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Directed Evolution of Novel Biosynthetic Pathways: Overexpression of Leucine Aminopeptidase allows an *Escherichia coli* Proline Auxotroph to Grow Faster on Prolinamide

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Abstract

Selection of plasmids which confer a rapid growth phenotype on prolinamide from a library maintained in an *Escherichia coli* proline auxotroph reveals aminopeptidase I to be responsible for the growth; the enzyme has been overproduced, purified to homogeneity, characterised and crystallised in a form suitable for structure determination.

Introduction

As part of a programme aimed at investigating modes of biosynthetic pathway evolution we have recently assessed the growth of an *E. coli* proline auxotroph on a variety of proline precursors. In previous papers we have described the growth of an *Escherichia coli* proline auxotroph, strain X7026, on the unnatural precursor Δ^1-pyrroline-2-carboxylic acid[1,2,3,4] and recently we have extended the approach to a wide variety of alternative proline precursors[5]. Using prolinamide **2** as a simple precursor we have selected plasmids from mutagenised and wild-type libraries that confer a rapid growth phenotype on this substrate. To determine which enzyme/s in *E. coli* are capable of converting **2** to proline **1**, Fig. 1, we have analysed the inserts in these selected plasmids.

2 **1**

Fig. 1. Conversion of Prolinamide 2 to Proline 1

Results and Discussion

Plasmid DNA was isolated from seven colonies that displayed an enhanced growth phenotype on prolinamide **2**. Restriction endonuclease digestion analysis quickly revealed that all the plasmids contained a common insert but aligned in either direction and with differing amounts of upstream and downstream DNA, Fig. 2. Given the fact that the polylinker-derived *Kpn* I site was still present in all the plasmids and that the common region of the insert contained a *Kpn* I site, we were able to employ a rapid deletion strategy to obtain internal sequence. Accordingly, plasmids 2 and 8 were cut with *Kpn* I and religated to produce plasmids bearing deletions extending from the insert *Kpn* I site in either direction. The partial insert sequence obtained from these deleted plasmids using universal primer was identical with that reported by Sherratt's group for the *xerB* gene[6].

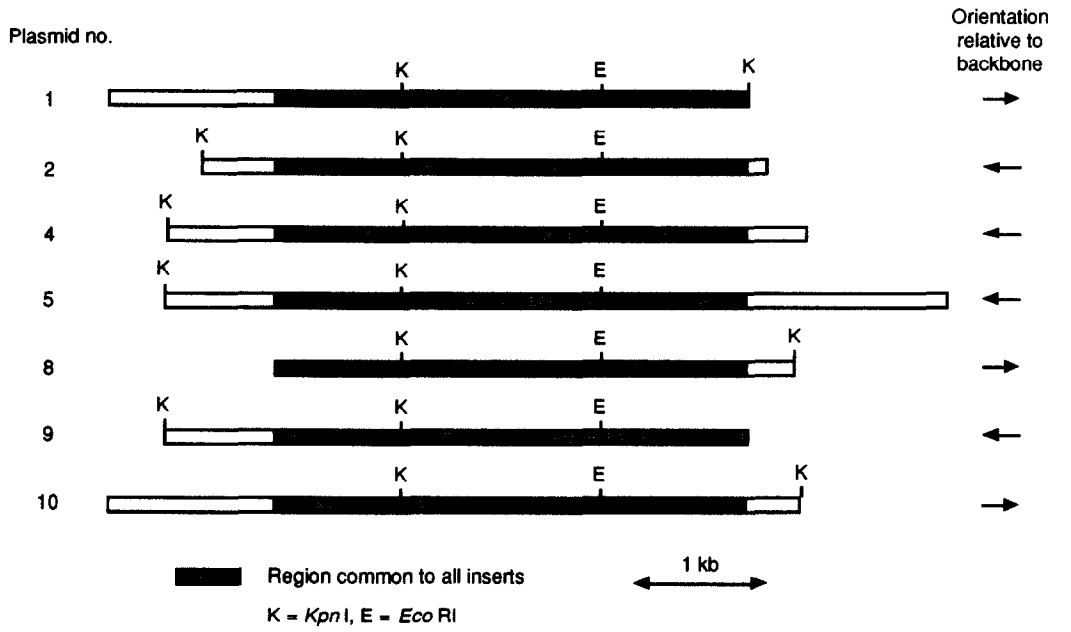

Fig. 2. Selected Plasmid Inserts

On the basis of sequence similarities, Sherratt's group concluded[6] that the *xerB* gene product was the same as aminopeptidase I of *E. coli* isolated by Vogt[7]. The finding that overexpression of an aminopeptidase increased growth of our proline auxotroph on prolinamide **2** initially seemed unsurprising. Vogt did not assess the activity of aminopeptidase I with proline derivatives but, on the basis of an extensive substrate specificity study, concluded that in its substrate profile the enzyme closely resembled leucine aminopeptidase[7]. Other workers have measured the rates of hydrolysis of proline derivatives by leucine aminopeptidase and have found that for enzymes from four different sources that the optimal substrate tested, LeuLeu is hydrolysed between 330 and 950 times as fast as **2**[8]. Vogt found that *E. coli* aminopeptidase I hydrolyses tripeptides faster than dipeptides (MetLeuGly 190 times faster than LeuLeu[7]) thus it appeared that **2** is most likely a very poor substrate for the enzyme we selected (>10,000 times more slowly hydrolysed than a good tripeptide substrate). Measurement of specific activities towards leucine or proline *p*-nitroanilides in crude lysates suggests that plasmid no. 2 directs about 100 fold overexpression of aminopeptidase I.

