Cloning of the Aspartase Gene (*aspA*) of *Escherichia coli*

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The aspartase gene (*aspA*) of *Escherichia coli* has been isolated in two plasmids, pGS73 and pGS94, which contain segments of bacterial DNA (12.5 and 2.8 kb, respectively) inserted into the *tet* gene of the vector pBR322. The plasmids were constructed by sequential sub-cloning from a larger ColE1-*frd*⁺ hybrid plasmid. The location of the *aspA* gene confirmed predictions based on a correlation between the genetic and restriction maps of the corresponding region. The aspartase activities of plasmid-containing *aspA* mutants were amplified four- to sixfold relative to *aspA*⁺ parental strains. The *aspA* gene product was tentatively identified as a polypeptide of *M*_r 55 000, which is somewhat larger than previous estimates (*M*_r 45 000 to 48 000) for aspartase.

INTRODUCTION

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyses the reversible conversion of L-aspartate to fumarate and ammonia. The enzyme has been isolated from *Escherichia coli* strains B and W; it has a molecular weight of 170 000 to 193 000 and comprises four apparently identical subunits (Rudolph & Fromm, 1971; Suzuki *et al.*, 1973).

In *E. coli* aspartase participates in at least two metabolic processes: the regeneration of oxaloacetate as amino-acceptor for enabling growth on glutamate, and the formation of fumarate and succinate during anaerobic growth on glucose. The catabolism of glutamate involves transamination with oxaloacetate and oxidation of the resultant 2-oxoglutarate via the Krebs cycle. The oxaloacetate that serves as the primary amino-acceptor is converted to aspartate and then regenerated by a 'deamination cycle' involving aspartase, fumarase and malate dehydrogenase. Thus, *aspA* and *aspC* mutants lacking either aspartase or aspartate aminotransferase are unable to grow on glutamate as the sole source of carbon and energy (Marcus & Halpern, 1969*a*). The importance of aspartate aminotransferase plus aspartase as an alternative route from oxaloacetate to fumarate and succinate during glucose fermentation was discovered in studies with malate dehydrogenase mutants and this role was supported by the observation that malate dehydrogenase is repressed whereas aspartase is derepressed during anaerobic growth (Courtright & Henning, 1970).

The aspartase gene (*aspA*) has been located at 94.1 min in the *E. coli* linkage map by transduction when phage P1 (Spencer *et al.*, 1976; Guest & Nice, 1978; Bachmann, 1983). It lies between *mel* and *mop* (*groE*), very close to *mop* (90% cotransduction) and quite close to *ampCA* (71% cotransduction) and *frdA* (67% cotransduction), the gene order being: *mel-aspA-mop(groE)-ampCA-frdDCBA*. Several segments of this region of the chromosome have been isolated in phages and plasmid vectors. These include the artificially-constructed λ transducing phages, $\lambda groE$ (Georgopoulos & Hohn, 1978; Hendrix & Tsui, 1978), $\lambda frdA$ and derivatives of $\lambda frdA$ having extended transducing ranges, $\lambda frd amp$ and $\lambda frd amp groE$ (Cole & Guest, 1980*a*), and the ColE1-*amp*⁺ *frd*⁺ hybrid plasmids isolated from the gene bank of Clarke & Carbon (1976): pNU1 (Edlund *et al.*, 1979), which is probably identical to pGS1 and pLC16-43 (Guest, 1981); and pLC43-46 (Lohmeier *et al.*, 1981). Studies with these phages and plasmids have provided a restriction map for the corresponding 25 kb segment of bacterial DNA and the

positions of the *mop* (*groE*), *amp* and *frd* genes have been defined by selective labelling in transcription-translation systems and by nucleotide sequence analysis (Tilly *et al.*, 1981; Cole & Guest, 1980*b*; Cole, 1982; Cole *et al.*, 1982; Jaurin & Grundström, 1981; Grundström & Jaurin, 1982; Neidhardt *et al.*, 1983). A diagram correlating the genetic and restriction maps is presented in Fig. 1, and it indicates that the *aspA* gene should lie within the region of DNA that has been cloned. This paper reports the location, isolation and expression of the *aspA* gene of *E. coli*.

