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

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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 sasA-60 (semialdehyde sensitive) mutation in A. nidulans results in toxicity of catabolic precursors of L-glutamic γ-semialdehyde (or its internal Schiff base L-Δ1-pyrroline-5-carboxylate) and succinic semialdehyde, apparently without affecting the catabolic pathways concerned. As sasA-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 sasA-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 prnB 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 prnA–prnD–cis-acting regulatory region–prnB–prnC (Arst & MacDonald, 1975, 1978). prnD and prnC are the structural genes for proline oxidase and Δ1-pyrroline-5-carboxylate (P5C) dehydrogenase, respectively; prnB is probably the structural gene for the principal L-proline permease; and prnA 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<sup>d</sup>) affecting the expression of at least prnB map in the regulatory region (Arst & MacDonald, 1975; Arst, MacDonald & Jones, 1980). Deletion of the regulatory region considerably but incompletely reduces expression of prnC in cis, suggesting that the prnC product P5C dehydrogenase is synthesised from both a dicistronic prnB prnC 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<sup>−</sup> 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 & Seriver, 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),
Deletion mutations in Aspergillus gene cluster

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 x 10^5 progeny were tested. If no proline-utilizing progeny were recovered from at least 2 x 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 pantoB-100, sf-211 and mahA-10 (Arst, 1977b; Arst & MacDonald, 1978) segregated in each cross, and many crosses involved strains carrying prnd mutations (Arst & MacDonald, 1975; Arst et al. 1980b) 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, 1977b; Arst & MacDonald, 1975, 1978; Arst et al. 1980b; Arst, Bailey & Penfold, 1980a; Jones et al. 1981).
(a) sasA-60

This mutation was obtained fortuitously during N-methyl-N'-nitro-N-nitrosoguanidine (NTG) induced (Alderson & Hartley, 1969) reversion of the pppA-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 sasA-60 have been selected because of their reduced growth on proline-containing media, e.g. in the experiment in which prnA-46 and -49 were selected (see below). The allelism of these mutations to sasA-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 sasA-60 is described in detail in the Results and Discussion section.

(b) alnA-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. alnA-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), alnA-1 would appear to lead to loss of a highly specific catabolic L-alanine transaminase. This transaminase is apparently not involved in supplementation of ileA- 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 ileA-1 alnA-1 double mutants. Recessive in diploids, alnA-1 is located in linkage group VII but it recombines freely with gatA-1, leading to loss of GABA transaminase (Arst, 1976; Bailey, Arst & Penfold, 1980) and otaA-2, leading to loss of ornithine δ-transaminase (Piotrowska, Sawicki & Wegienski, 1969; Arst & MacDonald, 1975; Arst, 1977a) as well as with mutations in the prn cluster.

(c) prnA- mutations

prnA-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 proA-6 sasA-60
Deletion mutations in Aspergillus gene cluster

sF-211fwA-1 (L-proline requiring, hypersensitive to P5C and succinic semialdehyde toxicities, inhibited by a metabolite derived from sulphate, fawn conidial colour). prnA-46 and -49 are ethyl methanesulphonate induced (Alderson & Clark, 1966) mutations selected in a strain of genotype yA-2 pantoB-100 prn\(\delta\)-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. prnA-79 and -80 are spontaneous mutations selected in the same way as prnA-15, etc. but in strains of genotypes proA-6 sasA-60 mahA-10 (hypersensitive to methylammonium) sF-211 and proA-6 sasA-60 prn\(\delta\)-22, respectively. prnA-101 is a spontaneous mutation selected in a strain of genotype biA-1 pabaA-1 fwA-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 prnC-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. prnA-121 was selected in the same way as prnA-79 but at 25 °C. prnA-154 and -155 are NTG induced mutations selected in the same way as prnA-46 and -49 but in a strain of genotype yA-2 alX-4 (lacking allantoinase) pantoB-100. prnA-217 is a spontaneous mutation selected in a strain of genotype yA-2 proB-9 (L-proline requiring) prnD-156 (lacking proline oxidase and therefore unable to catabolize L-proline) pantoB-100 because it confers resistance to the toxicity of 50 mM L-proline (due to prnD-156) in appropriately supplemented minimal medium containing 1% (v/v) ethanol as carbon source and 595 \(\mu\)M uric acid as nitrogen source at 25 °C.

\((d)\) prnB\(\beta\)-mutations

prnB-81 and -82 are spontaneous mutations selected in the same experiment as prnA-80. prnB-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 proA-6 sasA-60 prn\(\delta\)-22.

