Co-synthesis of Penicillin Following Treatment of Mutants of *Aspergillus nidulans* Impaired in Antibiotic Production with Lytic Enzymes

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Mycelia from four mutants of *Aspergillus nidulans* impaired in penicillin production at separate genetic loci were treated with an enzyme complex capable of lysing cell walls, then mixed in all possible paired combinations and grown in osmotically buffered penicillin production media, containing 2-deoxyglucose and an unrefined mixture of polyoxins to prevent cell wall regeneration. The culture filtrates were assayed after 6 d and significant penicillin yields were observed in four of the six possible combinations. None of these pairs produced penicillin when grown together as normal mycelium, suggesting that intermediates of the penicillin biosynthetic pathway unable to diffuse from untreated mycelium could do so from enzyme-treated mycelium when cell wall regeneration was inhibited. A general method is thus available for examining biochemical pathways with mutants accumulating intermediates unable to cross the cell wall barrier.

INTRODUCTION

If two mutants blocked at different steps in the biosynthetic pathway of an antibiotic are grown together, an intermediate from the mutant blocked at a later stage could diffuse to the other mutant so that antibiotic synthesis is achieved. Such co-synthesis has been demonstrated in *Streptomyces coelicolor* (Kirby *et al.*, 1975; Rudd & Hopwood, 1979) but previous attempts to use this method in fungal systems have failed. Bonner (1947) grew all possible pairs of 55 mutants of *Penicillium notatum* impaired in penicillin production, but no significant penicillin production was observed. Pairs of antibiotically inactive mutants of *Cephalosporium acremonium* examined by Nash *et al.* (1974) did not co-synthesize penicillin N or cephalosporin C, although presumptive heterokaryons between certain inactive pairs did show some antibiotic synthesis, suggesting that the intermediates of β -lactam antibiotic synthesis could not diffuse across the cell wall barrier. The growth together of pairs of strains of *Penicillium chrysogenum* (Normansell *et al.*, 1979) impaired in penicillin synthesis at different genetic loci did not result in any significant penicillin production.

The formation of protoplasts of filamentous fungi by treatment with lytic enzymes is well documented (Peberdy, 1979) and such protoplast preparations have been employed in biochemical (Nomi *et al.*, 1978; Dutton & Anderson, 1978), genetic (Dales & Croft, 1977) and morphological investigations (Gibson & Peberdy, 1972).

To overcome the permeability barrier of the cell wall, protoplasts of *P. chrysogenum* and *C. acremonium* have been used in biochemical studies of penicillin and cephalosporin biosynthesis involving the uptake of radioactive intermediates (Fawcett *et al.*, 1973).

Unfortunately protoplasts exist only for 2 to 3 h before cell wall regeneration begins, and hence in order to use them in co-synthesis experiments, it is necessary to inhibit cell wall regeneration for a significant period.

We have now developed a system using known inhibitors of cell wall synthesis and osmotically fragile mycelium (rather than protoplasts) in which antibiotic co-synthesis by certain pairs of strains of *A. nidulans* carrying mutations impairing penicillin production (*npe*) at different loci can be demonstrated. The mutations involved – *npeA*, *B*, *C* and *D* – complement each other in diploids and hence probably produce blocks at different points in penicillin biosynthesis.

METHODS

Organisms. Strain NRRL 194 of *Aspergillus nidulans* (the wild-type strain originating from Glasgow University) and a number of derivatives were used (Table 1). *Streptomyces cacaoi* var. *asoensis* (strains ATCC 19093 and ATCC 19094) were obtained from the American Type Culture Collection. *Trichoderma harzianum* (strain CBS 354.33) was kindly provided by Drs R. B. G. Dales and J. H. Croft, University of Birmingham.

