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Nucleotide sequence and organization of *dnaB* gene and neighbouring genes on the *Bacillus subtilis* chromosome

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ABSTRACT

A region of the Bacillus subtilis chromosome containing dnaB, a gene essential for the initiation of chromosomal replication in B. subtilis, was cloned and nucleotide sequence of some 5000bp determined. The region consists of 4 open reading frames (ORFs) possibly comprising a single transcriptional unit. Two dnaB mutations, dnaB27 and dnaB19 were located within the first ORF which would give rise to a protein of 472 amino acids. The dnaB27 mutation involves codon at the position of 122 (replacement of Asp by Asn) close to a DNA binding domain and the dnaB19 codon 379 (replacement of Ala by Thr) close to a region rich in charged amino acids which may be alpha helical. The third ORF in the same transcriptional unit would produce a hydrophobic protein which might be involved in the DNA-membrane binding function of dnaB gene. No homologous Escherichia coli genes have been found.

INTRODUCTION

Initiation of chromosomal replication in Bacillus subtilis requires de novo synthesis of protein and RNA. Many mutants have been isolated in which the initiation of replication is specifically inhibited at non-permissive temperatures (initiation mutants) (1). These mutations map to several loci on the chromosome. The most well studied initiation mutations are those that map in the dnaB locus between polA and citF at around 250° of the 360° chromosomal map. Some 16 mutations have been identified at or near the locus (2-8). All except one, dnaB19, are located close together and are assumed to be within a single cistron. A relatively large recombination index between dnaB19 and any one of the clustered mutations suggested that dnaB19 is located in a second cistron (8). All mutations including dnaB19 affect initiation of chromosomal replication. In some cases, like in dnaB27, the effect of the temperature shift is reversible, thus, after incubation of the mutant cells at non-permissive temperature for a certain period of time, upon temperature shift down to permissive temperature a new round of chromosomal replication is initiated. This initiation requires de

novo synthesis of RNA but not protein (4,6). Some dnaB mutations affect replication of a temperate phage and a plasmid in addition to chromosomal replication. Ten mutations abolished induction of prophage SP02 by 6-hydroxyazouracil (7). Another dnaB mutation, dna-1, inhibited replication of a plasmid pUB110 and its derivatives at non-permissive temperatures (9). In contrast, dnaB19 shows no effect on the plasmid replication (9). These results indicate that the gene in the dnaB locus plays a key role in the initiation of replication of the chromosome and of extra-chromosomal replicons.

To get further insight into the structure and function of the dnaB gene and its products, we have cloned DNA fragments which transform the two distant mutations, dnaB27 and dnaB19, and fragments in their vicinity. Determination of the nucleotide sequence of about 5 kbp revealed 4 complete and 2 partial open reading frames (ORF). The four complete ORFs may constitute a single transcriptional unit. The most 5' proximal frame of the 4 was identified as the dnaB gene because both dnaB27 and dnaB19 mutations fell within this ORF.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacillus subtilis CRK2000(leu8, trpC. thyAB) (10), CRK2001(leu8, trpC, thyAB, dnaB27) (6) and BD54(metB5, ile-1, dnaB19) (3) were used as sources for DNA and recipients for transformation. Escherichia coli HB101 and JM105 were used as hosts for plasmid and M13 phage vectors. Plasmid pJH101 (11) and M13 phage derivatives, M13mp18 and M13mp19, were used for cloning of B. subtilis chromosomal fragments.

Chemicals. α-^{32}P-dCTP (PB10205, 3000Ci/mmole) and nick translation kit were purchased from Amersham International plc (Buckinghamshire, England). T4 DNA ligase, restriction endonucleases and M13 sequencing kit were from Takara Shuzo Co. Ltd. (Kyoto, Japan).

