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Isolation of Plasmids Carrying the Arginine Repressor Gene *argR* of *Escherichia coli* K12

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Summary. A plasmid carrying the arginine repressor gene (*argR*) of *Escherichia coli* was obtained out of the *Clarke and Carbon* colony bank which contains clones of individual ColE1 plasmids carrying fragments of the *E. coli* chromosome. A shortened derivative of this plasmid was isolated and analyzed by restriction mapping and by cloning fragments of it into plasmid pBR322. The *argR* gene was located on a 2 kb fragment.

Introduction

The arginine repressor protein is the central regulatory element in arginine biosynthesis in *Escherichia coli*. In *E. coli* K12 it controls at least 10 genes located at different sites on the chromosome. The repressor protein has been partially purified (Kelker et al., 1976) and it was shown that L-arginine is the co-repressor (Cunin et al., 1976).

In order to have a better understanding of the arginine repressor gene I set out to isolate a plasmid carrying the *argR* gene from the colony bank of Clarke and Carbon (1976). In this paper I report the isolation of such a plasmid as well as the isolation and properties of a shorter derivative obtained by an enrichment procedure. Hybrid plasmids of pBR322 (Bolivar et al., 1977) carrying the *argR* gene are described and a physical map of the inserted region is presented.

Materials and Methods

Bacterial Strains. EC113(λAZ-7); F⁻, *Δ(pro-lac), argD, argR, aroE, rpsL,* lysogenic for λAZ-7 (Eckhardt, 1977); EC146(λAZ-7); F⁻, ΔDE5 *(proB-argF-lac), argD, argR, relAl, supE44, thi* (derived from X7026 of J. Beckwith); JA200: F⁺, *ΔtrpE5, thy, lacY, thr, leu, recA,* (Clarke et al., 1976; this strain contains the plasmids of the Clarke and Carbon plasmid bank).

Media. For genetic work minimal medium A was used, for plasmid preparations M9 with glycerol as carbon source was used (Miller, 1972). The media were supplemented with appropriate vitamins (1 μg/ml) and aminoacids (50 μg/ml) and 1% of a carbon source. X-gal(5-bromo-4-chloro-3-indolyl-β-D-galactoside) plates have been described elsewhere (Miller, 1972).

Genetic Techniques. The plasmids of the Clarke and Carbon bank were mated into strain EC113(λAZ-7) by replicating the donors onto a lawn of EC113(λAZ-7) on minimal medium, supplemented with L-arginine, L-proline and aroE-mixture (L-tyrosine, L-phenylalanine, L-typtophane, 20 μg/ml each, p-amino-benzoic acid, p-hydroxy-benzoic acid 5 μg/ml each). The plates were incubated for 8 h at 37° C in order to express the ArgR⁺ phenotype. The mating plates were then replicated onto MacConkey-lactose plates containing streptomycin (100 μg/ml) to prevent growth of the donor and colicin E1 to select against the recipient. The ColE1 transconjugants formed either red (argR⁻) or white (argR⁺) patches.

Isolation and Purification of Plasmids. Preparation of plasmids was usually carried out with or without chloramphenicol amplification. The cells were lysed using lysozyme and subsequently Triton X-100 following a modified procedure in Meagher et al. (1977).

Enzymes and Assays. T4 ligase, *Hae*II, and *Hin*cII restriction endonucleases were purchased from New England Biolabs, Inc. and the assays were performed according to their instructions. The digests were carried out overnight at 37° C except for the ligation which was performed at 16° C.

Gel Electrophoresis. Electrophoresis was performed in vertical slab gel apparatus using 0.8% agarose (type ME, Marine Colloids, Inc.) unless specified otherwise in electrophoresis buffer (89 mM trisborate pH 8.2 with 2.5 mM Na_2EDTA). The DNA samples were precipitated in 2 volumes EtOH at −70° C, vacuum dried and resuspended in electrophoresis buffer with 5% Ficoll 400,000 and 0.3% bromphenol blue.

Transformation. The cells were transformed using a modification of the procedure of Clarke and Carbon (1976) employing 75 mM $CaCl_2$. The $CaCl_2$ was removed by centrifugation and the cells were resuspended and 20× diluted into growth medium.

