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Cloning and expression of the *Pst* I restriction-modification system in *Escherichia coli*

(restriction enzymes/molecular cloning/protein overproduction/DNA sequence recognition/periplasmic proteins)

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ABSTRACT **Here we report the cloning and preliminary characterization of the *Pst* I restriction-modification system of *Providencia stuartii* 164. Transformants of *Escherichia coli* carrying the *Pst* I gene system inserted into the cloning vector pBR322 were selected on the basis of acquired resistance to bacteriophage λ infection. *Pst* I endonuclease was detected in osmotic shock fluid from each of the resistant clones. Plasmid and chromosomal DNA from these clones could not be digested by *Pst* I, indicating that the gene for the corresponding modification enzyme had also been cloned and was being expressed. The smallest recombinant plasmid encoding both activities, pPst201, contains an insert of approximately 4000 base pairs. *In vitro* transcription studies indicate that this DNA fragment also contains the endogenous promoter(s) of the system. When pPst201 was introduced into a minicell-producing strain of *E. coli*, two new proteins, 32,000 and 35,000 daltons, were synthesized. We have assigned these to the *Pst* I modification (methylase) and restriction enzymes, respectively. The active form of the restriction enzyme is a dimer, as determined by gel filtration. Constructed transformants of *P. stuartii* 164 that carry the *Pst* I system inserted into pBR322 produce approximately 10 times more *Pst* I endonuclease activity than does the native strain.**

Restriction enzymes have become essential tools for the manipulation and characterization of DNA molecules. The restriction enzymes themselves, however, have not been as well characterized. Little is known of the molecular basis by which these proteins recognize the specific nucleotide sequence at which they cleave double-stranded DNA or of the basis for control of expression of the genes for the restriction enzyme and its cognate methylase. The recognition mechanism is of particular interest because general features of this process may apply to proteins of diverse function that interact with DNA.

In this report we describe the cloning and preliminary characterization of the class II restriction-modification system of *Providencia stuartii* 164 (*Pst* I). Our interests in cloning the *Pst* I system were to obtain an overproducing strain that would facilitate isolation of the restriction enzyme and methylase in the large quantities required for detailed physical studies including x-ray crystallographic analysis and to isolate the genes for the restriction enzyme and methylase in order to study the molecular mechanism by which their expression is controlled. The selection of transformants carrying the *Pst* I system was based on acquired resistance to bacteriophage λ infection as used by Smith and his collaborators in cloning the *Hha* II system (1). The conditions of selection were modified, however, so that only transformants that carried and expressed the restriction-modification system were resistant to infection. The cloned genes are contained within a 4.0-kilobase (kb) *Hin*dIII fragment which, from *in vitro* transcription studies, also appears to contain the endogenous promoter(s) and hence regulatory elements of the system. Transformants of *P. stuartii* 164 that carry the *Pst* I system inserted into pBR322 produced approximately 10 times more of the *Pst* I endonuclease than did the native strain.

MATERIALS AND METHODS

Enzymes and Radiolabeled Reagents. Restriction enzymes were purchased from Bethesda Research Laboratories (Bethesda, MD) and New England Bio-Labs. T4 DNA ligase was obtained from Bethesda Research Laboratories. Bacterial alkaline phosphatase was from Sigma. L-[^{35}S]Methionine was purchased from Amersham and [α-^{32}P]CTP was from New England Nuclear.

Plasmids and Bacterial and Phage Strains. *E. coli* K-12 strain HB101 (r^-m^-recA) was obtained from H. W. Boyer. *P. stuartii* 164 was from the collection of R. J. Roberts. The minicell-producing strain of *E. coli* K-12, χ1411, was obtained from R. Curtiss III. The plasmid pBR322 and recombinant plasmids were isolated by the CsCl/ethidium bromide procedure (2). Phage λcI60 was generously supplied by M. Feiss. All experiments using recombinant DNA were performed in accordance with the guidelines of the National Institutes of Health.

