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Characterization of a new choline locus in *Aspergillus nidulans* and its significance for choline metabolism

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SUMMARY

A new locus for choline-requirement has been identified in *Aspergillus nidulans* and designated *choC3*. It has been located to linkage group VIII at a position $31{\cdot}9 \pm 2{\cdot}2$ map units to the left of the *sD* locus.

It is proposed that this locus codes either for the enzyme that catalyses the transmethylation of phosphatidyl monomethylaminoethanol to phosphatidyl dimethylaminoethanol or for a molecule essential for the synthesis or function of this enzyme.

1. INTRODUCTION

Little is known about choline metabolism in *A. nidulans* in comparison with several other microorganisms such as *Neurospora crassa*, *Saccharomyces cerevisiae* and *Agrobacterium tumefaciens* (Scarborough & Nyc, 1967*a*, *b*; Steiner & Lester, 1970; Kaneshiro & Law, 1964). The major part of the work on choline metabolism has been carried out by these workers and by other groups using rat liver microsomes (Kennedy & Weiss, 1956; Bremer & Greenberg, 1959; 1960, 1961). In *A. nidulans* however, apart from an investigation showing that some choline mutants deprived of choline developed abnormally (Markham & Bainbridge, 1978), the few metabolic studies have been largely concerned with the role of choline in sulphate metabolism (Arst, 1968, 1971; Gravel, 1976). For many years only one locus affecting choline requirement (*choA1*) was known in this organism (Pontecorvo *et al.* 1953), but recently Waldron & Roberts (1974) have detected a second locus (*choB*).

This work reports the identification of a further choline locus and an investigation of the response of strains carrying mutations at each of the three loci, to various precursors of intermediates known to be involved in choline metabolism.

2. METHODS

(i) *Organisms.* Nine strains of *A. nidulans* were used (Table 1). Strains BWB152, BWB167, BV121, PM1 and PM3 are from the Queen Elizabeth College collection. Strains FGSC421 and FGSC489 were obtained from the Fungal Genetics Stock

Center, Arcata, California 95521, U.S.A. Strain CS48 was kindly provided by Dr C. F. Roberts and strain G83A arose by spontaneous reversion of the *galC* mutation, from strain G83 which was obtained from the Glasgow University collection. Strain BV121 carries the novel choline-requirement mutation that has been designated *choC3* in accordance with the proposals of Clutterbuck (1973) and also an uncharacterized temperature-sensitive mutation *ts-121*. It was first isolated by Dr B. P. Valentine (1975) from strain BWB152 by treatment with the mutagen *N*-methyl-*N'* nitro-*N*-nitrosoguanidine. Strain PM1 was isolated from the haploidization of a diploid between strains BV121 and BWB167. Strain PM3 is a recombinant from a meiotic cross between strains PM1 and G83A. PM1 and PM3 do not carry the *ts-121* mutation. All strains were maintained on CM plates.

Table 1. *Strains of* A. nidulans *used. Gene symbols are those of Clutterbuck (1974)*

Strain	Genotype
BWB152	*pabaA1, biA1*
BWB167(G95)	*yA; AcrA1; galA1; pyroA4; facA303; sB3; nicB8; riboB2*
BV121	*pabaA1, biA1, ts-121; choC3*
PM1	*yA; pyroA4; sB3; nicB8; choC3*
PM3	*pabaA1, biA1; fwA1, choC3*
FGSC421	*riboA1; sD85*
FGSC489	*riboA1, biA1; dilA1; choA1*
CS48	*wA3; pyroA4; choB (cs48)*
G83A	*pabaA1; fwA1, facB101, riboB2*

(ii) *Media.* The basic media were prepared by the methods of Pontecorvo *et al.* (1953), except that minimal medium (MM) was supplemented with 50 ml/l. of trace element solution, see below. The complete medium (CM) as modified by MacKintosh & Pritchard (1963) was supplemented with 25 ml/l. of trace element solution and with the standard vitamin solution.

The trace element solution consisted of (g/l. distilled water): EDTA $Na_2 2H_2O$, 12·0; $MgSO_4.7H_2O$, 5·0; $CaCl_2$, 1·0; $ZnSO_4.7H_2O$, 0·4; $MnSO_4.4H_2O$, 0·4; $CuSO_4.5H_2O$, 0·1; $FeSO_4 \cdot 7H_2O$, 2·0; Na_2SO_4, 8·5; $NaMoO_4 \cdot 2H_2O$, 0·1.