DNA preparation, cloning and transformation. Chromosomal DNA was prepared from B. subtilis cells as described previously (12). Construction of a B. subtilis chromosomal DNA library and isolation of clones, clone pMP66-29 and pMP33-25, which could transform dnaB19 mutation will be described elsewhere (13). Cloning of regions left side of the clone pMP66-29 was carried out as follows: Southern hybridization of the chromosomal DNA from the dnaB mutants and wild-type cells by the left end portion of the clone pMP66-29 as the probe showed that the clone pMP66-29 reacts with a 2.5 kbp EcoRI fragment (Fig.1). Therefore chromosomal DNA from mutant and wild-type cells were

digested by EcoRI and fragments corresponding to about 2.5 kbp were recovered from a low-melting agarose gel. They were inserted into M13mp19 and phages containing the left end-portion of the clone pMP66-29 were identified by plaque hybridization. Transformation of B. subtilis cells with plasmid DNAs was according to Dubnau et al. (14).

Determination of the nucleotide sequence. The detailed map of restriction sites in the cloned fragments was constructed (Fig. 1). Using these restriction sites, sub-fragments were recloned in M13mp18 or in mp19 to use for sequence determination. Length and direction of nucleotides sequenced by the dideoxy chain termination method are shown in Fig. 1.

RESULTS

Cloning of fragments from the dnaB region of the chromosome.

From a B. subtilis DNA bank, constructed in the vector pJH101 (11) two partially overlapping DNA fragments, clone pMP33-25 and pMP66-29 (see Fig.1) which could transform dnaB19 were cloned (13). Experiments with dnaB27 as a recipient showed that only the clone pMP66-29 had the ability to transform dnaB27 (Fig.1).

Preliminary determination of the nucleotide sequence of the clone

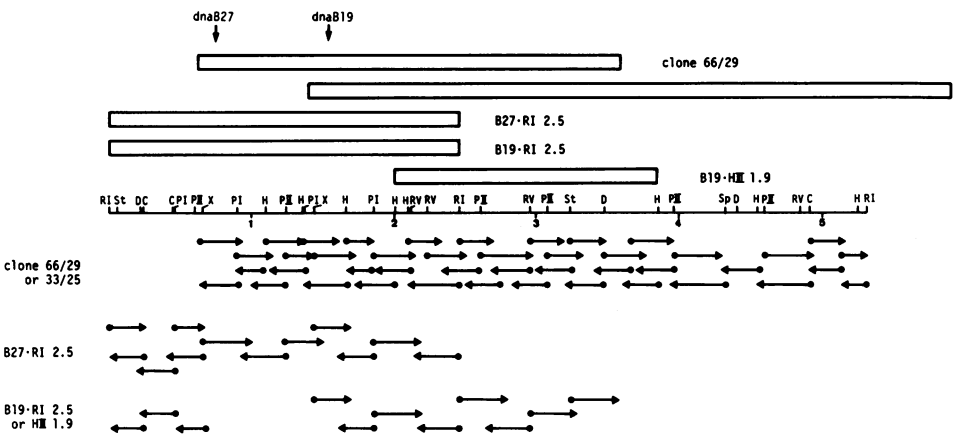


Figure 1. DNA fragments cloned from the dnaB region, restriction site map and strategy for sequence determination.
Cloning of various fragments illustrated by open square are described in the text. Restriction enzyme cleavage sites of each fragment were determined and constructed on the scaled map (numbers are kbp). Symbols for enzymes are: C: ClaI, D: DraI, H: HindIII, PI: PstI, PII: PvuII, RI: EcoRI, RV: EcoRV, Sp: SphI, St: StuI, X: XhoI. Below the map arrows indicate length and direction of sequences determined by the dideoxy chain termination method. Approximate positions of mutation sites for dnaB27 and dnaB19 are shown.

pMP66-29 revealed that the left end of the clone pMP66-29 was within a long open reading frame (ORF). In order to determine the structure of the complete ORF as well as of the 5'-upstream regulatory region, a complete EcoRI fragment was cloned using a truncated EcoRI fragment at the left end of the clone pMP66-29 as a probe. Two fragments, clones B27/RI2.5 and B19/RI2.5, were cloned from chromosomal DNA of mutants dnaB27 and dnaB19, respectively, inserted into M13mp18 vector (Fig. 1). Attempts to clone the same fragment from $dnaB^+$ strain, B. subtilis 168 CRK2000, have been failed.