Transformation and Colony Screening Procedures. *P. stuartii* 164 DNA (60 μg), isolated according to the procedure of Marmur (3), was partially digested with 4–30 units of *Hin*dIII for 30 min at 37°C. The DNA from each digest was pooled and centrifuged on a 10–40% sucrose gradient in 30 mM Tris·HCl, pH 8.1/10 mM EDTA/1 M NaCl for 20 hr at 25,000 rpm in an SW 27 rotor. Fractions collected by puncturing the bottom of the tube were examined by agarose gel electrophoresis, and fractions that contained predominantly fragments 3–10 kb long were pooled and precipitated with ethanol. Hybrid plasmids of pBR322 were constructed by ligating the sized *Hin*dIII-cut *P. stuartii* DNA (2.0 μg) to phosphatase-treated *Hin*dIII-cut pBR322 (1.6 μg) by using T4 DNA ligase. The reaction was allowed to proceed for 12 hr at 16°C in 200 μl of 66 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/10 mM dithiothreitol/200 μM ATP containing 50 mg of bovine serum albumin per ml.

DNA from the ligation reaction (0.5 μg) was added to approximately 2.5×10^9 HB101 cells that had been treated with $CaCl_2$ according to the method of Wensink *et al.* (4). The protocol for transformation described by Bolivar and Backman (5) was followed, after which 0.9 ml of L broth was added to each reaction mixture. After 30 min the entire reaction volumes were plated on two 15-cm LB agar plates containing ampicillin (200 μg/ml). Approximately 2000 transformants were obtained per plate.

The transformants were screened against bacteriophage λ as follows. Two milliliters of λ lysate (10^{10} plaque-forming units/ml) were added to 1.6×10^5 cells from a subculture of the *Pst* I genomic library in 0.6 ml of L broth containing 0.2% maltose;

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Abbreviation: kb, kilobase.

the suspension was allowed to incubate for 10 min at 37°C. The sample was then serially diluted, and aliquots containing 10^4 cells were layered on LB agar plates containing ampicillin (200 μg/ml). Approximately 0.1% of the transformants gave rise to normal-appearing colonies.

Pst I endonuclease activity released by osmotic shock from transformed and native strains was assayed as described by Smith *et al.* (6). The substrate used in the assay was the plasmid p82-6B (7) which contains two *Pst* I recognition sites.

Analysis of Plasmid-Directed Protein Synthesis in Minicells. The minicell-producing strain of *E. coli*, χ1411, was transformed with pBR322 and recombinant plasmids carrying the *Pst* I restriction-modification system as described above for HB101 cells. Small-scale plasmid isolations from the χ1411 transformants and subsequent restriction enzyme analyses showed that the plasmids were not altered during their maintenance in χ1411. Minicells were isolated by sucrose density gradient centrifugation and were incubated for 30 min at 37°C with aeration to degrade endogenous mRNA (8). The proteins made within the minicells were labeled with L-[^{35}S]methionine (specific activity, 800 mCi/mol; 1 Ci = 3.7×10^{10} becquerels). Cells were lysed as described (9), and lysates were run on a 10–18% gradient NaDodSO$_4$/polyacrylamide gel (10). Gels were stained with Coomassie brilliant blue, destained, dried, and exposed for autoradiography for 12 hr at room temperature on Kodak XR-5 film.

RESULTS

Selection of Clones Carrying the *Pst* I Restriction-Modification System. Transformants of HB101 carrying the *Pst* I system were selected on the basis of acquired resistance to infection by bacteriophage λ. Of the *E. coli* transformants containing cloned *P. stuartii* 164 DNA, about 0.1% of the cells formed normal-appearing confluent colonies after infection in liquid culture with λ at a titer of 7.7×10^9 plaque-forming units/ml. Approximately 0.4% of the cells formed flat patchy colonies, clearly heavily infected by λ. Osmotic shock fluid obtained from seven randomly selected λ resistant colonies contained *Pst* I restriction enzyme activity (Fig. 1). Osmotic shock fluid from an infected colony and from the nontransformed host HB101 had no *Pst* I activity (lanes 8 and 9, respectively). Apparently, only those cells that expressed the *Pst* I restriction enzyme were resistant to λ at the titer used in the selection procedure.

Characterization of Recombinant Plasmids. Plasmids were isolated from five of the recombinant clones that produced the *Pst* I restriction enzyme and digested with *Hin*dIII to cut out the inserted DNA fragment (Table 1). Three of the plasmids (pPst101, pPst102, and pPst 105) had identical inserts, 5.9 kb long, which contained an internal *Hin*dIII site that divided the DNA into a 1.9-kb and a 4.0-kb fragment. Both of these fragments were contained within the inserts of the larger plasmids pPst103 and pPst 104.

