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CLONING AND RESTRICTION ANALYSIS OF A BACILLUS SUBTILIS DNA FRAGMENT CARRYING CAR GENE, CODING FOR CARBAMOYL PHOSPHATE SYNTHETASE ENZYME.

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Kew Words: Recombinant DNA, restriction enzymes, restriction analysis, *B. subtilis car* gene.

ABSTRACT

A fragment of 1229 bp known to carry *B. subtilis car* gene has been cloned in pBR322 to construct recombinant pAKC plasmid. Prior to nucleotide sequencing, restriction analysis of this fragment has been carried out as a prerequisite.

Of the employed 13 restriction enzymes for restriction analysis, only the following produced varying number of restriction sites which have been shown to reside on the insert : Acc I, Taq I, and Rsa I just one; Alu I and Sau 3A, two; Msp I, three; Hae III, four; hence, a restriction map of the cloned insert has been drawn up accordingly.

INTRODUCTION

Carbamoyl phosphate, a common precursor of the arginine and pyrimidine pathways, is synthesized in *Escherichia coli* and related organisms from glutamine, ATP, and CO_2 by a single enzyme (Pierard *et al.*, 1964; Pierard *et al*, 1965), carbamoyl phosophate synthetase [Carbondioxide: L–glutamine amido–ligase (ADP–forming, carbamate phosphorylating): EC. 6.3.5.5].

The enzyme consists of two subunits (Trotta *et al*, 1971), the products of the adjacent genes *carA* and *carB* (Mergeay *et al*, 1974). They constitute an operon oriented from *A* to *B*. *CarA* encodes a small subunit of Mw–42,000 that carries the glutamine–binding site; *carB* encodes a subunit of Mw–130,000 that catalyzes the synthesis of carbamoyl phosphate from NH_3. The two genes have already been cloned and sequenced to near completion (Piètte *et al*, 1984).

Eukaryotes generally have two different synthetases: one specific for the arginine pathway and the other for the pyrimidine pathway (Jones, 1972). Although many systems regulating gene expression

in *E. coli* and *Salmonella typhimurium* have been analysed in considerable molecular detail, very little comparable information is available in other prokaryotes. *Bacillus subtilis* is a sporulating, gram–positive soil bacterium which might offer hope of interesting differences from enterobacterial species. Few *Bacillus* genes concerned with vegetative growth have been studied at the molecular level (Losick and Pero, 1981). However, no information has been available either of comparable sequences or of regulatory macromolecules for metabolic systems homologous with well–studied operons in *E. coli* or *S. typhimurium.*

Therefore, arginine metabolism in *B. subtilis* has been studied as a model of this system (Harwood and Baumberg, 1977; Mountain and Baumberg, 1980). A cluster of *B. subtilis* genes of arginine biosynthesis has, thereafter, been cloned and expressed in *E. coli* as an obvious prerequisite for molecular analysis. The hybrid plasmid pUL720, harbouring an insert of 12,000 bp in length, comprising eight arginine genes has been cloned into pBR322 plasmid vehicle. The cloned insert has been subcloned even further to enable the detailed molecular analyses of *B. subtilis* arginine genes (Mountain *et al*, 1984).

I describe here the construction and the restriction analysis of pAKC recombinant plasmid harbouring the insert carrying *B. subtilis argD–carA,B* genes, as a prerequisite for the nucleotide sequencing and the molecular analysis of controlling site(s) for *B. subtilis car* gene(s).

MATERIALS AND METHODS

Strains. E. coli strain of DHI (F⁻*gyrA recA relA endA thi hsdR hsdM supE)* was used for the transformation experiments as well as the preparation of recombinant plasmid DNAs of all types constructed in the course of this work. Routine maintenance and growth of *E. coli* strains was as in Kadıkıran and Baumberg (1980). Antibiotic concentrations employed were: ampicillin, 25μg / ml and tetracycline, 15 μg / ml.

