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DIRECTED EVOLUTION OF NOVEL BIOSYNTHETIC PATHWAYS: GROWTH OF AN *ESCHERICHIA COLI* PROLINE AUXOTROPH ON Δ^1-PYRROLINE-2-CARBOXYLIC ACID.

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ABSTRACT.

A directed approach to the evolution of novel biosynthetic pathways is exemplified by the selection of plasmids which enhance the growth of an *Escherichia coli* proline auxotroph on an unnatural precursor, Δ^1-pyrroline-2-carboxylic acid **2**.

INTRODUCTION.

A major goal of Bioorganic Chemistry is the realisation of new methodologies for the production of natural and unnatural products. In the last twenty years the use of whole cells and isolated enzymes, both wild-type and mutant, has burgeoned and more recently the possibility of using alternative biological catalysts such as catalytic antibodies and ribozymes has been realised. Many problems still remain with these new technologies however and, in a large number of cases, conventional organic synthesis, despite its expense and attendant environmental problems, still has to be relied on. Indeed, some chemistry is unlikely ever to be achieved using biological catalysts because of the incompatibility of certain functional groups with the conditions required for function of biological macromolecules. Despite this latter *caveat*, there are still a number of organic transformations which can be envisioned to be biologically feasible but for which a biological catalyst remains to be found. Current biological catalysts are the result of Darwinian evolution by mutation and selection for novel or improved function and we have been interested in ascertaining whether presumed modes of biosynthetic pathway evolution can be mimicked to produce new biosynthetic pathways to materials of interest currently made by conventional synthesis. In an attempt to demonstrate the feasibility of this approach we have focussed, initially, on the simple natural metabolite, proline. In this and the following papers we describe preliminary results of our efforts to introduce novel proline biosynthetic pathways to the bacterium, *Escherichia coli*.

BIOSYNTHETIC PATHWAY EVOLUTION.

The literature abounds with theories of biosynthetic pathway evolution and an extensive discussion is not appropriate at this juncture, suffice it to say that from a potentially exploitative point of view the theory of retrograde biosynthetic pathway evolution first propounded by

Horowitz in 1945[1] seemed a good starting point. In essence the Horowitz hypothesis involves the acquisition of biosynthetic steps backwards in a situation where the chemical intermediates already exist in the environment. Thus if a compound, C is used by an organism and biological use renders it scarce then there will be a strong competitive advantage for those organisms which evolve to catalyse the conversion of a more abundant compound, B to C. In time, B likewise becomes depleted and an advantage accrues to those organisms that evolve to catalyse the conversion of another abundant material, A into B and thence into the essential C.

In order to evaluate this possible mode of evolution for proline synthesis there are two basic requirements; an organism that cannot already synthesise proline and one or more chemical syntheses of proline which proceed through stable intermediates and involve transformations which are potentially biologically feasible. We have chosen to use a proline auxotroph of *Escherichia coli* as the organism and chemical syntheses which have as their last step, the reduction of Δ^1-pyrroline-2-carboxylic acid **2** to proline.

DIRECTED EVOLUTION OF NEW BIOSYNTHETIC PATHWAYS TO PROLINE.

The natural biosynthetic pathway to proline[2], Fig. 1, involves phosphorylation of glutamate and subsequent reduction to give γ-glutamyl semialdehyde which cyclises, dehydratively, giving Δ^1-pyrroline-5-carboxylic acid **1**. This cyclic imine is then reduced to proline by the *pro* C gene product.

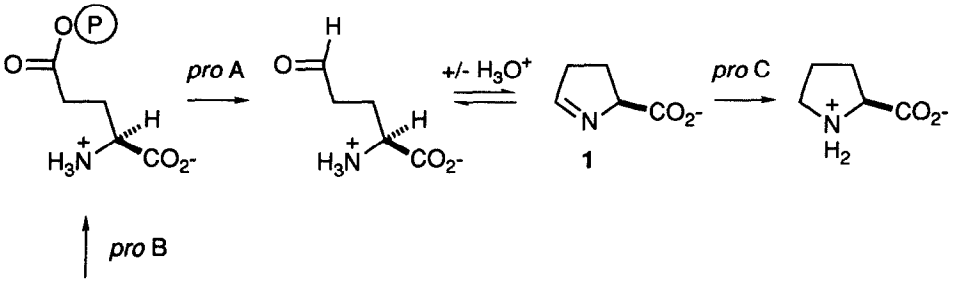

Fig. 1 Proline Biosynthesis in *Escherichia coli*

The first two steps of the biosynthesis cannot operate in *E. coli* X7026[3] in which the *pro* A and *pro* B genes are deleted and this strain thus has an obligate requirement for proline to allow growth. Our choice of Δ^1-pyrroline-2-carboxylic acid **2** as the initial unnatural proline precursor was influenced by various factors notably the presumed biological plausibility of the reduction to proline and the flexibility of synthetic routes to **2**, Fig. 2[4].

Our initial strategy then was to mutate *E. coli* strain X7026 and to select for mutants which could grow on this precursor. Any such mutants could then be remutagenised and selected for growth on chemical precursors to **2**. Repetition of this process, we envisaged, would allow us to build up a multi-step biosynthetic pathway to proline by this "teaching" procedure[5].