At this point it appeared that both the 1.9-kb and 4.0-kb fragments may be required for expression of the restriction-modification phenotype. However, subcloning experiments showed that the 4.0-kb *Hin*dIII fragment contained the entire *Pst* I system and that cells transformed with this piece of DNA were as resistant to infection by λ as were cells transformed with the entire 5.9-kb insert (data not shown). A partial restriction map of the 4.0-kb insert from the plasmid pPst201 is shown in Fig. 2.

Modification of Host DNA. Recombinant clones producing the *Pst* I restriction enzyme must also express a corresponding modification activity to protect their own DNA from digestion. The cloning vector pBR322 contains a single *Pst* I site. Digestion of the recombinant plasmid pPst105 with *Bam*HI and *Pst* I established that this site is protected within pPst105 (Fig. 3). *Bam*HI cleaves pPst105 at a single site located within pBR322 (lane 1). The linear molecule that is produced by *Bam*HI digestion is not susceptible to further cleavage by *Pst* I (lane 2). The control experiments in lanes 3 and 4 demonstrate that the *Pst* I endonuclease remains active in the incubation mixture containing pPst105.

Chromosomal DNA isolated from HB101 transformed with

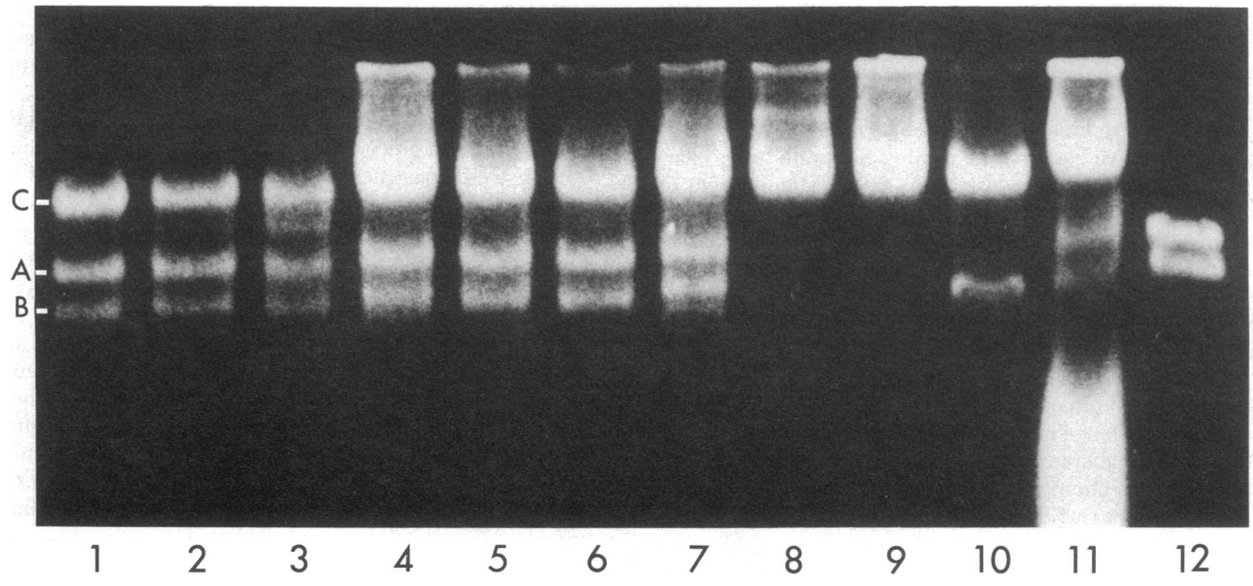

FIG. 1. Assay of *Pst* I endonuclease activity in osmotic shock fluids. The substrate in the assay was the plasmid p82-6B (7) which yields two characteristic DNA fragments (A and B) upon digestion with *Pst* I. The sources of the osmotic shock fluids were as follows: lanes 1–7, individual transformants resistant to infection by λ; 8, a λ sensitive transformant; 9, *E. coli* HB101, the nontransformed host bacteria; 10, *E. coli* RY13, the source of the restriction enzyme *Eco*RI; and 11, *P. stuartii* 164. Lane 12 is p82-6B after digestion with *Pst* I endonuclease purified from *P. stuartii* 164. Band C in lanes 1–11 includes chromosomal DNA released by the small and variable amount of cell lysis that occurs in the osmotic shock procedure. DNA bands were visualized by fluorescence with ethidium bromide.

