The EMBO Journal vol.8 no.5 pp.1623 – 1627, 1989

xerB, an *Escherichia coli* gene required for plasmid ColE1 site-specific recombination, is identical to *pepA*, encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase

Colin J.Stirling[1], Sean D.Colloms, John F.Collins[2], George Szatmari and David J.Sherratt

Institute of Genetics, University of Glasgow, Church Street, Glasgow G11 5JS and [2]Biocomputing Research Unit, Department of Molecular Biology, Kings Buildings, University of Edinburgh, Edinburgh EH9 3JR, UK

[1]Present address: Department of Biochemistry, University of California, Berkeley, CA 94720, USA

Communicated by D.J.Sherratt

The heritable stability of ColE1 is dependent on a site-specific recombination system which acts to resolve plasmid multimers into monomers. This plasmid stabilizing recombination system requires the presence in *cis* of the ColE1 *cer* region, plus at least two *trans*-acting factors encoded by the *xerA* and *xerB* genes of *Escherichia coli*. The *xerB* gene has been cloned and sequenced and found to encode a polypeptide with a calculated mol. wt of 55.3 kd. The predicted amino acid sequence of this protein exhibits striking similarity to that of bovine lens leucine aminopeptidase (53 kd). The biological significance of this similarity is corroborated by genetic and biochemical evidence which suggests that *xerB* is identical to the *E.coli* and *S.typhimurium pepA* genes that encode aminopeptidase A.

Key words: plasmid ColE1/stable inheritance/*cer* site-specific recombination/*xerB*/*pepA* genes/aminopeptidase

Introduction

The stability of high copy number plasmids in *Escherichia coli* can be greatly reduced by the formation of plasmid multimers by homologous recombination. The natural plasmid ColE1 is stabilized by a site-specific recombination system which serves to resolve unstable plasmid multimers into their monomeric form (Summers and Sherratt, 1984). This system requires the presence in *cis* of the 250 bp ColE1 *cer* locus (Summers and Sherratt, 1984, 1988), plus the *trans*-acting products of the *E.coli xerA* and *xerB* genes (Stirling *et al.*, 1988a). The *xerA* gene has been cloned and sequenced and found to correspond to the previously defined *argR* gene (Stirling *et al.*, 1988b); accordingly we have adopted the *argR* nomenclature in place of *xerA*. We have demonstrated that ArgR protein (plus its co-repressor L-arginine) binds to a specific sequence within *cer* located ~100 bp upstream of the cross-over region. There is no evidence that the Xer activity of ArgR is mediated by transcriptional repression, and we have postulated that the bound ArgR may play a role in the formation of a topological intermediate essential for the formation of a productive synaptic complex (Stirling *et al.*, 1988b).

The DNA sequence of '*xerA*' with its consequent identification as *argR*, has provided us with a crucial step forward in the elucidation of the *cer* recombination system. However, in addition to a requirement for ArgR, *cer*-specific recombination also exhibits an absolute dependence upon the product of the *E.coli xerB* gene. In an effort to further characterize this second function we have cloned the *xerB* gene and now report its complete nucleotide sequence, plus the purification of the 55.3 kd XerB polypeptide. To our surprise the XerB protein sequence exhibits striking similarity to that of the bovine leucine aminopeptidase (Cuypers *et al.*, 1982a). This sequence similarity provided the inspiration to investigate aminopeptidase mutants of *Salmonella typhimurium*. These experiments led us to conclude that *xerB* represents the *E.coli* homologue of the pepA (aminopeptidase A) gene of *S.typhimurium* (Miller and MacKinnon, 1974; Miller, 1987), which in turn is likely to be identical to the aminopeptidase I of *E.coli* isolated by Vogt (1970).

