The Genetic Location of Three Mutations Impairing Penicillin Production in *Aspergillus nidulans*

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Three mutations impairing penicillin production in *Aspergillus nidulans*, *npeB*, *npeC* and *npeD*, have been located on linkage groups III, IV and II, respectively, and positioned relative to other loci on these chromosomes.

INTRODUCTION

Both *Aspergillus nidulans* and *Penicillium chrysogenum* elaborate penicillin and have been used in genetic and biochemical studies of the biosynthesis and over-production of this antibiotic (Macdonald & Holt, 1976). Although only producing small amounts, *A. nidulans* has several advantages over *P. chrysogenum* as an organism with which to study the genetics of penicillin production. Foremost are the wealth of genetic information available and the existence of well-characterized multiple mutant strains to analyse genetic events in this organism (Clutterbuck, 1974).

Following treatment with a variety of different mutagens, strains of *A. nidulans* with altered abilities to elaborate penicillin have been isolated (Edwards *et al.*, 1974; Holt *et al.*, 1976; Ditchburn *et al.*, 1976). Three mutations which raised penicillin yield, *penA*, *B* and *C*, have been assigned to positions on linkage groups VIII, III and IV, respectively (Ditchburn *et al.*, 1976). Mutants impaired in penicillin production (*Npe*) have also been isolated and allotted to one of four complementation groups, *A*, *B*, *C* and *D*; over two thirds were members of complementation group *A* (Edwards *et al.*, 1974). Additionally, the lack of penicillin production in certain wild-type isolates of *A. nidulans* was shown to be the result of changes at this same *npeA* locus (Cole *et al.*, 1976). The *npeA* gene was located on linkage group VI (Holt *et al.*, 1976) and subsequent investigations by Makins *et al.* (1981) and Makins & Holt (1982) suggested it controlled the formation of the tripeptide α -aminoadipylcysteinylvaline, a precursor of penicillin, and was probably analogous to *npeY* in *P. chrysogenum* (Normansell *et al.*, 1980). While mutants with lesions at the *npeA* locus have been the subject of considerable attention, relatively little is known about mutations in complementation groups *B*, *C* and *D*. This paper reports studies on strains with mutations in these latter three complementation groups.

METHODS

Strains. Details are given in Table 1. The gene symbols used are those of Clutterbuck (1974). The criterion for classifying a mutant as *Npe* was reduction of penicillin yield to 10% or less that of its parent without affecting nutritional requirements, growth rate or gross morphology.

Media. Minimal media (MM), complete media (CM) and sporulation media (SPM) were based on those of Pontecorvo *et al.* (1953). The penicillin production media (PPM) was described by Holt & Macdonald (1968).

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Table 1. *Genotypes and linkage data for the strains of Aspergillus nidulans used*

Genes determining (1) resistance: *actA*, actidione; *acrA*, acriflavine; *iodA*, iodoacetate; *facA*, fluoracetate; (2) conidiospore colour: *chaA*, chartreuse; *wA*, white; *yA*, yellow; (3) nutritional requirements: *biA*, biotin; *nicB*, nicotinamide; *pabaB*, *p*-aminobenzoic acid; *phenA*, phenylalanine; *proA*, proline; *pyroA*, pyridoxine; *riboA*, *riboB*, riboflavin; (4) inability to utilize: *cnxA*, nitrate; *galA*, galactose; *sC*, sulphate; (5) decreased enzyme activity: *palC*, alkaline phosphatase; (6) suppressor: *suBproA*, suppresses *proA*; (7) altered penicillin yield: *penB*, *penC*, raised titre; *npeB*, *npeC*, *npeD*, reduced titre; (8) morphology: *sgp*, slow growing non-perithecial.