Nucleotide sequence of the dnaB region: identification of putative ORF.

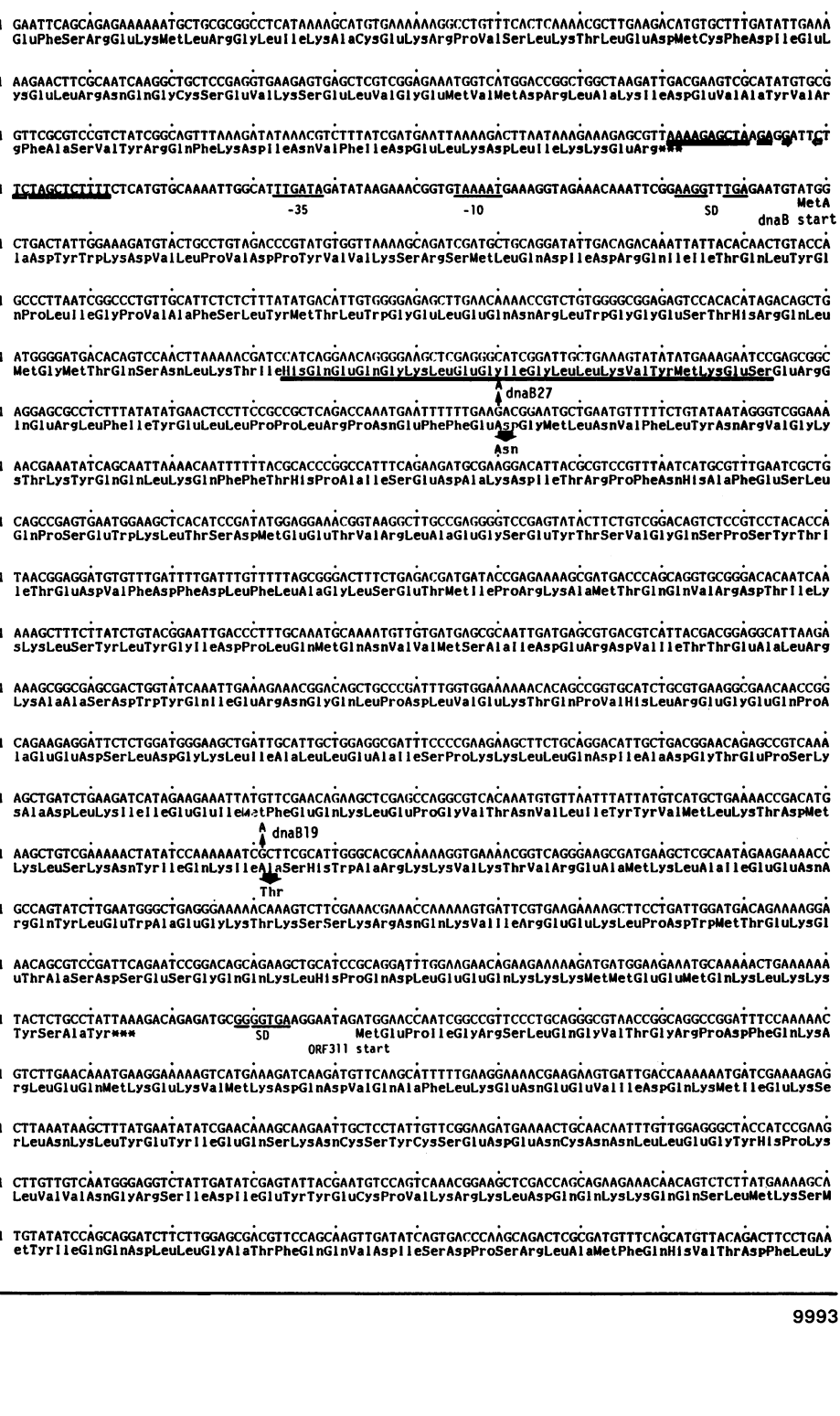
Nucleotide sequence of the wild type DNA was determined using clone pMP66-29 and pMP33-25, subcloned in M13. The portion further left of the clone pMP66-29 was determined using DNA cloned from the two mutants. Both strands were sequenced except for two small non-coding regions which could be cloned only in one direction in the vector (see strategy in Fig. 1).