Results

Defining the minimum XerB complementing sequence

A 45 kb cosmid clone (pB4), containing the wild-type *xerB* gene was cloned and identified by virtue of its complementing the Xer phenotype of DS947 *xerB*1 (Stirling *et al.*, 1988a). The complementation test employed involved the transformation of the test cosmid into DS947 *xerB*1 carrying the compatible 2-*cer* reporter plasmid pCS210. pCS210 is

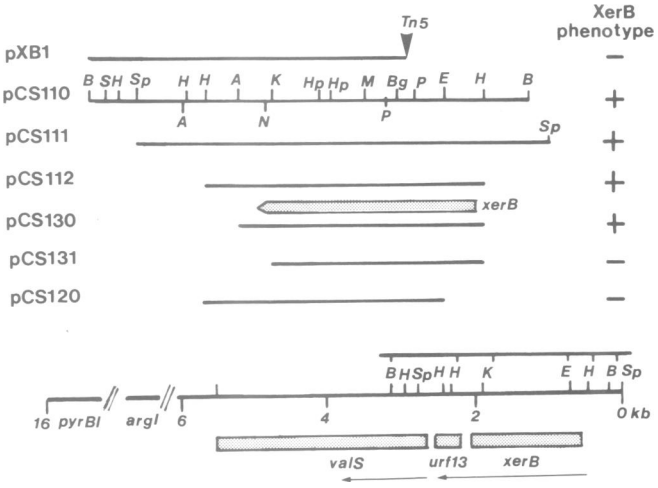

Fig. 1. Determination of a minimal functional *xerB* gene by complementation and its position on the *E.coli* chromosome. The top section of the figure shows the XerB phenotype of plasmids encoding the segments of DNA indicated. The *xerB* gene is indicated by the hatched box. The bottom section shows the map of 16 kb of *E.coli* chromosomal DNA at ~96.5 min, indicating the *xerB*, *urf13* and *valS* genes, and the downstream *argI* and *pyrBI* genes. Directions of transcription of the *xerB*, *urf13*, *valS* cluster are shown (→). Immediately above the 16 kb section of the chromosome is the corresponding region of the map of Kohara *et al.* (1987). B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; Sp, *Sph*I; A, *Acc*I; N, *Nar*I; K, *Kpn*I; Hp, *Hpa*I; M, *Mlu*I; P, *Pvu*II; E, *Eco*RI.

a derivative of pACYC184 which encodes tetracycline resistance (Tc^r) and which carries a functional *lacZ* gene inserted between two *cer* sites. The Xer-dependent resolution of pCS210 *in vivo* results in the deletion of *lacZ* from the replicon; this deletion may be monitored directly by agarose gel electrophoresis of plasmid DNA, or indirectly by the phenotypic expression of *LacZ* on X-gal-containing medium (Stirling *et al.*, 1988a).

In order to identify the minimum *xerB*-complementing sequence we have sub-cloned various restriction fragments from pB4 into suitable plasmid vectors. Each sub-clone was tested for *xerB*-complementing activity by transformation into DS947 *xerB*1 (+pCS210), and transformants selected on minimal medium containing Tc, Ampicillin (Ap) and X-gal. The results of complementation were scored by colony colour (blue = Xer^-, white = Xer^+), and are summarized in Figure 1; in each case the Xer phenotype was confirmed by agarose gel electrophoresis of plasmid DNA to ensure that partial complementation phenotypes were not overlooked (data not shown). The minimum sequence identified as retaining the complementing activity was the 1692 bp *Hin*dIII−*Acc*I fragment in pCS130. The complete nucleotide sequence of the slightly larger *Hin*dIII fragment (1921 bp) from pCS112 has been determined and found to contain a 503-codon open reading frame (ORF) which would encode a polypeptide of 55.3 kd. The data presented in Figure 1 strongly suggest that this ORF encodes the complementing function. The correspondence between the restriction map

