Genet. Res., Camb. (1981), **38**, *pp.* 171–195
With 3 text-figures
Printed in Great Britain

A Method for the Selection of Deletion Mutations in the L-Proline Catabolism Gene Cluster of *Aspergillus nidulans*

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(*Received* 14 *January* 1981 *and in revised form* 18 *March* 1981)

SUMMARY

Interest in the selection of mutations affecting L-proline catabolism in *Aspergillus nidulans* is heightened by the involvement of one of the very few examples of a cluster of functionally related genes in an eukaryote and by an increasing awareness of the biological phenomena in which proline and proline catabolism participate. The *sas*A-60 (semialdehyde sensitive) mutation in *A. nidulans* results in toxicity of catabolic precursors of L-glutamic γ-semialdehyde (or its internal Schiff base L-Δ^1-pyrroline-5-carboxylate) and succinic semialdehyde, apparently without affecting the catabolic pathways concerned. As *sas*A-60 is unlinked to the *prn* gene cluster, specifying the gene products necessary for L-proline catabolism and as L-proline, a precursor of L-glutamic γ-semialdehyde, is highly toxic to *sas*A-60 strains, this forms the basis of a powerful positive selection technique for obtaining a number of types of *prn* mutations. Many of these *prn* mutations can be directly classified according to the gene product(s) affected on the basis of growth phenotype with respect to L-arginine and L-ornithine utilization, proline-dependent resistance to certain toxic amino acid analogues and effect on supplementation of proline auxotrophies. The availability of both a positive selection technique and an extensive nutritional screening system has enabled the identification of fourteen spontaneous deletion mutations, recognized as extending into the *prn*B gene, specifying the principal L-proline permease, and into at least one other *prn* gene. These deletion mutations have been partially characterized both genetically and biochemically. In particular their use has greatly facilitated fine-structure mapping of the *prn* cluster and aided studies of the regulation of *prn* gene expression.

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1. INTRODUCTION

In the ascomycete fungus *Aspergillus nidulans* the genes involved in L-proline catabolism (see pathway in Fig. 1) form a cluster in linkage group VII. The gene order is *prn*A–*prn*D–*cis*-acting regulatory region–*prn*B–*prn*C (Arst & MacDonald, 1975, 1978). *prn*D and *prn*C are the structural genes for proline oxidase and Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, respectively; *prn*B is probably the structural gene for the principal L-proline permease; and *prn*A is almost certainly a positive acting regulatory gene whose diffusible product mediates induction of the synthesis of the other *prn* gene products by L-proline (Arst & MacDonald, 1978; Jones, Arst & MacDonald, 1981). *Cis*-acting regulatory mutations (designated prn^d) affecting the expression of at least *prn*B map in the regulatory region (Arst & MacDonald, 1975; Arst, MacDonald & Jones, 1980*b*). Deletion of the regulatory region considerably but incompletely reduces expression of *prn*C in *cis*, suggesting that the *prn*C product P5C dehydrogenase is synthesised from both a dicistronic *prn*B *prn*C messenger and an overlapping messenger which might be mono-, tri- or tetracistronic (Arst & MacDonald, 1978).

Analysis of the organization and regulation of the *prn* cluster will benefit in proportion to the number of types of *prn* mutations available for study. Here we describe a system for the selection and classification of a number of types of prn^- mutations, including several classes of deletion mutation. The phenotypes of a number of deletion mutations selected using this system have been determined, and a series of heterozygous deletion crosses has enabled construction of a fine-structure genetic map of the *prn* cluster.

Beyond their immediate importance in facilitating analysis of a rare example of an eukaryotic gene cluster, the selective techniques we describe might find application in other experimental systems. The availability of a variety of techniques for selecting mutations affecting proline catabolism might be especially welcome now that there is an increasing awareness of the diversity of biological phenomena which involve proline and its catabolism. For example, in both mammalian cells (Phang, Downing & Yeh, 1980) and insect muscle (Balboni, 1978; Pearson, Imbuga & Hoek, 1979) there is evidence that the interconversion of proline and P5C plays an important role in energy production. It has further been proposed that this cycle forms the basis for a mutually beneficial metabolic interaction between erythrocytes and tissue cells (Phang, Yeh & Hagedorn, 1981). There are clinically defined abnormalities associated with hyperprolineaemia resulting from hereditary deficiency of proline oxidase or P5C dehydrogenase in humans and other mammals (Blake, 1972; Valle, Phang & Goodman, 1974; Rosenberg & Scriver, 1974). Proline can apparently act as a cryoprotectant for cultured plant cells (Withers & King, 1979) which accords with a positive correlation between proline accumulation and freezing tolerance in a number of plant species (reviewed by Stewart & Larher (1980)). An extensive array of literature presents evidence that proline accumulation functions in osmoregulation in bacteria (Measures, 1975; Dhavises & Anagnostopoulos, 1979; Csonka, 1980),

yeast (Ho & Miller, 1978) and higher plants (reviewed by Moore (1975), Stewart & Larher (1980) and a number of authors in the volume edited by Rains, Valentine & Hollaender (1980)). Of course, it is also possible that, through analogy, these selective techniques can be extrapolated to yield selective techniques for mutations affecting catabolism of metabolites other than proline.

2. MATERIALS AND METHODS

(i) *Genetic techniques and growth testing*

The genetic techniques described by Pontecorvo *et al.* (1953), McCully & Forbes (1965) and Clutterbuck (1974) were employed. Growth testing of *A. nidulans* has been described by Arst & Cove (1969, 1973). The minimal media described by Cove (1966) were used throughout. Unless otherwise specified, these contained 1 % (w/v) D-glucose as carbon source and were incubated for growth at 37 °C.

Deletion mapping in the *prn* cluster has been described previously (Arst & MacDonald, 1978). Proline-utilizing progeny were recovered by top-layering ascospore suspensions into glucose–minimal solid medium (Cove, 1966), appropriately supplemented and containing (final concentrations) 5 mM L-proline as sole nitrogen source and 0·08 % (w/v) sodium deoxycholate (Mackintosh & Pritchard, 1963) to restrict colony diameter. Top-layered plates were then incubated 2–3 days at 37 °C except for crosses involving cryosensitive *prn*$^-$ mutations, which were incubated 4–5 days at 25 °C. Recovery of *prn*$^+$ recombinants in crosses involving leaky *prn*$^-$ mutations was facilitated by the inclusion of 15–20 mM-Cs^+ (as the chloride) in the medium. Cs^+ accentuates reduced nitrogen source utilization (Rand & Arst, 1977; Jones *et al.* 1981). The total progeny of at least five (but more in crosses of low fecundity) hybrid cleistothecia were tested in this way so that $1–3 \times 10^5$ progeny were tested. If no proline-utilizing progeny were recovered from at least 2×10^5 tested, it was concluded that the deletion mutation fails to recombine with the *prn*$^-$ mutation in repulsion. In crosses where *prn*$^+$ progeny were recovered, the expected segregation of markers other than *prn*$^-$ mutations was confirmed. At least one and usually two or three of the flanking markers *panto*B-100, *s*F-211 and *mah*A-10 (Arst, 1977*b*; Arst & MacDonald, 1978) segregated in each cross, and many crosses involved strains carrying *prn*^{d} mutations (Arst & MacDonald, 1975; Arst *et al.* 1980*b*) which map in the centre of the cluster. In each case, the distribution of these markers amongst proline-utilizing progeny conformed to that predicted from the established map order.

(ii) *Mutations*

With the exceptions listed below markers carried by strains of *A. nidulans* used here have been described previously (Arst & Cove, 1973; Clutterbuck, 1974; Arst, 1977*b*; Arst & MacDonald, 1975, 1978; Arst *et al.* 1980*b*; Arst, Bailey & Penfold, 1980*a*; Jones *et al.* 1981).