Plasmid DNA Preparation. Plasmid DNAs were purified by the alkaline / SDS method of Ish–Horowicz and Burke (1981).

Enzymes. Restriction endonucleases were purchased from Bethesda Research Labs, used according to the manufacturer's instructions and inactivated by phenol extraction followed by ethanol pre-

cipitation. T4–DNA ligase was purchased from New England Biolabs and used according to the protocol of Bolivar and Backman (1979).

Electrophoresis. Electrophoresis of DNA was performed in buffer containing 45mM Tris / HCl, 20mM Na–acetate pH 8.1, using 0.8 % agarose gels (Sigma type II) in a horizontal submarine apparatus at approximately 8 V / cm. For restriction analysis λ Hind III and ∅x174 Hae III digests were visualised using a long–wave UV–transilluminator; when needed, excised with a scalpel and purified by the electroelution procedure of McDonnel *et al* (1977).

Transformation. E. coli was transformed by the method of Mandel and Higa (1970), except when maximal transformation was required in which circumstances the procedure of Hanahan (1983) was employed.

RESULTS

p5BH2 plasmid, which is a derivate of pUL720 carrying eight *B. subtilis* arginine genes, was known to harbour *car* region on a Hind III–Hpa I fragment of 1229 bp in length.

An attempt was made to cleave the fragment and clone in pBR322. The strategy adopted to accomplish this goal was first to purify the 1229 bp fragment of p5BH2 and then ligate it with double–digested pBR322. An obvious choice for the latter was to use Hind III and PVu II digestions to produce one sticky and one blunt–end Hind III–Pvu II to enhace the ligation frequency (Fig. 1). The consructed recombinant plasmid was named pAKC. AK stands for the author's initials and C for *Car* gene of B. subtilis.

Preparation of the insert DNA. 1.5 μg of p5BH2 DNA was double digested with Hpa I and Hind III enzymes consecutively since the latter is a robust enzyme, in a total reaction mixture of 45 μl. The digestion mix was run on a gel and 1229 bp fragment was cut out using a sharp–cutter on a UV–transilluminator (Fig. 2). The removed piece of gel was placed in a dialysis tubing and the fragment was electroeluted for 15–20 minutes. The contents of the dialysis tubing was centrifuged and the gel debris was removed. The fragment was then purified by two rounds of phenol extraction followed by ethanol precipitation, overnight at –20°C.

Preparation of the vector DNA. pBR322 DNA was digested with Pvu II followed by Hind III cleavages to match the fragment to be