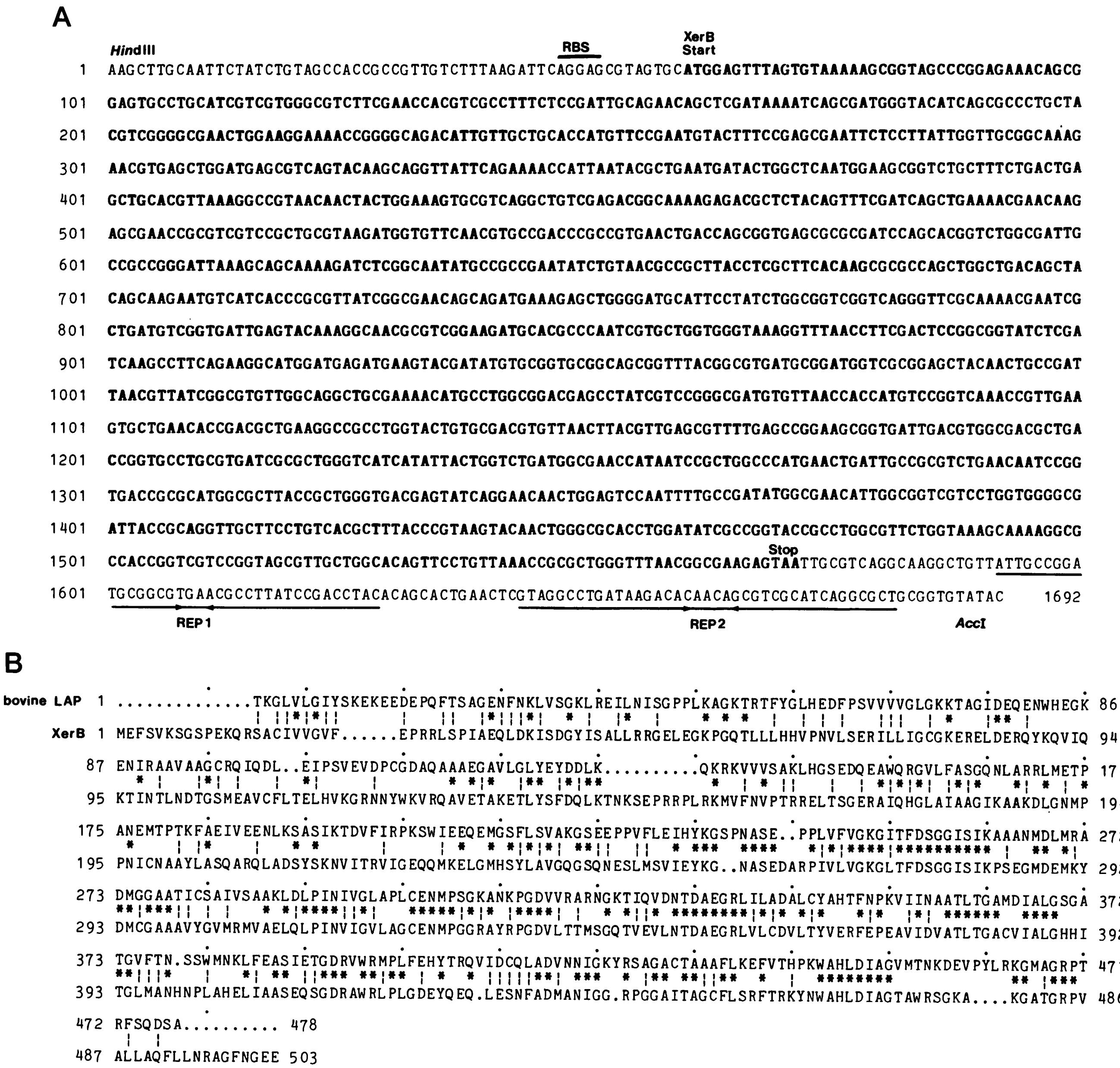

Fig. 2. (**A**) DNA sequence of the 1692 bp *Hin*dIII−*Acc*I fragment containing the *xerB* gene. Nucleotides encoding XerB protein are in bold face. The presumptive ribosome binding site is indicated (RBS) as are the two *REP* sequences (Stern *et al.*, 1984; Gilson *et al.*, 1987) downstream of *xerB*. *urf*13 initiates 39 bp 3′ of the *Acc*I site (unpublished). (**B**) Protein sequence of XerB compared with that of bovine lens leucine aminopeptidase (Cuypers *et al.*, 1982a,b). Identical amino acid residues are indicated by * and similar residues by |. Overall there is 31% identity and a further 22% of similar residues. This match was initially found using the Best Local Similarity Algorithm of Smith and Waterman (1981) on an ICL 64 × 64 Distributed Array Processor using the implementation protocol described by Collins *et al.* (1988).

of pXB1 (containing the cloned *xerB*1 mutant locus) with those of pCS110 and pCS112 indicates that we have indeed cloned the wild-type *xerB* locus and not some extra-genic suppressor. Furthermore, this comparison confirms that the 503-codon *xerB* gene is disrupted by Tn*5* insertion in the *xerB*1 mutant (Figure 1).