Fig. 2 shows the complete nucleotide sequence of 5313 bp covering the two EcoRI fragments. As mentioned above the sequence of the wild type DNA begins from nucleotide 630 and the upstream sequence was determined from the two mutant DNAs. Since the two sequences were found to be identical with each other, we concluded that they represent the wild type sequence. It is obvious that only one of the six frames is used as the coding frame in this region (Fig. 3). Five ORFs in this region, all in one direction 5' to 3' from left to right, are preceded by initiation codons and sequences resembling Shine and Dalgarno sites (Fig. 2 and 3). Typical promoters for the σ^{43} RNA polymerase (15) are found, one upstream from ORF472 and the other in between ORF281 and a truncated ORF at the right end. That both are promoters is supported by the fact that these regions containing the putative promoters were clonable only in one direction in M13 vectors. Although there is no typical termination signal downstream from ORF281, we assume that the four ORFs, ORF472, ORF311, ORF213 and ORF281 constitute one transcriptional unit. An additional promoter-like structure is found between ORF213 and ORF281 (Fig. 2).

In an extensive search for homologous ORF in E. coli using the NBRF Protein Data Bank (release 7.0) only the last and truncated ORF showed significant homology with 5' portion of the ORF for threonine-tRNA synthetase (thrS) (16).

Identification of dnaB ORF by determining location of mutation dnaB27 and dnaB19.

Two EcoRI fragments, clone B27/RI2.5 and B19/RI2.5, cloned from


-35
-10
SD
MetA
dnaB start
dnaB27
Asn
dnaB19
Thr
SD
ORF311 start

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2301 AAGCTATAATGAGACGGGAAAAGGGAAAGGATTATATTTATACGGGAAATTTGGGGTAGGAAAAACGTTTATGCTCGCTGCAATTGCCAATGAGCTGGCG
     sSerTyrAsnGluThrGlyLysGlyLysGlyLeuTyrLeuTyrGlyLysPheGlyValGlyLysThrPheMetLeuAlaAlaIleAlaAsnGluLeuAla

2401 GAAAAAGAGTATTCCTCCATGATTGTTTATGTGCCTGAATTTGTGAGAGAGCTTAAGAATTCGCTGCAAGACCAGACATTGGAAGAGAAGCTCAATATGG
     GluLysGluTyrSerSerMetIleValTyrValProGluPheValArgGluLeuLysAsnSerLeuGlnAspGlnThrLeuGluGluLysLeuAsnMetV

2501 TAAAGACCACACCCGTATTAATGCTTGATGACATCGGAGCAGAATCAATGACAAGCTGGGTAAGGGATGAGGTCATCGGAACGGTGCTTCAGCACAGAAT
     alLysThrThrProValLeuMetLeuAspAspIleGlyAlaGluSerMetThrSerTrpValArgAspGluValIleGlyThrValLeuGlnHisArgMe

2601 GTCCCAGCAGCTGCCAACGTTCTTTTCTTCTAATTTCTCACCTGACGAGCTGAAGCATCATTTTACGTATTCGCAGAGGGGAGAAAAAGAAGAAGTAAAA
     tSerGlnGlnLeuProThrPhePheSerSerAsnPheSerProAspGluLeuLysHisHisPheThrTyrSerGlnArgGlyGluLysGluGluValLys

2701 GCTGCAAGATTAATGGAGCGGATATTATATTTGGCTGCTCCGATCCGCCTTGACGGAGAAAACCGCCGACATCCATAATTGCTGTGGTTCAAAGGGGAAG
     AlaAlaArgLeuMetGluArgIleLeuTyrLeuAlaAlaProIleArgLeuAspGlyGluAsnArgArgHisPro***