(*a*) sas*A-60*

This mutation was obtained fortuitously during *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) induced (Alderson & Hartley, 1969) reversion of the *ppp*A-7 (loss of transaldolase resulting, *inter alia*, in inability to utilize pentoses (Hankinson, 1974)) marker in a strain also carrying *pabaA*-1 (*p*-aminobenzoate auxotrophy) on appropriately supplemented minimal medium containing 1% (w/v) D-xylose as sole carbon source and 10 mM-ammonium (chloride) as nitrogen source at 37 °C. Other mutations having the same phenotype as *sas*A-60 have been selected because of their reduced growth on proline-containing media, e.g. in the experiment in which *prn*A-46 and -49 were selected (see below). The allelism of these mutations to *sas*A-60 has not been tested, but they can apparently be induced with a frequency similar to that of mutations in any one of the *prn* genes. The phenotype of *sas*A-60 is described in detail in the Results and Discussion section.

(*b*) aln*A-1*

This mutation was selected following ultraviolet mutagenesis of a strain of genotype *pabaA*-1, after replica plating (Mackintosh & Pritchard, 1963), as resulting in inability to utilize 5 mM L-alanine as nitrogen source. *aln*A-1 strains cannot utilize L-alanine as carbon or nitrogen source but are indistinguishable from the wild type for utilization of a wide range of other carbon and nitrogen sources. As the compounds tested include carbon sources probably catabolized via pyruvate (D(−)- and L(+)-lactate), carbon and nitrogen sources catabolized via glutamate (L-proline, L-ornithine, L-aspartate, γ-amino-*n*-butyrate (GABA) and L-glutamate itself – see Arst, Parbtani & Cove, 1975), and other nitrogen (and, where applicable, carbon) sources with some structural similarity to L-alanine (glycine, L- and D-α-amino-*n*-butyrate, L-serine, L-threonine and β-alanine), *aln*A-1 would appear to lead to loss of a highly specific catabolic L-alanine transaminase. This transaminase is apparently not involved in supplementation of *ile*A$^-$ L-isoleucine auxotrophies resulting from loss of threonine dehydratase (MacDonald, Arst & Cove, 1974) by L- or D-α-amino-*n*-butyrate (Arst & Cove, 1973) because such supplementation occurs normally in *ile*A-1 *aln*A-1 double mutants. Recessive in diploids, *aln*A-1 is located in linkage group VII but it recombines freely with *gat*A-1, leading to loss of GABA transaminase (Arst, 1976; Bailey, Arst & Penfold, 1980) and *ota*A-2, leading to loss of ornithine δ-transaminase (Piotrowska, Sawicki & Weglenski, 1969; Arst & MacDonald, 1975; Arst, 1977*a*) as well as with mutations in the *prn* cluster.

(*c*) prn*A*$^-$ *mutations*

*prn*A-15, -16, -17, -27, -29 and -38 are spontaneous mutations selected as conferring resistance to 50 mM L-proline in appropriately supplemented minimal medium containing (final concentrations) 1% (v/v) ethanol as carbon source and 595 μM uric acid as nitrogen source at 37 °C in a strain of genotype *pro*A-6 *sas*A-60

*s*F-211*fw*A-1 (L-proline requiring, hypersensitive to P5C and succinic semialdehyde toxicities, inhibited by a metabolite derived from sulphate, fawn conidial colour). *prn*A-46 and -49 are ethyl methanesulphonate induced (Alderson & Clark, 1966) mutations selected in a strain of genotype *y*A-2 *panto*B-100 *prn*^{d}-20 (yellow conidial colour, D-pantothenate requiring, derepressed L-proline transport), using replica plating, because they result in inability to utilize 5 mM L-proline as nitrogen source in appropriately supplemented glucose-minimal medium at 37 °C. *prn*A-79 and -80 are spontaneous mutations selected in the same way as *prn*A-15, etc. but in strains of genotypes *pro*A-6 *sas*A-60 *mah*A-10 (hypersensitive to methylammonium) *s*F-211 and *pro*A-6 *sas*A-60 *prn*^{d}-22, respectively. *prn*A-101 is a spontaneous mutation selected in a strain of genotype *bi*A-1 *paba*A-1 *prn*C-61 *fw*A-1 (biotin requiring, *p*-aminobenzoate requiring, lacking P5C dehydrogenase and therefore unable to catabolize L-proline, fawn conidial colour) as conferring resistance to the toxicity (due to *prn*C-61) of 10 mM L-proline on appropriately supplemented glucose-minimal medium with 10 mM-NO_3^- (as the Na^+ salt) as nitrogen source at 37 °C. *prn*A-121 was selected in the same way as *prn*A-79 but at 25 °C. *prn*A-154 and -155 are NTG induced mutations selected in the same way as *prn*A-46 and -49 but in a strain of genotype *y*A-2 *al*X-4 (lacking allantoinase) *panto*B-100. *prn*A-217 is a spontaneous mutation selected in a strain of genotype *y*A-2 *pro*B-9 (L-proline requiring) *prn*D-156 (lacking proline oxidase and therefore unable to catabolize L-proline) *panto*B-100 because it confers resistance to the toxicity of 50 mM L-proline (due to *prn*D-156) in appropriately supplemented minimal medium containing 1 % (v/v) ethanol as carbon source and 595 μM uric acid as nitrogen source at 25 °C.

(*d*) prn*B*$^{-}$ *mutations*

*prn*B-81 and -82 are spontaneous mutations selected in the same experiment as *prn*A-80. *prn*B-109 is a spontaneous mutation selected as conferring resistance to 5 mM L-proline on appropriately supplemented glucose-minimal medium with 10 mM ammonium (as the (+)-tartrate) as nitrogen source at 37 °C in a strain of genotype *pro*A-6 *sas*A-60 *prn*^{d}-22.

(*e*) prn*C*$^{-}$ *mutations*

*prn*C-180 and -181 are spontaneous mutations selected in a strain of genotype *y*A-2 *pro*A-6 *panto*B-100 as conferring resistance to the toxicity of 5 mM D-serine in the presence of 5 mM L-proline on appropriately supplemented glucose-minimal medium containing 10 mM ammonium (as the (+)-tartrate) as nitrogen source at 37 °C. This resistance to D-serine which is conditional upon the presence of L-proline is discussed in section i (*d*) of the Results and Discussion.

(*f*) prn*D*$^{-}$ *mutations*

*prn*D-62 was selected in the same experiment as *prn*A-46 and -49. *prn*D-90 was selected by Mr D. W. Tollervey (unpublished). It is an ultraviolet-induced mutation selected as allowing utilization of 5 mM L-alanine as nitrogen source on appropriately

supplemented glucose–minimal medium at 37 °C in a strain of genotype *bi*A-1 *paba*A-1 *are*A^{r}-18 (biotin requiring, *p*-aminobenzoate requiring, lacking a positive acting regulatory molecule mediating nitrogen metabolite repression and consequently unable to utilize nitrogen sources other than ammonium). That the *prn*D-90 mutation is itself responsible for the weak suppression of *are*A^{r}-18 for L-alanine utilization was confirmed by selection of another probable *prn*D^{-} mutation in this way (D. W. Tollervery, unpublished data) and by co-segregation of this property with inability to utilize L-proline (as a carbon source because *are*A^{r} mutations prevent its use as a nitrogen source) and L-proline-dependent resistance to D-serine (see section i (*d*) of Results and Discussion). Weak suppression of *are*A^{r} mutations for L-alanine utilization seems to be a general property of *prn*D^{-} mutations because *prn*D-66, -67 and -156 all have this effect in double mutants with *are*A^{r}-1. Even more marginal suppression of *are*A^{r}-1 for L-alanine utilization is exerted by *prn*C-61 and -64. The metabolic basis for such suppression is unclear, but it does resemble L-proline-dependent D-serine resistance (section i (*d*) of Results and Discussion) in that it requires functional *prn*A and *prn*B alleles. Thus no suppression occurs in *are*A^{r}-1 *prn*D-156 *prn*B-216 triple mutants or in *are*A^{r}-1 strains carrying the *prn*-306 or -309 deletion mutations (see below). Similarly no suppression occurs in *are*A^{r}-1 *prn*C-61 *prn*A-101 or *are*A^{r}-1 *prn*C-61 *prn*B-102 triple mutants. Unlike the conditional D-serine resistance conferred by *prn*D^{-} and *prn*C^{-} mutations, the weak suppression of *are*A^{r} mutations for L-alanine utilization is not dependent upon the presence of L-proline in the growth medium.