MM was supplemented as required with vitamins and with choline chloride when necessary to a final concentration of 20·0 mg/l. medium. The inhibitor acriflavin was added to CM as required to a final concentration of 50 mg/l. medium. Sodium acetate and galactose were used when necessary in place of glucose in MM at a concentration of 1% (w/v). The media were solidified with 1·5% (w/v) Davis agar.

(iii) *Incubation temperature.* Cultures for all experiments involving strain BV121 were incubated at 30 °C because it carries an uncharacterized temperature sensitive mutation which at 37 °C severely impairs growth. Genetic analysis using strains free from this mutation was done at 37 °C. Strain CS48 is cold sensitive and, in order to obtain expression of its *choB* mutation, cultures for all experiments involving this strain were incubated at 25 °C.

(iv) *Genetic analysis.* Genetic techniques were essentially those of Pontecorvo *et al.* (1953) except that haploidization was induced with chloral hydrate using the method of Singh & Sinha (1976). All yellow sectors so obtained and all green sectors unable to grow on MM were analysed for the various markers by the fixed pattern multineedle replicator technique of Roberts (1959). Ploidy of the sectors was determined using the criterion of spore volume, which was measured on a Coulter counter (Kubitshek, 1969) model Z_{B1} connected to a Coulter Channelyzer C-1000 (Coulter Electronics, Dunstable, Beds.) using samples of approximately 20000–25000 conidia from each sector. The conidia were suspended in a solution consisting of (g/l. distilled water): NaCl, 4·0; KCl, 0·1; Na_2HPO_4, 0·575; KH_2PO_4, 0·1; NaN_3, 0·5. This solution served to fix the sample and increase the conductivity of the suspension.

(v) *Growth responses to precursors of intermediates of choline metabolism.* The growth responses of strains were tested on MM supplemented with precursors of various intermediates of the pathway of choline metabolism. The investigation was based on the pathway proposed by Bremer, Figard & Greenberg (1960). Strain FGSC489 was inoculated onto the same media for comparison. The basic medium used was MM supplemented with the vitamins riboflavin, pyridoxin HCl, *p*-amino benzoic acid and biotin. Precursors of choline metabolism were added to the following concentrations (mg/l. medium): choline chloride (Sigma), 20·0; ethanolamine (B.D.H.), 10·0; *N*-methylaminoethanol (B.D.H.), 12·5; *N*,*N*-dimethylaminoethanol (Sigma), 15·0; L-α-glycerophosphorylcholine (Sigma), 20·0; L-serine, 20·0.

The concentrations used were designed to provide molar concentrations of precursors slightly higher than the concentration of choline chloride added to the media (143 μM). The concentration of choline supplied is in excess of the required amount which is about 5 mg/l. medium (36 μM) for normal growth on plates.

3. RESULTS

The nutritional requirement of *A. nidulans* strain BV121 was identified as a requirement for choline by the standard procedure of observing growth responses to variously supplemented MM plates.

(i) *Complementation test.* Heterokaryons between BV121 and FGSC489 at 30 °C and between BV121 and CS48 at 25 °C were formed on MM lacking choline. In both cases faster growing diploid sectors arose from the heterokaryons. This demonstrates that the BV121 choline-requirement locus is different from both the *choA1* and *choB* loci and is probably non-allelic. An alternative explanation could be that the mutations were in separate subunits of a multimeric protein coded by a single gene.

(ii) *Location of the new locus to its linkage group.* Analysis of the genotypes of 97 haploid sectors obtained from the diploid BV121 + BWB167, showed the new choline mutation to segregate with *riboB2*$^+$ and independently of all other markers, so locating it to linkage group VIII (Clutterbuck, 1974). The *choA1* gene has

previously been located to linkage group VII (Käfer, 1958) and the *choB* gene to linkage group VI (Waldron & Roberts, 1974) therefore confirming that the mutation carried by BV121 is at an entirely different locus.