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Results

Identification and Properties of a Hybrid Plasmid Carrying the argR Gene. The colony bank of Clarke and Carbon (1976) was screened for plasmids carrying the $argR^+$ allele. Generally it is not possible to select for $argR^+$ offspring in an *argR* background. To facilitate the identification of a functional $argR^+$ gene a special strain was used as recipient into which the plasmids of the colony bank were transferred by mating. In this strain EC113(λAZ-7) the ArgR phenotype can be recognized in two ways. It carries the *lacZ* gene fused to the *argA* operator on λAZ-7 (Eckhardt, 1977). The strain is Lac^+ on MacConkey-Lactose medium if its is *argR*. The presence of an $argR^+$ allele however changes it to Lac^- because the *lacZ* gene is repressed by the arginine repressor and arginine present in MacConkey medium. Strain EC113(λAZ-7) has furthermore a *proA* deletion and its proline requirement is suppressed under derepressed conditions by a leaky *argD* mutation. Proline synthesis occurs through the early steps of the arginine pathway (Eckhardt et al., 1975). This strain grows on minimal medium (MM)+proline, MM+arginine and MM+arginine+proline. An $argR^+$ derivative however does not grow on MM+arginine because the suppressive proline pathway is repressed by the arginine repressor and arginine resulting in proline auxotrophy. The donor strains carrying the hybrid plasmids were mated with strain EC113(λAZ-7) (see methods) and offspring were scored for their Lac phenotype on MacConkey-lactose medium. Of the 2,000 strains of the colony bank only transconjugants of clone pLC32–38 gave Lac^- phenotype.

The Lac^- offspring was furthermore inhibited on MM+arginine. Both criteria for the presence of an active repressor are therefore fullfilled. The isolation of pLC32–38 DNA was unexpectedly difficult; the plasmid was hardly amplifiable by the chloramphenicol treatment unlike other ColE1 derivatives and the overall yield was less than 1 µg/liter of cell culture (5×10^8 cells/ml). Plasmid pLC32–38 was therefore not particularly suitable for our work. The length of its DNA was estimated by gel electrophoresis to be 22 kb. To explain the unexpected behaviour of the plasmid we reasoned that its size might be responsible for its low copy number. Efforts were thus directed to isolate a shortened derivative of pLC32–38.

Isolation of a Short Derivative of pLC32–38. The following enrichment procedure was set up to isolate a shortened plasmid with a high copy number. Plasmid pLC32–38 was grown in strain EC113(λAZ-7) and chloramphenicol was added in late exponential phase for 12 h. The plasmid DNA was isolated and used to retransform strain EC113(λAZ-7) selecting for colicin immunity. Approximately 10^4 colicin immune transformants were pooled, grown up and treated again with chloramphenicol. The plasmid DNA was isolated and used to retransform strain EC113(λAZ-7). Individual colicine immune $argR^+$ colonies were isolated and screened for plasmid size using a rapid screening method (Eckhardt, 1978). Of 120 colonies analyzed one colony had a plasmid considerably shorter than pLC32–38. This plasmid which still contained the entire *argR* gene was designated pEC101 and used for further analysis. Its size was determined to be 5.1 kb using gel analysis of restriction endonuclease digests. Since it is even shorter than the original ColE1 plasmid (6.34 kb) from which pLC32–38 is derived it must have lost ColE1 DNA in addition to chromosomal DNA. The yield of pEC101 DNA is comparable to that of ColE1 although chloramphenicol treatment does not increase it dramatically. The yield is 50–100 µg/l, much higher than that of pLC32–38.