\((e)\) prnC\(\gamma\)-mutations

prnC-180 and -181 are spontaneous mutations selected in a strain of genotype yA-2 proA-6 pantoB-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.

\((f)\) prnD\(\gamma\)-mutations

prnD-62 was selected in the same experiment as prnA-46 and -49. prnD-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 biA-1 pabaA-1 areA'-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 prnD-90 mutation is itself responsible for the weak suppression of areA'-18 for L-alanine utilization was confirmed by selection of another probable prnD- 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 areA' 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 areA' mutations for L-alanine utilization seems to be a general property of prnD- mutations because prnD-66, -67 and -156 all have this effect in double mutants with areA-1. Even more marginal suppression of areA'-1 for L-alanine utilization is exerted by prnC-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 prnA and prnB alleles. Thus no suppression occurs in areA'-1 prnD-156 prnB-216 triple mutants or in areA'-1 strains carrying the prn-306 or -309 deletion mutations (see below). Similarly no suppression occurs in areA'-1 prnC-61 prnA-101 or areA'-1 prnC-61 prnB-102 triple mutants. Unlike the conditional D-serine resistance conferred by prnD- and prnC- mutations, the weak suppression of areA' mutations for L-alanine utilization is not dependent upon the presence of L-proline in the growth medium.

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

(g) prn deletion mutations

All fourteen prn deletions are spontaneous mutations selected in sasA-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 proA-6 sasA-60 sF-211 fvaA-1, proA-6 sasA-60 prn-22, and proA-6 sasA-60 prn-22 prnB-109, respectively. prn-303 through -313 were selected in a strain of genotype proA-6 sasA-60 mahA-10 sF-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. 1980a; 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 prnA-, prnB-, prnC- or prnD- 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 pabaA-1. In addition the prn-303, -307, -311 and -312 strains carry the fawn conidial colour mutation fwA-1 and the prn-303, -305, -306, -307, -308, -309, -310, -311, -312 and -313 strains carry mahA-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 \( \text{L-[U-}^{14}\text{C]} \)proline (Radiochemical Centre) was measured as described by Arst et al. (1980b).

(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, Seazzocchio & 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 10 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 sasA-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 (1976b) 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 prnB permease, provide a positive selection technique for obtaining prnB~ 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. prnC~ mutants are extremely inhibited by L-proline, presumably because they accumulate the highly reactive compound L-P5C (Arst & MacDonald, 1978; Arst et al. 1980a; Jones et al. 1981). prnD~ 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 prnC~ and prnD~ mutants is sufficient to permit facile selection of spontaneous prnA~ and prnB~ mutations (Arst & MacDonald, 1978; Arst et al. 1980a; Materials and Methods section above). Whilst such use of prnC~ and prnD~ 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 \( sasA-60 \) (semialdehyde sensitive).

The growth of \( sasA-60 \) 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 \( \gamma \)-semialdehyde (or its internal Schiff base L-P5C – see Fig. 1) and 2-pyrrolidone and \( \gamma \)-amino-\( n \)-butyrate (GABA) which can be converted to succinic semialdehyde (see Fig. 2). There is no direct evidence that \( sasA-60 \) strains are more susceptible to semialdehyde toxicity than the wild type, but this interpretation is a working hypothesis which is consistent both with the \( sasA^- \) phenotype and with the classes of resistance mutations which can be obtained using an \( sasA-60 \) strain.

The basis for this apparent semialdehyde sensitivity is unclear. It is likely to result from a loss of function because \( sasA-60 \) is recessive in diploids and mutations having the \( sasA-60 \) 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,
Deletion mutations in Aspergillus gene cluster

aldA. aldA− 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, aldA− mutations also lead to toxicity of L-proline, 2-pyrrolidone and GABA, albeit to a much lesser degree than sasA-60. Thus aldA− mutations probably enhance sensitivity to the toxicity of acetaldehyde rather than block its oxidation. As aldA− 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. sasA-60 apparently has no effect on acetaldehyde toxicity because it does not affect growth on ethanol or ethylammonium as carbon source. aldA− mutations are additive with sasA-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 aldA− and sasA− mutations affect different functions. The two genes are unlinked: whereas aldA is in linkage group VIII (Page, 1971), haploidisation analysis (McCully & Forbes, 1965) has located sasA-60 to linkage group V.