Domestic codes	Linkage group ...	I	II	III	IV	V	VI	VII	VIII
G27	<i>biA</i>	+	+	+	+	+	+	+	<i>chaA</i>
G30	<i>yA</i>	<i>acrA</i>	<i>galA</i>	<i>pyroA</i>	<i>facA</i>	<i>sB</i>	<i>nicB</i>	<i>riboB</i>	
G107	<i>biA</i>	<i>wA</i>	+	<i>penC</i>	+	+	<i>nicB</i>	+	
G109	+	<i>wA</i>	<i>penB</i>	<i>pyroA</i>	<i>facA</i>	+	+	<i>riboB</i>	
G121	Prototroph bearing <i>sgp</i> (linkage group unknown)								
GH49	<i>yA</i>	+	(<i>npeB</i>)	<i>pyroA</i>	+	+	+	<i>cnxA</i>	
GH50	<i>yA</i>	+	+	(<i>npeC</i>) <i>pyroA</i>	+	+	+	<i>cnxA</i>	
GH117	<i>biA</i>	<i>wA</i> (<i>npeD</i>)	+	+	+	+	+	+	
GH119	<i>biA</i>	<i>wA</i>	+	(<i>npeC</i>)	+	+	+	+	
GH124	<i>biA</i>	<i>wA</i>	(<i>npeB</i>)	+	+	+	+	<i>cnxA</i>	
GH163	+	<i>wA</i>	+	<i>palC</i> <i>pabaB</i>	+	+	+	+	
				<i>pyroA</i>					
GH165	<i>riboA</i> <i>proA</i>	<i>wA</i>	<i>sC</i> <i>galA</i>	+	+	+	+	+	
	<i>biA</i>		<i>actA</i> <i>phenA</i>						
			<i>suBproA</i>						
GH168	+	<i>iodA</i> <i>acrA</i>	+	+	+	+	+	+	
GH170	<i>biA</i>	<i>wA</i>	+	<i>penC</i>	+	+	<i>nicB</i>	+	

Haploidization agents. Benlate [methyl(1-butylcarbamoyl)-2-benzimidazole carbamate] was obtained from Dupont Chemicals Co. Ltd, and Thiram (tetramethylthiuram disulphide) from BDH.

Genetic techniques. Except where indicated, parasexual and sexual crosses and the characterization of the resulting progeny were performed according to the procedures of Pontecorvo *et al.* (1953). The growth of segregants on diagnostic media (MM supplemented with various combinations of required nutrients or CM with the addition of certain normally toxic chemicals) was scored numerically on a 1 to 5 scale, by reference to the growth of the wild-type (5) and the mutant strains (1) inoculated side by side on the same agar medium. These data along with the penicillin titres of each segregant were then processed by a data-sorting computer program (written by J. F. M.), which calculated and displayed the frequencies of recombination for every pairwise combination of marker genes segregating in a cross, having first assigned each segregant a particular numerical *npe* or *pen* genotype on the basis of its penicillin titre. In addition, this program could produce an analysis of the penicillin titre distribution with respect to each marker gene, and the average titre of all segregants showing a particular genotype. Strains with characteristics that were intermediate between those associated with mutant or wild-type alleles could be excluded from analysis or be processed as having either the mutant or the wild-type phenotype in consecutive runs of the program.

Penicillin yield testing. Penicillin production was assessed using the method of Holt & Macdonald (1968).

RESULTS

The following pairs of strains were crossed parasexually: GH124 (*npeB*) × G30; GH119 (*npeC*) × G30; and GH117 (*npeD*) × G30. Strain G30 is labelled on all eight linkage groups. Following haploidization of the heterozygous diploids on CM containing 1 µg benlate ml⁻¹ for the first cross, or CM containing 2.5 µg thiram ml⁻¹ (Upshall & Johnson, 1981) for the second and third crosses, segregants were isolated and tested for penicillin yield and for the genetic markers borne. The results showed that *npeB* was located on chromosome III, *npeC* on chromosome IV, and *npeD* on chromosome II (Table 2). The few segregants isolated showing recombination between *npeB* and *galA* and between *npeD* and *acrA* or *wA* (numbers italicized in Table 2) were probably the result of coincident mitotic crossing over.

After a sexual cross had established that *npeB* was more than 50 map units from *galE*, *adI* and *methH*, all located on the left arm of chromosome III, an analysis of the progeny from a sexual

Table 2. Frequency of segregation of various markers on known linkage groups with *npeB*, *npeC* or *npeD*

Segregants were isolated following induced haploidization of the heterozygous diploids in three paraxial crosses between strains G30 and GH124 (*npeB*), G30 and GH119 (*npeC*), and G30 and GH117 (*npeD*). The difference in the total numbers of segregants analysed for each pair of markers results from the exclusion of segregants which were not unequivocally scored as being of either genotype (see Methods). The figures in *italic* are the percentage of recombinants which were probably the result of coincident mitotic crossing over.