*prn*D-108 and -129 are spontaneous mutations whose selection was identical to that of *prn*A-79 *prn*D-158 is a spontaneous mutation selected in the same experiment as *prn*A-15, etc.

(*g*) prn *deletion mutations*

All fourteen *prn* deletions are spontaneous mutations selected in *sas*A-60 (see below) strains for resistance to 50 mM L-proline on appropriately supplemented minimal medium containing 1 % (v/v) ethanol as carbon source and 595 μM uric acid as nitrogen source at 37 °C. All deletion mutations selected and recognized by the procedure outlined in the Results and Discussion section were given sequential allele numbers beginning with 300. Allele numbers for other *prn* mutations carry no special significance. *prn*-300, -301 and -302 were selected in strains of genotype *pro*A-6 *sas*A-60 *s*F-211 *fw*A-1, *pro*A-6 *sas*A-60 *prn*^{d}-22, and *pro*A-6 *sas*A-60 *prn*^{d}-22 *prn*B-109, respectively. *prn*-303 through -313 were selected in a strain of genotype *pro*A-6 *sas*A-60 *mah*A-10 *s*F-211. Further details of the selection, recognition and characterization of *prn* deletion mutations are given in the Results and Discussion section.

(iii) *Gene assignments for* prn^{-} *mutations*

Allocation of *prn*$^{-}$ mutations to one or more of the four *prn* genes is based upon three criteria: (1) phenotype, as described previously (Arst & MacDonald, 1975, 1978; Arst *et al.* 1980*a*; Jones *et al.* 1981) and further in the Results and Discussion

section (2) map position, as determined previously (Arst & MacDonald, 1978) and more accurately in Fig. 3. (3) complementation responses using a set of four diploids constructed with a standard *prn*A$^-$, *prn*B$^-$, *prn*C$^-$ or *prn*D$^-$ allele respectively in repulsion. For all *prn*$^-$ mutations except *prn*-313, this also served to demonstrate recessivity because full complementation was observed with at least one of the standard *prn*$^-$ mutations. For *prn*-313 it was additionally necessary to construct a *prn*-313/*prn*$^+$ diploid to confirm that *prn*-313 is recessive.

(iv) *Growth of mycelia in shaken liquid culture for use in uptake studies and* in vitro *analysis*

Mycelia were grown for 8 h at 37 °C or 21 h at 25 °C in shaken liquid minimal medium (Cove, 1966) supplemented with (final concentrations) 10 μg l.$^{-1}$ biotin, 2 mg l.$^{-1}$ *p*-aminobenzoic acid, 595 μM uric acid (as nitrogen source) and 1 % (w/v) D-glucose (as carbon source). L-proline, at a final concentration of 5 mM, was added at 6 h at 37 °C and at 16 h at 25 °C to induce the *prn* activities as indicated. All strains grown in liquid culture for uptake studies, enzyme assays, thermal denaturation studies or crossed immunoelectrophoresis carry the *p*-aminobenzoate auxotrophy *paba*A-1. In addition the *prn*-303, -307, -311 and -312 strains carry the fawn conidial colour mutation *fw*A-1 and the *prn*-303, -305, -306, -307, -308, -309, -310, -311, -312 and -313 strains carry *mah*A-10, leading to methylammonium hypersensitivity.

(v) *Enzyme assays*

Mycelia were harvested as described by Cove (1966) and ground with an equal weight of acid-washed sand in a chilled mortar for several minutes to prepare cell-free extracts. Ten volumes of ice-cold extraction buffer (100 mM tris-HCl, pH 8·5, containing 500 mM sucrose) were then added and the mixture ground for several additional minutes to give a smooth paste. The mixture was centrifuged at 12000 ***g*** for 15 min at 4 °C in an MSE High Speed 18 centrifuge and the supernatant taken for enzyme assays or crossed immunoelectrophoresis. Proline oxidase (EC 1.4.3.2) was assayed by the method of Arst & MacDonald (1975) except that assays were done at 25 °C. P5C dehydrogenase (EC 1.5.1.12) was assayed in 1 cm cuvettes in a Pye Unicam SP 8000 double beam spectrophotometer at 25 °C. In a total volume of 1 ml the assay mixture contained 100 μl cell-free extract, 3·0 μmole NAD, 70 μmole tris-HCl, pH 8·5, and 50 μmole β-mercaptoethanol. The reaction was started by adding 600 nmole DL-P5C and the increase in absorbance at 340 nm was measured against a reference cell containing all components of the assay except P5C.

DL-P5C was synthesized either from DL-α-amino-δ-hydroxyvaleric acid (Cylco Chemical Corporation) by the method of McNamer & Stewart (1974) or from DL- and DL-*allo*-δ-hydroxylysine (Sigma London Chemical Co., Ltd.) by the method of Williams & Frank (1975). After reaction with *o*-aminobenzaldehyde, the concentration of DL-P5C was estimated colorimetrically.

The biuret method (Layne, 1957) was used to determine soluble protein in extracts.

(vi) *Proline transport measurements*

Uptake of L-[U-^{14}C]proline (Radiochemical Centre) was measured as described by Arst *et al.* (1980*b*).

(vii) *Thermal denaturation studies*

Mycelia were grown at 25 °C under induced conditions (*vide supra*). Cell-free extracts were prepared and incubated at 60 °C for up to 45 min in a water bath. Samples were withdrawn at regular time intervals and kept on ice for at least 10 min before being assayed for remaining P5C dehydrogenase activity. Regression lines were drawn through plots of the logarithm of per cent remaining activity against time and used to calculate half-lives. Results are the mean of three independent determinations.

(viii) *Production of P5C dehydrogenase antisera*

A description of the purification of P5C dehydrogenase will be published separately. The final step involved polyacrylamide gel electrophoresis. Purified P5C dehydrogenase was recovered by excising, from unstained sections of the polyacrylamide gel, the region corresponding to the activity band in stained duplicate sections (following Sealy-Lewis, Scazzocchio & Lee, 1978) and thoroughly homogenizing the gel slice in ice-cold 100 mM tris-HCl buffer, pH 8·5. Antisera were raised by a modification of the method of Harboe & Ingild (1973). Two New Zealand White female rabbits were injected intrascapularly on day 0 of the immunization programme with 1·0 ml of a 1:1 mixture of purified enzyme preparation and Freund's Complete Adjuvant (Difco Laboratories). Injections were repeated using Freund's Incomplete Adjuvant on days 14, 28 and 42. 20 ml of blood was collected from the marginal ear vein on day 50 and again on days 63 and 84, following 'booster' injections of antigens on days 56 and 77. Blood samples were left at room temperature overnight to ensure complete clotting. Sera were obtained as supernatants after centrifugation at 5000 ***g*** for 10 min, stored at −20 °C and used without further purification.

(ix) *Crossed immunoelectrophoresis*

Crossed immunoelectrophoresis using P5C dehydrogenase antisera was done by a modification of the method of Clarke & Freeman (1966) in which both first and second dimension electrophoretic runs were carried out on the same plate with no transfer step, as described by Lewis (1975). Gel preparation, running and staining followed methods of Axelsen, Kroll & Weekes (1973). The concentration of antisera used was 0·5 % (v/v) and gels contained 0·8 % (w/v) agarose in 50 mM tris-barbitone buffer, pH 8·6.

3. RESULTS AND DISCUSSION

(i) *A system for the selection and classification of* prn$^-$ *mutations*

(*a*) *The* sas*A-60 mutation*

One of the highest priorities for the selection of further classes of mutations in the *prn* cluster is a method to obtain deletion mutations. Recently 1,2,7,8-diepoxyoctane has been used to induce a high proportion of deletion mutations in *Neurospora crassa* (Ong & De Serres, 1975) and *A. nidulans* (Hynes, 1979; G. C. Ong & C. Scazzocchio, unpublished data), but at the time this work was undertaken, it seemed likely that spontaneous mutations would afford the highest probability of deletions, following results using prokaryotes (e.g. Schwartz & Beckwith, 1969; Ratzkin & Roth, 1978) and the work of Cove using *A. nidulans* (later published in Cove (1976*b*) and, after more extensive work, in Tomsett & Cove (1979)). However, it was expected (and subsequently confirmed) that even amongst spontaneous mutations, deletions would form only a small proportion. Thus it was imperative to devise a powerful positive selection technique capable of yielding large numbers of spontaneous *prn*$^-$ mutations.