Cloning of the argR Gene into Plasmid pBR322. Because pEC101 does not have a suitable marker to ensure persistence of the plasmid in the cell, the *argR* gene was transferred to pBR322 (Bolivar et al., 1977), allowing at the same time a rough localization of the *argR* gene. pEC101 did not show a single restriction site suitable for cloning. *Hae*II restriction digests were used for cloning, although pBR322 has eleven and pEC101 four *Hae*II sites which makes pBR322 not a particularly good choice as cloning vehicle. The rationale was that judging from the restriction map there was a good chance that only two contiguous *Hae*II fragments would be necessary for a viable cloning vehicle which carries the β-lactamase *(bla)* gene for ampicillin resistance (Ap^R). The partial digest of pBR322 would therefore yield predominantly vectors suitable for cloning. The ligation and transformation using partial *Hae*II digests of pEC101 and pBR322 was performed as described in Material and Methods. The partial digests were empirically determined, for pEC101 conditions were used which gave the maximal amount of indermediates, for pBR322 a near complete digest was used. The ligated fragments were transformed into strain EC113(λAZ-7) and Ap^R colonies were selected on MacConkey-lactose plates containing 20 µg/ml ampicillin. Over 60% of the offspring were $ArgR^+$ and of the remaining $ArgR^-$ colonies 70% carried a plasmid larger than pBR322 as judged by gel electrophoresis. The frequency of hybrid formation between pEC101 and pBR322 was therefore about 90%. About 10% of the AP^R colonies retained tetracycline resistance (Tc^R) indicating the presence of at least 80% of the pBR322 plasmid.

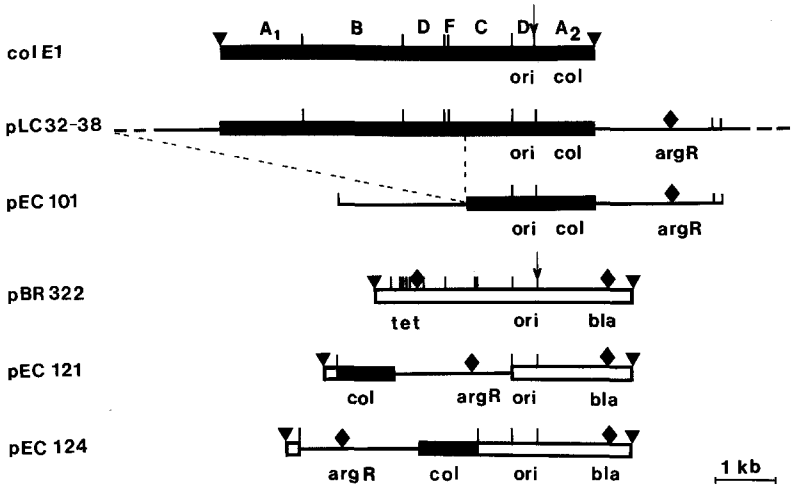

Fig. 1. Restriction map of pEC101, its derivatives pEC121 and pEC124 and the parental plasmids ColE1 and pBR322 showing the *Hae*II (|), *Hin*cII (◆) and *Eco*RI (▼) recognition sites. All plasmids are lined up to the common sequence bearing the identical *Hae*II site (↓) in ColE1 and pBR322 to the right side of the origin of replication (Oka et al. (1979), Sutcliffe (1978b)). ColE1 and pBR322 and its derivatives are linearized at their *Eco*RI sites, pEC101 at an arbitrarily chosen *Hae*II site. The map of pLC32–38 is hypothetical and was tentatively derived from pEC101. The deletion which leads to the formation of pEC101 is indicated (--------). The *Hae*II fragments are labeled according to Oka (1977). The genes and sites were abbreviated as follows: *Col*: ColE1 immunity; *tet*: tetracycline resistance; *bla* ampicillin resistance; *argR*: arginine repressor; *ori*: origin of replication of colE1 resp. pBR322. (=) indicates DNA derived from pBR322, (■) DNA from ColE1 and (—) chromosomal DNA

This was a high frequency of hybrids considering the fact that hybrid plasmids were not selected. The plasmids were screened for their size to identify the smallest recombinants. Two plasmids pEC121 and 124 were selected for further studies based on their small size (5.1 and 5.6 kb resp.).

