Commun. Fac. Sci. Univ. Ank. Serie C
V. 6. pp. 169-182 (1988)

CLONING AND NUCLEOTIDE SEQUENCE ANALYSIS OF A B. SUBTILIS DNA FRAGMENT CARRYING CAR GENE CODING FOR CARBAMOYL PHOSPHATE SYNTHETASE ENZYME

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Key Words: Recombinant DNA; sequencing vectors; dideoxy nucleotide sequence of *B. subtilis car* gene.

ABSTRACT

A *B. subtilis* DNA fragment (1229 bp) known to carry *car* gene coding for carbamoyl phosphate synthetase enzyme was cloned in pBR322 and the restriction analysis of this fragment was carried out (Kadıkıran, 1988a).

The 1229 bp fragment was then cloned in M13mp8 and M13 mp9 sequencing vectors. Sequencing was carried out by the dideoxy chain termination method. Subcloning was employed to insure the fidelity of sequencing.

Of the 1229 bp, 796 bp have been documented while 433 bp stretch of DNA corresponding to the left of centre on the fragment has not been included due to the further clarifications needed.

Restriction site positions on the fragment for the restriction enzymes used for restriction analysis have been refined further.

Probable promoter consensus sequences for −35 promoter up-stream, −10 Pribnow-box, +1 start signal sequences have been proposed.

INTRODUCTION

Bacillus subtilis is a sporulating, gram-positive soil bacterium which might offer hope of interesting differences from enterobacterial species. Only few *Bacillus* genes concerned with vegetative growth have been analysed at the molecular level (Losick and Pero, 1981).

Escherichia coli and *Salmonella typhimurium*, have been investigated in considerable molecular detail. Arginine metabolism in *E. coli* and its regulation has already been worked out (Kadıkıran and Baumberg, 1980; Baumberg and Kadıkıran, 1977; Bretscher and Baumberg,

1976). The nucleotide sequence of the arginine genes in *E. coli* have been investigated to near completion (Nyunoya and Lusty, 1983; Piètte *et al*, 1984).

As part of a team, working on the molecular analysis of *B. subtilis* arginine genes, I have attempted to carry out sequence analysis of a *B. subtilis* fragment carrying *car* gene, as demonstrated by complementation analysis (Mountain *et al*, 1986), which codes for the carbamoyl phosphate synthetase enzyme [Carbon dioxide; L-glutamine amidoligase (ADP-forming, carbamate phosphorylating); E.C. 6.3.5.5].

The restriction analysis, as reported by Kadıkıran (1988a), has paved the way for the subcloning of *B. subtilis car* gene fragment to increase the efficiency and accuracy of nucleotide sequencing.

MATERIALS AND METHODS

Strains, Sequencing vectors and Chemicals

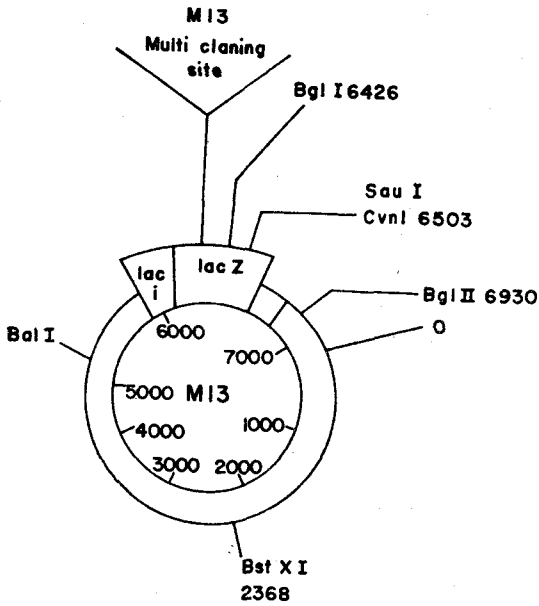
Strains E. coli strain of DHI (F^-*gyrA recA relA endA thi hsdR hsdM supE)* was used as the recipient in transformations for the preparation of pAKC plasmid DNA. pAKC plasmid carrying the *B. subtilis car* gene was constructed as described in Kadıkıran (1988a).

