the hybridization products of pLS11, which is detected in *B. subtilis* IFO 3022 and is 8.6 kb in size, with pUH1 revealed a heteroduplex molecule containing 1.11and 2.14-kb double-stranded DNA termini (6). We thought that these double-strand regions might play an important role in polyglutamate production and DNA replication.

This communication reports that the 2.0-kb *Bst*EII DNA fragment corresponds to the replication origin of Natto plasmid pUH1 and contains a 999-bp open reading frame, a promoter for the open reading frame, and a possible replication origin upstream of the promoter. Significant homologies were observed between the amino acid sequence predicted from the 999-bp open reading frame and those of the similar putative proteins encoded by the *B. amyloliquefaciens* plasmid pFTB14 (13) and *Staphylococcus aureus* plasmid pC194 (3, 7).

To facilitate identification of the replication region of



FIG. 2. Structure and replication activity of the modified fragments of the 2.0-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. Modifications were made by deletion with restriction enzymes or insertion of a few base pairs (closed triangle), using the Klenow fragment, after digestion with the indicated restriction enzyme or by *Bal*31 digestion (B) from the *Bgl*II site of pBB2.

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BstEll
5'-ogtaaccGGACCGTAGGG/
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250 CAAAGCCAAGAACTGTTGCAAGGCTCGTTGAGAATAAAGAATGCTTTTCAGGATGCTTAGAATCGTTTCTGAGAGCTTC 300 AAATAAAAAAAGATGACCTTTTATAGGGGGGAAGCTCTTAAAATTGAATGTAGGGGCATTTAAAACACGTTTAAAAAATAAAA 350 AAAGCAGACTCTTTAGAGTCCGCCTTGTTATTTTTAACCCAGTGCTCCATTTTCGGCTGTTTGGAAATCTTTTGAGATG 450 CCATCCATTTTCTTTGTTCCATGAAAAAAGTGCTTTTGGATGCTTAAAAAAGGCTTTTTCGTATAAAAAAGCC 500 GATYTTTGAAAAAAAAATCTCCCTGCGGGGGAAGAATGGTTTTGATCTTTGGGTTTTAGGTTTTAAAAAAACCGGGCTG 600 TTTTCAGCCGGCTTTTTTTCGATTTTGGCGGGAGCCGAAATCGGGTCTTTTCTTATCTTGATACTATAGAAACATCTC 700 (-38) AAGGCGAAAAAATAGCCCCCCATCCCTTATTTGTCAAGGGTTTGACGGCTTT<mark>ITGACA</mark>TGTAGAAACTCCTTCCGC<mark>TATT</mark> ATG ATT ATA TCA TCC TTG AGG ACA AGA CCG CAA CAG GTA AAA Met 11e 11e Ser Ser Leu Arg Thr Arg Pro G1n G1n Val Lys AGC GGG ATT GGA AGG GGA 900 AAA AGA GAC GGA CGA ATC TTA TGG CTG AGC ACT ATG AAG CTT TAC AGA GTA AAA CTG GTA Lys Arg Asp Giy Arg iie Leu Trp Leu Ser Thr Het Lys Leu Tyr Arg Vai Lys Leu Vai 74C CTT ACT ATG CCA AAA AAG CTG AGA AAT TGT GCA GTT GTG CGG AAT GTC TTT CGT TTA Tyr Leu Thr Met Ala Lys Lys Leu Arg Asn Cys Ala Val Val Arg Asn Val Phe Arg Leu 1050 AAC GAG ACC GGG AGA GGC AAA TTA AAG TTG TAT CAA GCT CAG TTT TGT AAA GTG AGG TTA Asn Giu Thr Arg Arg Arg Lys Leu Lys Leu Tyr Gin Ala Gin Phe Cys Lys Val Arg Leu 0 TGC CCG ATG TGT GCG TGG CGT AGG TCT TTA AAA ATT GCT TAT CAT AAT TAA TTA ATC GTT Cys Pro Met Cys Ala Trp Arg Arg Ser Leu Lys Ile Ala Tyr His Asn Lys Leu Ile Val GAG GAA GCG AAT CGG CAG TAC GGT TGT GGA TGG ATT TTT CTC ACA CTG ACG GTT CGG AAT Glu Glu Ala Asn Arg Gln Tyr Gly Cys Gly Trp lle Phe Leu Thr Leu Thr Val Arg. Asn GTC GAG GGT GAC GGA TTA AAA CCC ATG ATT GCT GAC ATG ATA GGA TGG AAC CGC CTT Val Glu Gly Asp Gly Leu Lys Pro Met 11e Ala Asp Met Met Lys Gly Trp Asm Arg Leu 1250 TTC GCA TAT AAA CGA GTT AAG GTA GCG ACT TTA GGT TAT TTC AGA GCT TTA GAG ATT ACC Phe Gly Tyr Lys Arg Val Lys Val Ala Thr Leu Gly Tyr Phe Arg Ala Leu Glu 11e Thr . 1300 AAA AAT CAC GAA GAA GAT ACA TAT CAT CCG CAT TTT CAT GTG TTG TTG CCT GTG AAG AAA Lys Asn His Glu Glu Asp Thr Tyr His Pro His Phe His Val Leu Leu Pro Val Lys Lys 0 AGC TAT TTT ACT CAC AAT TAC ATT AAG CAG TCT GAG TGG ACG AGC TTA TGG AAA AGG GCG Ser Tyr Phe Thr His Asn Tyr 11e Lys G1n Ser G1u Trp Thr Ser Leu Trp Lys Arg Ala ATG AAA CTG GAC TAC ACG CCG ATT GTT GAT ATC CGA AGA GTC AAG GAA AGA GCT AAA ATT Met Lys Leu Asp Tyr Thr Pro lle Val Asp lle Arg Arg Val Lys Gly Arg Ala Lys lle GAT GCC GAA CAG ATT GAG AGC GAT GTG CGG GAA GCC ATG ATG GAG CAA AAA GCT GTT CTT Asp Ala Glu Gin Ile Glu Ser Asp Val Arg Glu Ala Met Met Glu Gin Lys Ala Val Leu 1550 GAA ATC TCT AAA TAT CCG GTT AAA GAT ACG GAT GTT GTG CGC GGC AAT AAG GTG ACA GAA Glu Ile Ser Lys Tyr Pro Val Lys Asp Thr Asp Val Val Arg Gly Asn Lys Val Thr Glu 1600 GAC AAT CTG AAC ACG GTG TTŤ TAT TTG GAT GAT GCG CTT TČT CGC CGC CGĞ CTT ATT GGT ASp Asn Lew Asn Thr Val Phe Tyr Lew Asp Asp Ala Lew Ser Arg Arg Arg Lew Ile Giy 0 TAC GGT GGC ATC TTG AAG GAA ATT CAT AAA GAA CTA AAC CTC GGT GAT GCG GAG GAC Tyr Gly Gly lie Leu Lys Glu lie His Lys Glu Leu Asn Leu Gly Asp Ala Glu Asp GAT CTC GTC AAG ATT GAG GAA GAA GAT GAC GAG GTG GCG AAC GAA GCA TTT GAA GTT ATG Asp Leu Val Lys Ile Glu Glu Glu Asp Asp Glu Val Ala Asn Glu Ala Phe Glu val Met SCT TAC TGG CAT CCA GGC ATT AAA AAT TAC ATA ATC AGA TAA TCAGATAAAAAGCAGGCGTTGTT Vla Tyr Trp His Pro Gly 11e Lys Asn Tyr 11e 11e Arg \*\*\* 1850 CCTGCTITTTITATACTCTAATAGTCAAATCAAGAGTTAATTTTAGATGTAATTGTGAGAATTAGAGTGGCTGACCAGT ATTTGAÄACTTCTTGGGCTACTTTCTTAACTTTATATAAACTATGTATATATGTGTTGTTTTTTCTATTATTTTGAT 2000 BstEll 2044 ATTATTTACAAGTATTGAAATTTTGCTAGGAGGGAAAGTTTTTATGGttacc-3