Restriction Analysis of the argR Plasmids. The restriction maps of the *argR* plasmids and the parental plasmids ColE1 and pBR322 are given in Fig. 1. The *Hae*II restriction fragments are shown in Fig. 2 and their size listed in Table 1.

pEC101 gives four *Hae*II fragments. The arrangement of the fragments was determined by partial digestion (data not shown). It was established that the small and the large fragments alternate, resulting in the restriction map shown in Fig. 1 line 3. The origin of the different *Hae*II bands was determined as follows. The 0.34 kb fragment is shared with the parental ColE1 (Fig. 2, column 2 and 3). It is known to carry the origin of replication of ColE1 (Oka, 1978). This fragment is the only one shared with ColE1, the remaining *Hae*II fragments of ColE1 are therefore at least partially deleted. The 3.0 kb fragment of pEC101 carries the *argR* gene since it is the only band appearing in pEC121 and 124. The same fragment contains ColE1 DNA too, since it carries colicin E1 immunity (see below). Colicin E1 immunity is located on the A_2 fragment of an *Eco*RI·*Hae*II double digest (Oka, 1978), as shown in Fig. 1, line 1. Bearing in mind that the Clarke and Carbon plasmids were constructed by cleavage of ColE1 at the *Eco*RI site and by insertion of the chromosomal fragments into this site through complementary dA and dT sequences added by terminal transferase (Clarke et al., 1976) the 3.0 kb *Hae*II fragment contains the A_2 fragment, the dA-dT region and chromosomal DNA including the *argR* gene. The ColE1 part of the fragment lies adjacent contiguously to the 0.34 kb fragment as in the original ColE1 (Fig. 1, lines 1 and 3). The 0.16 kb fragment contains chromosomal DNA only and the 2.45 kb fragment contains chromosomal and ColE1 DNA. The ColE1 DNA originated from the C fragment of ColE1 and comprises about 0.7 kb, based on other restriction analysis (data not shown). The ColE1 part of this fragment lies adjacent to the left side of the 0.34 kb fragment. This fragment is a result of the deletion in pLC32–38 which must have occurred in the C fragment of the ColE1 part and somewhere in the chromosomal DNA, thereby deleting about 17 kb (Fig. 1, line 2 and 3).

Plasmids pEC121 and 124 both contain the *argR* gene; the main difference between them is that the *argR* fragment is inserted in opposite directions in the two plasmids (see below). Their organization was determined as follows. Both plasmids share the 3.0 kb fragment with pEC101 (Fig 2, lines 3 through 5). Since both plasmids are $ArgR^+$, this fragment has to contain the *argR* gene. All other fragments are shared by pBR322. Both contain the 430 bp fragment carrying the origin of replication of pBR322 and the 1.87 kb fragment containing the *bla* gene. pEC124

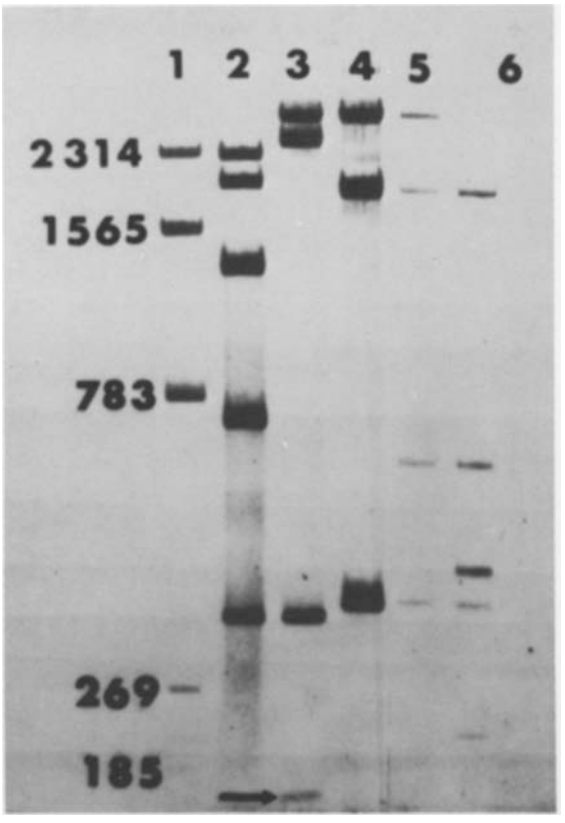

Fig. 2. *Hae*II restriction endonuclease digest of (1) ϕX174; (2) ColE1 (3) pEC101; (4) pEC121; (5) pEC124; (6) pBR322. The size of the molecular standard (ϕX174) is given on the left side (in bp). Electrophoresis was performed in a gel containing 1.7% Acrylamide, 0.085% Bis (N,N′methylenebisacrylamide) and 0.5% agarose. The arrow points out the position of the 160 base pair fragment of pEC101