E. coli JM 103 (F^-*lacZ* $\Delta M15$ *thi strA recA endA sbcB*15 *hsdR*4) was used for the transformations with M13mp8 and M13mp9 recombinant phages (Messing and Vieira, 1982). M13mp8 and M13mp9 sequeucing vector phages (Fig. 1) were employed for cloning the inserts in both orientations (Messing, 1983). Stock cultures of JM103 was maintained on minimal salts agar medium supplemented with thiamine. JM103 competent cells were grown in 2 x TY liquid medium containing: 1.6 % tryptone, 1 % yeast extract and 0,5 % NaCl in 1000 ml of H_2O. When needed 1.5 % agar (Difco No. 1) was added to the medium. H-top agar, employed in transformation of competent JM103 cells, contained: 1 % tryptone, 0.8 % NaCl and 0.4 % agar in 1000 ml of H_2O.

Sequencing vectors ss-M13mp phages were employed as sequencing vectors.

Chemicals Isopropyl–beta–D–thiogalactopyronaside (IPTG) and 5–bromo–4–chloro–3–indolyl–beta–D–galactoside (X–gal) were purchased from Sigma. Deoxyribonucleotides (dNTPs) were purchased from PL–Biochemicals or Sigma. Dideoxyribunucleotides (dd–NTPs) and sequencing primers were purchased from Bethesda, α–32p–dATP

a)



b)

5'-ACCATGATTACGAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTTGGCACTGGCCGTCGTTTTACAACG -3' M13mp8

EcoRI BamHI SalI PstI HindIII
SmaI AccI
XmaI HincII

5'-ACCATGATTACGCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCACTGGCCGTCGTTTTACAACG -3' M13mp9

HindIII PstI SalI BamHI EcoRI
AccI SmaI
HincII XmaI

<---------- Direction of DNA Synthesis ----------

Figure 1. a) M13mp sequencing vector phage DNA, b) Nucleotide sequence in multicloning sites (mcs) of M13mp8 and M13mp9 sequencing vector phage DNAs.

was obtained from New England Nuclear (NEN); restriction enzymes as well as the Klenow enzyme were all purchased from Bethesda. Sigma –cote and bind–silena were obtained from Sigma. X–ray films used for autoradiography were of Kodak quality.

Cloning, T-tracking, Electrophoresis, DNA Sequencing.

Cloning was carried out by using M13mp8 and M13mp9 double stranded (ds) vector phage DNAs (Fig. 1). The genetic assay for the selection of recombinants was based on the failure of the recombinant phages to complement *LacZ* $\Delta M15$ mutation in JM103 host bacterium to give rise to *Lac* Z^+ phenotype (α–complementation) (Messing, 1981). Transformants of non–hybrid M13mp phages were detected as blue plaque formers on indicator plates, containing X-gal and IPTG at final concentrations of 40 and 20 $\mu g/\mu l$, respectively. Transformants harbouring the recombinant M13mp8 and M13mp9 phages were identified as colourless plaque formers. The insertion of a DNA fragment into the multicloning site (mcs) in M13mp phages impairs the expression of *lac* gene; hence, the indicator X-gal is not utilized and the plaques remain colourless.

T-tracking method was employed, prior to sequencing, for distinguishing the recombinants from one another to insure that the clones to be used in sequencing were not the replicas. The DNA synthesis in T-tracking was carried out in just one reaction tube, containg dd-TTP together with four dNTPs (see, DNA sequencing). Hot–chasing was continued by the addition of α–^{32}p–dATP.

Electrophoresis DNA electrophoresis was performed as described in Kadıkıran (1988a). Sequencing electrophoresis was carried out at 28 mA, 1600 volts for 30-90 minutes as required. Normal sequencing gels were poured as described in Maniatis *et al* (1982). Gradient gels were prepared according to a new protocol developed at Cambridge (Biggin *et al*, 1983).