Media. The penicillin production medium (PPM) was as described by Holt & Macdonald (1968). Osmotically buffered penicillin production medium (osmo-PPM) was PPM supplemented with KCl (70 g l⁻¹). Osmo-PPM with the addition of freeze-dried unrefined polyoxins (2.5 g l⁻¹) and 2-deoxyglucose (0.25 g l⁻¹) was referred to as protoplast penicillin production medium (proto-PPM). *Aspergillus* minimal medium (AMM) and *Aspergillus* complete medium (ACM) were based on the formulations of Pontecorvo *et al.* (1953). Osmo-AMM and proto-AMM contained the same additives as osmo-PPM and proto-PPM.

The *Trichoderma* enzyme production medium (TEPM) was derived from the recipe of Peberdy & Isaac (1976). Heat-sterilized mycelium of *A. nidulans* strain NRRL 194 from a 48 h shake culture in liquid ACM partially replaced the laminarin (see later). The medium consisted of: glucose, 3 g; laminarin, 1 g; chitin (BDH), 5 g; bacteriological peptone (Oxoid), 1 g; (NH₄)₂SO₄, 1.4 g; KH₂PO₄, 2 g; MgSO₄·7H₂O, 0.3 g; CaCl₂·6H₂O, 0.3 g; trace element solution (Vogel, 1964), 1 ml; mycelium from *A. nidulans*, 5 g wet wt; tap water to 1 l. The mixture was dispensed in 500 ml quantities in 2 l conical flasks and sterilized by autoclaving. When cool, 2.5 ml 40 % (w/v) sterile urea (Oxoid) was added to each flask. *Streptomyces* media were based on those of Isono *et al.* (1967). *Streptomyces* seed medium (SSM) comprised: soluble starch, 10 g; glucose, 10 g; soybean meal, 20 g; yeast extract, 10 g; NaNO₃, 2 g; K₂HPO₄, 2 g; distilled water to 1 l. The pH was adjusted to 6.8 with NaOH. *Streptomyces* polyoxin production medium (SPPM) was the same as SSM except that the soluble starch content was increased to 90 g and the yeast extract to 40 g. Protoplast buffer solution (PBS) contained (per litre) 50 g KCl, 31.2 g NaH₂PO₄·2H₂O, and 28.3 g Na₂HPO₄, and was used to protect protoplasts and enzyme-treated mycelium from osmotic shock and pH changes during their preparation and subsequent manipulation.

Preparation of laminarin. Fronds of the seaweed *Laminaria digitata* were dried for 1 week at 37 °C and then milled to a fine powder. Approximately 100 g was added to 1 l water and the mixture was heated to 70 °C and maintained at this temperature for 15 min with occasional stirring. The resulting slurry was centrifuged (300 g for 10 min); the supernatant was decanted, allowed to cool and then an excess of ethanol was added. Laminarin precipitated as a fine white powder which was harvested by filtration and dried in an oven at 60 °C. The usual yield was about 20 % (dry weight).

Preparation of lytic enzyme complex from *Trichoderma harzianum*. The strain was grown for 3 d at 25 °C in continuous light on slopes of TEPM solidified with 4 % (w/v) Oxoid no. 3 agar. A spore suspension prepared from these slopes was then inoculated into 500 ml TEPM in 2 l flasks, to give a final concentration of approximately 10⁶ spores ml⁻¹. Flasks were incubated for 100 h at 30 °C on a rotary shaker (200 rev. min⁻¹) illuminated by four 60 W fluorescent striplights, and then the contents were gravity filtered through glass microfibre filters (Whatman GF/F). The culture filtrate was centrifuged (4000 g for 15 min) and the clear supernatant was decanted off and freeze-dried in 28.5 ml McCartney bottles. The enzyme was re-constituted by adding 2 ml PBS and shaking in a vortex blender. It was then sterilized by membrane filtration. This enzyme mixture, containing chitinase and a variety of β -glucanases, is referred to as 'polystripping'.