METHODS

Bacterial strains. The strains of *E. coli* K12 are listed in Table 1. The aspartase mutation (designated *aspA23*) came from CS8ASP/23, a mutant of a glutamate-permease-constitutive strain (CS8, *gluC8*) that was originally isolated by Marcus & Halpern (1969*b*). A series of derivatives of W3110 and Ymel (W/G and W/Gasp, and Y/G and Y/Gasp) containing the *gluC* and *aspA* mutations were later constructed by P1 transduction (Spencer *et al.*, 1976). For the work to be described, the *aspA* mutants were cured by replacing the P1 prophage with a temperature-inducible prophage (P1*cmlclr100*), and then isolating heat-resistant P1-sensitive derivatives. Recombination deficient strains were isolated by screening Tet^R transductants obtained with a P1 lysate prepared on an *srl::Tn10 recA* mutant (Kleckner *et al.*, 1977).

Strains containing the ColE1-*frd*⁺ hybrid plasmids, pLC16-43 and pLC43-46 (Edlund *et al.*, 1979; Guest, 1981; Lohmeier *et al.*, 1981) in JA200 were obtained from a copy of the Clarke & Carbon (1976) colony bank kindly provided by Dr R. A. Cooper. Strain C600 was used for routine phage preparation and assay, and its *recA* λ^R derivative (JRG1370) was used as the transformation recipient for plasmid construction. Other strains used for special purposes were: AB2480, the 'maxicell' host, and GM242, the host for producing plasmid DNA that is susceptible to *Bcl*I digestion.

Bacteriophages. Populations of recombinant λ phages containing *Hind*III and *Eco*RI fragments of *E. coli* DNA in appropriate vectors (λ NM540, λ NM761, λ 781 and λ 816) were kindly provided by Dr N. E. Murray or prepared according to Borck *et al.* (1976). The λ *frdA* phages, λ G1F and λ G40F, have been described previously (Cole & Guest, 1980*a*) as have the λ *groE* phages, λ W3 and λ H13a (Georgopoulos & Hohn, 1978), which were kindly provided by Dr C. Georgopoulos.

Media. The minimal medium E of Vogel & Bonner (1956) was used with glucose (11 mM) or monosodium L-glutamate (30 mM) as substrates. The medium was supplemented to satisfy the requirements of specific auxotrophs and sometimes enriched with Difco Bacto nutrient broth (0.2%, w/v). The complete media used for routine growth and maintenance were L and LG broth, and BBL medium was used for assaying λ phages (Guest *et al.*, 1983): these were supplemented with the antibiotics, ampicillin (50 μ g ml⁻¹), tetracycline (20 μ g ml⁻¹) or chloramphenicol (25 μ g ml⁻¹), as required for selective purposes. Media were solidified with Difco Bacto agar at 15, 10 or 6.5 g l⁻¹.

Genetic methods. The transduction procedures used for phage (Cole & Guest, 1980*a*) and phage P1 (Spencer *et al.*, 1976) have been described previously.

Isolation and manipulation of DNA. The methods used for isolating phage and plasmid DNA have been described previously as have the methods for restriction endonuclease digestion, agarose gel electrophoresis and DNA ligation (Guest *et al.*, 1983).

Table 1. *Strains of E. coli* K12

Strain	Genotype or derivation
JRG817	<i>gluC8 aspA23 trpR iclR</i> (P1); formerly W/Gasp
JRG818	<i>gluC8 trpR iclR</i> (P1); formerly W/G
JRG821	<i>gluC8 aspA23 supE57 supF58</i> (P1); formerly Y/Gasp
JRG822	<i>gluC8 supE57 supF58</i> (P1); formerly Y/G
JRG1113	P1-sensitive derivative of JRG821
JRG1114	P1-sensitive derivative of JRG817
JRG1476	<i>srl::Tn10 recA</i> derivative of JRG1114
JRG1480	<i>srl::Tn10 recA</i> derivative of JRG1113
JA200	F ⁺ , <i>thr-1 leuB6 trpE63 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 supE44 (glnV44)</i>
C600	<i>thr-1 leuB6 thi-1 supE44 tonA21 lacY1</i>
JRG1370	<i>thr-1 leuB6 thi-1 supE44 tonA21 lacY1 recA</i> λ^R
GM242	F ⁻ , <i>dam-3 recA1 sin-2 thr-1 leuB6 proA2 his-4 metB1 lacY1 galK2 ara-14 tsx-33 phi-1 deoB6 supE44 rpsL260</i>
AB2480	<i>uvrA6 recA13 pro-2 thi-1 lac rpsL</i>