DNA sequencing was done by the dideoxy chain termination method (Sanger, 1977). Sequencing was carried out in a set of four reaction tubes by using the Klenow fragment of *E. coli* DNA polymerase I to extend a "universal" primer (17 bp) hybridized with the single stranded (ss) M13mp DNAs in the presence of four deoxynucleoside triphosphates (Hu and Messing, 1982). Hot chasing was continued by the addition of α–^{32}p–dATP or α–^{35}S–dATP to the reaction mixture.

The reaction was terminated in a random fashion by the incor poration of dideoxynucleotide analogues (dd-NTPs) of the four DNA nucleotides, since only one each of the four dd-NTPs was added to the four reaction tubes in dd-ATP, dd-GTP, dd-CTP and dd-TTP order. Chains of varying lengths, all terminated with the same base, were generated in each reaction tube. They were then separated by high resolution polyacrylamide–urea gel electrophoresis (Sanger *et al*, 1978). Following autoradiography, the cloned DNA sequence can be read out directly from the bottom to the top on the autoradiograph. α–^{32}p–dATP or α–^{35}S–dATP were employed for chasing new DNA synthesis. Both normal and buffer–gradient gels were employed to improve the resolution in the autoradiography as recommended (Sanger *et al*, 1980; Biggin *et al*, 1983).

RESULTS

Cloning of the 1229 *bp fragment, carrying B. subtilis car gene, in M*13*mp*8 *and M*13*mp*9 *sequencing phage vectors.*

The 1229 bp Hpa I–Hind III fragment was cloned in ds–M13mp8 and ds-M13mp9, double stranded replicative form (RF) of vector phages, in two orientations in order to carry out the nucleotide sequencing from both ends of the cloned fragment. Two approaches were adopted in order to accomplish the cloning of the fragment(s) by using the double–stranded replicative form of M13mp8 and M13mp9 phage vectors:

1– Cloning of 1229 bp fragment in M13mp phages.

2– Cloning of subfragments in M13mp phages.

Insert DNA Preparations:

a) The 1229 bp fragment was excised from p5BH2 DNA (50 μg) by Hpa I and Hind III double digestion and then electroeluted from the gel as described by Kadıkıran (1988a). Electroeluted insert DNA concentration obtained was 0.2 μg/μl.

b) 100 μl of 1229 bp fragment (0.2 μg/μl) was subjected to 90 units of Hae III digestion; followed by 6 units of Acc I digestion, to produce sufficiently small sized subfragments conducive to cloning short stretches of the fragment to be sequenced, in 150 μl reaction

mix at 37°C for 5 hours. Following ethanol precipitation overnight at –20°C, Hae III and Acc I digestion products of 1229 bp fragment was resuspended in 10 μl of ligation buffer.

Vector DNA preparations:

Two batches of both M13mp8 and M13mp9 phage DNAs were double–digested in two different combinations.

a) 3 μg of M13mp8 and M13mp9 double stranded DNAs were separately subjected to double digestions; first, with 6 units of Sma I restriction enzyme for 15 hours at 37°C and then with 6 units of Hind III enzyme at 37°C for 5 hours in a final volume of 15 μl to match one sticky–end and a blunt–end on the 1229 bp insert DNA. The double digestions resulted in the removal of 25 bp stretch of DNA from their multicloning sites and the linearization of the vector phage DNAs (Fig. 1b).

b) 3 μg of double–stranded vector M13mp8 and M13mp9 DNAs were digested in the following three combinations to accommodate a series of Hae III and Acc I digestion subfragments of 1229 bp fragment:

Sma I : for cloning Hae III–Hae III subfragments.
Sma I + Hind III : for cloning Hae III–Hind III subfragments.
Sma I + Acc I : for cloning Hae III–Acc I subfragments.

Following ethanol precipitation overnight at –20°C, the double digested vector DNAs in (a) and (b) were separately resuspended in 10 μls of ligase buffer.