2801 GAACTGAGGGTATGAAACGAAAAACGGCTGTCAAATGGCTGGCAGTGCTTGCAGGTGCTGGTTTACTCTATTGGGGAAATAAAACGTATTTGAATGTATC
     SD          MetLysArgLysThrAlaValLysTrpLeuAlaValLeuAlaGlyAlaGlyLeuLeuTyrTrpGlyAsnLysThrTyrLeuAsnValSe
        ORF213 start

2901 GCCGAAAGAAATCAGGGTATGGGTATTGTCGTTCGGAGTCTTTGCACCTCTGATGTTTATCGGGATATCCATCGTCAGGCCACTTGTTTTATTTCCGGTG
     rProLysGluIleArgValTrpValLeuSerPheGlyValPheAlaProLeuMetPheIleGlyIleSerIleValArgProLeuValLeuPheProVal

3001 TCTGTTATTTCAATAGCTGGCGGACTTGCGTTTGGCCCGCTTCTCGGCACGCTTTATACGTTATTCGGTTCGATGTGCGCTTCAGCTGTTTCGTTTTTTG
     SerValIleSerIleAlaGlyGlyLeuAlaPheGlyProLeuLeuGlyThrLeuTyrThrLeuPheGlySerMetCysAlaSerAlaValSerPhePheA

3101 CGGCTGGTTTGTTTTCGGCGAAAAAGAACGGGCATTACGAAAGGCTTGAAGCCATACAGAAGCAAATGGAGGATAACGGATTTTTCTATATCTTTCTTTT
     laAlaGlyLeuPheSerAlaLysLysAsnGlyHisTyrGluArgLeuGluAlaIleGlnLysGlnMetGluAspAsnGlyPhePheTyrIlePheLeuLe

3201 GAGAATCCTGCCGATCAATTTTGATTTCGTCAGCTATGCGGCAGGCCTTTCCAATGTCAAAGCGCTGCCGTATTTTGCCGCAACGGCTGTGGGAATCATC
     uArgIleLeuProIleAsnPheAspPheValSerTyrAlaAlaGlyLeuSerAsnValLysAlaLeuProTyrPheAlaAlaThrAlaValGlyIleIle

3301 CCTGGGACGATTGCGCTTAATGTGCTGGGTGCGAGCTTTTTGGCTGGCAATCTGCCCGCTTTCTTTATGGTGCTCGCTTTGTATATCGTGTTTATCTCAT
     ProGlyThrIleAlaLeuAsnValLeuGlyAlaSerPheLeuAlaGlyAsnLeuProAlaPhePheMetValLeuAlaLeuTyrIleValPheIleSerL

3401 TGCCGTTCATCTTCAGAAAGAAAATGCAAAACTTGTTCCAAGAATCAAATTAAGATTTGCCAATGACCTTTACGTCTATTTTAAAAACATCCCCCATATA
     euProPhePhePheArgLysLysMetGlnAsnLeuPheGlnGluSerAsn***          -35          -10

3501 CTTGTAACAGATGCCGTAAGGGGGGACAAACATGCTTGAAATAATCTTTGAAGATGACCATGATGCAGCCGCTTTTTTACATCTCATTCAGCATTCGGAC
                          SD          MetLeuGluIlePheGluAspAspHisAspAlaAlaAlaPheLeuHisLeuIleGlnHisSerAsp
                             ORF281 start

3601 GATCGGAACAATATCATTGTCCGTGAAGGCATACGAAAAATCGGGATTGAAAAAGCGAATCCGGCTTTTCCTATTCAGCGTTTTATGGAGCCGATTCTGG
     AspArgAsnAsnIleIleValArgGluGlyIleArgLysIleGlyIleGluLysAlaAsnProAlaPheProIleGlnArgPheMetGluProIleLeuV

3701 TCAAATTTTTTTTGGAATGCAAAGAAGACGAGCATATGCTGTCATTGATTGAGGAAACGTATTGTTTCACGGACCAGGATGAACAGCAGCAAATCCTTCA
     alLysPhePheLeuGluCysLysGluAspGluHisMetLeuSerLeuIleGluGluThrTyrCysPheThrArgAspGlnAspGluGlnGlnGlnIleLeuGl