(iii) *Mapping of the new locus.* Analysis of the meiotic recombinants of a cross between strains FGSC421 and PM3 provided the data given in Table 2. This allowed the order of the three genes to be determined as *choC–sD–fwA* and the linkage distances between the genes were calculated to be *choC–sD*, $31{\cdot}9 \pm 2{\cdot}2$ map units, *sD–fwA*, $24{\cdot}3 \pm 2{\cdot}1$ map units and *choC–fwA*, $56{\cdot}1 \pm 4{\cdot}3$ map units.

Table 2. *Genotypes of a random sample of recombinant ascospores from a hybrid cleistothecium produced by a cross between* A. nidulans *strains PM3 and FGSC421*

Genotype	Number of ascopores		Recombinant class
choC + fwA + sD +	132 101	233	Parental
choC sD + + + fwA	47 47	94	Single crossover (choC–sD)
+ sD fwA choC + +	23 38	61	Single crossover (sD–fwA)
choC sD fwA + + +	14 30	44	Double crossover
Total		432	

(iv) *Growth responses to precursors of intermediates of choline metabolism.* The growth responses of *choA1* strains to several precursors of choline metabolism have previously been reported (Arst, 1968) and our results confirm these findings. Arst also reported that strains carrying the *choA1* mutation, when inoculated on unsupplemented MM, are capable of germinating and exhibiting slow growth before finally stopping and turning brown. This was also found for strain BV121 which was capable of growth at 30 °C for at least 36 h after inoculation. Growth was however very slow and point inocula failed to extend more than approximately 1 mm unless the inoculum density was very high. Strain CS48 was also capable of a restricted level of slow growth on MM at both 20 and 25 °C. Under these conditions this strain could extend further than the other strains and did not turn brown. As this is a cold-sensitive mutant it is probable that this was due to a slight leakiness of the mutation at the restrictive temperature.

The growth responses of the three strains are given in Table 3. The abbreviations given in this table and in Fig. 1. are used from now on. It can be clearly seen that the strains all differed in their growth responses to precursors. The differences occurred in the growth on supplements of ethanolamine (EA) and *N*-monomethylaminoethanol (MAE). BV121 can grow on neither of these supplements, FGSC489 only on MAE and CS48 on both. Hence the three mutations have been demonstrated to be phenotypically as well as genotypically different.

Table 3. *Growth of* A. nidulans *strains on MM plates supplemented with precursors of choline metabolism*

Precursor	Strain BV121 (*choC*)	FGSC 489 (*choA*)	CS48 (cho*B*)
Serine	−	−	−
EA	−	−	+
MAE	−	+	+
DAE	+	+	+
GPC	+	+	+
Choline	+	+	+

+ = normal growth, − = growth equivalent to that attained on unsupplemented MM. Abbreviations: EA = ethanolamine, MAE = *N*-monomethylethanolamine, DAE = *N*,*N*-dimethylethanolamine, GPC = glycerophosphoryl choline.