Ligation results:

Vector M13mp DNAs were ligated with insert DNAs in Va + Ia and Vb + Ib combinations. Ligations were carried out in 1:1 ratios for a single digested; 4:1 ratios for double–digested DNAs, respectively. Both *in vivo* and *in vitro* ligation controls were run in parallel to the actual ligations.

The ligation reactions were checked out on an agarose gel by running a small sample (1 μl) from the ligation mix (Fig. 2).

The rate of ligation was substantiated by the diminishing amount of insert DNA which was compatible with the intensity of a new band corresponding to the hybrid phage DNA as evident from an increase in size.

Transformation. 4–10 μl of the ligation mixtures were mixed with 150 μl of competent *E. coli* JM 103 cells and plated in H-top agar containing X-gal and IPTG, then poured on to 2 x TY plates and allowed to set on a levelling table. The plates were incubated overnight as inverted at 37°C.

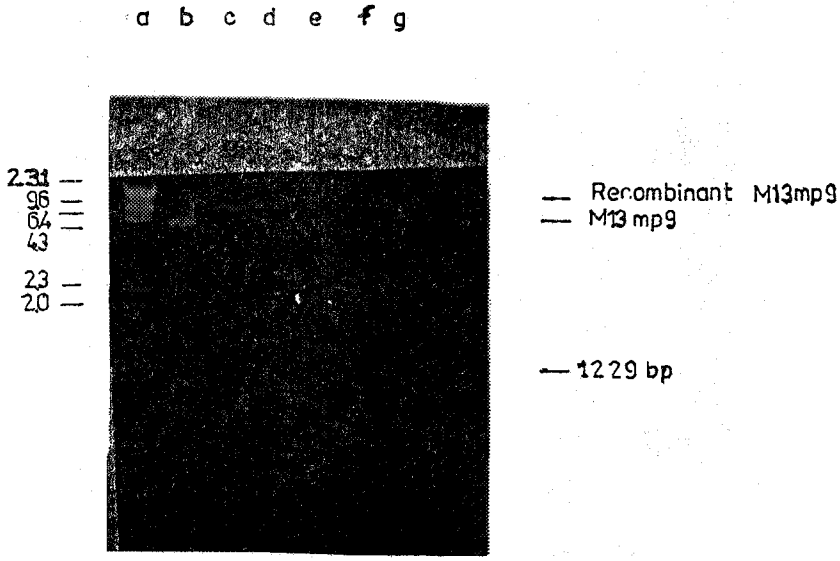


Figure 2. Ligation of M13mp9 vector DNA with 1229 bp insert DNA. a) λHind III digests as size standards, b) Undigested M13mp9 DNA, c) Sma I digested, linearized M13mp9 DNA, d) Sma I and Hind III double digested M13mp9 DNA, e) Ligation of Sma I and Hind III double digested vector DNA with Hind III and Pvu II digested 1229 bp insert-DNA as in vitro ligation, f) M13mp9 self-ligation as in vivo ligation control, g) 1229 bp insert DNA.

The white plaques were accounted for recombinant M13mp transformants while the blue plaques were considered as non–hybrid M13mp (self–ligated) transformants. The transformations gave rise to about 4000 recombinant white plaques per μg of ligated M13mp DNAs. Recombinant M13mp phages were then grown up for ss-DNA preparations.

A fresh JM 103 culture, grown in 2 x TY starting from a single colony, was infected with a single white plaque and grown up overnight at 37°C by vigorous shaking. The supernatant was employed for ss-phage DNA preparation as described by Messing (1983). Recombinant M13mp ss-DNAs were checked against ss-M13mp8 and ss-M13mp9

DNAs. The results clearly demonstrated the differences in size by slower electrophoretic mobility of the recombinants on % 0.8 agarose gel (Fig. 3).