Linkage group ...		I	II	III	IV	V	VI	VII	VIII	I	II	VIII
Segregants of a cross between strains G30 and:		Marker gene ... <i>yA</i>	<i>acrA</i>	<i>galA</i>	<i>pyroA</i>	<i>facA</i>	<i>sB</i>	<i>nicB</i>	<i>riboB</i>	<i>biA</i>	<i>wA</i>	<i>cnxA</i>
		<i>trans</i> *								<i>cis</i> *		
GH124 (<i>npeB</i>)	Parental types	NA	20	51	22	33	27	28	29	30	NA	27
	Recombinants	NA	30	1	27	25	29	22	26	23	NA	24
	Recombinants (%)	NA	60	2	55	43	51	44	47	44	NA	47
GH119 (<i>npeC</i>)	Parental types	19	23	20	44	16	21	20	16	23	26	NC
	Recombinants	14	17	19	0	24	18	15	22	15	18	NC
	Recombinants (%)	42	42	48	0	60	46	42	57	40	41	NC
GH117 (<i>npeD</i>)	Parental types	5	54	22	21	32	23	24	26	18	58	NC
	Recombinants	11	1	27	29	18	30	29	29	34	1	NC
	Recombinants (%)	68	2	55	58	36	56	54	52	66	2	NC

NA, Not analysed; NC, not carried in cross.

* Marker configuration with respect to the *npe* mutation carried in each cross.

cross between strains GH49 and GH165 indicated that *npeB* was closely linked to the suppressor mutation *suBproA* on the right arm of chromosome III. The presence of this latter marker was scored by performing test crosses between recombinants not requiring proline and strain G27 which has chartreuse-coloured conidia. Ascospores from individual cleistothecia were plated on CM and the resulting growth inspected for the presence of colonies with different conidial colours in ratios consistent with their having arisen from a hybrid cleistothecium. Colonies from ascospores of such hybrid cleistothecia were then tested for the presence or absence of a requirement for proline. When the proportion of proline-requiring strains to prototrophs was about one third, the original proline independence was considered to have been due to the presence of the suppressor mutations *suBproA*. Data from this cross provided evidence that *npeB* was located about 8 map units from *suBproA* and some 30 from *phenA* (see Table 3a), placing it on the same chromosome which bore *penB* (Ditchburn *et al.*, 1976) but on the opposite arm.

A cross between strains GH163 and GH119 established that *npeC* was located between *palC* and *pabaB* on chromosome IV (Table 3b). Previous work by Ditchburn *et al.* (1976) had located *penC* on chromosome IV at a distance less than 50 units from *pyroA*. A cross between strains GH117 and GH168 located *npeD* at a distance of 6 units from *iodA* (Table 3c) confirming the suggestion of Cole (1977) that *npeD* was on the left arm of chromosome II.

It was decided then to set up crosses of the following types: *penC* × *npeC*, *penB* × *npeB*, *penB* × *npeC* and *penC* × *npeB*. It was envisaged that from the first cross the distribution of penicillin titres among progeny might give an estimate of linkage between *penC* and *npeC* while the other crosses would give a measure of the inherent variation in penicillin titre among progeny from crosses between strains where the mutations affecting penicillin titre were more than 50 map units apart on homologues of the same chromosome (*penB* × *npeB*) or on different chromosomes (*penB* × *npeC* and *penC* × *npeB*).

Strains of *A. nidulans* bearing *penC* do not produce cleistothecia, but Ditchburn *et al.* (1976) showed that *penC* mimicked mutants designated *sgp* which grew slowly and did not have cleistothecia (Houghton, 1970) but where interallelic crosses yielded fertile hybrids. Therefore a sexual cross was made between strain G107 (*penC nicB biA wA*) and strain G121 (*sgp*). Since

Table 3. *Recombination frequencies among segregants from crosses made to position the mutations npeB, npeC and npeD*

Values are the percentage recombination between each pair of markers carried on the same linkage group as the relevant *npe* mutation. The figures in *italic* show the percentage recovery of each marker gene among the population of segregants.