'Maxicell' procedure. Polypeptides expressed from plasmid-encoded genes were labelled by the 'maxicell' technique of Sancar *et al.* (1979) details of which have been described previously by Shaw & Guest (1982).

Enzymology. Cultures were grown in LG broth (250 ml in shaken 21 flasks) for 12 h at 37 °C, starting with an exponential culture (5 ml) grown in the same medium. In order to limit the segregation of plasmid-free bacteria, relatively large inocula were used and D-ampicillin (100 µg ml⁻¹) was added, where possible. Cultures of mutant strains were also tested to ensure that significant reversion had not occurred. Ultrasonic extracts were prepared, dialysed against potassium phosphate buffer (0.04 M, pH 7.8), and assayed for protein according to Creaghan & Guest (1977). Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) was assayed at 20 °C by estimating the rate of ammonia produced from aspartate over periods of 5 to 30 min with a range of protein concentrations and several different extracts, according to Spencer *et al.* (1976). The specific activities are quoted in terms of µmol NH₃ released (mg protein)⁻¹ h⁻¹ at 20 °C.

Materials. Restriction enzymes were purchased from the Boehringer, BRL and New England Biolabs. T4 DNA ligase was from BRL and L-[³⁵S]methionine (80 to 125 µCi ml⁻¹; 2.96 to 4.625 MBq ml⁻¹) was purchased from Amersham.

RESULTS

Location and isolation of the *aspA* gene

The results of genetic studies with aspartase mutants of *E. coli* are summarized in Fig. 1. They indicate that the *aspA* gene should lie within 10 to 15 kb of the fumarate reductase genes (*frdABCD*) and quite close to the *mop* (*groE*) genes. These studies were hampered by having to

Fig. 1. Genetic and restriction maps of the *mel-frd* (94 min) region of the *E. coli* chromosome. The genetic map (a) is drawn to scale from phage P1 cotransduction frequencies for different pairs of markers (Spencer *et al.*, 1976; Guest & Nice, 1978); the map distances are shown in kb, derived from the cotransduction frequencies according to Wu (1966). The *ampA1* and *frdA* markers have been used as fixed points for constructing the map, and the results indicate that the aspartase gene (*aspA*) is located very close to *mop* in the *mel-mop* segment. The *ampA1* marker is an up-promoter mutation of chromosomal β -lactamase (*ampC*). The restriction map (b) shows cleavage sites for *Bam*HI (B), *Eco*RI (R), *Hind*III (H) and *Sal*I (S). The segments of DNA cloned in phages and plasmid vectors are indicated by the horizontal lines and plasmid vectors are shown as open boxes with *Pst*I targets (vertical lines) to identify the relative orientations of the cloned fragment and vector, where known. The vectors and sizes (kb) of cloned fragments are indicated in parentheses. The positions of the *ampC* and *frd* genes, as defined by nucleotide sequence analysis, are shown above the restriction map and they provide the fixed points for the linear correlation between the genetic and restriction maps. The approximate positions of the *mop* genes are also indicated by bars corresponding in length to the molecular weights of their products.

rely on the glutamate-utilizing phenotype ($\text{Glt}^+/\text{Glt}^-$) for diagnosing the *aspA* genotypes of recombinants. The problems stem from the fact that *E. coli* K12 will not normally grow on glutamate because glutamate transport is severely repressed. This necessitates the use of strains that express the transport system constitutively. These strains have mutations in the operator locus (*gltC*) that is adjacent to the glutamate permease structural gene (*gltS*) and they render permease synthesis insensitive to the *gltR*-encoded repressor (Marcus & Halpern, 1969*c*). It is also observed that *aspA* mutants exhibit a pronounced tendency to revert on glutamate medium. Consequently, the *aspA* marker is best used non-selectively and it is important to monitor for reversion at all stages. The same problems apply when devising strategies to clone the *aspA* gene by complementation.

