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Short Communication

In vitro Construction of Plasmids which Result in Overproduction of the Protein Product of the *araC* Gene of *Escherichia coli*

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Summary. Derivatives of the *Escherichia coli* drug resistance plasmid pMB-9 were constructed which contain the promotor from the lactose operon of *E. coli* fused to the *araC* gene of *E. coli*. *E. coli* possessing these plasmids contain about 50 times as much of the *araC* gene product as do cells with a wild-type *araC* gene and promotor.

To facilitate purification of the *araC* gene protein from *Escherichia coli*, we have sought a more concentrated source than the wild type bacteria. The accompanying paper (Steffen and Schleif, 1977) describes a lambda-arabinose transducing phage that directs cells to oversynthesize *araC* protein by a factor of about 100. This synthesis is roughly equivalent to that which could be produced by a single copy of the *araC* gene if it were fused to the lactose operon promotor. Thus such a fusion combined with a means of generating mutiple gene copies in each cell could yield still greater synthesis of *araC* protein. Here we describe the construction of plasmids that satisfy both requirements – they are present at 50 copies per cell (Fuller, Johnsrud and Gilbert, unpublished data), and the *araC* on each has been fused to the *lacZ* gene promotor.

A derivative of the drug resistance factor pMB-9 (Maniatis, et al., 1976), pOP-2, isolated by Fuller, Johnsrud and Gilbert (unpublished experiments) contains just a single cleavage site for the restriction endonuclease *Eco* RI about 200 nucleotides transcriptionally downstream from a functional lactose promoter. DNA inserted at this cleavage site ought to be transcribed from the lactose promotor. Additionally, pOP-2 contains the useful properties of its parent, pMB-9. It confers resistance to the antibiotic tetracycline. It is present in many copies per cell under normal growth conditions and is amplified in bacteria exposed to chloramphenicol.

DNA containing the *araC* gene was obtained from lambda-arabinose transducing phage (Lis and Schleif, 1973; Schleif, et al., 1971). The DNA was isolated from the phage by phenol extraction and digested with *Bam* HI or *Eco* RI nucleases as described by Haggerty and Schleif (1976). In some cases it was then sonicated to an average of about 1000 nucleotides in length. This DNA was used without isolation of the specific fragments carrying the *araC* gene.

pOP-2 DNA was isolated by the method of Tanaka and Weisblum (1975) and cleaved with endonuclease *Eco* RI. The fragments of transducing phage DNA were joined to the cleaved plasmid DNA by a modification of the method described by Lobban and Kaiser (1973), Jackson, et al. (1972) and Wensink, et al. (1974). The addition of polydeoxyadenosine and polydeoxythymidine tails to the DNA was done without the use of lambda exonuclease as described by Roychoudhury, et al. (1976). The polydA or polydT regions could serve as transcriptional termination signals. Therefore the plasmids were constructed with both orientations of polydA and polydT. As shown in Table 1, however, apparently RNA polymerase crosses a stretch of poydA as efficiently as it crosses a stretch at polydT. The polarity of the polydA/polydT tails makes no difference. EKI, PI containment was used for this work.

The plasmid DNA and lambda-arabinose DNA containing tails was annealed and immediately used

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Table 1. Properties of the pOP2–araC hybrid plasmids

Name of plasmid	Source of *araC* DNA	Strand of "Tail" read by RNA polymerase	% araC$^+$ plasmids		Units araC/mg. protein
			expected	found	
pDS1	*Eco* RI digested λ*paraC138*	polydT	25	2	40
pDS2	*Bam* HI digested, sonicated λ*paraC138*	polydA	1	1	3
pDS3	*Bam* HI digested λ*paraC138*	polydA	20	17	2
pDS4	*Bam* HI digested, sonicated λ*paraC138*	polydT	1	1.3	2
pDS5	*Eco* RI digested λ*paraC138*	polydT	25	2	3
pDS6	*Bam* HI digested λhy80d*ara*	polydA	10	8	60
pDS7	*Bam* HI digested λhy80d*ara*	polydA	10	8	80
pDS8	*Bam* HI digested λhy80d*ara*	polydA	10	8	50

DNA samples of *in vitro* constructed hybrids were used to transfect *araC*$^-$ cells. DNA extracted from the Ara$^+$ transformants was used to transform *araB*$^-$ cells that must be used in the assay of *araC* protein

to transfect DLS25 (*araC7025 endoI hsd*R *hsd*M *lac*$^+$ *tetr*^{S}) by a modification of the method of Mendel and Higa (1970) as described by Steffen (1977). The bacteria were plated on X-gal plates containing 20 μg/ml tetracycline. (X-gal plates are minimal glucose plates containing 40 μg/ml 5-bromo, 4-chloro, 3-indolyl β-D Galactoside). Cells containing a derivative of pOP-2 are able to grow in the presence of tetracycline, whereas the parent bacteria cannot. In addition, the multiple copies of pOP-2 in the cell, each containing a binding site for the lactose repressor, titrates lactose repressor off of the lactose operon and thereby induces it. This causes the colonies to indicate blue.