FIG. 3. Nucleotide sequence of the 2.0-kb BstEII DNA fragment. -10, -35, and SD indicate, respectively, plausible sequence for the -10 and -35 sequences and the ribosome binding site. The nucleotide sequences indicated by lowercase latters at the two termini originate from the BstEII site.

pUH1, we used the trimethoprim-resistant (Tmp<sup>r</sup>) dihydrofolate reductase gene of B. subtilis 168. A schematic presentation of the constructed plasmids is given in Fig. 1. The dihydrofolate reductase-coding gene was derived from a Tmp<sup>r</sup> strain, TTK24, of B. subtilis 168 (20) and has been cloned in plasmid pBR322 of Escherichia coli (2). DNAs from pTL12, carrying the Tmp<sup>r</sup> dihydrofolate reductase gene, which was constructed by Tanaka and Kawano (18), and pBR322 were both treated with EcoRI and HindIII, mixed, and ligated with T4 ligase; then pATE1 was constructed. Natto plasmid pUH1 was digested with BstEII, and then the ends were filled in with Klenow fragment to generate a blunt end. The DNA fragments were mixed and ligated at the AatI site of pATE1 with T4 ligase and then added to B. subtilis MI112 (19) protoplasts. Several Tmp<sup>r</sup> colonies were obtained on AA agar plates (18) containing trimethoprim (1 µg/ml), and one of them was used for further study. Plasmid pBB2 carried in such a Tmp<sup>r</sup> colony had a molecular mass of 8.4 kb (Fig. 1). The physical map of pBB2, using various restriction enzymes, is shown in Fig. 1.