(b) Selection of resistance mutations using sasA-60 strains

The toxicity of both L-proline and GABA to sasA-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, sasA-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 sasA-60 strains has yielded a variety of mutations reducing GABA transport including gabA− (Arst, 1976; Bailey, Penfold & Arst, 1979), intA− (Arst, 1976, 1981; Bailey et al. 1979) and pacC−
Alleles. Mutations in \textit{gatA}, the putative structural gene for GABA transaminase (Arst, 1976; Bailey \textit{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 \textit{et al.} 1980). Indeed \textit{gatA} strains have been used for the selection of mutations blocking GABA uptake or formation (Arst, Penfold & Bailey, 1978; Bailey \textit{et al.} 1979; Arst \textit{et al.} 1980a).

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

A crucial feature of the utility of sasA-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 sasA+ strains carrying the mutations selected upon outcrossing but allows sasA+ revertants to be classified in the initial screening of newly selected mutations.

\textbf{(c) The recognition of \textit{prnB} mutations}

In strains having a growth requirement for L-proline, \textit{prnB} 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, proA\textsuperscript{−} and proB\textsuperscript{−} auxotrophs (see Fig. 1) can be supplemented by low concentrations (e.g. 125 \mu M) of L-proline only if the major proline permease, specified by \textit{prnB}, is functional. Under these growth conditions, ammonium inhibits uptake of L-proline by one or more minor permeases (Arst \textit{et al.} 1980b) so that supplementation requires participation of the \textit{prnB} permease. Although \textit{prnB} mutations can be easily distinguished from \textit{prnA}, \textit{prnC} and \textit{prnD} mutations by their lack of effect on L-arginine- and L-ornithine-containing media, the recognition of loss of \textit{prnB} 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 \textit{prnB} phenotype. Use of a proA\textsuperscript{−} (or proB\textsuperscript{−}) sasA-60 double mutant for selection of proline resistant mutants enables unambiguous classification of \textit{prnB} functionality in every case. (It should be noted that, in the presence of glucose and ammonium, the low proline concentrations required for \textit{prnB} classification are not toxic to sasA-60 strains).

\textbf{(d) The use of toxic amino acid analogues}

Not all mutations capable of protecting sasA-60 strains against proline toxicity map in the \textit{prn} cluster. In addition to reversion to sasA\textsuperscript{+} whose recognition is outlined above, mutations pleiotropically reducing uptake of a number of amino acids available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S00166723080020516.
Deletion mutations in Aspergillus gene cluster

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. 1980b) or some component common to several amino acid permeases including the prnB 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 sasA-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, prnC and prnD mutations confer resistance to D-serine and another less toxic analogue, D-threonine. This characteristic facilitates classification of prnD mutations selected as protecting sasA-60 strains against proline toxicity. Unlike pleiotropic amino acid uptake mutations, prnD 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 prnD 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 prnB or a prnA mutation prevents this L-proline-dependent D-serine resistance of prnD strains. As prnA mutations reduce expression of the prnB permease (Arst & MacDonald, 1978), the basis for reversal of resistance by prnA mutations might be the same as that by prnB mutations. prnC mutants still show some proline-dependent D-serine resistance in the absence of the prnB permease, a finding which aids in the classification of deletion mutations extending from within prnB into prnC (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 alnA strains. alnA-1 probably leads to loss of a catabolic L-alanine transaminase (see Materials and Methods). otaA-2 strains, lacking ornithine δ-transaminase (Piotrowska et al. 1969; Arst & MacDonald, 1975; Arst, 1977a; 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 \textit{prnD}^{-} mutations such as \textit{prnD}-157 (Jones \textit{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 \textit{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 \textit{prn}\textsuperscript{-} mutations and provides a positive selection technique for obtaining \textit{prnC}^{-} as well as \textit{prnD}^{-} mutations (see Materials and Methods).

\textbf{(e) Classification of mutations in the \textit{prn} cluster selected as conferring L-proline resistance using a \textit{proA}-6 \textit{sasA}-60 strain}

Growth data in Table 1 compare \textit{proA}-6 \textit{sasA}-60 strains with all classes of L-proline resistant mutants thus far selected using them which carry a mutation in the \textit{prn} cluster. \textit{prnA}^{-}, \textit{prnB}^{-} and \textit{prnD}^{-} mutations result in unique phenotypes. Deletion mutations can be recognised directly provided they extend into \textit{prnB} and at least one other \textit{prn} gene. Such deletions combine the effect of \textit{prnB}^{-} 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 \textit{prn} genes.