Preparation of polyoxin mixture. Four 100 ml flasks each containing 25 ml SSM were inoculated with equal mixtures of spores of *Streptomyces cacaoi* var. *asoensis* strains ATCC 19093 and ATCC 19094 and incubated at 25 °C on a rotary shaker (200 rev. min⁻¹) for 3 d. These seed cultures were then used to inoculate 8 l SPPM in a 14 l fermenter vessel (Microform model MF1/H, New Brunswick Scientific Co.) and grown for 6 d at 25 °C while being stirred at 200 rev. min⁻¹ with an airflow through the mixture of 5 l min⁻¹. The broth was harvested by filtering through glass microfibre filters (Whatman GF/F). The filtrate

Table 1. *Strains of Aspergillus nidulans used*

Domestic code	Genotype*
G (NRRL 194)	Prototroph
GH79	<i>yA2; pyroA4; cnxA5; npeA0022</i>
GH117	<i>biA1; methG1; wA3; npeD0045</i>
GH119	<i>biA1; wA3; npeC007</i>
GH124	<i>biA1; wA3; cnxA5; npeB006</i>

* The gene symbols used are those of Clutterbuck (1974): *y*, yellow conidiospores; *w*, white conidiospores; *pyro*, *meth*, *bi*, requiring pyridoxine, methionine and biotin, respectively; *cnx*, lacking the enzymes nitrate reductase and xanthine dehydrogenase, characterized by the inability to utilize nitrate as a sole nitrogen source; *npe*, impairment of penicillin production (less than 10% of parental titre).

was centrifuged (5000 g for 15 min at 4 °C), and the supernatant was decanted and poured on to a column containing cationic exchange resin [Dowex 50W-X8, 20 to 50 US mesh (H)], left standing at 4 °C overnight and then eluted with 0.3 M-NH₄OH. The eluate was freeze-dried.

Production and culture of osmotically fragile mycelium of *A. nidulans*. One ml of a suspension of approximately 10⁶ spores ml⁻¹ from each strain of *A. nidulans* was added to 100 ml ACM in 500 ml conical flasks and incubated at 25 °C for 48 h on a rotary shaker (200 rev. min⁻¹). The culture was then centrifuged (4000 g for 5 min). The mycelium was resuspended in 5 ml 2 M-KCl in 0.1 M-phosphate buffer (pH 5.8) after which 1 ml of reconstituted polystripping was added along with 4 ml 10% (v/v) *Helix pomatia* juice (HPJ; from Uniscience, Cambridge). This mixture was incubated at 25 °C with gentle shaking for 1 h. The mycelium was then centrifuged (4500 g for 5 min), washed twice and re-pelleted in PBS. For the strains bearing different *npe* mutations, 1 g lots of mycelial pellets were mixed together in the presence of 6 ml PBS, in all pairwise combinations. Two ml of each mixture was added to three 100 ml polypropylene flasks each containing 18 ml proto-PPM. As controls, 2 g portions of unmixed mycelium from each strain were added to each of three flasks containing the same medium. After 6 d at 25 °C on a rotary shaker (220 rev. min⁻¹) the mycelium was harvested by gravity filtration (Whatman no. 113V fluted filter papers) and the filtrate was collected in 28.5 ml Universal bottles and assayed for penicillin.

Estimation of the effects of chemical inhibitors on cell wall regeneration in protoplasts of *A. nidulans*. Protoplasts of *A. nidulans* strain GH79 were prepared using the technique of Ferenczy *et al.* (1975), except that polystripping was used as a lytic enzyme instead of *Helix pomatia* juice. Protoplasts were harvested in PBS and their concentration was estimated by haemocytometer count. The suspension was inoculated into 10 ml proto-ACM in Universal bottles to give a final concentration of 10⁸ protoplasts ml⁻¹. These cultures were maintained at 20 °C and gently agitated using a Spiromix (Denley). Samples were taken at various times over several days and 0.1 ml portions were spread on ACM and osmo-ACM plates. Each plate was incubated for 2 d and the number of visible colonies was counted.