The first attempts to isolate the *aspA* gene involved screening pools of hybrid λ phages from *aspA* transducing phages using glutamate medium for selective purposes. Some evidence for *aspA* transduction was obtained but phages capable of complementing the *aspA* lesion were not found, suggesting that the intact and functional *aspA* gene is not released in fragments that can be accepted by the lambda vectors, viz: *Hind*III and *Eco*RI fragments ranging up to 16 kb. Transduction tests with previously characterized phages were either negative (λ *frd*) or inconclusive (λ *groE*).

Attempts to isolate the *aspA* gene by sub-cloning fragments of DNA from phages and plasmids containing the neighbouring *mop* (*groE*), *amp* and *frd* genes were more successful. These sources are illustrated in Fig. 1, and they include the ColE1-*frd*⁺ plasmids, pLC16-43 and pLC43-46 as well as several transducing phages, λ *mop* (*groE*) and λ *frd*. A basic restriction map of the entire region (25 kb) was obtained by analysing the large plasmid, pLC43-46, with four endonucleases. *Bam*HI, *Eco*RI, *Hind*III and *Sal*I (Fig. 1). This map is consistent with previous results obtained with the ColE1 plasmids and the λ phages. Furthermore, by taking account of the known positions of the *mop*, *amp* and *frd* genes and by expressing genetic linkage data in kb according to Wu (1966), a good correlation between the genetic and restriction maps was obtained (Fig. 1). This indicates that the *aspA* is located very close to the *mop* genes in the *mel*-*mop* segment. During the course of isolating the *aspA* gene, several new plasmids were constructed and these are shown in Fig. 1. They were all obtained by sub-cloning restriction fragments into appropriate sites in pBR322 or pBR325, and the sources of the bacterial fragments inserted into specific plasmids were: λ G1F, 4.9 kb *Hind*III (pGS39); and pLC43-46, 5.4 kb *Bam*HI-*Sal*I (pGS67), 5.0 kb *Sal*I-*Eco*RI (pGS71), 7.7 kb *Eco*RI (pGS72) and 12.5 kb *Sal*I-*Hind*III (pGS73).

The five newly constructed plasmids, plus pBR322 as a control, were tested for the presence of the *aspA* gene by transforming competent cultures of the *aspA* (JRG1113, JRG1114) and *aspA recA* (JRG1480, JRG1476) mutants and selecting independently for Amp^R and Glt^+ phenotypes. Some 20 to 50 Amp^R transformants and Glt^+ transformants (plus revertants) were then tested for co-inheritance of the alternative phenotype by replica plating. Only one plasmid, pGS73, gave reproducibly high frequencies of Amp^R Glt^+ cotransformation and it was concluded that the *aspA* gene is located in the 12.5 kb fragment of bacterial DNA cloned therein. In addition, the results suggest that the *aspA* gene contains, or is very close to, the *Eco*RI site in the 12.5 kb fragment, because there is no evidence for aspartase expression with the plasmids (pGS71 and pGS72) that contain the segments flanking this site. The results are entirely consistent with the location of the *aspA* gene predicted from genetic studies (Fig. 1).

Further attempts to define the position of the *aspA* gene were made following a detailed analysis of pGS73 for restriction targets that could be used in sub-cloning. A map of pGS73 based on cleavage patterns obtained with different combinations of 17 restriction enzymes is shown in Fig. 2. Some 29 sites were placed in the 12.5 kb *Sal*I-*Hind*III fragment of bacterial DNA and evidence for a further 13 sites, including several for *Hpa*I, *Pvu*II, *Pst*I and *Bcl*I, was obtained. The relative orders of some of the very close sites were not defined unambiguously. No sites were detected for *Sst*I and *Xho*I.