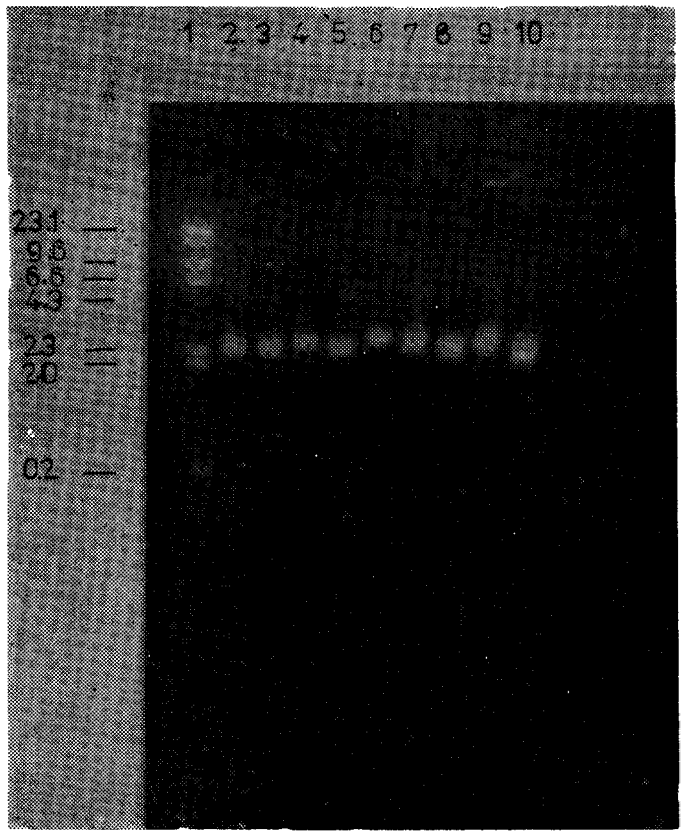


Figure 3. Agarose gel electrophoresis of recombinant ss-M13mp9 DNAs from three ligation combinations. Lane 1 is λHind III digests as size standards. Lanes 2, 3 and 4 are recombinants from Sma I digested vector and Hae III digested insert ligations. Lanes 5 and 10 are ss-M13mp9 DNAs as controls. Lane 6 is the recombinants M13mp9 carrying 1229 bp insert, used as a second control. Lanes 7, 8 and 9 are recombinants resulting from ligation between Sma I and Hind III digested M13mp9 vector DNA and Hae III–Hae III digests of 1229 bp insert -DNA.

The resulting recombinant ss-M13mp8 and ss-M13mp9 phages from all ligation combinations were then tested, in groups, for T-tracking. Recombinant ss-phage DNAs representatives of different ligation combinations, were selected by virtue of their T-tracking patterns, compared with the original ss-M13mp8 and ss-M13mp9 phage DNAs

as controls. Selected recombinants were employed for sequence analysis by the dideoxy method as described in materials and methods.

Prior to the actual DNA synthesis, 1 μl of 17 bp primer solution (2,5 μg/ml) was used for the hybridization reaction with 1,5 μl of recombinant M13mp ss-DNAs (0.2 μg/μl). The polymerization was continued in four reaction tubes containing; 1 μl stock dNTP solution, 0.5 μl of one of dd-NTPs, and 1.1 μl of α–^{32}p–dATP radioactive label (10 mCi/μl) in each.

The reaction tubes were labelled A, G, C and T corresponding to the kind of dd-NTP added (0.5 μl). 0.1 unit of Klenow enzyme was always added last to inititate the polymerization reaction. In all cases, hot chase was continued for 13–15 minutes, in a water-bath at 37°C, in a total reaction volume of 10–15 μl; then continued for an additional 15 minutes upon the addition of 2.5 μl cold–chase (d–NTPs only). Continuing DNA synthesis was terminated altogether by the addition of 2.5 μl of formamide dye solution.

The contents of the reaction mixtures were brought down to about 2–3 μl by placing them in a boiling water–bath for 10 minutes. 2–3 μl contents of four reaction tubes were loaded into the pockets in a sequencing gel in an order of A, G, C and T. Sequencing electrophoresis was continued as described in materials and methods.

Buffer gradient gels were employed to obtain maximum information from a single gel, particularly for sequencing long stretches of DNA. This eliminated the need to run reactions for different lengths of time, in order to obtain maximum information, since the banding pattern on a gradient gel bore an arithmetic relationship to molecular weight unlike the normal gel which demonstrated a logarithmic relationship to molecular weight.