3801 GCTCGCACACAGTATTATTGAAGGTGAAGCAGACGATCTTCCGTTTGAGCCGCTGAAGCTTTCACGGAAACAATCAATTTTGGATGAACTCCAAACGATC
     nLeuAlaHisSerIleIleGluGlyGluAlaAspAspLeuProPheGluProLeuLysLeuSerArgLysGlnSerIleLeuAspGluLeuGlnThrIle

3901 TGTTTAGAGGAAGGAGTATTTTACATTCGCTCCTTTCAGACCTTCCGACTTGGATCATACTATAAGCAGCTGAGAGATATTACAGAAGCAGCGATTGATG
     CysLeuGluGluGlyValPheTyrIleArgSerPheGlnThrPheArgLeuGlySerTyrTyrLysGlnLeuArgAspIleThrGluAlaAlaIleAspG

4001 AATATAAGATGGAACAGGAATATCAAAACTTTATTCAAACACTCAGAGACTATGTGGACGCCAAACAGCCGCGAATCAAAAAAGTGCATATTGTCCATGA
     luTyrLysMetGluGlnGluTyrGlnAsnPheIleGlnThrLeuArgAspTyrValAspAlaLysGlnProArgIleLysLysValHisIleValHisAs

4101 TGGTTCCTTTACATTATGGGAGCTGCGCTATGTGCCGGAACGGGAAAAAATGAAATACATAGACAGAAGATTTGTCCGGGATCATCCTATGTATATTGAT
     pGlySerPheThrLeuTrpGluLeuArgTyrValProGluArgGluLysMetLysTyrIleAspArgArgPheValArgAspHisProMetTyrIleAsp

4201 TCTCATTTGCTCGCGCCGCTGATTTCCATTGCGCCTGATGAAGTCGTGCTTTATACCGACCAGCCCGAGCACATGATGGCAAGGACCATTCAAAACGTAT
     SerHisLeuLeuAlaProLeuIleSerIleAlaProAspGluValValLeuTyrThrAspGlnProGluHisMetMetAlaArgThrIleGlnAsnValP

4301 TTCAAGAGAGAGTGGAAATCGTCCCGCTGCATGCTTTTACAGATGCAGAAATACCGGTGAAGCACTCGGAAGGATGAGGGCCGGCAGCCCTGTCTATTTAA
     heGlnGluArgValGluIleValProLeuHisAlaPheThrAspAlaGluIleProValLysHisSerGluGly***

4401 GGCTGTCGGTTTAAAAAAAAGGAAACGCGATCGTGTTGATTTTTGGATTGAACAATTTATAATACATAGGAGATTAAGAAAGACACACGTATTGATAAG
                                          -35          -10

4501 GACACGAAGGAATGATACCGGCTCAAAGAGAGGGAAGCCCTGGTGCAAGCTTCCCAGCTAAGGACCATTCGCTTTACCGCCTTTGAACTTCAGCTGTGAA
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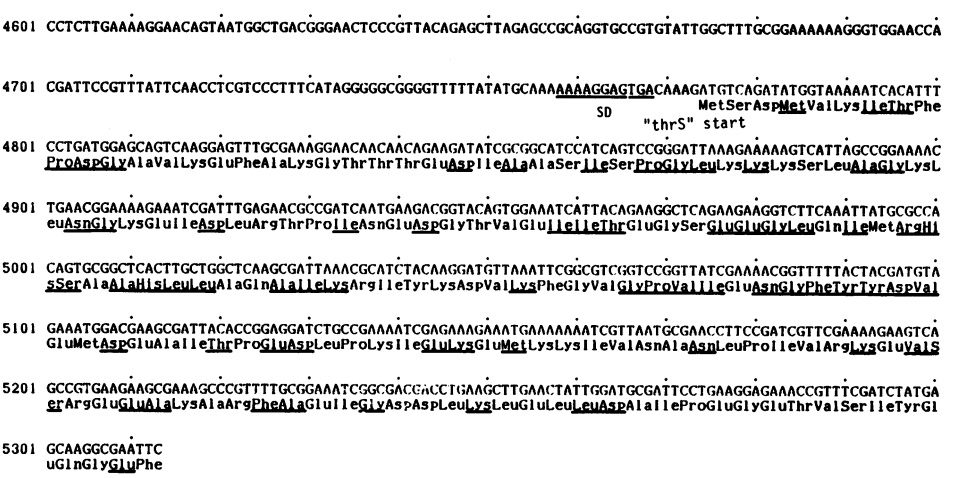