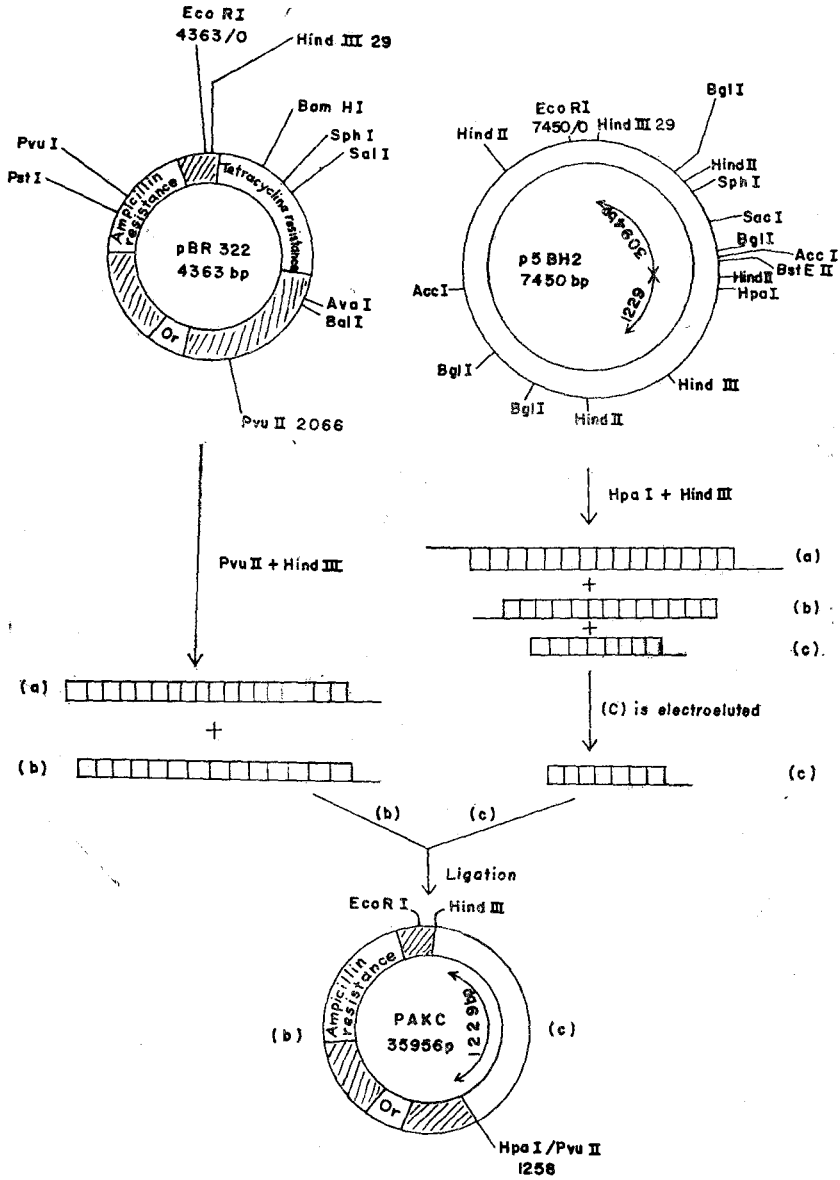

Figure 1. The construction of pAKC recombinant plasmid.

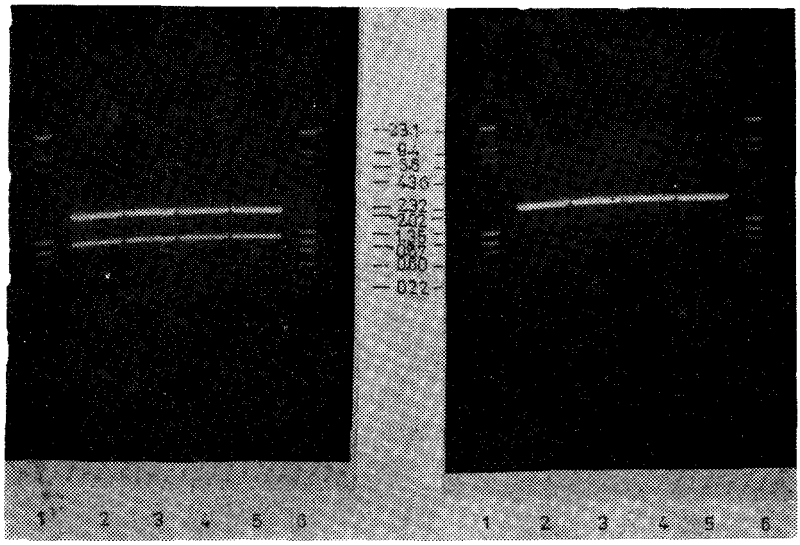

Figure 2. Preparative DNA agarose gel electrophoresis of Hind III and Hpa I double digestion products of recombinant pAKC plasmid.
a) Lanes 1 and 6 are Hind III and ∅x174 Hae III digests as size standards (sizes given in kbp). Lanes 2, 3, 4 and 5 are Hpa I and Hind III digestion products of p5BH2 DNA. The middle band is the 1229 bp *B. subtilis* fragment, carrying *car* gene.
b) It shows the excised middle band in (a), corresponding to the 1229 bp *B. subtilis* fragment.

cloned. However, sufficient time was allowed for the completion of digestion which was checked out by running 1 μl samples from the reaction mix on a gel. Pvu II and Hind III double digestion of pBR322 resulted in the production of two fragments:

1– 2237 bp fragment (2066–29) carrying *ApR* gene as well as the genes for plasmid replication of origin.