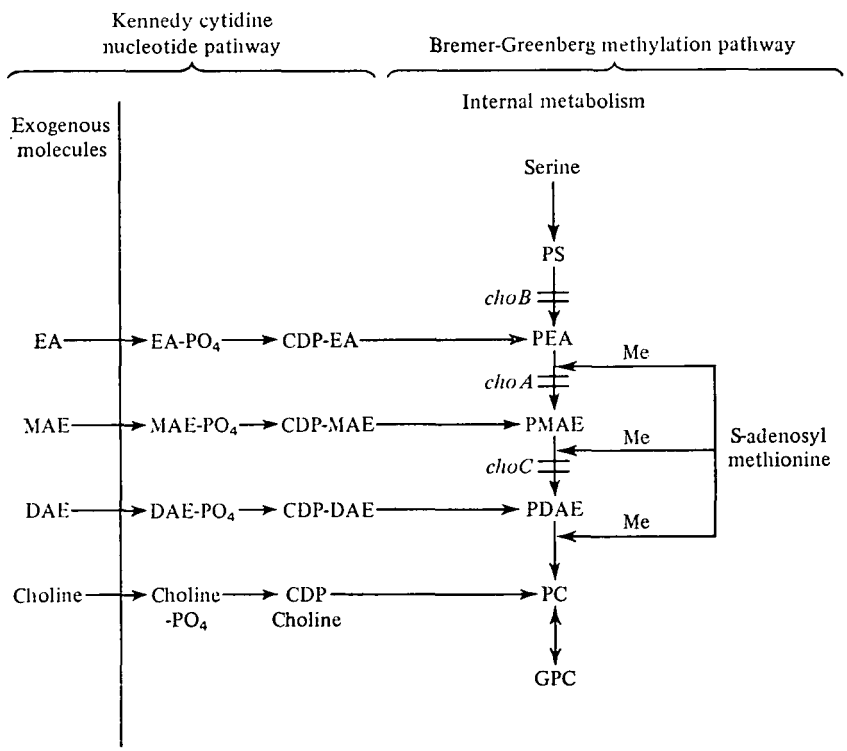

Fig. 1. Pathways of choline metabolism and the proposed sites of action of the *cho* loci in *A. nidulans*. Abbreviations: As in Table 3 with the addition of PS = phosphatidyl serine, PEA = phosphatidyl ethanolamine, PMAE = phosphatidyl monomethylaminoethanol, PDAE = phosphatidyl dimethylaminoethanol, PC = phosphatidyl choline (lecithin), CDP = cytidine 5′-diphosphate and Me denotes a methyl group.

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4. DISCUSSION

Growth responses to precursors of choline metabolism by strains carrying the three choline loci of *A. nidulans* make it possible to suggest their sites of action in the relevant pathway. It is reasonable to expect that the pathways demonstrated in rat liver microsomes by Bremer & Greenberg (1959, 1960); Bremer, Figard & Greenberg (1960) and Kennedy & Weiss (1956) are also present in *A. nidulans*, as they have been found to be present in all the organisms previously mentioned, with the exception that the cytidine nucleotide pathway is absent in *Agr. tumefaciens*. Positive responses obtained from the strains considered here to several of the precursors of the Bremer–Greenberg methylation pathway also support the probability of the presence of this pathway in *A. nidulans*. In these pathways the likely site of action of each of the choline loci would be as indicated in Fig. 1. So we suggest that each locus codes for the enzyme itself, or some molecule involved in the synthesis or function of the enzyme which carries out the transmethylation: of PS to PEA in the case of *choB*, of PEA to PMAE in the case of *choA* or of PMAE to PDAE in the case of *choC*. The sites of action of the *choA* and *choC* loci are therefore the same as those of the two *chol* loci of *N. crassa*, *choA* being the counterpart of *chol-1* and *choC* that of *chol-2* (Horowitz, 1946).

Each of the *A. nidulans* choline mutations can be relieved by supplementing the medium with the precursor of the intermediate subsequent to their site of action in the proposed pathway. This at first suggested that the four steps PS to PC were mediated by separate enzymes or that there was at least a separate molecule or enzyme site involved at each step. In *N. crassa* however, it has been suggested and subsequently supported on the basis of genetic, physical and kinetic evidence that the two steps PMAE to PC are carried out by a single enzyme (Scarborough & Nyc, 1967*a*, 1967*b*). There is similar evidence for the same feature of the pathway in rat liver microsomes (Rehbinder & Greenberg, 1965). The fact that the *A. nidulans* strain carrying the *choC3* mutation is relieved by DAE would appear to rule out this possibility in this organism, but the equivalent mutation in *N. crassa*, *chol-2*, was also found to be relieved by DAE (Horowitz, Bonner & Houlahan, 1945). The explanation for this is that *N. crassa* strains blocked in PC synthesis will incorporate PDAE and to a certain extent PMAE in place of PC (Crocken & Nyc, 1964). When supplied with DAE the *chol-2* mutant is able to convert it to PDAE via the Kennedy cytidine nucleotide pathway (Crocken & Nyc, 1964). The PDAE can be incorporated in place of PC, so allowing growth. It is therefore possible that the same feature of the pathway is present in *A. nidulans*. An analysis of the phospholipid content of *A. nidulans choC3* mutants grown on DAE supplemented medium would determine whether PC is replaced by PDAE as in *N. crassa*.

Direct investigation of choline metabolism in *A. nidulans* is now required to clarify the situation suggested by the circumstantial evidence provided here. The availability of three mutations should facilitate the study of these pathways, especially as the new mutation is in a locus that affects the site in the pathway at which the number of enzymes involved is in doubt. Also if the system is similar

to that in *N. crassa* the new mutation will provide a means of varying the phospholipid composition by growing the strain on media supplemented with various concentrations of the relevant precursors.

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