The *xerB* locus maps to within the 96–97 minute region of the *E.coli* linkage map (Stirling *et al.*, 1988a). A higher resolution map location can now be determined by searching the restriction map of the *E.coli* chromosome (Kohara *et al.*, 1987), for restriction 'fingerprints' compatible with that of the cloned *xerB* locus. The only convincing match identified is at 4560 kb on the map, equivalent to 96.5 min on the *E.coli* K12 linkage map (Figure 1). This comparison, confirmed by genetic mapping data, highlights the potential of the map of Kohara *et al.* in the prediction of chromosomal map location from simple restriction data.

Nucleotide sequence and characterization of xerB

The 1921 bp *Hin*dIII *xerB*$^+$ fragment from pCS112 was cloned in both orientations in M13mp19 to produce mCS112 and mCS113 respectively. A series of nested *Exo*III deletion derivatives of both mCS112 and mCS113 were then generated by the method of Henikoff (1984). These derivatives together with several specific sub-clones were then employed as DNA sequencing templates from which the complete nucleotide sequence has been derived for both strands using the method of Sanger *et al.* (1977). Analysis of the nucleotide sequence of this region indicates the presence of a single large ORF which would encode a polypeptide 503 amino acid residues in length, with a calculated mol. wt of 55.3 kd (Figure 2). The complementation data shown in Figure 1 indicates that this open reading frame represents the *xerB* gene. Minicell expression studies have confirmed that this sequence encodes a polypeptide with an apparent mol. wt of 55 kd (data not shown). Furthermore, by fusing this gene downstream of the inducible *tac* promoter, we have constructed a plasmid (pCS126) which leads to the isopropylthiogalactoside (IPTG)-dependent over-expression of a 55-kd polypeptide to a level ~5–10% of total cell protein (data not shown). Using crude extracts from over-expressing cells we have purified XerB to homogeneity exploiting the protein's insolubility in low-salt buffers at neutral pH. The N-terminal sequence of the purified protein has been determined for the first 20 amino acid residues and is entirely consistent with that predicted for the product of the *xerB* gene (Figure 2). Attempts to show specific binding of *XerB* to *cer* DNA have not been successful.

XerB shows striking similiarity to bovine lens leucine aminopeptidase

Initial seaches of databases (during 1986/87) revealed no obvious similarity of XerB to other proteins. Subsequently (April, 1988) a Distributed Array Processor search of protein databases (8177 proteins) using the 'Best Local Similarity' algorithm of Smith and Waterman (1981), as modified by Collins *et al.* (1988), revealed a highly significant and striking similarity between the C-terminal portion (residues 230–470) of XerB and the C-terminal portion (residues 210–451) of bovine lens leucine aminopeptidase (EC 3.4.11.1). The alignments of the whole proteins are shown in Figure 2B. Overall there is 31% identity of residues and a further 22% of related residues. The C-terminal portions of the two proteins show 52% identity, quite remarkable for proteins from such diverged organisms. These findings suggested that *xerB* might be identical to a gene encoding one of the characterized aminopeptidases of enteric bacteria. Examination of the published *E.coli* and *S.typhimurium* genetic maps revealed a locus, *pepA*, in *S.typhimurium* that encodes aminopeptidase A and which maps at 97 min, in the same relative position as *xerB* on the *E.coli* map. *pepA* is just one of five aminopeptidase genes that have been described in *S.typhimurium* (Miller and MacKinnon, 1974; Miller, 1987). Work on *E.coli* aminopeptidases has been more limited (Miller and Schwartz, 1978), though it appears that *E.coli* synthesizes aminopeptidases with the same general properties as those of *S.typhimurium*. Moreover, aminopeptidase A, encoded by the *pepA* gene in *S.typhimurium*, appears to have a counterpart in *E.coli* both at the genetic and biochemical level; this aminopeptidase probably being identical to aminopeptidase I characterized by Vogt (1970). Like Miller and Schwartz (1978), we describe this aminopeptidase as aminopeptidase A, and its gene as *pepA*.