Resolution was improved further when α–^{35}S–dATP was employed for radioactive–labelling in conjunction with buffer gradient gels (Fig. 4).

Following electrophoresis under high–current (28mA, 1600 volts) for 30–90 minutes, the gel was dried up at 80°C for 2 hours. When cooled, it was wrapped up with an X–ray film and stored in the dark. Next day the autoradiograph was developed. Storage period in the dark was 9–12 hours for α–^{32}P–dATP and 18–24 hours for α–^{35}S–dATP labelling. Each group of the four tracks on the autoradiograph were read out in the same order of A, G, C and T from the bottom to the top on a light source (Fig. 4).

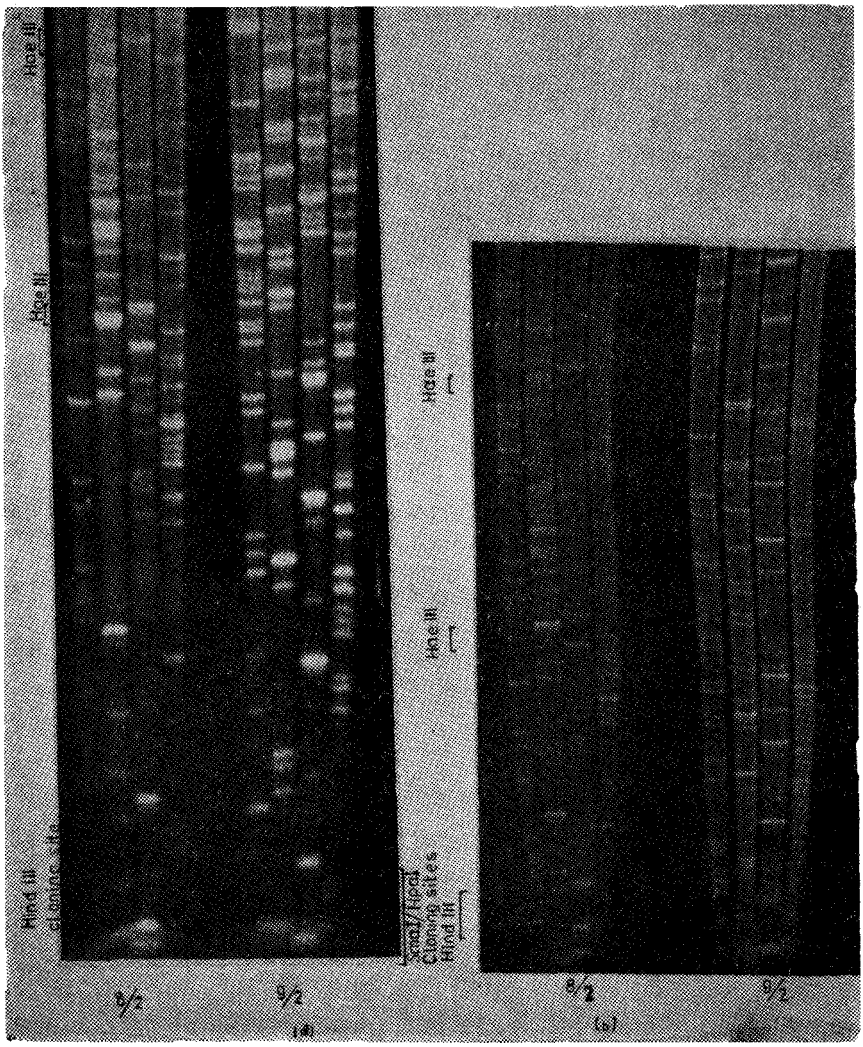


Figure 4. Autoradiographs showing sequencing by the dideoxy chain termination method; a) α-^{32}P-labelling, normal gel, b) α-^{35}S-Labelling, buffer gradient gel,

8/2 is the autoradiograph showing the 5′- end sequence of 1229 bp *B. subtilis* fragment cloned in M13mp8.