2– 2155 bp fragment (29–2066) harbouring *TtR* gene.

Both double digestion reactions were stopped by heating the reaction mixtures at 65°C for 5 minutes.

Ligation. 12 μl of 1229 bp fragment (0.2 μg/μl) was added to the ligation buffer containing 3 μl of the double digested pBR322 DNA (0.2 μg/μl) in 4: 1 ratio. Ligation was initiated by the addition of 6 μl T4–DNA ligase enzyme (4 units/μl) in a total reaction mixture of 30 μl. Ligation was checked out by running small samples on a gel. Ligation was stopped by phenol extraction followed by ethanol preci-

pitation. Dried up pDNA pellet in the joblin tube was resuspended in a small volume (20 μl).

Transformation. Transformation was carried out in the presence of necessary controls by adding 10 μl of the ligation mix into 150 μl of competent DHl cells, already treated with $CaCl_2$ and heat shocked at 43°C for 3 minutes, before plating out on a selective medium containing ampicillin (25 μg/ml). Transformants were later checked for tetracycline sensitivity (15 μg/ml). The recombinant plasmid carrying transformants were picked up by virtue of *ApR TtS* properties. Three of the tested transformants proved to possess the expected size of pAKC upon gel electrophoresis (Fig. 3).

One of the transformants carrying the recombinant pAKC (Fig. 3.d) was grown up in 2 liters of Lennox Broth (LB) for the large scale pAKC plasmid DNA preparation in Caesium chloride density gradient

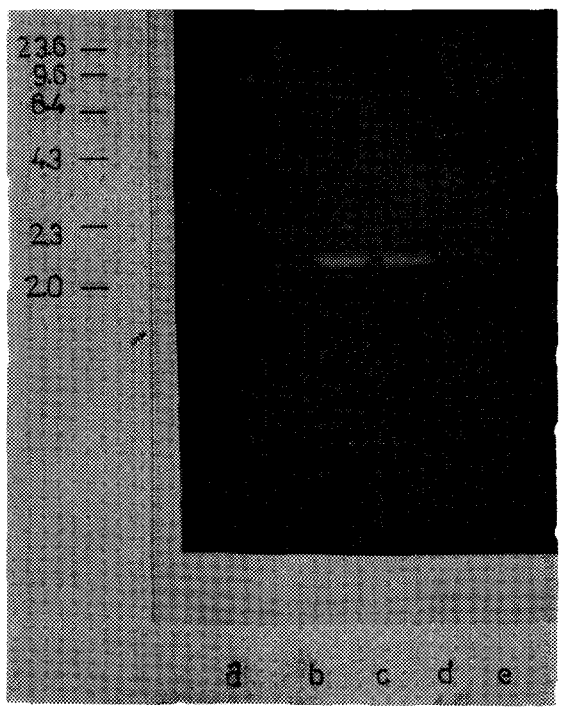

Figure 3. Recombinant pAKC transformants employed for large scale pDNA preparations. Lane a shows λ Hind III digests as size standards (sizes given in kbp). Lane e is pBR322 DNA (4363 bp) as control. Lanes b, c, and d are recombinant pAKC mini-DNA preparations using different transformants.

with ethidium bromide (0.5 μg/ml). 2ml of pAKC DNA (0.2 μg/μl) was obtained according to the protocol by Maniatis *et al* (1982).

Restriction analysis of the insert was carried out by employing a number of restriction enzymes recognizing 4 or 6 bases long specific restriction sites. This was implemented in two ways:

a) Purified 1229 bp fragment of pAKC, electroeluted from the gel after Hind III and Hpa I digestion, was further digested either by single restriction enzyme digestions or a combination of them, thereupon, as appropriate. In both cases, seven restriction enzymes with 6 bases long recognition sites: EcoRI, Bgl I, SacI, Bam HI, Sal I, Sph I, Acc I; and six restriction enzymes with 4 bases long recognition sites Rsa I, Alu I, Sau 3A, Msp I, Taq I, and Hae III have been employed to yield a good profile of digested fragments.

b) pAKC and pBR322 DNAs were simultaneously subjected to the same digestions, in parallel.