XerB is the E.coli homologue of the S.typhimurium pepA gene and is therefore equivalent to E.coli pepA

To investigate whether *xerB* is a peptidase gene, we determined the Xer phenotype of *S.typhimurium* peptidase-deficient strains (Miller and MacKinnon, 1974). This analysis used *S.typhimurium* rather than *E.coli* because of the more extensive genetic characterization of *pep* mutants in the former organism (Miller and MacKinnon, 1974;

Table I. Xer phenotype of peptidase mutants of *S.typhimurium*

Strain	Genotype	Xer phenotype	
		Assayed by loss of Cm^R 'reporter' gene	Assayed by DNA electrophoresis
CH44	del(*oppBC*)250	+	+
CH351	del(*oppBC*)250 *pepA*	−	−
CH356	del(*oppBC*)250 *tppB*	+	+
TN1301	del(*leuBCD*)485 *pepA*16 *pepB*11 del*sup*Q302(*proAB-pepD*) *pepP*1 *pepQ*1	−	−
TN1302	del(*leuBCD*)485 *pepB*11 del*sup*Q302(*proAB-pepD*) *pepP*1 *pepQ*1 *pepN*90	+	+
TN1303	del(*leuBCD*)485 *pepA*16 del*sup*Q302(*proAB-pepD*) *pepP*1 *pepQ*1 *pepN*90	−	−
TN996	del(*leuBCD*)485 *pepA*1 *pepB*1 *pepD*1 *pepN*10 *zjh*-829::Th1	−	−
TN1420	del(*leuBCD*)485 *pepA*16 *pepB*11 del*sup*Q302(*proAB-pepD*) *pepP*1 *pepQ*1 *pepN*90 *pepT*1 *metE*388 *zie*-822	−	−

Miller, 1987; Gibson *et al.*, 1984). The strains shown in Table I were transformed with the Ap^R 2-*cer* reporter plasmid pKS455, which loses *cat* on Xer-dependent resolution.

Transformants were tested for their Xer phenotype by testing for sensitivity to chloramphenicol and by agarose gel electrophoresis of plasmid DNA (Table I). All strains carrying a functional *pepA* gene are Xer^+, whereas the presence of a mutant *pepA* gene is sufficient to give an Xer^- phenotype. Mutations in other *pep* genes and in *tppB*, a tripeptide permease, gave a Xer^+ phenotype.

XerB protein has aminopeptidase activity

With the demonstration that the *xerB* and *pepA* loci are probably identical, aminopeptidase assays were performed on XerB protein purified some 12 months before we had any insight into its possible aminopeptidase function.

Purified XerB had a sp. act. of ~1000 U/mg protein at 37°C with leucine *p*-nitroanilide as a substrate. This sp. act. was about twice that obtained for porcine leucine aminopeptidase (supplied by BCL; EC 3.4.11.1) which should be similar to bovine lens aminopeptidase. This activity was dependent on Mn^{2+}, inhibited by Zn^{2+} and EDTA and was stable to 70°C incubation (5 min). These properties are similar to those reported by Vogt (1970) for *E.coli* aminopeptidase I, and indeed the heat stability of aminopeptidase A/I is characteristic for this enzyme (Vogt, 1970; Miller and Schwarz, 1978). More recently we have purified aminopeptidase A from an IPTG-induced $pCS126^+$ *E.coli* strain using a method similar to that of Vogt. The purified enzyme had a similar sp. act. to that reported above. Additionally, we assayed for leucine *p*-nitroanilide hydrolysing activity in crude heat-treated (70°C, 5 min) extracts of $xerB^-$, $xerB^+$ and IPTG-induced $pCS126^+$ ($xerB^+$) *E.coli*. Activities of 0, 14 and 600 U/mg protein were obtained confirming that $xerB^-$ *E.coli* are deficient in aminopeptidase A, and that a strongly expressed aminopeptidase A gene is present on the $XerB^+$ plasmid pCS126.

Discussion

The demonstration that *xerB*, a gene absolutely required for site-specific recombination at ColE1 *cer* and its analogues in other multicopy plasmids is identical to *pepA*, the gene for aminopeptidase A, is perplexing, particularly in view of the fact that there are several other aminopeptidases in both *E.coli* and *S.typhimurium* that have similar specificities *in vitro* (Miller and MacKinnon, 1974; Miller and Schwartz, 1978; Miller, 1987). We can only presume that *in vitro* substrate specificities do not accurately reflect *in vivo* specificity or function. Consistent with this view is the observation that in *S.typhimurium* the peptide analogue alafosphalin is a good substrate for PepA but not other aminopeptidases (Gibson *et al.*, 1984). The striking similarity of PepA to bovine lens aminopeptidase is also surprising, yet intriguing in view of the enzymatic activity and structural roles of other lens proteins (see below).