Estimation of the effects of chemical inhibitors on cell wall repair in enzyme-treated mycelium of *A. nidulans*. Osmotically fragile mycelium of *A. nidulans* strain GH79 was prepared as described above and 0.5 g (wet wt) samples were used to inoculate twelve 100 ml conical flasks, six containing 20 ml osmo-ACM and six containing 20 ml proto-ACM. These were incubated at 25 °C on a rotary shaker (200 rev. min⁻¹). At various times, flasks were removed from the shaker and the mycelium was harvested by centrifugation. A known wet weight (about 0.5 g) of each mycelial pellet was then added to 10 ml PBS in a 28.5 ml Universal bottle and gently agitated for 30 min on a Spiromix, after which the suspension was filtered through a sintered glass crucible (porosity 1). Samples of the filtrate at several dilutions were spread in 0.1 ml portions on to ACM and osmo-ACM agar plates. After 48 h incubation at 37 °C the number of colonies on each plate was counted.

Antibiotic assay. The penicillin content of the culture filtrates was measured using an agar well diffusion assay as described by Holt & Macdonald (1968). Fifteen samples were included in duplicate in each assay plate along with three duplicated standard concentrations of benzylpenicillin (1, 2 and 4 units ml⁻¹). The test organism was *Bacillus subtilis* strain NCTC 8236. After overnight incubation at 37 °C the diameters of the inhibition zones around each well were measured. The zone sizes were converted to benzylpenicillin equivalent units using a computer programme. To ensure that the antibiotic assayed was indeed penicillin, parallel assays were performed with a β -lactamase (BDH; EC 3.5.2.6) present in the agar at a concentration of 0.09 EU ml⁻¹ (1 EU hydrolyses 1 μ mol benzylpenicillin min⁻¹ at 25 °C).

Chemicals. Dimilin [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] was a gift from Durhar-Midox Ltd, Smarden, Kent. A pure sample of polyoxin D was a gift from the Kaken Chemical Co., Japan, and a sample of agricultural grade polyoxin D was from the Kuniai Chemical Co., Japan.

Fig. 1. Number of colonies of *A. nidulans* strain GH79 regenerating from a suspension of protoplasts incubated in liquid proto-AMM, when 0.1 ml portions were spread at various times on the surface of ACM (●) or osmo-ACM (○) agar plates.

RESULTS

Three inhibitors of cell wall synthesis were tested, either alone or in combination, for their ability to inhibit protoplast regeneration. The first, 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea (Dimilin), reported as an inhibitor of chitin synthesis in insects (Deul *et al.*, 1978), had no visible effect on the regeneration of cell walls by protoplasts of *A. nidulans*. The second compound, 2-deoxyglucose, which inhibits wall synthesis in both young yeast cells (Johnson, 1968) and yeast protoplasts (Farkas *et al.*, 1969) as well as retarding the synthesis of the α -glucan wall component of *A. nidulans* (Zonneveld, 1973), was found to delay the regeneration of cell walls by protoplasts of *A. nidulans* for up to 12 h. The third inhibitor tested was a group of closely related uracil nucleotide antibiotics termed polyoxins which are produced together in broth cultures of *Streptomyces cacaoi* var. *asoensis*. They competitively inhibit chitin synthesis (Hori *et al.*, 1974*a, b*), and the formation of protoplast-like bodies from mycelium of *Alternaria kikuchiana tanaka* growing in the presence of polyoxins has been described (Ishizaki *et al.*, 1974). Polyoxins A to J have no effect on the growth of *Aspergillus* species (Isono *et al.*, 1967), but a mixture of polyoxins was found to retard the regeneration of protoplasts from *A. nidulans* for up to 2 d.