The resulting restriction profiles of the digestions in (a) and (b) were evaluated with reference to pBR322 digestion profiles, since the latter has already been well characterized (Fig 4).

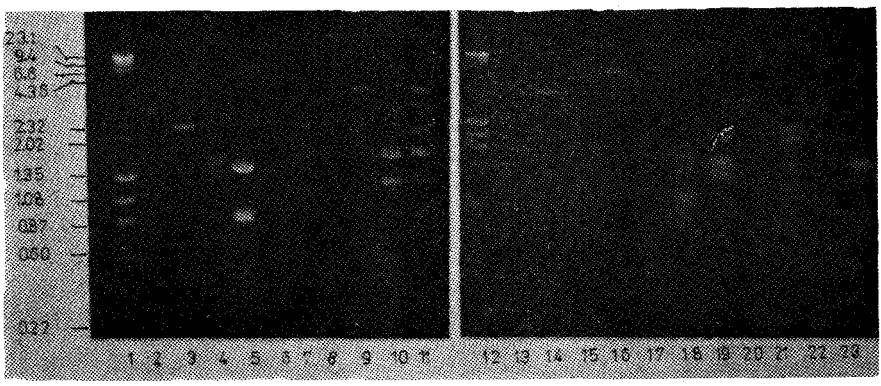

Figure 4. Agarose gel electrophoresis of restriction digests of recombinant pAKC and pBR 322 plasmids. Lanes 1 and 12 are λ Hind III and ∅X174 Hae III digests as size standards Lanes 2 and 13 are undigested pBR322 DNAs. Lanes 3 and 14 are undigested pAKC recombinant plasmids. Lanes 6 and 15 are EcoRI digests of pBR322; 9 and 16 are EcoRI digests of pAKC. Lane 7 is EcoRI and Rsa I, lane 8 is EcoRI digests of pBR322. Lane 19; is EcoRI and Rsa I, lane 11 is RsaI digests of pAKC. Lanes 17 and 18 are Alu I digests of pBR322 and pAKC, respectively. Lane 19 is Hae III digests of pAKC. Lanes 20 and 21 are Sau3A digests; lanes 22 and 23 are Msp I digests of pBR322 and pAKC.
Different position of λHind III and ∅X174 size standards in 1 and 12 reflect the time differences of electrophoresis.

The exact sizes of digestion products were determined as to the λ Hind III and ⌀x174 Hae III digests as size standards. When necessary, ambiguities for the exact positioning of the restriction sites were refined by employing double or triple digestions in various combinations. The combined restriction digestion profiles of the recombinant DNA yielded to construct sufficiently accurate restriction mapping of the insert in pAKC, accommodating *car* gene of *B. subtilis*, given in Figure 5.