The most powerful positive selection techniques are generally those in which mutations can be selected as conferring resistance to the inhibition of growth by a toxic compound. For example a toxic analogue of L-proline might, if a substrate for the *prn*B permease, provide a positive selection technique for obtaining *prn*B$^-$ mutations, some of which might be deletions extending into neighbouring genes. However, none of the available proline analogues is suitable for this approach in *A. nidulans*. Therefore, one obvious strategy is to search for a situation in which L-proline is itself toxic. However, like most of the products of primary metabolism, L-proline is not toxic to wild type *A. nidulans*, even at high concentrations. Nevertheless a selective method might be based on use of a mutant to which proline would be toxic. Using the yeast *Saccharomyces cerevisiae*, Meuris, Lacroute & Slonimski (1967) and Meuris (1969) have shown that mutations resulting in hypersensitivity to any of a variety of primary metabolites can be obtained and have identified several types of mechanisms responsible for such hypersensitivity.

Mutations resulting in considerable L-proline toxicity occur in the *prn* cluster itself. *prn*C$^-$ mutants are extremely inhibited by L-proline, presumably because they accumulate the highly reactive compound L-P5C (Arst & MacDonald, 1978; Arst *et al.* 1980*a*; Jones *et al.* 1981). *prn*D$^-$ mutants are subject to less but still considerable L-proline toxicity, showing that proline is itself toxic under conditions where it can be accumulated but not catabolized (Arst & MacDonald, 1978). Proline toxicity to both *prn*C$^-$ and *prn*D$^-$ mutants is sufficient to permit facile selection of spontaneous *prn*A$^-$ and *prn*B$^-$ mutations (Arst & MacDonald, 1978; Arst *et al.* 1980*a*; Materials and Methods section above). Whilst such use of *prn*C$^-$ and *prn*D$^-$ mutants enables several classes of double mutants to be obtained readily, it is not suitable as a general selection method for mutations in the *prn* cluster because of the difficulties of classifying the second *prn*$^-$ mutation in the

presence of the first and of separating the second *prn*$^-$ mutation so as to obtain singly mutant strains. For a general selection method, it would be desirable to have a mutation resulting in L-proline toxicity which (1) recombines freely with mutations in the *prn* cluster, (2) can be recognized in the absence of proline, enabling it to be scored in the presence of any *prn*$^-$ mutation, (3) has a phenotype as distinct as possible from that of any *prn*$^-$ mutation on proline-containing media so as to maximize sensitivity of screening *prn*$^-$ mutations, and (4) is subject to proline toxicity only after its conversion to P5C (or a further catabolite derived from P5C) so that it can be used for selection of mutations blocking proline catabolism as well as mutations preventing proline uptake. These criteria are fulfilled by a mutation designated *sas*A-60 (semialdehyde sensitive).

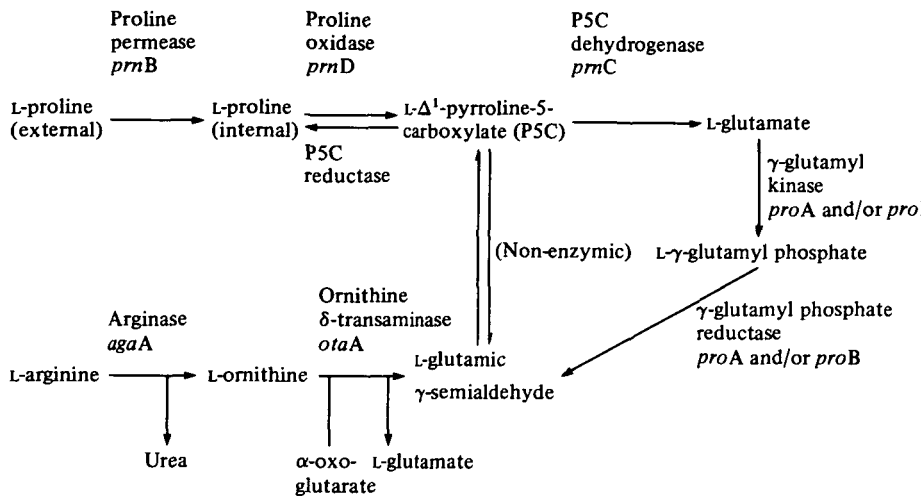

Fig. 1. Metabolic relationships between L-proline, L-arginine, L-ornithine and L-glutamate (Weglenski, 1966; Piotrowska *et al.* 1969; Arst & MacDonald, 1975, 1978).

The growth of *sas*A-60 strains is severely impaired by the presence of compounds such as L-proline, L-ornithine and L-arginine which can be converted to L-glutamic γ-semialdehyde (or its internal Schiff base L-P5C – see Fig. 1) and 2-pyrrolidone and γ-amino-*n*-butyrate (GABA) which can be converted to succinic semialdehyde (see Fig. 2). There is no direct evidence that *sas*A-60 strains are more susceptible to semialdehyde toxicity than the wild type, but this interpretation is a working hypothesis which is consistent both with the *sas*A$^-$ phenotype and with the classes of resistance mutations which can be obtained using an *sas*A-60 strain.

The basis for this apparent semialdehyde sensitivity is unclear. It is likely to result from a loss of function because *sas*A-60 is recessive in diploids and mutations having the *sas*A-60 phenotype (but whose allelism has not been tested) can be induced with a frequency similar to that for loss of function mutations in other genes (Arst, unpublished data). There is at least one other gene in *A. nidulans* where a common class of recessive mutations can apparently enhance aldehyde toxicity,

*ald*A . *ald*A$^-$ mutants were originally thought to be blocked in the oxidation of acetaldehyde to acetate because of their lack of growth on media containing ethanol or ethylammonium as carbon source (Page, 1971; Page & Cove, 1972). However, *ald*A$^-$ mutations also lead to toxicity of L-proline, 2-pyrrolidone and GABA, albeit to a much lesser degree than *sas*A-60. Thus *ald*A$^-$ mutations

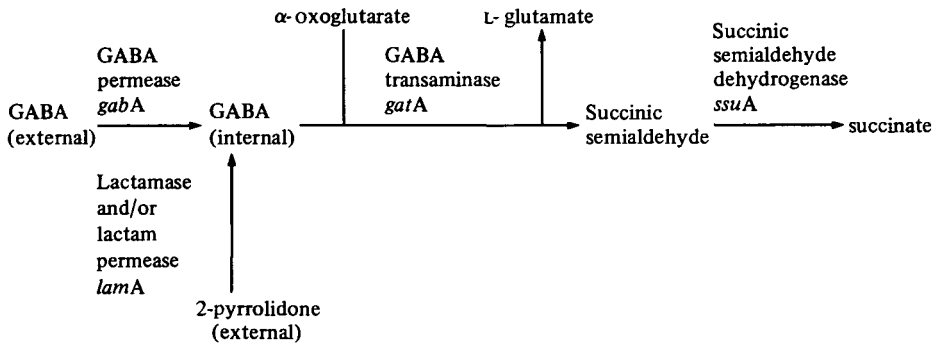

Fig. 2. Pathway for catabolism of GABA and 2-pyrrolidone in *A. nidulans* (Arst, 1976; Arst *et al.* 1978; Bailey *et al.* 1980).

probably enhance sensitivity to the toxicity of acetaldehyde rather than block its oxidation. As *ald*A$^-$ mutations apparently enhance sensitivity to the toxicities of acetaldehyde and, to a lesser extent, L-glutamic γ-semialdehyde and succinic semialdehyde, the *ald* mnemonic should probably now be understood as aldehyde sensitivity. *sas*A-60 apparently has no effect on acetaldehyde toxicity because it does not affect growth on ethanol or ethylammonium as carbon source. *ald*A$^-$ mutations are additive with *sas*A-60 in the degree of sensitivity of double mutants to precursors of L-glutamic γ-semialdehyde and succinic semialdehyde. This additivity, along with their different patterns of sensitivities, suggests that *ald*A$^-$ and *sas*A$^-$ mutations affect different functions. The two genes are unlinked: whereas *ald*A is in linkage group VIII (Page, 1971), haploidisation analysis (McCully & Forbes, 1965) has located *sas*A-60 to linkage group V.