9/2 is the autoradiograph showing the 3′- end sequence of 1229 bp *B. subtilis* fragment cloned in M13mp9.

Hind III hybrid clonning sites have been indicated on the autoradiographs.

The nucleotide sequences obtained from all types of recombinants were put together to establish the 796 bp nucleotide sequence of the 1229 bp fragment unequivocally (Fig. 5). The initial 504 bp sequences corresponded to the 5′–end, while 292 bp (937–1229) corresponded to the 3′–end of the fragment. The nucleotide sequences from positions 505 to 937 (433 bp) were not presented since the results appeared somewhat arbitrary.

Computer analysis for the complementary sequences and the translation of the known sequences were carried out (not shown here). In addition, possible sites for all known restriction enzymes were also checked out.

DISCUSSION

B. subtilis car gene coding for carbamoyl phosophate synthetase has been cloned in pBR322. The restriction mapping of the 1229 bp fragment, known to harbour *B. subtilis car* gene has already been completed (Kadıkıran, 1988a).

In this study, I have attempted to carry out the nucleotide sequencing of the 1229 bp fragment by the dideoxy chain termination method of Sanger (1981) using ss–M13mp phage vectors. The 1229 bp fragment has been cloned in ds–M13mp8 and ds–M13mp9 in two orientations. Subcloning of Hae III and Acc I digestion products of this fragment was also carried out as necessitated by a more reliable and refined sequence analyses.

Recombinant M13mp phages were picked up as white plaque formers and checked for an increase in their size by running on agarose gels, followed by T-tracking to distinguish among different types of inserts cloned in recombinant phages. The results of sequence analyses of the insert DNAs in all combinations both in M13mp8 and M13mp9 recombinant phages were carefully scrutinized. The sequencing analyses, insuring the appearence of cloning sites have given rise to elucidate about 796 bp of *B. subtilis* fragment (Fig. 5). The sequence corresponding to the 433 bp in length lying between positions 505 and 937 has been left out since the nucleotide sequence analysis of this region has produced some irregularities; therefore, further clarification is needed before the full sequence can be presented. Digestion of one of the Hae III–Hae III fragments, ca. 600 bp looks necessary in order to produce sizeable subclones and the use of α–^{35}S–dATP la-