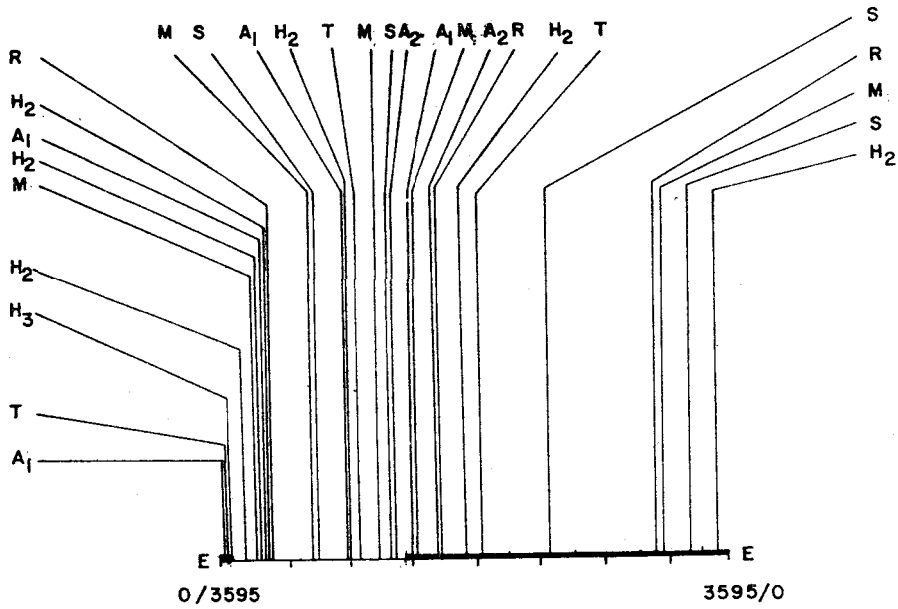

Figure 5. Restriction map of recombinant pAKC plasmid carrying car gene of B. subtilis. The restriction enzymes are Al, Alu I; A2, Acc I; F, EcoRI; Hl, Hpa I; H2, Hae III; H3, Hind III; M, Msp I; P, Pvu II; R, Rsa I; S, Sau3A; T, Taq I.
The heavy line indicates the vector DNA and the light line represents the insert DNA (1229 bp).

It was obvious from the restriction analysis that the first six of the seven restriction enzymes with 6 bases long recognition sites had no restriction sites; whereas, Acc I, Taq I and Rsa I demonstrated the presence of just one; Alu I and Sau 3A two; Msp I, three; Hae III, four restriction sites on the 1229 bp insert in pAKC recombinant plasmid DNA.

DISCUSSION

The evidence has been provided for a cluster of genes concerning arginine biosynthesis on a single 12000 bp EcoRI fragment of *B. subtilis* chromosome which was cloned in pUL720, complementing *E. coli* mutants defective for the first six enzymes of arginine biosynthesis and for carbamoyl phosophate synthetase A, B (or either subunit of the enzyme) as shown by Mountain *et al* (1984).

This study is a part of a team–work for determining the nucleotide sequence of the clustered all *B. subtilis* arginine genes. I, therefore, have attempted to clone the 1229 bp fragment which was considered to possess a secondary (weak) promoter as suggested by Mountain *et al* (1984).

Restriction mapping of the 1229 bp fragment has been necessitated for purposes of nucleotide sequencing (Kadıkıran, 1988 b). *B. subtilis* 1229 bp Hind III–Hpa I fragment has been cloned in Hind III and Pvu II digested pBR322 for the construction of recombinant pAKC plasmid (Fig. 1).

The restriction mapping of the insert is carried out by employing 13 restriction enzymes. Of the tested enzymes; Eco RI, Bgl I, Sal I, Bam H I, Sac I and Sph I did not not prove to have a recognition site; however, other restriction enzymes displayed the presence of varying number of recognition sites; Acc I, Taq I and Rsa I, one; Alu I and Sau 3 A, two; Msp I, three; and Hae III, four new restriction sites on 1229 bp fragment in pAKC recombinant plasmid as depicted in Figure 5.

The new restriction sites on the insert in pAKC have been verified by comparative analysis of single and double digestions of both pAKC and the purified 1229 bp insert DNAs. The results were re-evaluated in view of pBR322 digestion patterns, run in parallel (Fig. 4a–b). However, when necessary, triple digestions were also employed.

The results have been compiled together to produce a restriction map of the insert in pAKC. This has yielded sufficient information for the production of suitable sizes to be subcloned for the purposes of nucleotide sequencing (Fig. 5). Sequencing of the entire fragment which has already been undertaken (Kadıkıran, 1988 b) has resolved the obscurities involving the exact positioning of one Sau 3A, two Msp I restriction sites and proved the presence of a third Alu I site.

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