Table 1. *Hae*II restriction fragments (base pairs)

colE1[a]	pEC101	pBR322[b]	pEC121	pEC124
2324 (A)	3000	1875	3000	3000
1732 (B)	2450	622	1875	1875
1134 (C)	422	439	430	622
693 (D)	160	430		430
422 (E)		370		
340 (F)		227		
252 (G)		181		
170 (H)		83		
70 (I)		60		
		53		
		21		

[a] Calculated from Oka (1978)
[b] from Sutcliffe (1978a)

shares additionally a 622 bp fragment (Fig. 2, lines 4 through 6), allowing a simple differentiation between pEC121 and 124 based on their size. The organization of the pBR322 part of the plasmid is identical to that of the parental pBR322 (Fig. 1, line 4) as it was

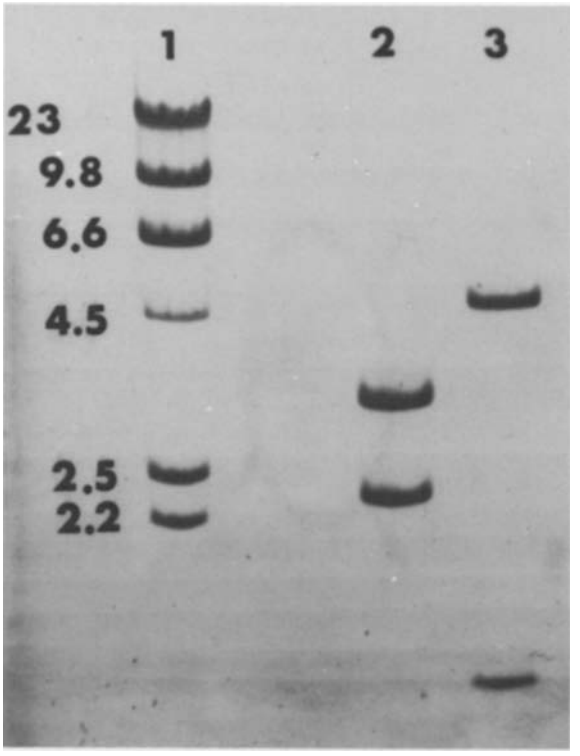

Fig. 3. *Hin*cII restriction endonuclease digest of (2) pEC121; (3) pEC124 and (1) *Hin*dIII digest of λ phage as molecular size standards

verified by further restriction analysis (data not shown).

The orientation of the *argR* fragment in pEC121 and 124 was determined as follows. The 3.0 kb *argR* fragment has an asymmetrical *Hin*cII site which lies 0.7 kb from the chromosomal end of the fragment. This was deduced from the fact that the sequence of the 1067 bp ColE1 part of the fragment is known and shows no *Hin*cII recognition site (Oka et al., 1979). Another *Hin*cII site is found in the *bla* gene of the pBR322 part, 685 bp from the joint between pBR322 and pEC101 DNA, (Sutcliffe, 1978b). The fragment sizes of a *Hin*cII digest thus allows to determine the orientation of the *argR* fragment (Fig. 3). pEC124 shows a 1.38 kb fragment (Fig. 3, line 3 and Table 2) which is composed of 685 bp pBR322 DNA and 700 bp chromosomal DNA from the *argR* fragment. In pEC124 the *argR* gene is therefore close to the *bla* gene of pBR322 (Fig. 1, line 6). pEC121 does not show a 1.38 kb fragment, but a 3.0 kb fragment comprised of 680 bp pBR322 DNA and 2.3 kb DNA from the 3.0 kb fragment. In this case the gene for colE1 immunity is close to the *bla* gene.