To define the boundaries of a functional unit of pUH1 replication, a 2.0-kb BstEII fragment of pUH1 was digested with selected restriction endonucleases to obtain a set of overlapping DNA fragments. Digests modified with Klenow fragment were ligated with pATE1, followed by introduction into *E. coli* by transformation and successive selection for ampicillin resistance. The plasmid DNA preparations containing each generated fragment were tested for replication activity in *B. subtilis*. The results are summarized in Fig. 2. Plasmid pBB2, which corresponds to fragment 1 in Fig. 2, and a derivative containing fragment 7 specified Tmp<sup>r</sup> in *B. subtilis*, but attempts to transform *B. subtilis* with recombinant plasmid preparations containing the small fragments (fragments 2 to 6 in Fig. 2) and inserted fragments (fragments 8 and 9) were unsuccessful.

The nucleotide sequence of the 2.0-kb BstEII fragment was determined by the method of Sangar et al. (14). Though the strategy is not shown, the nucleotide sequence was determined for both strands by using numerous restriction fragments to give enough overlapping regions. By examination of possible open reading frames, we found only one large frame (Fig. 2), designated rep, which consists of 999 bp and encodes a protein molecule with 333 amino acids and an  $M_{\rm r}$  of 39,074. The 5' upstream region of rep contains a 5'-AAGGAG-3' sequence (indicated as SD in Fig. 3) complementary to the 3' end of 16S rRNA (3'-OH-UCUUUC CUCCAGUAG-5') of B. subtilis (11) at nucleotides 777 to 782 for translation initiation. There is a 5'-TATTAT-3' sequence (-10 in Fig. 3) resembling a Pribnow box (12) at nucleotides 727 to 732, and at a site 24 bp upstream of this -10 sequence, there is a 5'-TTGACA-3' sequence resembling the -35 consensus sequence (12) of the B. subtilis gene. The observed distance (18 bp) between the -10 and -35 sequences accords well with that observed generally in B. subtilis genes (17 to 18 bp) (12).

Plasmid pBB2 was digested with BgIII and then treated for 20 min with exonuclease Bal31 under conditions in which about 50 bp was removed per min from each end of the DNA molecule. After ligation by T4 ligase, the DNA was introduced into *B. subtilis* by protoplast transformation. Tmp<sup>r</sup> transformants, which contained fragment 11 in Fig. 2, were obtained at high efficiencies with Bal31-generated deletions of 581 bp (as determined by nucleotide sequencing), while no transformants were obtained with a similar 653-bp deletion (fragment 10 in Fig. 2), suggesting that the 5' upstream portion is dispensable for replication. The interaction of the