One point from Table 1 requires further comment. Mutations in \textit{prnD}, the structural gene for proline oxidase (Jones \textit{et al.} 1981) affect growth responses to L-arginine and L-ornithine. These effects are even more striking when \textit{sasA}^{+} strains are compared: \textit{prnD}^{-} 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 \textit{\gamma}\textsuperscript{-} 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 \textit{proA}^{-} and \textit{proB}^{-} auxotrophies efficiently (Weglenski, 1966; Piotrowska \textit{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 \textit{prnD}^{-} 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 \textit{prnD}^{-} strains (Arst & MacDonald, 1978). Brandriss & Magasanik (1980) have shown that arginine catabolism proceeds wholly through proline in \textit{S. cerevisiae}. 

\url{https://www.cambridge.org/core/terms}. \url{https://doi.org/10.1017/S0016672300020516}. Downloaded from \url{https://www.cambridge.org/core}. IP address: 54.70.40.11, on 01 Mar 2019 at 20:56:37, subject to the Cambridge Core terms of use, available at \url{https://www.cambridge.org/core/terms}. https://doi.org/10.1017/S0016672300020516
Table 1. Growth responses of various prn<sup>-</sup> mutants selected in a strain of relevant genotype proA-6 sasA-60

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>5 mM L-proline</th>
<th>5 mM L-ornithine</th>
<th>5 mM L-arginine</th>
<th>125 μM L-proline + 10 mM NO&lt;sub&gt;3&lt;/sub&gt;⁻</th>
<th>10 mM NH&lt;sub&gt;4&lt;/sub&gt;⁺</th>
<th>5 mM D-serine</th>
<th>2.5 mM L-arginine + 10 mM acetamide</th>
<th>2.5 mM L-arginine + 5 mM GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0*</td>
<td>5</td>
<td>0*</td>
</tr>
<tr>
<td>(proA&lt;sup&gt;+&lt;/sup&gt; sasA&lt;sup&gt;+&lt;/sup&gt; prn&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0*</td>
<td>5</td>
<td>0*</td>
</tr>
<tr>
<td>proA&lt;sup&gt;-&lt;/sup&gt; sasA&lt;sup&gt;+&lt;/sup&gt; prn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0*</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0*</td>
<td>4</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>proA&lt;sup&gt;-&lt;/sup&gt; sasA-60 prn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0*</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0*</td>
<td>4</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>proA&lt;sup&gt;-&lt;/sup&gt; sasA-60 prnD&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>0*</td>
<td>1</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>proA&lt;sup&gt;-&lt;/sup&gt; sasA-60 prnD&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0*</td>
<td>0*</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ΔprnA → prnC</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0*</td>
<td>0*</td>
<td>2</td>
</tr>
<tr>
<td>ΔprnD → prnC</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0*</td>
<td>0*</td>
<td>2</td>
</tr>
<tr>
<td>ΔprnD → prnB</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0*</td>
<td>0*</td>
<td>2</td>
</tr>
</tbody>
</table>

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 <i>A. nidulans</i> by D. J. Cove, J. A. Pateman and their colleagues, but has only been alluded to in print relatively recently (e.g. Cove, 1976a). 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<sup>-</sup> 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<sup>-</sup>, prnB<sup>-</sup> and prnD<sup>-</sup> 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 \( \text{prnB}^- \) allele \( \text{prnB-6} \). These fourteen deletion mutations fall into four classes on the basis of the map locations of their endpoints (Fig. 3): (1) \( \Delta\text{prnB} \rightarrow \text{prnC} \), represented by \( \text{prn-307} \) and \( \text{-308} \), covering at least parts of \( \text{prnB} \) and \( \text{prnC} \), (2) \( \Delta\text{prnD} \rightarrow \text{prnB} \), represented