Figure 2. Nucleotide sequence of the dnaB region including two sites of mutations dnaB27 and dnaB19.
Nucleotide sequence and amino acid sequence of the putative open reading frames are shown. *** indicates termination codons. Two nucleotides shown above the sequence at 760 and 1531 were determined by sequencing relevant fragments from dnaB27 and dnaB19 mutant cells, respectively, as shown in Fig. 1. Changes in amino acids corresponding to these nucleotide changes are indicated. Putative signals for initiation of transcription (-10 and -35) and translation (SD) are underlined by thin solid lines. Possible termination signals of transcription, inverted repeats, are underlined by open square arrows. The stretch of amino acids characteristic for DNA binding function is indicated by a thick underline. In the case of "thrS" gene, amino acids common to E. coli thrS gene are underlined.

chromosomal DNA of mutants dnaB27 and dnaB19, respectively, were sequenced (Fig. 1). In addition a HindIII fragment which overlaps with and extends downstream of the clone B19/RI2.5 was cloned from dnaB19 chromosome and sequenced (Fig. 1).

Two base changes were found, one in dnaB27 DNA at nucleotide 760 and the other in dnaB19 DNA at nucleotide 1531. No other base changes were found within the region covered by the three mutant DNA fragments. Both of the two base changes are located in ORF472. The mutations caused amino acid changes, one from Asp to Asn at amino acid 122 in dnaB27, and the other from Ala to Thr at amino acid 379 in dnaB19. We therefore conclude that ORF472 is the dnaB gene itself.

DISCUSSION

DnaB gene is a key gene involved in the initiation of chromosomal replication in B. subtilis. Differences in the pleiotropic effects of the

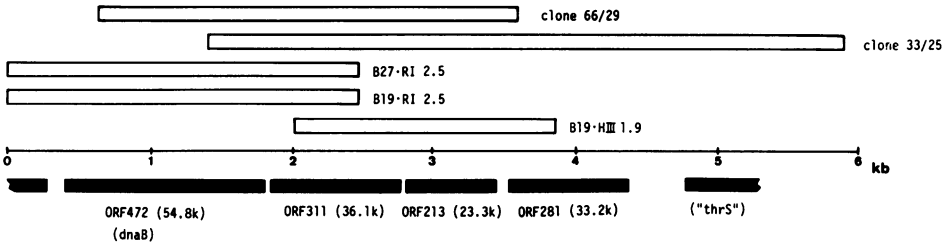

Figure 3. Open reading frames in relation to all possible stop codons in 6 possible reading frames.
Above the scale (numbers are kbp) all possible stop codons are listed in all reading frames. Frames 1 to 3 are left-to-right orientations. Below the scale putative open reading frames are indicated. Numbers below each frame are nucleotide numbers in Fig. 2. Numbers with ORF are number of amino acids in each frame. Calculated molecular weight of putative protein products are shown in parenthesis.

two mutations, which lie far apart, on plasmid replication have led to suggestions that they lie in different cistrons (17). The present results, based on the cloning and the determination of the nucleotide sequence of the dnaB region together with the identification of the location of the two mutations, dnaB27 and dnaB19, convincingly show that both lie in the same dnaB gene which is a single ORF coding for 472 amino acids.