		10	20	30 40	50	60
	M1	I SSI DTODO	VESCICPCER	DOGRTIWISTME	VRVKI VVI TMAKKI R	NCAVV
rep	MACCENDY				FROICADYYCKKAED	I SECA
repu	MISSENDI	RILEDRIAIS	********	(KANLMACHICAL	EKRIGAPTIGKKAER	LJEUN
RepB						
Protein A			MCYNME	EKYTEKKQRNQVF	QKFIKRHIGENQMDL	VEDCN
		70	80	90 100	110	120
	ONVEDING		A OFCEVEL CE	MCAUPPSIKIAY	HNKI TVEFANROVGC	GWIFI
rep	CHU CCKOC			MCAUDDELKIAN	UNKLITEEANBOYCC	CHIEL
repu	ENLSPKKL	PEIGRERET	(AMP CKYRLUP	MUAWKKSLKIAT	HNKLITELANKUTUC	GWIFL
RepB				·MKHGIQ	SUKVVAEVIKUKPIV	KWLFL
Protein A	TFLSFVAD	OKTLEKQKLYM	(ANSCKNRFCP	<b>VCAWRKARKDAL</b>	.GL SL MMQ Y I KQQ E K K	EFIFL
		130	140	150 16	0 170	180
<b>n</b> en	TITVONV	FORGI KPMIA	DMMKGWNRLF	GYKRVKVATI GY	FRAL FITKNHEFDTY	HPHFHV
rep	TLTVDNV		CHMCCEDVIE		EDALETTENHEEDTY	HOHEHV
repn	TL TYRAY	NOCKL NPULS	DEMMEGRANLE	UTKKYKI SVLOF	TRALEI TRANCEDIT	
керв	LIVKNVI	DGEELNKSLS	DMAQGERRMM	<b>NUTKKINKNLVG</b>	MRAIEVIINNKUNST	NUHMHY
Protein A	TLTTPNVN	ISDE-LENEIN	(RYNNSFRKL I	IKRKKVGSVIKGY	VRKLEITYNKKDNSY	понмни
		190	200	210 2	20 230	240
rep	LLPVKKS	F THNYIK	DSEWTSLWKRA	AMKLDYTPIVDIR	RVKGRAKIDAEOIES	DVREAM
PAD	LIPVKRNY	FGKNYIKO	AFWTSIWKRA	AMKIDYTPIVDIR	RVKGRVKIDAFOIFS	DVRFAM
Rend	IVCVEDTY	EVNTENVVN	KOUTOEUKKA	MEL DYDDNV		KNKKKC
Dente in A	LICVERT					VEMANN
Protein A	LIAVNKS	IF IDKKTTIS(	{UFMCDEM	KDA1012511	<b>AAAAAKIKANNUKE</b>	TEMAKT
	ž	250 2	260 2	270 280	) 290	300
rep	EQKAVLE	SKYPVKDTD	/VRGNKVTEDN	ALNTVFYLDDALS	RRRLIGYGGILKEIH	IKELNL
PAD	FORAVLET	SKYPYKTDD	VRGSKVTDDN	NUNTVEYIDDALS	ARRI IGYGGILKETH	IK FINI
Deep	TOSATOFT	AFVEVENTO	ENTDOFFYN	NI KRI SDI FECI H	OVDI TSYNCI I VETH	IN VI NI
керь	1034102	NKIPTKUIU-	- FMIDDEEK	ILKKLJULLLULN	INCREISIDUCERCIN	INNERL
Protein A	GKDSDIL	WK2K2L				
	3	310 3	320 3	330		
rep	GDAEDGDL	VKIEEEDDEV	ANEAFEVMAY	WHPGIKNYIIR-		
	GDAFGGDI	VELEFEDDEN	ANGAFEVMAN	WHPGTENYTIE-		
Deep	ODTEECDI	THINDOLEV	DEDEESTIAN			
керв	PRIFERRE	-INIDUDERA	VCDGF SI IAP	INDUCKNOTPIKE		
Protein A						

FIG. 4. Comparison of the amino acid sequences of *rep* of pUH1,  $rep_{14}$  of pFTB14, RepB of pUB110, and protein A of pC194. Amino acid identities with *rep* of pUH1 are indicated (\*). Amino acid numbers follow the sequence of the *rep* protein from the amino-terminal methionine to the carboxyl terminus. Gaps have been inserted to gain maximum matching. The one-letter amino acid code has been used.

initiator protein with its binding site on the DNA is a key step in the replication initiation process (4, 21). Therefore, the structure of the 2.0-kb *ori* sequence that is necessary and sufficient for the replication of the Natto plasmid pUH1 molecule contains a 999-bp open reading frame, *rep*, a promotor region for *rep* expression, and a putative replication origin for the plasmid, which is located upstream of the promoter between positions 581 and 653.

The amino acid sequence of the *rep* protein coding region of pUH1 was compared with a number of protein sequences registered in GenBank by using the homology search system of GENAS (10). Homologies between the predicted amino acid sequence of the rep protein of pUH1 and those of the rep protein of pFTB14, RepB of pUB110, and protein A of pC194 are illustrated in Fig. 4. A high similarity (71.8%) was found between the rep protein of pUH1 and  $rep_{14}$  of pFTB14 in B. amyloliquefaciens. The structure of the replication origin sequence of pFTB14 contains an open reading frame (rep), stretching for 1,017 bp, a promoter region for rep expression, and a possible replication origin for rep expression, which is located upstream of the promoter. The rep product is trans active and essential for plasmid replication (13). Compared with rep of pUH1, a low similarity (39.7%) was found for RepB of pUB110 (1) and for protein A (29.2%) of pC194 (8) in S. aureus. Khan et al. (9) determined 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 RepC protein of pT181 (9), the protein of E. coli plasmid R6K (4, 17, 18) does not have any significant homology in its amino acid sequences with those of the rep proteins of pUH1 and pFTB14 deduced from the open reading frame (data not shown). The Rep protein of Natto plasmid pUH1 shows high homology with replication proteins encoded by pFTB14, pC194, and pUB110, which originate from various gram-positive bacteria such as B. amyloliquefaciens and S. aureus. Taxonomic studies (based

on the 16S rRNA similarities) revealed that *Bacillus* and *S. aureus* strains are more related than *Streptococcus* and *Lactobacillus* strains (16). The plasmid homologies suggest an exchange of plasmid replicons by recent horizontal transfer through the different genera, including "natto" *Bacillus* species.

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