We now believe it likely that PepA protein, like the ArgR protein, is involved as an accessory factor in *cer*-site-specific recombination, since a variant *cer* site can function in the absence of both of these proteins (Summers, 1989). This variant *cer* site has an altered cross-over region and only requires ~50 bp of *cer* in the region of cross-over to function. Recombination at such sites has lost its directionality, no longer being biased toward intramolecular monomerization of multimers (Summers, 1989). This strongly indicates that normal intramolecular selectivity requires the 200 bp of *cer* 5′ of the cross-over in addition to accessory protein(s). This is analogous to several other site-specific recombination systems where accessory factors and their binding sites can provide selectivity and directional specificity by being involved in the formation of specific recombination synapses. For example, in lambda *int*-mediated recombination, integration host factor (IHF) and the factor for inversion stimulation (FIS) can each bind to the arms of *attP* to provide selectivity (Echols, 1986; Thompson *et al.*, 1987). For Gin/*gix* and related inversion systems, host protein FIS acts at the recombinational enhancer *sis* (Kahmann *et al.*, 1985), whereas Tn*3* resolvase acts as its own accessory factor by binding to subsites II and III in *res* (Boocock *et al.*, 1987).

The precise role of PepA as an accessory factor remains unclear. It could have a direct structural role in the recombinational synapse or PepA enzymatic activity could be involved directly or indirectly. For example, PepA could mediate the enzymatic activation of ArgR protein or some other protein component of the *cer* recombination process. Though exoproteolytic activation of proteins does not seem particularly likely, there are a few precedents for such activation. For example, glutamine phosphoribosylpyrophosphate amidotransferase requires an N-terminal Cys residue for its glutamine amide transfer function. This residue is generated by the removal of a single N-terminal Met (Tso *et al.*, 1982). Similarly, elongation factor Tu undergoes a N-terminal Ser acetylation after removal of a N-terminal Met (Arai *et al.*, 1980). Alternatively, PepA could have other, or additional enzymatic roles, e.g. endopeptidase, esterase or transferase activity directed at ArgR or other substrates. We are currently assaying for various types of PepA activity on ArgR. A more indirect role of PepA in *cer* recombination would result if its enzymatic activity were directed at peptides or other small molecules acting either as effectors or in signal transduction necessary for recombination.

The other possibility is that PepA has a direct structural role in the *cer* recombinational synapse, either by interacting with *cer* DNA and/or with other recombinational proteins. As yet we have failed to detect specific interactions between PepA and *cer* or ArgR. Nevertheless there are precedents for enzymes having structural roles that appear to be independent of enzyme activity. Intriguingly, this may be the case for many of the lens crystallins which have now shown to be, or have extensive similarity to, known enzymes (Wistow *et al.*, 1987; Doolittle, 1988; Hendriks *et al.*, 1988). For example, lactate dehydrogenase, arginosuccinate lyase, aldose reductase, enolase and glutathione S-transferase all correspond to crystallins. Moreover, α-crystallin shows similarity to small heat-shock proteins and bacterial spore coat proteins (Ingolia *et al.*, 1982; Wistow *et al.*, 1985). Though bovine lens aminopeptidase is not a major lens protein [we calculate ~0.1–0.5% of lens protein from the data of Hanson and Frohne (1977)], it could have a structural role in the lens; indeed our 'Distributed Array Procedure Search' shows some small but possibly significant similarity of bovine lens aminopeptidase and PepA with those parts of human heat-shock protein (hsp 27) and bacterial spore protein that are related to α-crystallin (Ingolia *et al.*, 1982; Wistow *et al.*, 1985).

The discovery that all mutants completely defective in *cer*-

recombination that we have isolated after Tn*5* mutagenesis map to *argR* (*xerA*) and *pepA* (*xerB*), genes that appear to specify essential accessory function, leads us to believe that the recombinase gene for *cer* recombination could be either essential for *E.coli* viability or that there could be duplicate genes. With this in mind we have isolated a Tn*5*-generated Xer mutant with a 'leaky' phenotype using one of our standard 2-*cer* reporter plasmids. This mutant defines a locus *xerC*, mapping at 85 min on the *E.coli* chromosome. A functional *xerC* gene has been isolated on a 1.2 kb fragment and the *xerC1*::Tn*5* mutant isolated is deficient for recombination on the variant *cer*-site of Summers (1989) that recombines in the absence of *argR* and *pepA* function. The *xerC* gene and its product are currently being characterized.