(a) *Recombination frequencies of markers carried on linkage group III among 211 segregants from the sexual cross of strains GH165 and GH49 (npeB)*

	<i>npeB</i>	<i>sC</i>	<i>galA</i>	<i>actA</i>	<i>phenA</i>	<i>suBpro</i>
<i>npeB</i>	51	49	46	36	30	8
<i>sC</i>		51	52	48	49	50
<i>galA</i>			48	10	16	38
<i>actA</i>				51	6	28
<i>phenA</i>					41	22
<i>suBpro</i>						53

(b) *Recombination frequencies of markers carried on linkage group IV among 187 segregants from the sexual cross of strains GH163 and GH119 (npeC)*

	<i>npeC</i>	<i>palC</i>	<i>pabaB</i>	<i>pyroA</i>
<i>npeC</i>	48	5	15	25
<i>palC</i>		54	30	40
<i>pabaB</i>			46	10
<i>pyroA</i>				44

(c) *Recombination frequencies of markers carried on linkage group II among 194 segregants from the sexual cross of strains GH168 and GH117 (npeD)*

	<i>npeD</i>	<i>iodA</i>	<i>acrA</i>	<i>wA</i>
<i>npeD</i>	51	6	41	50
<i>iodA</i>		51	35	50
<i>acrA</i>			48	25
<i>wA</i>				50

Table 4. *Distribution of penicillin titres among the progeny of crosses of types penC × npeC, penB × npeB, penB × npeC and penC × npeB*

Strains bearing *npeB* or *npeC* yielded 0.5 units penicillin ml⁻¹, the wild-type, 6 units ml⁻¹, strains bearing *penB*, 12 units ml⁻¹ and strains bearing *penC*, 20 units ml⁻¹.

Cross	No. of segregants in penicillin titre groups (units ml ⁻¹)				
	0-5	5-10	10-15	15-20	>20
GH170 (<i>penC</i>) × GH50 (<i>npeC</i>)	61	2	8	56	8
G109 (<i>penB</i>) × GH49 (<i>npeB</i>)	75	8	56	60	0
G109 (<i>penB</i>) × GH50 (<i>npeC</i>)	79	98	32	39	0
GH170 (<i>penC</i>) × GH49 (<i>npeB</i>)	138	48	67	33	0

neither was fertile, all the cleistothecia developing were considered to be hybrid. A pooled ascospore suspension was spread on nutritionally supplemented medium and after incubation (5 d, 37 °C) cleistothecia were evident on about 25% of the colonies; these were tested for penicillin yield and characterized for their auxotrophic requirements. An isolate having white conidia, requiring nicotinamide and biotin for growth, producing cleistothecia on SPM and with a penicillin titre of 16 units ml⁻¹ was designated GH170. The penicillin yield of this strain indicated that it bore *penC*.

Sexual crosses were then made as follows: GH170 (*penC*) × GH50 (*npeC*); G109 (*penB*) × GH49 (*npeB*); G109 (*penB*) × GH50 (*npeC*) and GH170 (*penC*) × GH49 (*npeB*). The full genotypes of these strains are shown in Table 1. Segregants were selected by their ability to grow on MM supplemented with pyridoxine and the results are given in Table 4.

While there was a broad distribution of penicillin yields among progeny from crosses of types *penB* × *npeC* and *penC* × *npeB*, only very few segregants from crosses of types *penB* × *npeB* and

Fig. 1. Genetic map of *Aspergillus nidulans*. Mutations concerned with the biosynthesis and overproduction of penicillin are in bold print and a few other genetic markers are shown to allow the location of these penicillin mutations. The results from Table 3 were used for the positioning of *npe* and *pen* mutants, and those from Clutterbuck (1981) for the positioning of other genetic markers.

Explanations of symbols: ○, centromere; *npeA*, *npeB*, *npeC*, *npeD*, impairing penicillin titre; *penA1*, *penB2*, *penC3*, increasing penicillin titre; *acrA*, *acrB*, acriflavine-resistant; *actA*, actidione-resistant; *apsA*, anucleate primary sterigmata; *chaA*, chartreuse-coloured conidia; *drkB*, dark coloured conidia; *galA*, galactose non-utilizing; *iodA*, iodoacetate resistant; *lacA*, lactose non-utilizing; *lysA*, lysine-requiring; *meaB*, methylammonium resistant; *methG*, methionine-requiring; *moC*, morphologically abnormal; *molA*, molybdate resistant; *nicC*, nicotinic acid-requiring; *nirA*, nitrite non-utilizing; *pabaB*, para-aminobenzoic acid-requiring; *palC*, alkaline phosphatase deficient; *palcA*, *palcB*, acid and alkaline phosphatase deficient; *phenA*, phenylalanine-requiring; *pyroA*, pyridoxine-requiring; *sbA*, sorbitol non-utilizing; *sC*, reduced inorganic sulphur-requiring; *suBpro*, suppressor of proline requirement; *thiA*, thiazole-requiring; *trypC*, tryptophan-requiring; *tsd*, temperature sensitive; *uX*, urea non-utilizing; *uaY*, uric acid non-utilizing; *veA*, velvet morphology; *wA*, white-coloured conidia.