This analysis suggested that digestion with *Bcl*I would release a 2.8 kb fragment containing the *Eco*RI site, that had been predicted to be in or close to the *aspA* gene, flanked by 1.7 kb and 1.1 kb segments of bacterial DNA. Accordingly, this fragment was isolated from a *Bcl*I digest of

Fig. 2. Restriction map of pGS73. The scale diagram of the linearized plasmid indicates the restriction sites that were identified for 15 restriction enzymes in both the vector DNA (pBR322, hatched region) and the 12.5 kb *SalI*–*HindIII* segment of bacterial DNA (open region). The numbers of each type of site are indicated in parentheses. The regions containing unplaced sites, *BclI* (2), *PstI* (3), *PvuII* (2) and *HpaI* (6), are indicated thus: (). No sites were detected for *XhoI* and *SstI*. The structure of the smaller plasmid, pGS94, is shown below pGS73: it contains the 2.8 kb *BclI* fragment inserted in the *BamHI* target of pBR322 with the orientation indicated.

pGS73 and sub-cloned into the *BamHI* site of pBR322 to generate plasmid pGS94 (Fig. 2). The orientation of the sub-cloned fragment was deduced by restriction analysis with *EcoRI* and the positions of the *AccI*, *AvaI* and *SphI* sites in the fragment were confirmed by restriction analysis. No plasmids with the fragment inserted in the opposite orientation were recovered. Plasmid pGS94 was tested for complementation of the *aspA* lesion, and, like pGS73, it gave reproducibly high frequencies of the *Amp^R Glt⁺* cotransformation with the *aspA* and *aspA recA* strains. Thus it would appear that the 2.8 kb *BclI* fragment of pGS94 possesses a functional *aspA* gene.

Studies with 'maxicells'

The 'maxicell' technique was used to investigate the gene products expressed from pGS73 and pGS94 and a typical autoradiogram is shown in Fig. 3. The polypeptides that appeared unique to pGS73 were classified as strongly expressed (M_r 61 000, 31 500, 21 000, 18 000, 16 000 and 11 000) and poorly expressed (M_r 56 500, 55 000, 51 000, 36 000, 33 000, 28 500 and 27 500). The coding capacity predicted for the bacterial insert of pGS73 would just be sufficient to encode these polypeptides if it is assumed that each labelled band represents a single polypeptide and if they are neither artefacts nor degradation products. By comparison, experiments with several independent isolates of pGS94 indicated that this plasmid encodes only one specific and relatively poorly expressed polypeptide, M_r 55 000 (Fig. 3). No preferential labelling of a polypeptide of this size was ever detected with pBR322. The simplest but not the sole

Fig. 3. Autoradiogram of ^{35}S -labelled polypeptides expressed from cloned genes. Plasmid-containing cultures were labelled for 2 h by the 'maxicell' procedure. The molecular masses of the polypeptides are shown in kDa. The tracks represent: 1, no plasmid; 2, pBR322; 3, pGS73; 4, pGS94.

interpretation of these observations is that the polypeptide of M_r 55 000 is the product of the *aspA* gene. It is also likely that two of the strongly expressed polypeptides, M_r 61 000 and 16 000, correspond to the *mopA* (*groEL*) and *mopB* (*groES*) gene products, respectively. These have been identified previously as polypeptides of M_r 65 000 and 15 000 (Tilly *et al.*, 1981) or M_r 57 000 and 14 000 (Neidhardt *et al.*, 1983). Several other products that are expressed from the 94 min region of the *E. coli* chromosome have been detected with pLC16-43 and pLC43-46 (Neidhardt *et al.*, 1983). Their sizes, derived from two-dimensional electrophoretic gel coordinates, are approximately, 35 000 (*ampC*), 27 000, 21 000 (two polypeptides), 13 000 and 11 700. Some of these may correspond to polypeptides detected with pGS73, because the fragment cloned in pGS73 is derived from pLC43-46.

Enzymological studies with plasmid-containing strains

The aspartase specific activities of plasmid-containing derivatives of several *aspA* strains were compared with the corresponding plasmid-free mutant and *aspA*⁺ strains. Typical sets of results are shown in Table 2. These were reproducible and it is clear that the aspartase activity is not only restored to the *aspA* mutants by pGS73 and pGS94, but amplified four- to sixfold relative to the corresponding *aspA*⁺ parental strains. These observations are consistent with *aspA* being the aspartase structural gene and with its location in the segment of DNA common to both plasmids.