(*b*) *Selection of resistance mutations using* sas*A-60 strains*

The toxicity of both L-proline and GABA to *sas*A-60 strains is sufficient that mutations conferring resistance can be selected extremely easily. However, both nitrogen metabolite (i.e. ammonium) repression (Arst & Cove, 1973) and carbon catabolite repression (Arst & Cove, 1973; Bailey & Arst, 1975; Arst & Bailey, 1977) protect, to some extent, *sas*A-60 strains against both compounds. Selection of resistance mutations is therefore facilitated by a medium containing derepressing carbon and nitrogen sources such as ethanol and uric acid, respectively.

The selection of GABA resistant derivatives of *sas*A-60 strains has yielded a variety of mutations reducing GABA transport including *gab*A$^-$ (Arst, 1976; Bailey, Penfold & Arst, 1979), *int*A$^-$ (Arst, 1976, 1981; Bailey *et al.* 1979) and *pac*C$^-$

(Arst *et al.* 1980*a*) alleles. Mutations in *gat*A, the putative structural gene for GABA transaminase (Arst, 1976; Bailey *et al.* 1980; see pathway in Fig. 2), cannot be selected using this procedure because loss of that enzyme results in even more extreme GABA toxicity (Arst, 1976; Bailey *et al.* 1980). Indeed *gat*A$^-$ strains have been used for the selection of mutations blocking GABA uptake or formation (Arst, Penfold & Bailey, 1978; Bailey *et al.* 1979; Arst *et al.* 1980*a*).

The classes of mutations which can be selected as conferring L-proline resistance to *sas*A-60 strains are outlined below. Every class of *prn*$^-$ mutation can be selected with the exception of *prn*C$^-$ mutations which, as noted above, results in very extreme (even greater than that of *sas*A-60) sensitivity to proline toxicity. Nevertheless certain categories of deletions extending into *prn*C (from other *prn* genes) can be selected using *sas*A-60. *prn*D$^-$ mutations can also be readily selected in this way because the degree of sensitivity to proline toxicity they confer (*vide supra*) is less than that conferred by *sas*A-60.

A crucial feature of the utility of *sas*A-60 for the selection of resistance mutations is the fact that it can be recognized on both GABA- and L-proline-containing media. This not only makes it easy to obtain *sas*A$^+$ strains carrying the mutations selected upon outcrossing but allows *sas*A$^+$ revertants to be classified in the initial screening of newly selected mutations.

(*c*) *The recognition of* prn*B*$^-$ *mutations*

In strains having a growth requirement for L-proline, *prn*B$^-$ mutations can be recognized independently of their reduced ability to catabolize proline. Arst & MacDonald (1975) showed that on media containing 1 % (w/v) D-glucose as carbon source and 10 mM ammonium as nitrogen source, *pro*A$^-$ and *pro*B$^-$ auxotrophs (see Fig. 1) can be supplemented by low concentrations (e.g. 125 μM) of L-proline only if the major proline permease, specified by *prn*B, is functional. Under these growth conditions, ammonium inhibits uptake of L-proline by one or more minor permeases (Arst *et al.* 1980*b*) so that supplementation requires participation of the *prn*B permease. Although *prn*B$^-$ mutations can be easily distinguished from *prn*A$^-$, *prn*C$^-$ and *prn*D$^-$ mutations by their lack of effect on L-arginine- and L-ornithine-containing media, the recognition of loss of *prn*B function in the presence of mutations affecting catabolism of arginine and ornithine as well as proline (as in the case of certain deletion mutations or double mutations) would be problematic if there were no independent *prn*B$^-$ phenotype. Use of a *pro*A$^-$ (or *pro*B$^-$) *sas*A-60 double mutant for selection of proline resistant mutants enables unambiguous classification of *prn*B functionality in every case. (It should be noted that, in the presence of glucose and ammonium, the low proline concentrations required for *prn*B classification are not toxic to *sas*A-60 strains).

(*d*) *The use of toxic amino acid analogues*

Not all mutations capable of protecting *sas*A-60 strains against proline toxicity map in the *prn* cluster. In addition to reversion to *sas*A$^+$ whose recognition is outlined above, mutations pleiotropically reducing uptake of a number of amino

acids can be selected in this way. These mutations might affect the minor permease(s) involved in proline uptake (Arst & MacDonald, 1975; Arst *et al.* 1980*b*) or some component common to several amino acid permeases including the *prn*B and/or minor proline permeases. Activities and/or components common to several amino acid transport systems in *S. cerevisiae* have been investigated by Grenson & Hennaut (1971), Roon, Levy & Larimore (1977), Roon, Meyer & Larimore (1977) and Penninckx, Jaspers & Wiame (1980).

Ordinarily, mutations which pleiotropically reduce uptake of a number of amino acids are easily recognized as reducing the utilization of those amino acids as carbon and/or nitrogen sources (e.g. Kinghorn & Pateman, 1975). However, because of the necessity to supplement the proline auxotrophy of the strain used for mutant selection and because growth of this strain on media containing L-proline, L-arginine, L-ornithine or GABA is impaired by *sas*A-60, pleiotropic amino acid uptake mutations are more easily detected as conferring resistance to amino acid analogues such as DL-*p*-fluorophenylalanine and/or D-serine.

The usefulness of D-serine extends, however, well beyond its role in the identification of pleiotropic amino acid uptake mutations. In the presence, but not in the absence, of L-proline, *prn*C$^-$ and *prn*D$^-$ mutations confer resistance to D-serine and another less toxic analogue, D-threonine. This characteristic facilitates classification of *prn*D$^-$ mutations selected as protecting *sas*A-60 strains against proline toxicity. Unlike pleiotropic amino acid uptake mutations, *prn*D$^-$ mutations do not confer D-serine resistance when the proline auxotrophy of the strain used for selection is supplemented with L-arginine (see Weglenski, 1966, and Fig. 1) rather than L-proline.

The basis for this conditional D-serine resistance resulting from *prn*D$^-$ mutations is unclear, but a reasonable hypothesis would be that proline accumulation in the absence of catabolism interferes with (e.g. inhibits) D-serine uptake. The presence of either a *prn*B$^-$ or a *prn*A$^-$ mutation prevents this L-proline-dependent D-serine resistance of *prn*D$^-$ strains. As *prn*A$^-$ mutations reduce expression of the *prn*B permease (Arst & MacDonald, 1978), the basis for reversal of resistance by *prn*A$^-$ mutations might be the same as that by *prn*B$^-$ mutations. *prn*C$^-$ mutants still show some proline-dependent D-serine resistance in the absence of the *prn*B permease, a finding which aids in the classification of deletion mutations extending from within *prn*B into *prn*C (see below).

L-proline-dependent D-serine resistance is not the only example of this form of conditional D-serine resistance. L-alanine which protects all strains to some extent against D-serine toxicity (presumably by competition for a common permease), exerts a much more effective L-alanine-dependent D-serine resistance in *aln*A-1 strains. *aln*A-1 probably leads to loss of a catabolic L-alanine transaminase (see Materials and Methods). *ota*A-2 strains, lacking ornithine δ-transaminase (Piotrowska *et al.* 1969; Arst & MacDonald, 1975; Arst, 1977*a*; see Fig. 1) are resistant to D-serine in the presence, but not in the absence, of L-ornithine.

The presence of ammonium or another alternative nitrogen source is not obligatory for the expression of proline-dependent D-serine resistance. Strains

carrying leaky *prn*D$^-$ mutations such as *prn*D-157 (Jones *et al.* 1981) lead to D-serine resistance when L-proline is the sole nitrogen source.

Little is known about the mechanism of D-serine toxicity in *A. nidulans*. Its toxicity is reversed by L-serine, suggesting that it might act as an L-serine analogue, but the only mutations leading unconditionally to D-serine resistance whose basis has been identified lead to pleiotropically defective amino acid uptake (Kinghorn & Pateman, 1975). Whatever the basis for proline-dependent D-serine resistance, it plays an important rôle in the classification of *prn*$^-$ mutations and provides a positive selection technique for obtaining *prn*C$^-$ as well as *prn*D$^-$ mutations (see Materials and Methods).