Colicin E1 Immunity. All plasmids described above were tested for colicin E1 immunity. The original

Table 2. *Hin*cII restriction fragments (in Kilobases)

pEC121	pEC124
3.0	4.5
2.2	1.38

Table 3. Frequency of spontaneous *argR*$^-$ plasmids of pEC121

Source[a]	Relevant genetic marker	Frequency of ArgR$^-$ Ap^R colonies among ArgR$^+$ Ap^R transformants[b] (%)
EC146	*argR recA*$^+$	5/321 (1.5)
EC145	*argR*$^+$ *recA*$^+$	3/386 (0.8)
EC115	*argR recA*	0/1081 (<0.1)

[a] Strain in which pEC121 was grown and isolated

[b] pEC121 was transformed into strain EC146(λAZ-7) for the determination of the ArgR$^-$ offspring, see text

pLC32–38 and its shortened derivative pEC101 were both colicin immune. Since the gene for colicin immunity lies on the *Eco*RI · *Hae*II A_2 of ColE1 (Oka, 1978) and is not found on plasmid pBR322, the hybrid plasmids pEC121 and 124 were also tested for colicin immunity. Both plasmids were found to be colicin immune, showing that the 3.0 kb *argR* fragment indeed carries the ColE1 A_2 fragment.

Stability of the argR Gene on Plasmids. It was found that all plasmids carrying the *argR*$^+$ gene show a considerable rate of conversion to ArgR$^-$. This is apparent especially in a strain like EC113(λAZ-7) plated on MacConkey-lactose plates or suitable X-gal plates containing arginine. After 2–3 days sectors of lactose fermentors resp. high β-galactosidase producers appear indicating an ArgR$^-$ phenotype. The approximate frequency of ArgR$^-$ AP^r transformants of pEC121 from different origin into strain EC146(λAZ-7) is given in Table 3. Since the rapid spreading ArgR$^-$ phenotype in this strain might be confused with an ArgR$^-$ transformant all candidates were carefully examined on X-gal plates (including arginine and other necessary growth requirements). Only those colonies were counted as ArgR$^-$ which showed an intense blue center in the colony after two days. ArgR$^-$ phenotype which arose during growth in EC146(λAZ-7) usually have a lighter blue center due to the fact that some cells in the center are still argR$^+$. The ArgR$^-$ transformants were analyzed for the presence of plasmids. In most cases plasmids of about twice the size of the original plasmid were found. The ArgR$^-$ phenotype of these plasmids can not be explained as a product of recombination of the plasmids with the chromosomal *argR* gene, since the frequency of the ArgR$^-$ plasmids is the same in an *argR*$^+$ and an *argR*$^-$ host strain. At this point it is not known if the increase in size brings about the ArgR$^-$ phenotype or if the increase in size is a mere coincidence. The ArgR$^+$ phenotype is more stable in a *recA* background indicating that this function is involved in the formation of the ArgR$^-$ plasmids. The exact nature of these plasmids is currently under investigation.

Discussion

With the isolation of plasmids carrying the *argR* gene it has become possible to study the repressor gene more closely and the plasmids will be of great value in determining the DNA sequence of this gene and to study its expression. Although the initially isolated plasmid pLC32–38 was not suitable it seems to be possible to isolate plasmids with more desirable properties using an appropriate selection. This is possible since variations e.g. by formation of deletions occur with a reasonable frequency in the plasmid population. In the case of the *argR* plasmid the situation was advantageous insofar as in pLC32–38 the *argR* gene was already very close to the origin of replication and therefore a large portion of the plasmid could be lost without affecting the desired properties. In the hybrid plasmids pEC121 and 124 the amount of chromosomal DNA present is reduced to only 1.9 kb of which 0.8–1 kb comprise the *argR* gene.

The most intriguing observation during this work with the *argR* plasmids was the rapid conversion to ArgR$^-$ phenotype under most conditions. Since the ArgR$^+$ phenotype is dominant it means that all *argR*$^+$ plasmids must segregate out in order to become ArgR$^-$ in an *argR* background. In a single colony on MacConkey medium over 100 individual ArgR$^-$ sectors and papillae might be seen under a dissecting microscope indicating that this conversion is rather frequent. It appears that *argR*$^-$ plasmids have a selective advantage over the *argR*$^+$ plasmid, but a reason for this has not been established. It is possible that the arginine repressor is inhibitory to the cells at to high concentration which would give the *argR*$^-$ plasmids a selective advantage. Studies are in progress to clarify this point.

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