Table 1. Cloned DNA fragments from transformants producing the *Pst* I restriction enzyme

Plasmid	Sizes of fragments, kb
pPst101	4.0; 1.9
pPst102	4.0; 1.9
pPst103	4.0; 4.0; 2.2; 1.9; 0.4
pPst104	4.0; 4.0; 1.9; 0.4
pPst105	4.0; 1.9

Recombinant plasmids isolated from transformants producing the *Pst* I restriction enzyme were digested with *Hin*dIII, and the cloned *Hin*dIII fragments were sized by agarose gel electrophoresis.

pPst105 was also found to be protected from digestion by *Pst* I (data not shown). By analogy with other restriction-modification systems, DNA protection is presumably due to specific methylation of one of the residues, either C or A, within the *Pst* I recognition sequence (11).

Transformation of *P. stuartii* 164: Construction of a Stable Overproducing Strain. *P. stuartii* 164 was transformed with the recombinant plasmid pPst105 encoding the *Pst* I system by the same protocol used for transformation of *E. coli*. Transformed cells were selected on the basis of acquired resistance to ampicillin. The total amount of *Pst* I endonuclease produced by this clone was measured after the cells were disrupted by sonication. The transformed cell line was found to synthesize approximately 50,000 units of enzyme per g of cells, compared to 5600 units per g produced by the native strain.* Neither the level of enzyme overproduction (approximately 10-fold) nor resistance to ampicillin was diminished with serial culturing. Restriction enzyme analyses of pPst105 recovered from *P. stuartii* showed that the plasmid had not been altered during its maintenance in this cell.

Gene Products of the *Pst* I Restriction-Modification System. χ1411, a minicell-producing strain of *E. coli*, was transformed with pBR322 and with pPst201. At least two polypeptide chains were synthesized in minicells transformed by pPst201 that were not made in minicells containing pBR322 (Fig. 4). The presence of the insert also appeared to affect the relative amount of synthesis of several proteins encoded for by pBR322, as found previously by using the plasmid vector RSF2124 (12). The two new polypeptide chains had molecular weights of approximately 32,000 and 35,000 as determined by comparison to marker proteins. The latter comigrated with a prominent component of a partially purified sample of the *Pst* I endonuclease that was enriched during the purification of the enzyme (lane 3). On this basis we have tentatively assigned the 35,000-dalton polypeptide to the restriction enzyme and the 32,000-dalton polypeptide to the methylase. The apparent molecular weight of the native form of the restriction enzyme, as determined by gel filtration through Sephacryl S-200, is 69,500 at concentrations of the enzyme between 5 and 10 μg/ml (data not shown). Therefore, under these conditions, the catalytically active form of *Pst* I is a dimer.