Materials and methods

Bacterial strains

Most *E.coli* strains for plasmid experiments were derivatives of *E.coli* K12 AB1157 (Bachman, 1972): DS903 is AB1157 *rec*F; DS941 is AB1157 *recF lacI*^{q} *lacZ del*M15 and DS947 is a *sup*^{0} *del*(*lac pro*) derivative of DS903. *S.typhimurium* strains are shown in Table I.

Plasmids and cosmids

Cosmid pB4 is described in Stirling *et al.* (1988a). Chromosomal fragments from pB4 were sub-cloned into either pUC18 (Vieira and Messing, 1982) or pCS100, a pUC8 derivative containing ColE1 *rom* (306-bp *Hpa*III fragment) at the unique *Nar*I site. A 3 kb XerB$^+$ *Bam*HI fragment was recovered in pCS100, giving plasmid pCS110 and a 2.8 kb XerB$^+$ *Sph*I fragment in pUC18 gave plasmid pCS111 (Figure 1). Sub-cloning and restriction fragment deletion gave pCS112, pCS130 and pCS131 (pUC18 derivatives) and pCS120 (an mp18 polylinker-containing Cm^R λdv plasmid derivative). pXB1 is a 9.5 kb pBR322 derivative containing at its *Bam*HI site the *xerB* sequences and part of Tn*5* as indicated in Figure 1.

pCS126 is an expression vector derived from pKK223-3 (Pharmacia) that expresses *xerB* (contained within a 1.9 kb *Hin*dIII fragment) from a *tac* promoter.

pCS210 is a Tc^r pACYC184 derived 2-*cer* reporter plasmid with a functional *lacZ* gene between the *cer* sites.

pKS455 is a Ap^r Cm^r pUC9-derived reporter plasmid containing a functional *cat* gene between two directly repeated *cer* sites (Stirling *et al.*, 1988a).

Bacterial growth media and conditions

LB broth (Maniatis *et al.*, 1982) was used for routine growth supplemented with agar, antibiotics, etc. where appropriate.

In vitro DNA manipulations

Conditions for enzymatic reactions were generally those recommended by the suppliers. Protocols were essentially as described by Maniatis *et al.* (1982). DNA sequencing was by the chain termination method of Sanger *et al.* (1977) using M13mp vectors (Norrander *et al.*, 1983). Exonuclease III (Henikoff, 1984) was used to generate deletions for sequencing.

Purification of the XerB product

This purification was performed before the XerB was suspected to be an aminopeptidase. IPTG Induction (2 h) of DS941 carrying pCS126 resulted in cells in which 5–10% of total protein was XerB. After sonication in buffer containing 25 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1.2 mM phenylmethylsulphonylfluoride (PMSF) and 1 mM benzamidine, XerB sediments with cell debris as a consequence of its insolubility in this low ionic strength buffer. XerB protein readily dissolves from this pellet in buffer containing 1 M NaCl, 50 mM Tris–HCl (pH 8.5) 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, and 1 mM benzamidine. After dialysis against the low ionic strength sonication buffer, *Xer*B was re-precipitated and then re-dissolved in 1 M NaCl buffer. This re-dissolved material provided 5–10 mg (100–200 nmol) of electrophoretically pure 55 kd XerB, as judged by Coomassie-stained SDS–polyacrylamide gels, from 1.5 g wet weight of cells (285 mg protein).

Aminopeptidase assays

The enzymatic substrate was leucine *p*-nitroanilide. Assays were at 37°C in 20 mM Tris–HCl pH 8.2, 100 mM KCl, 1 mM $Mg(OAc)_2$, 1 mM $MnCl_2$, 0.1 mM EDTA, 1 mM L-leucine *p*-nitroanilide. For the enzyme levels used, reactions were linear for >30 min. Reaction was measured by absorbance change at 400 nm. Peptidase units were expressed as μmoles of *p*-nitroaniline produced per milligram protein per min at 37°C.

Acknowledgements

We wish to thank David Summers (Cambridge) and our many colleagues in the Genetics Department for their interest, help and technical support in this project. We also thank Dr J.E.Fothergill (Aberdeen) for N-terminal sequencing of XerB and Drs C.Miller and C.F.Higgins for providing Pep$^-$ strains. Sue Halley helped prepare the manuscript. The experimental work was supported by the MRC and the SERC. The Biocomputing Research Unit thanks the Darwin Trust for support.

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Received on January 6, 1989