The amino acid sequence deduced from the nucleotide sequence shows two characteristic features. One is a stretch of 19 amino acids, from 80 to 99, which is homologous with a DNA binding domain common to many known DNA binding proteins (18)(Fig. 4). The dnaB27 mutation is located near such a sequence. The second feature is a long stretch at the C-terminal portion of the protein, from 380 to the terminus. This region is very rich in charged amino acid and hence hydrophilic and seemingly rich in alpha helix (19). The dnaB19 mutation affects the N-side edge of this region. These data suggest that the protein consists of at least two domains differing in structure and function.

At the moment there is no genetic evidence supporting the possibility of involvement of other protein components in the function of dnaB gene product. However, the apparent polycistronic nature of the transcriptional unit of the dnaB region suggests such a possibility. In this respect, the third ORF213 is intriguing because the putative product would be extremely hydrophobic with a positively charged N-terminal segment common to Gram-positive signal sequence (20). Binding of the replication origin of the chromosome has been ascribed to one of the dnaB functions (17). The

hydrophobic membrane protein like product of ORF213 may be involved in this function. Furthermore, the second ORF311 codes for an amino acid sequence, from amino acid 166 to 181, which is identical to the consensus sequence, LXGXXGXVXGKTXXXXI, found in putative nucleotide-binding sites of various proteins (21). Further study is necessary for elucidating possible functions of other ORFs in the same transcriptional unit with dnaB gene.

The phenotypes of dnaB mutants of B. subtilis are similar to those of dnaA mutants of E. coli (6). In both cases initiation but not elongation of the chromosome is affected. The temperature sensitivity of some of the mutations is reversible. During incubation of the mutants at non-permissive temperatures potential for initiation of the chromosomal replication is accumulated and then used upon the temperature down shift. The accumulated potential no longer requires de novo protein synthesis but it is absolutely dependent on RNA synthesis. From these results dnaB has been assumed to be a counterpart of E. coli dnaA. We found that there is no homology between the putative dnaB protein and the E. coli dnaA protein. On the other hand we have recently reported discovery of a gene at the replication origin of the B. subtilis chromosome that is highly homologous with E. coli dnaA (22,23). The gene is named tentatively as "dnaA" with quotation mark. Although the function of the "dnaA" is not known, indirect evidence is highly suggestive for the involvement of the gene product in initiation of the chromosomal replication (23). If there are two initiation genes, dnaA and dnaB, in B. subtilis, is there any E. coli counterpart for B. subtilis

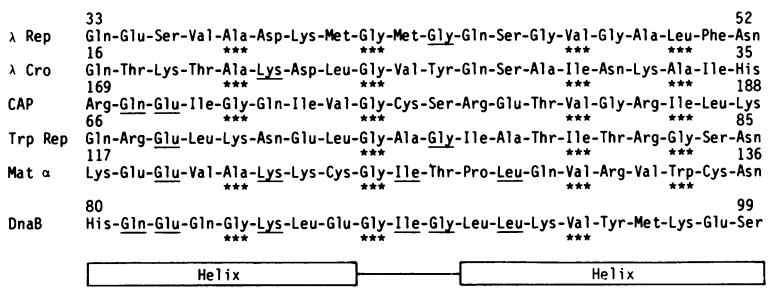

Figure 4. Comparison of a putative DNA binding sequence of the DnaB protein with those of well known DNA-binding domains.
A stretch of 19 amino acids, from 80 to 99, of the DnaB protein is aligned with DNA-binding domains of various proteins. Amino acids with *** are most highly conserved amino acids in 25 proteins compared (18). In addition, other amino acids identical with the DnaB sequence in corresponding positions are underlined.

dnaB? We found that E. coli dna genes, dnaA, B, J, K, T, Q and a gene for RNaseH are not homologous with dnaB. A search for homology with other E coli genes using the NBRF protein data bank has failed to find the E. coli counterpart. Preliminary experiments to find dnaB-homologous sequence in E. coli by Southern blot hybridization using the dnaB gene as a probe revealed discrete EcoRI bands of some homology. Cloning of the E. coli counterpart is now underway.

During the preparation of this manuscript, Dr. N. Sueoka communicated to us personally that they obtained the same results on the nucleotide sequence of the B. subtilis dnaB gene.

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