Table 2. *Aspartase activities of plasmid-containing strains*

Ultrasonic extracts of organisms grown in LG broth were prepared and assayed as described in Methods. Specific activities for aspartase are expressed as $\mu\text{mol NH}_3$ released $(\text{mg protein})^{-1} \text{ h}^{-1}$.

Strain and relevant genotype	Aspartase specific activity with plasmid:			
	None	pBR322	pGS73	pGS94
JRG818 (<i>gltC aspA</i> ⁺)	0.52	—	—	—
JRG1476 (<i>gltC aspA recA</i>)	0.05	0.07	2.71	3.02
JRG822 (<i>gltC aspA</i> ⁺)	0.97	—	—	—
JRG1480 (<i>gltC aspA recA</i>)	0.02	0.04	4.27	5.29

DISCUSSION

The *aspA* gene of *E. coli* has been isolated in two derivatives of pBR322, pGS73 and pGS94, by sequential sub-cloning from a large ColE1-*frd*⁺ hybrid plasmid. The position of the gene fully supported the cloning strategy that was based on the gene location, predicted by correlating earlier genetic linkage data with a restriction map of the corresponding segment of bacterial DNA. The evidence supporting the successful isolation of the gene was based on the nutritional complementation of *aspA* mutants and the restoration and amplification of aspartase activity in plasmid-containing mutant strains. Earlier attempts to demonstrate the presence of the *aspA* gene in the ColE1-*frd*⁺ source plasmid (pLC43-46) had been equivocal due to several factors including the instability of the *aspA* mutation and the lack of a convenient selection for plasmid maintenance. However, most of the problems were overcome by transferring the DNA to pBR322 in order to exploit ampicillin-resistance for selective purposes. Earlier failures to isolate the *aspA* gene from the λ gene banks could also be explained if the critical *Eco*RI target lies within the *aspA* coding region or a region that is essential for *aspA* expression, and if the corresponding *Hind*III fragment (>12.5 kb) exceeds the capacity of the vector phages.

The simplest interpretation of the results is that the *aspA* gene product is the polypeptide of M_r 55000 which is encoded by both pGS73 and pGS94. However, this polypeptide is somewhat larger than has been found or predicted for the aspartase monomer (45000 to 48000) from studies with the isolated enzyme (Rudolph & Fromm, 1971; Suzuki *et al.*, 1973) and the possibility that the polypeptide labelled with pGS94 represents the product of an *aspA-tet* fusion has not been excluded. This would mean that one of the other polypeptides labelled with pGS73 could correspond to the *aspA* gene product. In the present work, no direct evidence linking aspartase function with the M_r 55000 product was obtained and it is conceivable that the *aspA* product may not have been detected in the 'maxicells', but the coding capacity of the smaller plasmid (pGS94) would limit the size of any further product to less than 30000. It should also be noted that the aspartase structural gene was originally defined in studies with a temperature-sensitive mutant CS8ASP^{ts} and although the mutations of CS8ASP23 and CS8ASP^{ts} were shown to be in the same region, there was no evidence to indicate whether they affected the same gene (Marcus & Halpern, 1969*b*). Consequently there remains the formal possibility that the gene, isolated by virtue of its ability to complement the CS8ASP23 lesion, need not necessarily be the aspartase structural gene but a regulatory gene, or some other gene that affects the expression of aspartase. Nevertheless, the simplest interpretation of the results is that the aspartase structural gene has been isolated and its product identified. Current studies aimed at defining the nucleotide sequence of the cloned gene will provide the primary structure of its product for comparison with the isolated aspartase protein. The aspartase gene appeared not to be strongly expressed and the degree of amplification in the plasmid-containing strains was modest (four- to sixfold). Studies on the regulation of aspartase synthesis should be facilitated by the isolation of the gene. Also, by coupling the gene to stronger promoters, it should be possible to obtain cultures that are highly enriched for aspartase and these could be important for the industrial production of L-aspartate, using immobilized cells (Nishida *et al.*, 1979).

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