(*e*) *Classification of mutations in the* prn *cluster selected as conferring* L*-proline resistance using a* pro*A-6* sas*A-60 strain*

Growth data in Table 1 compare *pro*A-6 *sas*A-60 strains with all classes of L-proline resistant mutants thus far selected using them which carry a mutation in the *prn* cluster. *prn*A$^-$, *prn*B$^-$ and *prn*D$^-$ mutations result in unique phenotypes. Deletion mutations can be recognised directly provided they extend into *prn*B and at least one other *prn* gene. Such deletions combine the effect of *prn*B$^-$ mutations on supplementation of the proline auxotrophy with the reduction in utilization of L-arginine and L-ornithine characteristic of mutations in the other three *prn* genes.

One point from Table 1 requires further comment. Mutations in *prn*D, the structural gene for proline oxidase (Jones *et al.* 1981) affect growth responses to L-arginine and L-ornithine. These effects are even more striking when *sas*A$^+$ strains are compared: *prn*D$^-$ mutations strongly reduce utilization of L-arginine and L-ornithine as carbon and/or nitrogen sources. Why this should occur is not necessarily obvious from the pathway shown in Fig. 1 but there is nevertheless a straightforward explanation.

L-arginine and L-ornithine are catabolized to L-glutamic γ-semialdehyde and its internal Schiff base L-P5C (see Fig. 1). L-P5C can be either oxidized, via P5C dehydrogenase, to yield L-glutamate or reduced, via P5C reductase, to yield L-proline whose catabolism would require proline oxidase. The ability of L-arginine and L-ornithine to supplement *pro*A$^-$ and *pro*B$^-$ auxotrophies efficiently (Weglenski, 1966; Piotrowska *et al.* 1969; Bartnik & Weglenski, 1974) shows that a significant proportion of the L-P5C derived from catabolism of exogenous L-arginine and L-ornithine must be reduced to L-proline. The reduced growth of *prn*D$^-$ mutants on L-arginine- and L-ornithine-containing media confirms that a substantial fraction of the catabolism of these compounds proceeds via L-proline. The actual proportions catabolized via L-proline are difficult to estimate from growth properties alone because of the toxicity of L-proline to *prn*D$^-$ strains (Arst & MacDonald, 1978). Brandriss & Magasanik (1980) have shown that arginine catabolism proceeds wholly through proline in *S. cerevisiae*.

Table 1. *Growth responses of various* prn$^-$ *mutants selected in a strain of relevant genotype* pro*A-6* sas*A-60*

Relevant genotype	Growth score on appropriately supplemented glucose-minimal medium containing: 5 mM L-proline	5 mM L-ornithine	5 mM L-arginine	5 mM L-proline + 10 mM NO_3^-	125 μM L-proline + 10 mM NH_4^+	5 mM L-proline + 10 mM NH_4^+ + 5 mM D-serine	2·5 mM L-arginine + 10 mM acetamide + 5 mM D-serine	2·5 mM L-arginine + 5 mM GABA
Wild type (*proA*$^+$ *sasA*$^+$ *prn*$^+$)	5	5	5	5	5	0*	0*	5
proA-6 *sasA*$^+$ *prn*$^+$	5	5	5	5	4	0*	0*	5
proA$^+$ *sasA*-60 *prn*$^+$	0*	2	4	0*	5	0*	0*	2
proA-6 *sasA*-60 *prn*$^+$	0*	2	4	0*	4	0*	0*	2
proA-6 *sasA*-60 *prnA*$^-$	0	2	2	5	4	0*	0*	2
proA-6 *sasA*-60 *prnB*$^-$	1	2	4	5	1	0*	0*	2
proA-6 *sasA*-60 *prnD*$^-$	0	1	2	3	4	5	0*	2
proA-6 *sasA*-60 Δ*prnA* → *prnC*	0	1	2	5	1	0*	0*	2
proA-6 *sasA*-60 Δ*prnD* → *prnC*	0	1	2	4	1	0*	0*	2
proA-6 *sasA*-60 Δ*prnD* → *prnB*	0	1	2	4	1	0*	0*	2
proA-6 *sasA*-60 Δ*prnB* → *prnC*	0	1	1	4	1	3	0*	2

Growth scores were recorded after 2 days' incubation at 37 °C, but are not necessarily equivalent on different media. Scores range on a scale from 0 to 5. 0*, denotes that the level of growth is substantially less than that shown by a wild type strain on a medium lacking a nitrogen source (growth score = 0) due to the toxicity of a component of the growth medium. This distinction between a nitrogen-starved and an inhibited morphology has been made since the earliest days of the study of nitrogen metabolism in *A. nidulans* by D. J. Cove, J. A. Pateman and their colleagues, but has only been alluded to in print relatively recently (e.g. Cove, 1976*a*). A comparable effect can be seen with carbon source utilization where a systematic study of agar utilisation has been made (Page, 1971; Payton, McCullough & Roberts, 1976). In addition, there are slight morphological differences which enable further distinctions beyond those made in the Table but which are difficult to quantify or describe. For example, the morphology of an *sasA*-60 strain is different to that of an *sasA*-60 *prnA*$^-$ double mutant on a medium containing L-ornithine as nitrogen source, although, as shown above, the amounts of growth are similar. A discussion of the value of morphological criteria in growth testing is given by Arst (1981). In the above Table, *prnA*$^-$, *prnB*$^-$ and *prnD*$^-$ refer to non-leaky, complete loss of function mutations in the respective genes.

(ii) *Genetic characterization of fourteen deletion mutations selected using the system*

Table 2 shows diploid complementation responses of strains carrying each of the deletion mutations selected thus far. As expected on the basis of growth properties, all of these deletions fail to complement with the standard *prn*B⁻ allele *prn*B-6. These fourteen deletion mutations fall into four classes on the basis of the map locations of their endpoints (Fig. 3): (1) Δ*prn*B → *prn*C, represented by *prn*-307 and -308, covering at least parts of *prn*B and *prn*C, (2) Δ*prn*D → *prn*B, represented

Table 2. *Complementation responses of* prn *deletion mutations in diploids*

	Complementation response	
Relevant genotype of diploid	25 °C	37 °C
prn⁺/*prn*⁺ (wild type)	+ + +	+ + +
prn-300, -301, -302, -303, -306, -309, -310 or -311/*prn*A-1	+ + +	+ + +
prn-300, -301, -302, -303, -306, -309, -310 or -311/*prn*B-6	−	−
prn-300, -301, -302, -303, -306, -309, -310 or -311/*prn*C-61	±	+ +
prn-300, -301, -302, -303, -306, -309, -310 or -311/*prn*D-156	−	−
prn-304, -305 or -312/*prn*A-1	+ + +	+ + +
prn-304, -305 or -312/*prn*B-6	−	−
prn-304, -305 or -312/*prn*C-61	−	−
prn-304, -305 or -312/*prn*D-156	−	−
prn-307 or -308/*prn*A-1	+ + +	+ + +
prn-307 or -308/*prn*B-6	−	−
prn-307 or -308/*prn*C-61	−	−
prn-307 or -308/*prn*D-156	+ + +	+ + +
prn-313/*prn*A-1	−	−
prn-313/*prn*B-6	−	−
prn-313/*prn*C-61	−	−
prn-313/*prn*D-156	−	−
*prn*D-156 *prn*B-216/*prn*A-1	+ + +	+ + +
*prn*D-156 *prn*B-216/*prn*B-6	−	−
*prn*D-156 *prn*B-216/*prn*C-61	+ + +	+ + +
*prn*D-156 *prn*B-216/*prn*D-156	−	−

Scores represent complementation rather than growth responses for utilization of 5 mM L-proline as nitrogen source after 4 days' incubation at 25 °C or 2 days' incubation at 37 °C. Mutations in any one *prn* gene complement mutations in the other three *prn* genes fully. Complementation responses of *prn*D⁻ *prn*B⁻ double mutation are shown for comparison to those of Δ*prn*D → *prn*B mutations. + + +, full complementation; + +, slightly reduced complementation; +, reduced complementation; ±, very little complementation; −, no complementation. Although this Table shows reduced complementation between Δ*prn*D → *prn*B mutations and *prn*C⁻ mutations for utilization of L-proline as nitrogen source, the same phenomenon can be shown for utilization of L-proline as carbon source, utilization of L-arginine and L-ornithine as nitrogen sources and expression of L-proline-dependent D-serine resistance on ammonium as nitrogen source. In these cases, as in the data shown above, the reduction in complementation response is greater at 25 °C than at 37 °C and *prn*D⁻ *prn*B⁻ double mutations complement fully with *prn*C⁻ mutations.