Blue colonies from the above plates were tested for their ability to grow on minimal arabinose plates. The fraction of colonies which are Ara$^+$ is reported in Table 1. If fragments of DNA could be randomly placed in plasmids, we expect the fraction of colonies which are Ara$^+$ to be the inverse of the number of fragments into which the λ-arabinose transducing phage was broken.

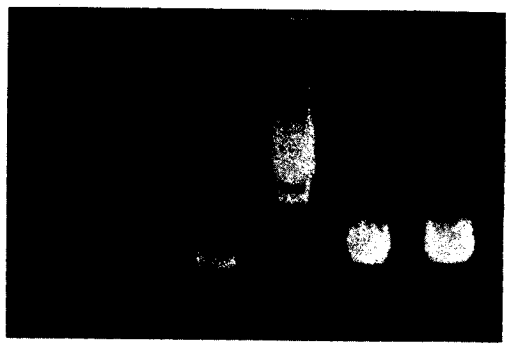
Plasmids were extracted from the Ara$^+$ colonies and used to transfect DLS26 (*araB7026 endoI hsd*R *hsd*M *lac*$^+$ *tetr*^{S}) to *lac*$^-$ *tet*^{r}. These colonies were grown and assayed for *araC* protein as described by Steffen and Schleif (1977).

Two classes of transformed bacteria were found, Table 1, one class containing high concentrations of *araC* protein and one class containing low concentrations. The classes could represent plasmids with two orientations of the inserted *araC* gene. Those oriented in the same direction as the *lac* promoter would yield the higher *araC* level. To test this hypothesis, we picked two plasmids (pDS1 and pDS5) of identical construction, one of which produced a high level of *araC* protein and one of which produced a low level. The purified plasmid DNA was digested with site specific endonucleases and electrophoresed in agarose gels as described by Haggerty and Schleif (1976). The results are shown in Figure 1. First, as expected the two plasmids are identical except for the orientation of the *araC* containing DNA on the plasmid. This confirms the hypothesis that the different levels of *araC* protein synthesis programmed by the plasmids is due to orientation of *araC* relative to *lacP*. The electrophoresis results also show that the plasmids do not contain the entire *araC Eco* RI fragment from the phage. Most likely the DNA was cut at low efficiency at a secondary site and transformants were selected which possessed this shorter fragment. This explanation is consistent with our finding that transformation of *araC* cells by plasmids containing *Eco* RI cut λ*araC* DNA yields abnormally low in numbers of AraC$^+$ transformants compared to AraC$^-$ transformants. Most likely a phage λ gene on the *araC* fragment, perhaps *cI*, *N*, or *cro*, is lethal.

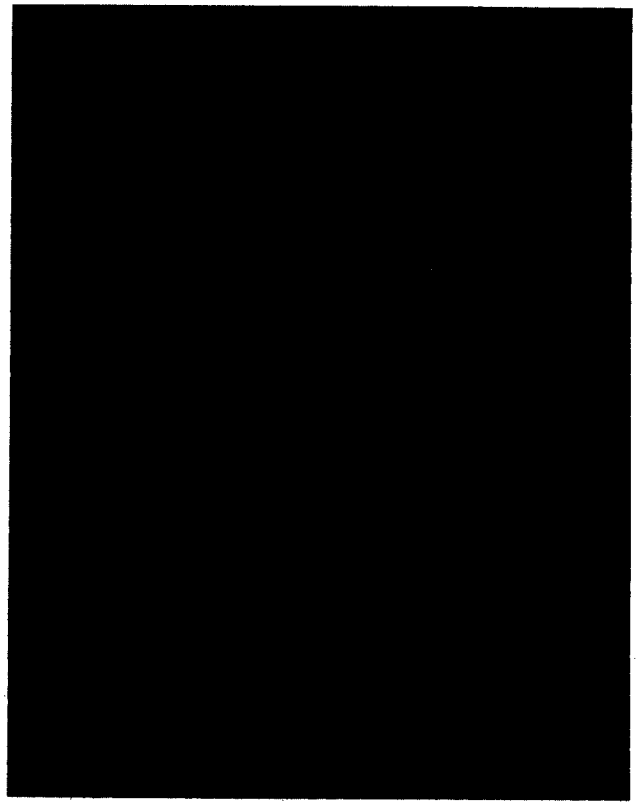
The 50-fold hypersynthesis of *araC* protein we measured is far below the level of 5000 expected on the basis of gene dosage and the relative activities of the *araC* promoter and *lacZ* promotors (Casadaban, 1976). This does not result from few copies of the plasmid per cell since the plasmid does titrate out free *lac* repressor. Since the plasmid contains about 200 nucleotides of undetermined origin between the inserted *araC* gene and the *lacZ* promotor (Fuller, Johnsrud and Gilbert, unpublished results) transcription could be appreciably attenuated before reaching the *araC* gene. Also, attenuation could occur in the polydA or polydT connector region, although this attenuation would have to occur equally on polydA and polydT tracts.