Combinations of Dimilin with 2-deoxyglucose and Dimilin with polyoxins showed no greater inhibition of regeneration than did 2-deoxyglucose or polyoxins on their own, but combinations of 2-deoxyglucose and polyoxins acted synergistically and retarded the regeneration of protoplasts for up to 6 d.

When protoplasts were incubated in liquid proto-AMM (osmotically buffered minimal medium to which 2-deoxyglucose and polyoxin had been added) and spread at intervals on to the surface of ACM or osmo-ACM agar plates, the difference between the number of colonies growing on each medium reflected the number of protoplasts which had not regenerated a cell wall (Fig. 1). On ACM, the latter would lyse and only protoplasts which had produced a new cell wall would give rise to colonies; while on osmo-ACM, all cell particles surviving would yield individual colonies. Although the viability of protoplasts incubated in proto-AMM was progressively reduced, they still exhibited some osmotic fragility even beyond 5 d (Fig. 1). This effect was not observed when pure or agricultural polyoxin D was used in combination with 2-deoxyglucose, indicating that a polyoxin other than polyoxin D, or a combination of polyoxins, with 2-deoxyglucose, was responsible for the inhibition of cell wall regeneration.

Protoplasts of mutants of *A. nidulans* bearing *npeA*, *B*, *C* or *D* were cultured in pairs in proto-PPM but no increase in penicillin production was detected in any combination of mutants. One possibility was that none of the intermediates of antibiotic synthesis from one

Fig. 2. Concentration of protoplasts of *A. nidulans* strain GH79 (determined by the difference in numbers of colonies developing on ACM and on osmo-ACM) released per gram (wet wt) of enzyme-treated mycelium when transferred to PBS after various periods of incubation in proto-ACM (●) or osmo-ACM (○).

strain were able to reach the sites of synthesis in the other. However, it was also possible that protoplasts were either unable to enter secondary metabolism or were unable to withstand the mechanical stress of submerged shake culture – certainly vigorous shaking of protoplasts in PBS can cause extensive lysis.

We therefore decided to investigate the effects of polyoxins and 2-deoxyglucose on cell wall regeneration in osmotically fragile mycelium. It was presumed that treatment with a lytic enzyme complex would lead to cell wall degradation but that protoplasts would not form if the osmotic pressure of the environment was kept sufficiently above that of the cell contents. Dilution of the osmotic environment would allow the release of protoplasts so long as the cell wall still contained discontinuities. Mycelium of strain GH79, treated so as to render it osmotically fragile, was grown in proto-ACM or osmo-ACM for different periods then transferred to PBS and gently agitated for 30 min. Following this, samples were plated on ACM and osmo-ACM. The difference in colony counts on these two plating media indicated the number of viable protoplasts in each sample. Protoplasts were present in every sample where the osmotically fragile mycelium had been cultured in proto-ACM, while samples in which the culture had taken place in osmo-ACM did not contain significant numbers of protoplasts at any time after 24 h of incubation (Fig. 2). This suggests that discontinuities in the mycelial cell wall, which could act as sites for protoplast release, were repaired during culture in osmo-ACM, and that this repair process was inhibited during culture in proto-ACM.

In the hope that such osmotically fragile mycelium would have sufficient rigidity to cope with the stress of submerged shake culture, and also be able to enter secondary metabolism, a further series of experiments was done. The penicillin yields of strains grown singly and in pairs, with or without polystripping and HPJ treatment, in osmo-PPM or in proto-PPM are shown in Table 2. Statistical tests for co-synthesis were made by comparing the yields of each mixture with that of its higher yielding component grown alone, under each set of conditions. After enzyme treatment followed by growth in proto-PPM, significant co-synthesis was detected in three pairs of mutants (*npeA/B*, *npeA/C*, *npeA/D*) with some possible co-synthesis in the *npeB/C* combination. Penicillin co-synthesis was not detected in osmo-PPM, or when proto-PPM had been inoculated with untreated mixtures of mycelia, indicating that both lytic enzyme treatment and the presence of polyoxin and 2-deoxyglucose were required for this process.