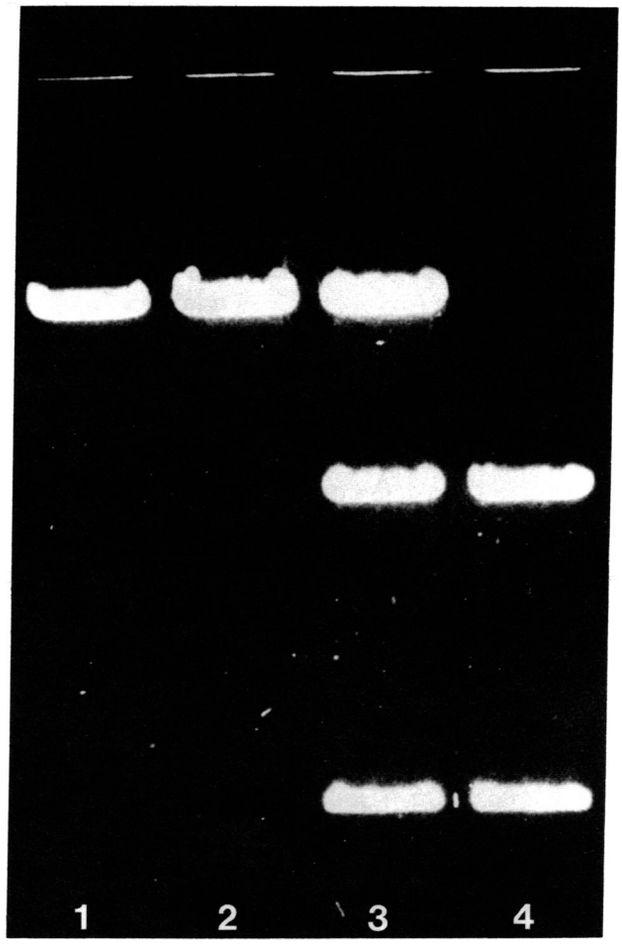

FIG. 3. Host cell modification of plasmid DNA encoding the *Pst* I system. The *Pst* I recognition site within the cloning vector pBR322 is protected from digestion in the recombinant plasmid pPst105. Lanes: 1, pPst105 digested with *Bam*HI; 2, pPst105 digested with *Bam*HI and *Pst* I; 3, pPst105 and pBR322 digested with *Bam*HI and *Pst* I; 4, pBR322 digested with *Bam*HI and *Pst* I. DNA bands were visualized by fluorescence with ethidium bromide.

***In Vitro* Transcription of the *Pst* I Restriction-Modification System.** *In vitro* transcription was assayed by the procedure described by Chelm and Geiduschek (13) in which ternary complexes among the DNA template (generally a specific restriction fragment), RNA polymerase, and short, newly synthesized RNA chains are identified by electrophoresis on agarose gels. Ideally,

* Preliminary studies indicate that transformants of *E. coli* that carry the *Pst* I system produce less enzyme than do native *P. stuartii* 164 (N. Pomato and D. V. Hendrick, Bethesda Research Laboratories, personal communication).

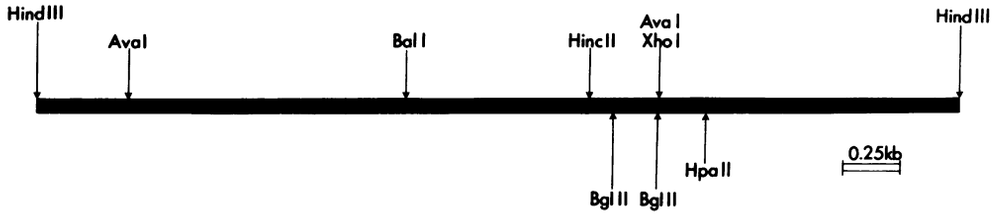

FIG. 2. Partial restriction map of the 4.0-kb insert that contains the *Pst* I restriction-modification system. The hexanucleotide recognition sites for the restriction enzymes *Eco*RI, *Sal* I, *Hpa* I, *Bam*HI, and *Pvu* II are not present within the DNA sequence.