<table>
<thead>
<tr>
<th>Relevant genotype of diploid</th>
<th>Complementation response</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{prn}^+ / \text{prn}^+ ) (wild type)</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prn-300} ), -301, -302, -303, -306, -309, -310 or -311/( \text{prnA-1} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prn-300} ), -301, -302, -303, -306, -309, -310 or -311/( \text{prnB-6} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-300} ), -301, -302, -303, -306, -309, -310 or -311/( \text{prnC-61} )</td>
<td>±</td>
</tr>
<tr>
<td>( \text{prn-303} ), -304, -305 or -312/( \text{prnA-1} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prn-304} ), -305 or -312/( \text{prnB-6} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-304} ), -305 or -312/( \text{prnC-61} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-304} ), -305 or -312/( \text{prnD-156} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-307} ) or -308/( \text{prnA-1} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prn-307} ) or -308/( \text{prnB-6} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-307} ) or -308/( \text{prnC-61} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-307} ) or -308/( \text{prnD-156} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prn-313} )/( \text{prnA-1} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-313} )/( \text{prnB-6} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-313} )/( \text{prnC-61} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prn-313} )/( \text{prnD-156} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prnD-156} )/( \text{prnB-216}/\text{prnA-1} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prnD-156} )/( \text{prnB-216}/\text{prnB-6} )</td>
<td>-</td>
</tr>
<tr>
<td>( \text{prnD-156} )/( \text{prnB-216}/\text{prnC-61} )</td>
<td>+ + +</td>
</tr>
<tr>
<td>( \text{prnD-156} )/( \text{prnB-216}/\text{prnD-156} )</td>
<td>-</td>
</tr>
</tbody>
</table>

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 \( \text{prn} \) gene complement mutations in the other three \( \text{prn} \) genes fully. Complementation responses of \( \text{prnD}^- \text{prnB}^- \) double mutations are shown for comparison to those of \( \Delta\text{prnD} \rightarrow \text{prnB}^- \) mutations. + + +, full complementation; + +, slightly reduced complementation; +, reduced complementation; ±, very little complementation; —, no complementation. Although this Table shows reduced complementation between \( \Delta\text{prnD} \rightarrow \text{prnB}^- \) mutations and \( \text{prnC}^- \) 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 \( \text{prnD}^- \text{prnB}^- \) double mutations complement fully with \( \text{prnC}^- \) mutations.
Deletion mutations in Aspergillus gene cluster

by prn-300, -301, -302, -303, -306, -309, -310 and -311, covering at least parts of prnD and prnB and all of the cis-acting regulatory region, (3) ΔprnD → prnC, represented by prn-304, -305 and -312, covering at least parts of prnD and prnC and all of prnB and the cis-acting regulatory region and (4) ΔprnA → prnC, represented by prn-313, covering at least parts of prnA and prnC and all of prnD,

<table>
<thead>
<tr>
<th>prnA</th>
<th>prnB</th>
<th>prnC</th>
</tr>
</thead>
<tbody>
<tr>
<td>15*,</td>
<td>16, 17,</td>
<td>29*, 33</td>
</tr>
<tr>
<td>27*,</td>
<td>28*, 45, 46,</td>
<td>48, 60, 47, 49,</td>
</tr>
<tr>
<td>154,</td>
<td>63, 79*,</td>
<td>108*, 66, 156,</td>
</tr>
<tr>
<td>155,</td>
<td>121*, 200*</td>
<td>129* 67 157* 158</td>
</tr>
</tbody>
</table>

prnB and the cis-acting regulatory region. In principle, one further class designated ΔprnA → prnB, covering at least parts of prnA and prnB and all of prnD 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 prnB 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

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 prnd mutations (Arst & MacDonald, 1975; Arst et al. 1980b). prn-305 and -308 do not recombine with any of the prnC 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.
complementation responses shown in Table 2, with the qualification that all eight deletions of the $\Delta prnD \rightarrow prnB$ class show reduced complementation with $prnC^-$ mutations, especially at 25 °C. This phenomenon was reported by Arst & MacDonald (1978), who showed that it apparently occurs with any $prnC^-$ 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 $prnC$ product P5C dehydrogenase and as $prnD^- prnB^-$ double mutation results in no such effects, Arst and MacDonald suggested that it results from inability to synthesise a dicistronic $prnB$ $prnC$ messenger in $\Delta prnD \rightarrow prnB$ mutants because of the absence of the cis-acting regulatory region where it would be initiated. The fact that such deletions do not abolish $prnC$ 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 $\Delta prnA \rightarrow prnB$ deletion complements a $prnC^-$ mutation to the same extent as $\Delta prnD \rightarrow prnB$ deletions do (K. K. Sharma & H. N. Arst, Jr., unpublished data). Because deletion mutations of the $\Delta prnA \rightarrow prnB$ class eliminate $prnA$ function, P5C dehydrogenase levels cannot be a measure of $prnC$ expression in such mutants. Therefore the complementation responses would eliminate the possibility that a tricistronic $prnD$ $prnB$ $prnC$ messenger accounts for any significant proportion of $prnC$ expression in wild-type strains.