In the absence of polyoxin and 2-deoxyglucose, penicillin production by the wild-type strain is complete after 5 d submerged shaken culture; however, in proto-PPM containing

Table 2. *Penicillin production by strains of A. nidulans grown singly or in pairs, for 6 d in osmo-PPM or proto-PPM, with or without pretreatment of the mycelia with lytic enzymes*

		Penicillin production (benzylpenicillin equivalent units ml ⁻¹)							
Strain(s)	Relevant genotype	From untreated mycelia in osmo-PPM		From polystripping/HPJ-treated mycelia in osmo-PPM		From untreated mycelia in proto-PPM		From polystripping/HPJ-treated mycelia in proto-PPM	
		Titre Significance*		Titre Significance*		Titre Significance*		Titre Significance*	
		Titre	Significance*	Titre	Significance*	Titre	Significance*	Titre	Significance*
G	Wild-type	4.3		4.2		3.65		2.1	
GH79	<i>npeA</i>	0.18		0.1		0.22		0.23	
GH117	<i>npeD</i>	0.25		0.2		0.29		0.26	
GH119	<i>npeC</i>	0.4		0.2		0.42		0.34	
GH124	<i>npeB</i>	0.0		0.0		0.01		0.02	
GH79/GH124	<i>npeA/npeB</i>	0.22	NS	0.1	NS	0.2	NS	0.88	1 %
GH79/GH119	<i>npeA/npeC</i>	0.54	NS	0.1	NS	0.5	NS	1.73	0.1 %
GH79/GH117	<i>npeA/npeD</i>	0.37	NS	0.1	NS	0.31	NS	1.23	0.1 %
GH124/GH119	<i>npeB/npeC</i>	0.58	NS	0.2	NS	0.58	NS	0.95	5 %
GH124/GH117	<i>npeB/npeD</i>	0.48	NS	0.3	NS	0.46	NS	0.44	NS
GH119/GH117	<i>npeC/npeD</i>	0.28	NS	0.2	NS	0.2	NS	0.3	NS

* Non-significance (NS), or significance at the confidence level shown, of penicillin production by mixed cultures determined after comparison with the higher yielding component of the mixture grown under the same conditions.

these inhibitors an extra 24 h growth was required to reach the maximum titres. The penicillin yield of untreated mycelium of the wild-type strain was slightly less (71.5 %) after culture in proto-PPM than in osmo-PPM. This depression of yield and extension of the required culture time could be due to toxic effects of 2-deoxyglucose. After enzyme treatment, mycelium from the wild-type strain cultured in osmo-PPM elaborated nearly identical amounts of penicillin to the untreated mycelium in the same medium, indicating that this treatment does not by itself significantly inhibit penicillin production. However, in the presence of polyoxin and 2-deoxyglucose, enzyme-treated mycelium produced only half the penicillin elaborated in their absence, suggesting that osmotically fragile mycelium is more susceptible to the toxic effects of these chemicals. The different treatments and culture conditions had no significant effect on the penicillin yields of the mutant strains grown alone.

DISCUSSION

Failure to observe β -lactam antibiotic synthesis in paired growth of mutants (Bonner, 1947; Nash *et al.*, 1974) has been attributed to the impermeability of fungal cells to intermediates of antibiotic synthesis (Macdonald & Holt, 1976). If the cell wall were responsible for the impermeability then protoplasts might be expected to allow the passage of the intermediates, but this expectation was not realized in our investigations or in preliminary experiments of Anné (1977), who suggested that the cytoplasmic membrane was the main barrier in *P. chrysogenum*. In this context it is worth noting the experiments of Fawcett *et al.* (1973) who investigated the capabilities of protoplasts of *Cephalosporium acremonium* and *P. chrysogenum* to take up intermediates of β -lactam antibiotic biosynthesis. Protoplasts of *C. acremonium* incorporated L- α -amino adipic acid and valine, but only the latter was absorbed by *P. chrysogenum*. The successful production of penicillin in paired cultures of *A. nidulans*, described here, may indicate a further innate difference between the cytoplasmic membranes of different organisms or be caused by some experimental factor such