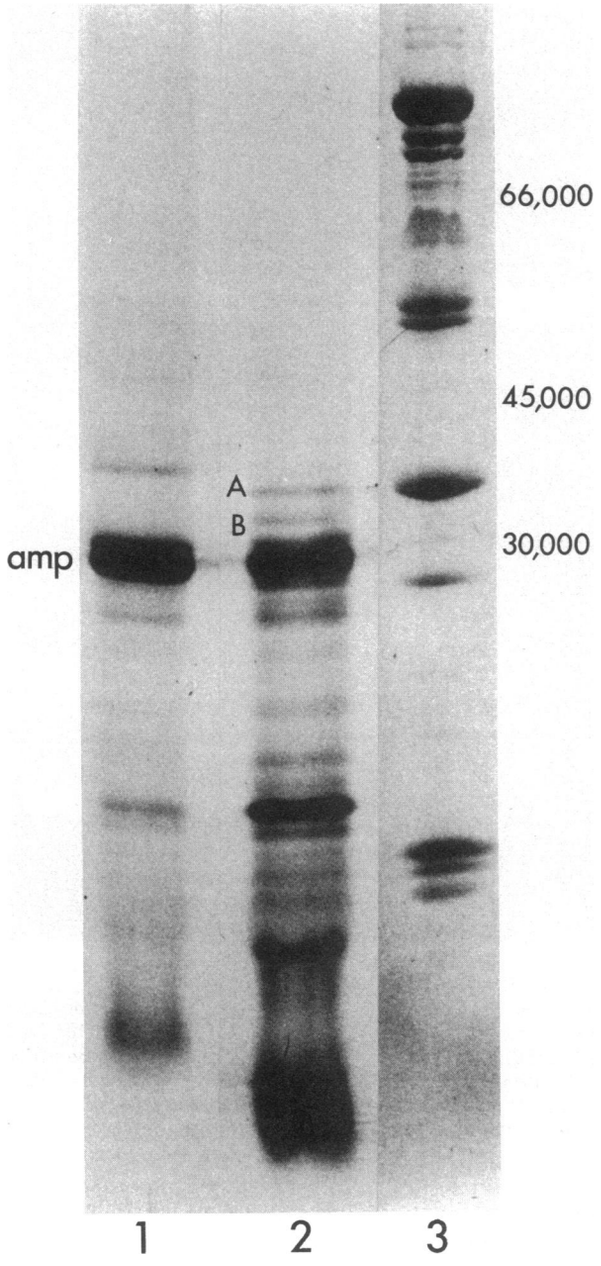

FIG. 4. Plasmid-directed protein synthesis in minicells. Minicells were incubated in the presence of L-[^{35}S]methionine to label newly synthesized plasmid-encoded proteins. Minicell lysates and a partially purified sample of the *Pst* I endonuclease were electrophoresed on a 10–18% gradient NaDodSO$_4$/polyacrylamide gel. The gel was stained with Coomassie brilliant blue, dried, and exposed for autoradiography. Lanes 1 and 2 are from the autoradiogram of the gel and correspond to minicell lysates prepared from transformants containing pBR322 and the recombinant plasmid pPst201 encoding the *Pst* I system, respectively. Lane 3 is the Coomassie blue stain of the partially purified *Pst* I endonuclease. Two new polypeptides (A and B) having molecular weights of 32,000 and 35,000 were synthesized in minicells containing pPst201 but not in minicells containing pBR322. The larger polypeptide comigrated with a prominent component of the partially purified *Pst* I restriction enzyme. The molecular weight calibration was derived from electrophoresis of a standard set of marker proteins not shown in the figure. amp, Product of the ampicillinase gene of pBR322.

ternary complexes would be formed only with restriction fragments that contained an active promoter site. RNA synthesis is carried out in the presence of one or more [α-^{32}P]ribotriphosphates so that the ternary complexes may be localized within the agarose gel by autoradiography.

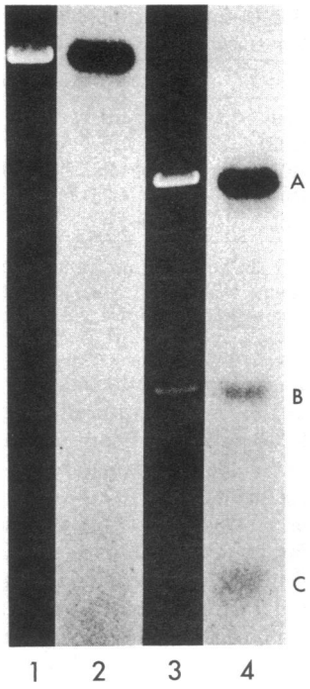

FIG. 5. *In vitro* transcription of the *Pst* I restriction-modification system. Lanes: 1 and 3, ethidium bromide fluorescence of the 4.0-kb DNA fragment encoding the *Pst* I system and the fragment after digestion with *Ava* I, respectively. 2 and 4, autoradiograms of ternary complexes of *E. coli* RNA polymerase, DNA, and nascent RNA chains formed on DNA templates in lanes 1 and 3, respectively. Ternary complexes were labeled with [α-^{32}P]CTP. The intensity of the band due to ternary complexes formed on the 2.3-kb *Ava* I fragment (A) is more than 12-fold greater than that due to ternary complexes formed on the 1.3-kb *Ava* I fragment (B), as determined by densitometric scanning of the autoradiogram at 550 nm. The 0.4-kb *Ava* I fragment (see Fig. 2) was not detected by ethidium bromide fluorescence in this gel but did give rise to a discernible level of transciption complex (C). The ternary complexes formed on the 0.4-kb and 1.3-kb *Ava* I fragment serve as endogenous controls for the level of nonspecific initiation of transcription.