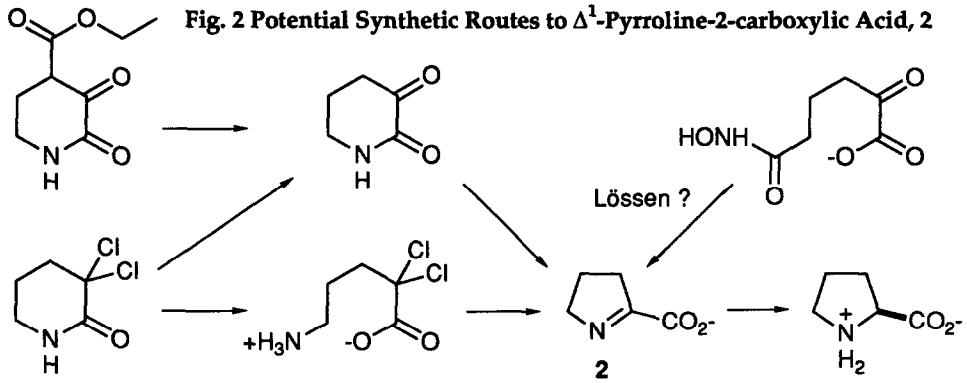

Fig. 2 Potential Synthetic Routes to Δ^1-Pyrroline-2-carboxylic Acid, 2

RESULTS & DISCUSSION.

As a control experiment we plated *E. coli* X7026 on minimal plates containing either proline ($50\mu gml^{-1}$), **2**[6] ($80\mu gml^{-1}$) or no additives. Remarkably the bacteria showed significant growth on the plates containing **2** although colonies were not as large as those on the plates containing proline. It thus appeared that without mutagenesis the first imposed step of our unnatural biosynthesis of proline could already be catalysed by *E. coli*. In an attempt to establish which gene was responsible for the growth on **2** we prepared a library of *E. coli* X7026 DNA in a high copy number vector. We reasoned that by introducing this library back into *E. coli* X7026 and plating on **2** we should observe enhanced growth of colonies harbouring plasmids containing the responsible gene.

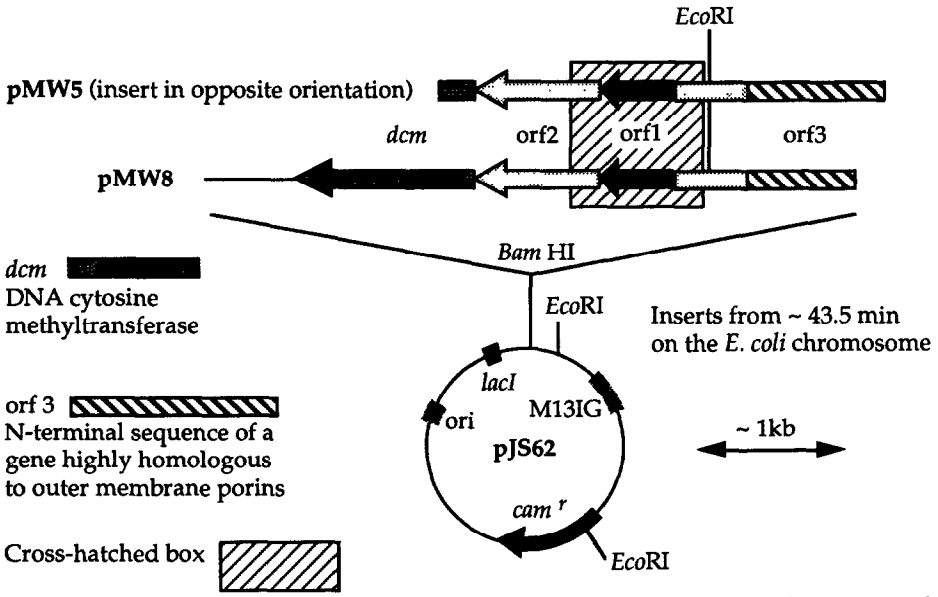

Fig. 3 Map of Plasmids Associated with Increased Growth on 2

The library was constructed by subcloning 5-10kb *Sau* 3A fragments of *E. coli* X7026 chromosomal DNA into the *Bam* HI site of the high copy-number plasmid, pJS62[7]. Transformation of the ligation mixture into *E. coli* NM554[8] gave a library of $4x10^4$ colonies >95% of which harboured insert containing plasmids. Pooled plasmid DNA was used to transform *E. coli* X7026 giving $2x10^4$ colonies which were then plated on minimal medium containing **2** and arginine to prevent selection for *arg* D mutations[9]. Following purification by streaking and retransformation of DNA from positives, several plasmids were obtained which were consistently associated with enhanced growth on **2**. Restriction endonuclease mapping showed two distinct species, pMW5 and pMW8, which contained inserts of 3.2kb and 4.4kb respectively. These contained a 2.9kb region common to both plasmids but aligned in opposite directions with respect to the plasmid backbone and with differing amounts of flanking sequence Fig. 3.

Extensive biochemical and molecular biological analyses to be reported elsewhere have suggested that the open reading frames in the insert common to pMW5 and pMW8 do not encode a reductase but are instead associated with a change in outer membrane permeability to **2** and certain other compounds[10]. We therefore conclude that permeability is limiting for growth of the proline auxotroph, X7026 on **2**.

Results of our studies with other 'unnatural' proline precursors will be reported in due course.

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