In this communication we describe construction of a series of plasmids containing a *lacP-araC* fusion. These plasmids provide a source of *araC* protein similar in concentration to the lambda-arabinose transducing phage described in the accompanying paper (Steffen and Schleif, 1977). In addition, this work points out some considerations affecting constructing of hybrid plasmids. First, that use of fragments of

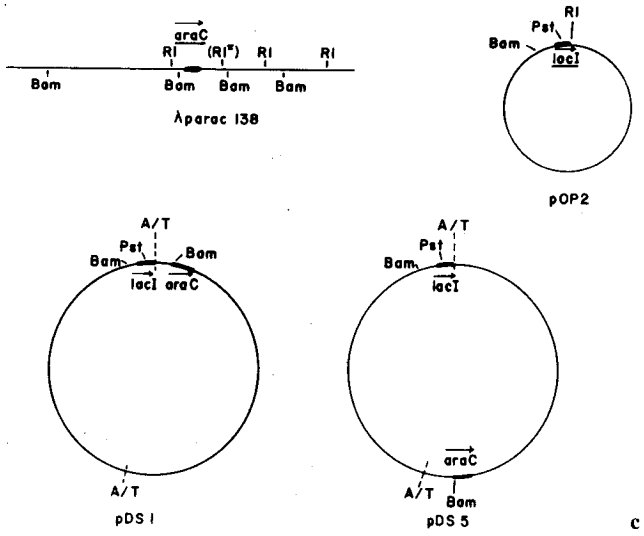
lambda phage as part of such plasmids involves complications due to the presence of phage genes. Some of these genes appear to be lethal. Consequently, fragments must be chosen with care in order to avoid such genes. Scond, when the goal of such work is to functionally fuse a promotor to a gene in order to increase its transcription, careful consideration must be given to the material located between the promoter and the gene. It appears from our work that substantial attenuation of transcription can occur over distances of a few hundred nucleotides.



a



b



c

Fig. 1a–c. Structure of pDS1 and pDS5 as determined by restriction enzyme cleavage.

a Agarose gel electrophoresis of cleaved and uncleaved pMB9 and pOP2: location of the *Pst* I (from Providencia Stuarti) site on pOP2.

1. Undigested pMB9; 2. pMB9 digested with *Pst* I; 3. pMB9 digested with *Eco* RI; 4. Undigested pOP2; 5. pOP2 digested with *Pst* I; 6. pOP2 digested with *Eco* RI.

The presence of a site for *Pst* I cleavage in pOP2 and its absence on pMB9 show that this site is on the DNA inserted into pMB9 to make pOP2. This conclusion is confirmed by the double digest of pOP2. Since no apparent further cleavage occurs with the double digest, the two sites must be very close together.

b Agarose gel electrophoresis of cleaved and uncleaved pDS1 and pDS5: analysis of their structure.

1. Undigested pDS1; 2. pDS1 digested with *Pst* I; 3. pDS1 digested with *Bam* HI; 4. pDS1 digested with both *Pst* I and *Bam* I; 5. Undigested pDS5; 6. pDS5 digested with *Pst* I; 7. pDS5 digested with *Bam* HI; 8. pDS5 digested with both *Pst* I and *Bam* HI.

Interpretation of these results is diagramed in part c) of this figure.

Tracks are numbered left to right. Electrophoresis is from top to bottom.

c Diagramatic explanation of structures of pDS1 and pDS5.

The cleavage sites of *Eco* RI and *Bam* HI on λ*paraC138* were determined by Haggerty (Dennis Haggerty, unpublished results). The location of the *Bam* HI and *Eco* RI sites on pOP2 determined by Fuller et al. (Fuller, F. Johnsrud, L. and Gilbert, W., unpublished results). The location of the *Pst* I site is given in part a) of this figure.

Bam HI
↓: *Bam* HI cleavage sites
Pst I
↓: *Pst* I cleavage sites
Eco RI
↓: *Eco* RI cleavage sites
(*Eco* RI*)
↓: Hypothetical secondary *Eco* RI cleavage site required for the formation of pDS1 and pDS5.
—: λ*ara* phage DNA
—: pOP-2 DNA
→: Lactose operon DNA containing *lacI* inserted into pMB-9 to produce pOP-2.
A/T: polydA:polydT joints joining pOP-2 to λ*ara* DNA
⟵: the *araC* gene, the "sense" direction indicated by the arrow *araC*.

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