In the experiment described in Fig. 5, the 4.0-kb fragment carrying the *Pst* I restriction-modification system as well as the *Ava* I digest of this fragment (see Fig. 2) were used as templates for *in vitro* transcription. The yield of ternary complex obtained with the entire 4.0-kb fragment and with the 2.3-kb *Ava* I fragment were comparable; that obtained with the 1.3-kb *Ava* I fragment was much less (<10%) (see legend to Fig. 5). The ternary complex formed with the 1.3-kb *Ava* I fragment is probably due to nonspecific initiation of transcription and, therefore, serves as an endogenous control for the amount of RNA synthesis directed by the other templates. These results strongly suggest that the 4.0-kb insert contains the native promoter(s) of the *Pst* I system and that the promoter(s) lie within the 2.3-kb *Ava* I fragment. As such, it should be possible to use the cloned system to study the physiological control of the expression of the restriction enzyme and methylase genes.

DISCUSSION

The *Pst* I restriction enzyme initially was thought to be encoded on a plasmid because it was found in only one clinical isolate, *P. stuartii* 164, and not in several other strains of *P. stuartii* examined (6). However, more recent studies indicate that *P. stuartii* 164 does not contain a plasmid (C. I. Kado, personal

communication). Therefore, the *Pst* I restriction-modification system must be located on chromosomal DNA and is presumably present in only one copy per cell. The transformants of *P. stuartii* that we have constructed that carry the *Pst* I system on the multicopy plasmid pBR322 produce a 10-fold greater amount of the restriction enzyme than that made in the native strain. With further manipulation, it should be possible to construct clones that produce even greater yields of the enzyme to facilitate further the large-scale purification of the protein. For example, from the autoradiogram shown in Fig. 4 it would seem that, if the gene for the restriction enzyme could be linked directly to the end of the coding region for the signal sequence (14) of the ampicillinase gene of pBR322, the synthesis of the restriction enzyme would be markedly increased. In addition, this should greatly simplify the purification of the enzyme because most if not all of the protein would then be secreted into the periplasmic space (15).

As in the native organism, a fraction of the *Pst* I restriction enzyme produced in the transformants is transported into the periplasmic space and can be released by osmotic shock of the cells. The signal sequence (14) which codes for the transport of the enzymes into the periplasmic space of *P. stuartii* 164 therefore must also be recognized at the cytoplasmic membrane of *E. coli*. The periplasmic space is clearly a strategic location for the restriction enzyme to intercept foreign DNA. The restriction enzyme located within this extracytoplasmic compartment may be particularly important for expression of the restriction phenotype and may be required for resistance against the very high titer of phage used in the selection procedure.

At the titer of λ used for selection, only those transformants that carried and expressed the restriction-modification system were resistant to infection. Colonies were also formed from about 0.4% of transformants that did not carry the *Pst* I system, but these were clearly heavily infected by λ and readily distinguished from the normal-appearing colonies that arose from cells having the restriction-modification system. Subsequently, we have found that the efficiency of plating of transformants containing the *Pst* I system is approximately 0.4 in the presence of λ at the titer used in the selection procedure.

There are two conditions that must be met in order for a restriction-modification system to be cloned and expressed in a new host. It is essential that the genes for the restriction enzyme and methylase be very closely linked and that, after the transformation, the methylase be expressed in advance of the restriction enzyme to protect the DNA of the host. Three cellular restriction-modification systems have been shown to satisfy these criteria: (*i*) *Eco*RI which occurs naturally on a plasmid (16), (*ii*) *Hha* II cloned by Smith and his collaborators (1), and (*iii*) *Pst* I. The close linkage of the genes in these systems may be required for their coordinated expression and suggests that they are transcribed as a polycistronic message. If the sequential expression of the genes after transformation is related to the physiological control of their expression, one would anticipate that other restriction-modification systems would be organized similarly.

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