penC × *npeC* gave intermediate penicillin titres, on initial tests, to suggest that they were recombinants bearing the wild-type alleles of the *pen* and *npe* mutations. Most were low or high in penicillin yield indicating that they carried the *pen* or the *npe* mutations, respectively. Among progeny from crosses of types *penB* × *npeB* and *penC* × *npeC* none of these few segregants which appeared to bear the wild-type alleles of the *pen* and *npe* mutations could be classified unequivocally as having intermediate penicillin titres on repeat tests.

A heterozygous diploid was synthesized between strain GH124 bearing *npeB* and having a penicillin titre of 0.1 units ml⁻¹ and strain G109 bearing *penB* and having a titre of 15 units ml⁻¹. The diploid yielded 12 units ml⁻¹. A second heterozygous diploid was made between strain GH50 bearing *npeC*, with a titre of 0.3 units ml⁻¹ and strain G107 bearing *penC* with a titre of 16 units ml⁻¹. It had a yield of 15 units ml⁻¹.

DISCUSSION

Three mutations *npeB*, *npeC* and *npeD*, impairing penicillin production, have been located on the genetic map of *A. nidulans*. Previous investigations located mutation *npeA* (Holt *et al.*, 1976), and three mutations increasing penicillin production, *penA*, *penB* and *penC*, have been mapped by Ditchburn *et al.* (1976). The positions of these seven mutations on the genetic map of *A. nidulans* are shown in Fig. 1.

All four *npe* mutations were shown to be recessive (Edwards *et al.*, 1974) but while *penA* behaved as a recessive mutation, *penB* was dominant and *penC* was semi-dominant (Ditchburn *et al.*, 1976). Our results, where diploids were synthesized between a strain bearing *npeB* and one bearing *penB* and between a strain bearing *penC* and one bearing *npeC*, were not in conflict with those of Edwards *et al.* (1974) or Ditchburn *et al.* (1976).

The inability to isolate *pen*⁺ *npe*⁺ recombinants, as measured by their penicillin titre, from crosses between *penB* and *npeB* strains is clearly not evidence of close linkage between *penB* and *npeB* as these genes have already been shown to be more than 50 map units apart. There may be processes acting against production or isolation of certain recombinants in these crosses. Consequently it is not possible to conclude that the absence of wild-type recombinants in the *penC* × *npeC* cross indicates close linkage of these two genes, and the relative positioning of the two mutations awaits a more accurate mapping of *penC*. If the mutations *penC* and *npeC* were contiguous, it would be of considerable interest indicating that both positive and negative mutations with respect to antibiotic titre could occur at the same locus; this would offer a genetic basis for explaining earlier work with *Streptomyces viridifaciens* (Dulaney & Dulaney, 1967), where it was shown that strains which reverted to production of tetracycline and were derived from mutants not yielding this antibiotic could have their antibiotic titre restored to that of the grandparent strain. What is even more important was that, in some cases, the antibiotic titres of the revertants were higher than that of the grandparent strain (Dulaney & Dulaney, 1967; Dhar & Bose, 1969).

That enzymes altered from their original configuration, perhaps by sequential nonsense and missense mutations, can have enhanced properties relative to the wild-type enzyme is supported by studies on the enzyme glutamate dehydrogenase in *Neurospora crassa*. Following the isolation of enzyme-negative mutants, revertants were selected from these, some of which yielded enzymes with increased heat stability relative to the original wild-type enzyme (Fincham, 1973). Similarly, an enzyme involved in the biosynthesis of an antibiotic could be altered by two successive mutations in such a way that the outcome was for the doubly mutated strain to elaborate more antibiotic than its grandparent strain.

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