by *prn*-300, -301, -302, -303, -306, -309, -310 and -311, covering at least parts of *prn*D and *prn*B and all of the *cis*-acting regulatory region, (3) Δ*prn*D → *prn*C, represented by *prn*-304, -305 and -312, covering at least parts of *prn*D and *prn*C and all of *prn*B and the *cis*-acting regulatory region and (4) Δ*prn*A → *prn*C, represented by *prn*-313, covering at least parts of *prn*A and *prn*C and all of *prn*D,

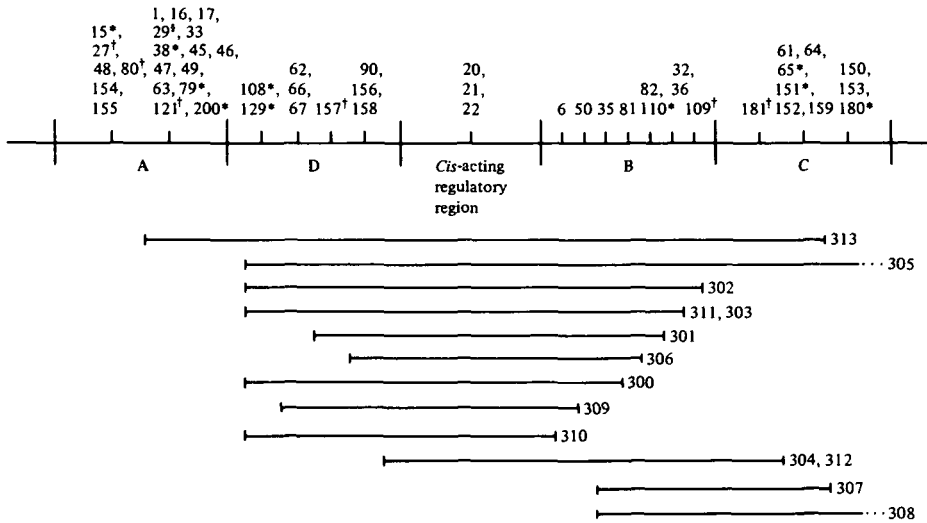

Fig. 3. Deletion map of the *prn* gene cluster. Within each gene and the regulatory region, the positioning of mutations (indicated by allele numbers) is based solely on whether or not recombination occurs with the fourteen deletion mutations *prn*-300 through *prn*-313. It is therefore possible (and quite likely) that mutations shown at the same map position can recombine with each other. The allele numbers in the regulatory region refer to prn^d mutations (Arst & MacDonald, 1975; Arst *et al.* 1980*b*). *prn*-305 and -308 do not recombine with any of the *prn*C mutations tested so their right-hand endpoints are not defined. In choosing mutations to map, preference has been given to conditional mutations so that the mutations shown on the map are not a random sample. *, denotes thermosensitive mutations; †, denotes cryosensitive mutations; §, denotes mutations which are leaky at both 25 and 37 °C.

*prn*B and the *cis*-acting regulatory region. In principle, one further class designated Δ*prn*A → *prn*B, covering at least parts of *prn*A and *prn*B and all of *prn*D and the *cis*-acting regulatory region ought to be capable of selection using the system. Very recently one such mutation has been obtained (K. K. Sharma & H. N. Arst, Jr., unpublished results). Whereas $prnA^-$, $prnB^-$ and $prnD^-$ mutations have characteristic phenotypes which usually allow their immediate classification (Table 1), phenotype differences amongst the five classes of deletion mutations eliminating function of *prn*B and at least one additional *prn* gene are more subtle and usually definitive classification must be based on complementation tests and fine-structure mapping.

A fine-structure map constructed using the fourteen deletion mutations is shown in Fig. 3. The recombination data depicted in Fig. 3 are consistent with the

complementation responses shown in Table 2, with the qualification that all eight deletions of the Δ*prn*D → *prn*B class show reduced complementation with *prn*C⁻ mutations, especially at 25 °C. This phenomenon was reported by Arst & MacDonald (1978), who showed that it apparently occurs with any *prn*C⁻ allele and that it is also observable when the complementation plates contain L-ornithine rather than L-proline as nitrogen source. As this reduced complementation correlates with reduced levels of the *prn*C product P5C dehydrogenase and as *prn*D⁻ *prn*B⁻ double mutation results in no such effects, Arst and MacDonald suggested that it results from inability to synthesise a dicistronic *prn*B *prn*C messenger in Δ*prn*D → *prn*B mutants because of the absence of the *cis*-acting regulatory region where it would be initiated. The fact that such deletions do not abolish *prn*C expression altogether (as judged from both complementation tests and enzyme assays) was interpreted to indicate the existence of at least one overlapping transcript initiated outside the central regulatory region. This overlapping transcript(s) might be mono-, tri- and/or tetracistronic. However, the recently isolated Δ*prn*A → *prn*B deletion complements a *prn*C⁻ mutation to the same extent as Δ*prn*D → *prn*B deletions do (K. K. Sharma & H. N. Arst, Jr., unpublished data). Because deletion mutations of the Δ*prn*A → *prn*B class eliminate *prn*A function, P5C dehydrogenase levels cannot be a measure of *prn*C expression in such mutants. Therefore the complementation responses would eliminate the possibility that a tricistronic *prn*D *prn*B *prn*C messenger accounts for any significant proportion of *prn*C expression in wild-type strains.

Arst & MacDonald (1978) also suggested that the differences in degree of *prn*C expression in Δ*prn*D → *prn*B mutants shown between growth at 25 °C and growth at 37 °C could be explained by a differential temperature effect on the synthesis and/or translation of the various transcripts. This would predict that *prn*C expression in the wild type occurs predominantly via the *prn*B *prn*C dicistronic messenger at 25 °C with expression via the overlapping transcript(s) predominating at 37 °C.

(iii) *Biochemical characterization of certain deletion mutants*

The Δ*prn*D → *prn*B mutations *prn*-300, -301 and -302 have previously been partially characterized biochemically (Arst & MacDonald, 1978). Data in Tables 3, 4, 5 and 6 concern the biochemical phenotypes of the remaining eleven deletion mutations.

Data in Table 3 confirm that all eleven deletion mutations lead to loss of the proline-inducible major L-proline permease specified by *prn*B. The consistent lowering of residual L-proline uptake levels by proline induction in the *prn*B-6 and deletion strains seen in Table 3 can presumably be attributed to inhibition of L-[U-^{14}C]proline uptake via the minor proline permease(s) by preloaded unlabelled L-proline added for induction. A similar effect has been observed when measuring residual GABA uptake in *gab*A⁻ mutants lacking the GABA permease (Bailey *et al.* 1979).

Data in Table 4 confirm that all deletions with the exception of those in the

Table 3. *Relative* L-*proline transport by wild type and various* prn *deletion mutants*

Relevant genotype	Relative L-proline uptake activity			
	25 °C growth		37 °C growth	
	Uninduced	Induced	Uninduced	Induced
Wild type (*prn*$^+$)	36	100	40	100
*prn*B-6	31	27	24	17
prn-303	29	10	16	10
prn-304	36	21	32	28
prn-305	30	9	30	13
prn-306	40	9	42	24
prn-307	17	9	18	15
prn-308	17	15	18	16
prn-309	32	25	14	8
prn-310	37	26	16	11
prn-311	40	26	35	16
prn-312	29	15	31	28
prn-313	22	19	30	20

Rates of L-[U-^{14}C]proline uptake were determined at the growth temperature. Results are expressed as a percentage of the appropriate induced wild type value and are the mean of three independent determinations. A *prn*B$^-$ mutant is included for comparison.