Arst & MacDonald (1978) also suggested that the differences in degree of $prnC$ expression in $\Delta prnD \rightarrow prnB$ 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 $prnC$ expression in the wild type occurs predominantly via the $prnB$ $prnC$ dicistronic messenger at 25 °C with expression via the overlapping transcript(s) predominating at 37 °C.

(iii) Biochemical characterization of certain deletion mutants

The $\Delta prnD \rightarrow prnB$ 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 $prnB$. The consistent lowering of residual L-proline uptake levels by proline induction in the $prnB$-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 $gabA^-$ 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

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>25 °C growth</th>
<th>37 °C growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>Wild type (prn⁺)</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>prnB-6</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>prn-303</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>prn-304</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td>prn-305</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td>prn-306</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>prn-307</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>prn-308</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>prn-309</td>
<td>32</td>
<td>25</td>
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<tr>
<td>prn-311</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td>prn-312</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>prn-313</td>
<td>22</td>
<td>19</td>
</tr>
</tbody>
</table>

Rates of L-[U-¹⁴C]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 prnB⁻ mutant is included for comparison.

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

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>25 °C growth</th>
<th>37 °C growth</th>
</tr>
</thead>
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<td>Induced</td>
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<td>wild type (prn⁺)</td>
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<tr>
<td>prnD-156 prnB-216</td>
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<td>0</td>
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<td>prn-303</td>
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<tr>
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<tr>
<td>prn-313</td>
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Results are expressed as a percentage of the appropriate induced wild type value and are the mean of at least three independent determinations.
ΔprnB → prnC class, prn-307 and -308, abolish proline oxidase. The fact that the two ΔprnB → prnC mutations lead to constitutive expression of proline oxidase with further inducibility beyond induced wild type levels is consistent with the phenotype of fully mutant prnC 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

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>25 °C growth</th>
<th>37 °C growth</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>wild type (prn⁺)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>prnD-156 prnB-216</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>prn-303</td>
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</table>

Results are expressed as a percentage of the appropriate induced wild type value and are the mean of at least three independent determinations. A prnD⁻ prnB⁻ double mutant is included for comparison to ΔprnD → prnB 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

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>25 °C growth</th>
<th>37 °C growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>wild type (prn⁺)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>prn-303</td>
<td>-</td>
<td>+</td>
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<tr>
<td>prn-304</td>
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<td>-</td>
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<td>prn-305</td>
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<td>prn-306</td>
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<td>+</td>
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<td>prn-312</td>
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<td>-</td>
</tr>
<tr>
<td>prn-313</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, 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 ΔprnD → prnC (prn-304, -305 and -312), ΔprnB → prnC (prn-307 and -308) and ΔprnA → prnC (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 ΔprnD → prnB 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 ΔprnD → prnB mutants

One possible way in which ΔprnD → prnB mutations might affect apparent expression of the prnC 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 prnD might still permit aggregation of wild type P5C dehydrogenase with a mutant proline oxidase, the vast majority of deletion mutations in prnD (including all ΔprnD -*prnB mutations if prnD 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 ΔprnD → prnB/prnC− diploids possess one wild type prnD gene and one wild type prnC 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 prnD− mutations and prnC− mutations: no prnD− mutation tested fails to complement with prnC-61 (and with other prnC− mutations where these have been tested). Nor have any dominant prnC− or prnD− mutations been found. Deletion of the prnC gene by ΔprnB → prnC mutations is fully complemented by prnD− 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 ΔprnD → prnB 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 ΔprnD → prnB 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 ≈ 0.1 for prn-300 v. wild type; P ≈ 0.14 for prn-306 v. wild type).

Table 7. Thermal stability of P5C dehydrogenase from wild type and four ΔprnD → prnB mutants

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>P5C dehydrogenase half-life at 60 °C (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (prn⁺)</td>
<td>15.0 ± 5.5</td>
</tr>
<tr>
<td>prn-300</td>
<td>26.2 ± 8.1</td>
</tr>
<tr>
<td>prn-301</td>
<td>16.1 ± 4.9</td>
</tr>
<tr>
<td>prn-303</td>
<td>14.3 ± 7.3</td>
</tr>
<tr>
<td>prn-306</td>
<td>22.1 ± 3.8</td>
</tr>
</tbody>
</table>

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.

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REFERENCES


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