as the presence of polyoxins affecting membrane permeability. Certainly the protoplasts should be regarded as a stressed system. When compared with intact cells, protoplasts of higher plants show altered metabolism with respect to leucine uptake, electrical potential, protein synthesis, RNAase activity and photosynthesis (Burgess, 1978). Possibly such metabolic stress reduced the permeability of the cytoplasmic membrane, and this was responsible for the failure to observe co-synthesis when protoplasts were grown together. Osmotically fragile mycelium could be under less constraint than protoplasts and so permit the passage of intermediates across the cell membrane.

The inability of polyoxins to affect the growth of spores or intact mycelium of *Aspergillus* species (Isono *et al.*, 1967) while having obvious effects on protoplast cell wall regeneration might be due to the protoplast affording greater access to the active site of these compounds.

Edwards *et al.* (1974) described the isolation and genetic analysis of 28 mutants of *A. nidulans* impaired in penicillin production. Of these, 20 were impaired at the same genetic locus, *npeA*, and the others were members of at least four different complementation groups. Certain wild-type isolates of *A. nidulans* do not produce penicillin and this has been shown to be due to a mutation at the *npeA* locus (Cole *et al.*, 1976). Studies with *P. chrysogenum* (Normansell *et al.*, 1979) have revealed the existence of five distinct genetic loci, designated *npeV*, *W*, *X*, *Y* and *Z*, mutations at which result in impairment of penicillin production. Of 16 mutants, nine belonged to the same complementation group, *npeY*. Biochemical differences between mutants of these five complementation groups were investigated and strains carrying the mutation *npeY* were unable to produce the tripeptide aminoadipylcysteinylvaline, which was shown by Arnstein & Morris (1960) to be a precursor of penicillin. Sermonti (1959) isolated from *P. chrysogenum* nine mutants impaired in penicillin production, eight of which were in one complementation group. He suggested that this locus controlled the condensation of the three amino acids, α -aminoadipic acid, cysteine and valine. In *A. nidulans* most mutations resulting in impaired penicillin production are at *npeA*, while in *P. chrysogenum* most are at *npeY*. It is possible that *npeA* is functionally similar to *npeY* and hence that mutations at *npeA* also result in an inability to form Arnstein's tripeptide, in which case the supply of this compound to strains bearing *npeA* could result in penicillin production. In all the combinations of mycelia grown in proto-PPM after treatment with the enzyme complex polystripping and HPJ, significant penicillin production was observed when one of the component strains bore a mutation in *npeA*. This suggests that mutant strains bearing *npeB*, *C* or *D* are blocked later in the penicillin pathway than those of *npeA*, so that early intermediates could pass from the former to the latter, and permit production of the antibiotic. If this were due to the diffusion of tripeptide then it may be possible to isolate this compound from strains carrying *npeB*, *C* or *D* mutations, treated with polystripping and HPJ, and grown in proto-PPM. Co-culture of strains of *P. chrysogenum* carrying a *npeY* mutation with strains of *A. nidulans* bearing a mutation in *npeB*, *C* or *D* could also result in the production of penicillin. Whether or not the substance is α -aminoadipylcysteinylvaline, clearly some compound (or compounds) is capable of diffusing from certain strains of *A. nidulans* to others and there acting as an intermediate in penicillin biosynthesis. The method of co-synthesis which has been described in this paper presumably results from the passage of the intermediate through enzyme-induced discontinuities in the mycelial wall, which are stabilized by the presence of polyoxin and 2-deoxyglucose. The method is apparently well suited to the examination of biosynthetic pathways in fungi with mutants releasing intermediates to which the cell wall is impermeable.

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