To investigate the hydrolysis of **2** further and to obtain structural information we required a high yielding source of the *E. coli* enzyme. To construct a higher producing source to facilitate purification we made use of PCR. An *Nco* I site incorporating the ATG initiation codon, was introduced at the 5'-terminus of the gene and a *Bam* HI site at the 3'-terminus by use of 5' and 3' internal mismatch primers[9]. The fragment was amplified from plasmid no. 2 and subcloned into the *Nco* I/*Bam* HI sites of pCE711[4] giving pJA101, Fig. 3. Upon IPTG induction of *E. coli* NM554 [pJA101], aminopeptidase I was overexpressed to the extent of about 20% of total cell protein (80% of low salt insoluble protein).

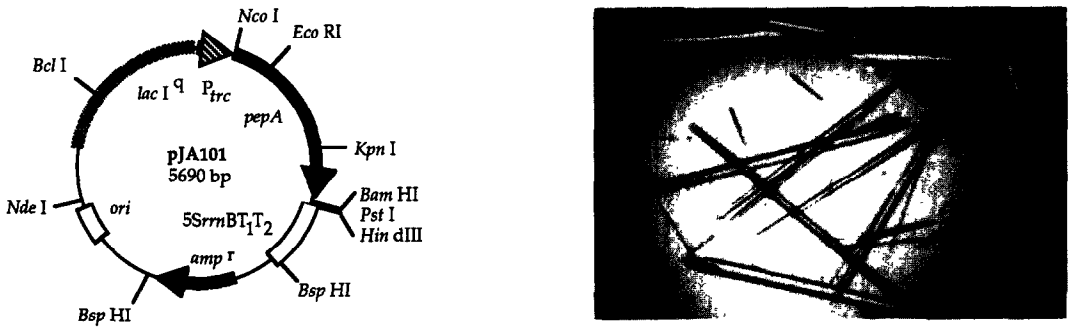

Fig. 3. *xerB* Expression Vector pJA101 and Crystals of Aminopeptidase I

Insolubility in low salt and moderate thermal stability are known properties of the enzyme and could be conveniently exploited to purify the enzyme using a method similar to that of Vogt although, because of the high level of overexpression, no chromatography was necessary to obtain apparently homogeneous enzyme. Using *E. coli* NM554 [pJA101] we can obtain > 40mg of purified material per litre of initial culture. In order to obtain structural information to complement our kinetic studies we have crystallised aminopeptidase I from buffer containing 100-250mM KCl and 0.5-2.0% PEG 3350 this gives needle shaped crystals over 150μm in length suitable for preliminary X-ray crystallographic analysis, Fig. 3 (other conditions result in much smaller needles *ca.* 30μm in length similar to those first described by Vogt[7]; some of these smaller crystals can be seen in the plate in Fig. 3). To investigate whether the gene we isolated by selection was mutant or wild-type we have compared the kinetic properties of aminopeptidase I derived from pJA101 with that derived from the original Sherratt clone pCS126[6]. Using recrystallised enzyme we have determined the Michaelis-Menten parameters for aminopeptidase I from both sources with proline and leucine *p*-nitroanilides. The values (pJA101 derived material: leucine *p*-nitroanilide; v_{max} = 7.1 I.U., K_m = 0.7mM, proline *p*-nitroanilide: v_{max} = 7.7 I.U., K_m = 2.0mM, pCS126 derived material: leucine *p*-nitroanilide; v_{max} = 6.3 I.U., K_m = 0.9mM, proline *p*-nitroanilide: v_{max} = 3.1 I.U., K_m = 2.4mM) are within experimental error suggesting that the aminopeptidase I from pJA101 does not contain a functionally significant mutation. It thus appears that 100 fold overexpression of aminopeptidase I is sufficient to confer an increased growth phenotype on prolinamide **2** to an *E. coli* proline auxotroph.

Having successfully cloned and expressed *xerB* we are seeking to investigate the potential for gene mutations to improve the enzyme's activity with respect to prolinamide **2** for which it was selected. In order to maximise the advantage of beneficial mutation of this nature it is necessary to reduce the level of expression of the encoding gene. Expression of *xerB* from pJA101 in the absence of IPTG is sufficient to confer strong growth on media containing prolinamide due to the 'leaky' nature of the *trc* promoter and therefore beneficial mutations would confer no further advantage. Accordingly we have deleted the promoter by restricting pJA101 with *Bcl I* and *Nco I*, filling-in and recircularisation. The resulting plasmid, pJA102, when transformed into the *xerB*$^-$ host, DS957[6] conferred slightly increased growth on **2** relative to a control using a similar plasmid lacking *xerB*. pJA102 mutagenised by passage through strain MW1000 in minimal media containing **2** has now been introduced into DS957. Results of experiments involving selection of DS957[pJA102mut] on **2** will be reported in due course.

Acknowledgements

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References & Footnotes

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9 Oligonucleotides were synthesised by Val Cooper using standard phosphoramidite chemistry on an Applied Biosystems 380B DNA Synthesiser (non-complementary regions are shown underlined):

5' internal mismatch primer: 5'-GATTCAGGAGCGTAGTCCATGGAGTTTAG-3'

3' internal mismatch primer: 5'-GGCGTTCACGCCGGATCCGGCAATAACAG-3'.

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