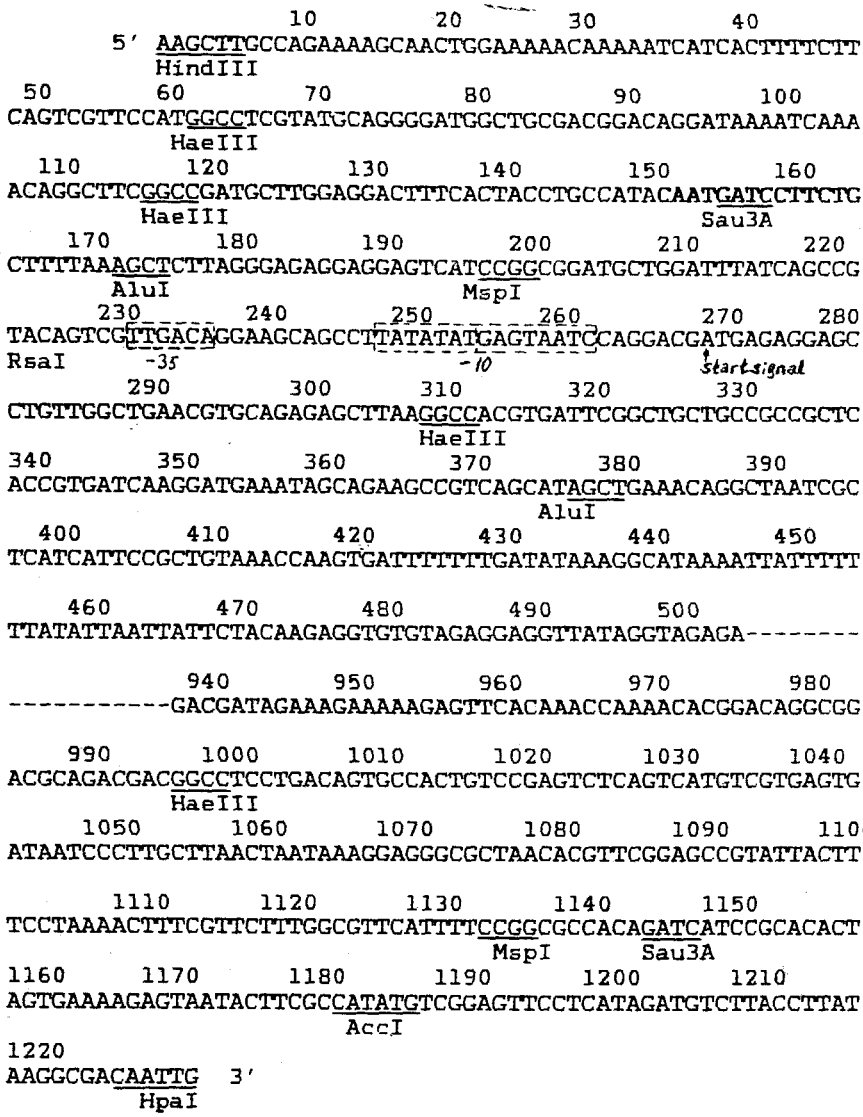


Figure 5. Nucleotide sequence of 1229 bp fragment, carrying *B. subtilis car* gene, determined by the dideoxy chain termination method.
Restriction sites have been indicated. Probable –35 promoter up-stream, –10 Pribnow box, +1 start signal and Shine-Delgarno (S-D) sequences have been underlined. 5'- end of the fragment ca. 200 bp corresponds to the 3'-end of *argD* preceding *car* gene carried on the 1229 bp *B. subtilis fragment.*

belling with buffer–gradient gels should overcome the difficulties for sequencing of this region.

The presented data appears compatible with the restriction analyses of the cloned 1229 bp fragment as reported in the preceding article (Kadıkıran, 1988a). As a result, the sequence analysis has added to refining the restriction mapping positions since it has elaborated further the exact positioning of the restriction sites for the enzymes exploited in this study with a marginal deviation of ∓ 10–15 bp which provides a solid evidence in support of restriction analysis.

One each of the restriction sites for Msp I (518), Sau 3A (545), Alu I (898), and Taq I (920) fall into 505–937 region; therefore, they are not shown in Figure 5.

In the preceeding article, the presence of two Alu I sites has been reported (Kadıkıran, 1988a). However, it now appears that there are three Alu I sites on the main fragment. A careful examination of the sequences reveals the fact that the appearance of two fragment upon Alu I digestion falls in line with the results, since two of Alu I digests are of similar size which must have given rise to a doublet in Alu I digestion of the fragment.

Nucleotide sequences running from either end of the cloned main fragment were carefully scrutinized for the presence of consensus sequences for *B. subtilis car* gene. Mountain *et al* (1986) have reported that the initial 5′–end of the 1229 bp fragment also comprises a part of the terminal sequences for *argD* gene which precedes *car* gene on the fragment.

The region from 230 to 237 harbours up-stream –35 region, most probably in *GTTGACAG* sequence, while –10 region consensus sequence (Pribnow box) appears within 247–262 nucleotide positions and the probable sequence is *TATATATGAGTAATC* and the +1 start signal A in ATG at position 269, 6 bases down–stream from –10 region. These findings all appear to be in accord with the previously reported compiled promoter consensus sequences (Watson, 1984). The significance of the presence of two possible –35 and –10 regions, both in tandem, appears quite intriguing and will be subject to further study, since the same phenomenon has also been reported in *E. coli car B* gene promotor sequences by Piette *et al* (1984).

ACKNOWLEDGEMENTS

This work was carried out in Dr. S. Baumberg's Lab in Genetics Department of Leeds University, Leeds, England.

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