Table 4. *Relative activities of proline oxidase in wild type and various* prn *deletion mutants*

Relevant genotype	Relative proline oxidase activity			
	25 °C growth		37 °C growth	
	Uninduced	Induced	Uninduced	Induced
wild type (*prn*$^+$)	0	100	2	100
*prn*D-156 *prn*B-216	0	0	0	0
prn-303	0	0	0	0
prn-304	0	0	0	0
prn-305	0	0	0	0
prn-306	0	0	0	0
prn-307	90	131	115	273
prn-308	95	144	97	343
prn-309	0	0	0	0
prn-310	0	0	0	0
prn-311	0	0	0	0
prn-312	0	0	0	0
prn-313	0	0	0	0

Results are expressed as a percentage of the appropriate induced wild type value and are the mean of at least three independent determinations.

https://doi.org/10.1017/S0016672300020516 Published online by Cambridge University Press

Δ*prn*B → *prn*C class, *prn*-307 and -308, abolish proline oxidase. The fact that the two Δ*prn*B → *prn*C mutations lead to constitutive expression of proline oxidase with further inducibility beyond induced wild type levels is consistent with the phenotype of fully mutant *prn*C⁻ mutations reported previously (Arst & MacDonald, 1978; Jones *et al.* 1981). These effects, which are probably trivial consequences of inducer accumulation, are discussed elsewhere (Jones *et al.* 1981).

Table 5. *Relative activities of P5C dehydrogenase in wild type and various* prn *deletion mutants*

Relevant genotype	Relative P5C dehydrogenase activity			
	25 °C growth		37 °C growth	
	Uninduced	Induced	Uninduced	Induced
wild type (*prn*⁺)	0	100	0	100
*prn*D-156 *prn*B-216	0	95	0	120
prn-303	0	23	0	79
prn-304	0	0	0	0
prn-305	0	0	0	0
prn-306	0	16	0	27
prn-307	0	0	0	0
prn-308	0	0	0	0
prn-309	0	58	0	65
prn-310	0	39	0	69
prn-311	0	31	0	48
prn-312	0	0	0	0
prn-313	0	0	0	0

Results are expressed as a percentage of the appropriate induced wild type value and are the mean of at least three independent determinations. A *prn*D⁻ *prn*B⁻ double mutant is included for comparison to Δ*prn*D → *prn*B mutants.

Table 6. *Presence or absence of cross-reacting material (CRM) to wild-type P5C dehydrogenase antiserum in extracts of wild type and various* prn *deletion strains*

Relevant genotype	P5C dehydrogenase CRM			
	25 °C growth		37 °C growth	
	Uninduced	Induced	Uninduced	Induced
wild type (*prn*⁺)	−	+	−	+
prn-303	−	+	−	+
prn-304	−	−	−	−
prn-305	−	−	−	−
prn-306	−	+	−	+
prn-307	−	−	−	−
prn-308	−	−	−	−
prn-310	−	+	−	+
prn-311	−	+	−	+
prn-312	−	−	−	−
prn-313	−	−	−	−

+, immunoprecipitate formed; −, no immunoprecipitate formed. Purified wild type P5C dehydrogenase was included as an additional check on the antiserum used.

Relative P5C dehydrogenase levels in deletion stains are shown in Table 5 whilst Table 6 shows whether or not cross-reacting material (CRM) to wild type P5C dehydrogenase is present in strains carrying each of the deletion mutations except *prn*-309. Deletions of the Δ*prn*D → *prn*C (*prn*-304, -305 and -312), Δ*prn*B → *prn*C (*prn*-307 and -308) and Δ*prn*A → *prn*C (*prn*-313) classes abolish P5C dehydrogenase, both as activity and as CRM. In agreement with earlier data for *prn*-300, -301 and -302 (Arst & MacDonald, 1978), deletion mutants of the Δ*prn*D → *prn*B class (*prn*-303, -306, -309, -310 and -311) have reduced P5C dehydrogenase levels with the effect more drastic at 25 °C than at 37 °C.

(iv) *Thermal stability of P5C dehydrogenase from* Δprn*D* → prn*B mutants*

One possible way in which Δ*prn*D → *prn*B mutations might affect apparent expression of the *prn*C gene located in *cis* to them without affecting either the regulation of P5C dehydrogenase synthesis or the primary structure of the enzyme would be if proline oxidase and P5C dehydrogenase exist *in vivo* as an aggregate which stabilizes P5C dehydrogenase against degradation. Although many missense mutations in *prn*D might still permit aggregation of wild type P5C dehydrogenase with a mutant proline oxidase, the vast majority of deletion mutations in *prn*D (including all Δ*prn*D → *prn*B mutations if *prn*D be transcribed outward from the *cis*-acting regulatory region) would preempt the possibility of aggregation.

There is, however, already considerable evidence against aggregation of the two enzymes. Firstly, this model is difficult to reconcile with the apparently *cis*-acting nature of the reduced complementation: as Δ*prn*D → *prn*B/*prn*C$^-$ diploids possess one wild type *prn*D gene and one wild type *prn*C gene, the formation of considerable quantities of an aggregate composed of the two wild type enzymes should be possible. Especially at 25 °C, there must be very little if any such wild type aggregate because only minimal complementation is observed. Secondly there is no evidence for any lack of complementation between *prn*D$^-$ mutations and *prn*C$^-$ mutations: no *prn*D$^-$ mutation tested fails to complement with *prn*C-61 (and with other *prn*C$^-$ mutations where these have been tested). Nor have any dominant *prn*C$^-$ or *prn*D$^-$ mutations been found. Deletion of the *prn*C gene by Δ*prn*B → *prn*C mutations is fully complemented by *prn*D$^-$ mutations (Table 2) and certainly does not lower proline oxidase levels (Table 4).

Enzyme localization data provide even more convincing evidence against aggregation. Proline oxidase and P5C dehydrogenase show no tendency to copurify and are probably located in the mitochondrial membrane and cytosol, respectively (Jones, 1980; D. W. MacDonald & S. A. Jones, unpublished results).

Data in Table 7 provide still further evidence against a stabilization by aggregation model. There is certainly no evidence for decreased stability of P5C dehydrogenase in crude extracts at 60 °C from any of four Δ*prn*D → *prn*B mutants as compared to the wild type. The validity of making such comparisons in crude extracts is supported by the fact that the thermal stability of wild type P5C dehydrogenase does not change appreciably upon 108-fold purification from crude extracts (Jones, 1980). Not only is there no evidence for decreased thermal stability

of P5C dehydrogenase from Δ*prn*D → *prn*B mutants, but also two such mutations, *prn*-300 and -306, appear to enhance P5C dehydrogenase thermal stability. These apparent increases are, however, not convincing when t tests are used to examine the significance of the differences between the means ($P \cong 0{\cdot}1$ for *prn*-300 *v.* wild type; $P \cong 0{\cdot}14$ for *prn*-306 *v.* wild type).

Table 7. *Thermal stability of P5C dehydrogenase from wild type and four* Δprn*D* → prn*B mutants*

Relevant genotype	P5C dehydrogenase half-life at 60 °C (min)
Wild type (*prn*$^+$)	$15{\cdot}0 \pm 5{\cdot}5$
prn-300	$26{\cdot}2 \pm 8{\cdot}1$
prn-301	$16{\cdot}1 \pm 4{\cdot}9$
prn-303	$14{\cdot}3 \pm 7{\cdot}3$
prn-306	$22{\cdot}1 \pm 3{\cdot}8$

One standard deviation of the half-life is also indicated.

Of course, it could still be argued that degradation of P5C dehydrogenase in intact cells, probably resulting principally from the action of proteases, bears little resemblance to thermal inactivation in cell extracts. Nevertheless in conjunction with all of the other evidence against stabilization of P5C dehydrogenase by aggregation with proline oxidase, it is sufficiently convincing that we feel that this possibility can now be safely disregarded.

We thank the Royal Society for the Smithson Research Fellowship (H.N.A.), the Medical Research Council for a studentship (S.A.J.) and the Science Research Council for grants to Professor J. M. Thoday (C.R.B. and H.N.A.) and H.N.A. We are grateful to Liz Workman and Shelagh Cousen for technical assistance, to Claudio Scazzocchio for help with immunological techniques and comments on the manuscript, and to Don MacDonald for useful discussions.

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