Mutants of *Aspergillus nidulans* with increased resistance to the alkylating agent, \( N\)-methyl-\( N'\)-nitro-\( N\)-nitrosoguanidine

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**Summary**

The isolation and characterisation of mutants of *Aspergillus nidulans* showing resistance to MNNG is described. Such isolates were stable through prolonged subculture in the absence of the selective agent, and resistance segregated as an allele of a single gene in meiotic and mitotic analysis. MNNG-resistant strains showed an increase in resistance to EMS and UV irradiation but no cross-resistance to MMS was detected. Possible mechanisms of resistance to alkylating agents are discussed.

In several prokaryotic and eukaryotic systems, mutants which show an increased sensitivity to a variety of mutagenic agents have been isolated as potentially showing defects in DNA-repair systems (e.g. Sargentini and Smith, 1985). In particular, sensitivity to the alkylating agents has received much attention owing to the clear carcinogenic activity of the compounds and the understanding of the specific damage they induce at a molecular level (Saffhill et al., 1985). Sensitive mutants also offer the prospect of screening gene banks for functional DNA-repair genes to complement such defects.

In contrast, resistance to mutagenic agents has been comparatively poorly studied, and only isolated reports of the characterisation of such mutants can be found. Mutants of *E. coli* resistant to alkylating agents showed lower MNNG induced and lower spontaneous mutability (Zamenhof et al., 1966). Later, Sedgwick and Robins (1980) described two classes of *E. coli* mutants resistant to alkylating agents, one of which proved to be constitutive for the adaptive response. Hence, study of resistance to alkylating agents can lead to an understanding of the control of expression of DNA-repair genes.

Lower eukaryote systems may provide more realistic models than prokaryotes for the behaviour of human cells subjected to alkylating agent damage. They also offer the potential of profuse sporulation, predominantly haploid life cycles and rapid sexual cycles for the production and characterisation of DNA-repair mutants. Whilst several studies have reported the isolation of mutants of *Aspergillus nidulans* showing increased sensitivity to alkylating agents (e.g. Käfer and Mayor, 1986; Swirski et al., 1988), we could...
find no reports of the isolation of resistant mutants. We describe here a preliminary analysis of the isolation and characterisation of mutants resistant to MNNG in this filamentous fungus.

Materials and methods

Strains

Liverpool strains L19 (yA2; pyroA4), L20 (pabaA1; wA3) and L130 (yA2; pyroA4; uvsC14) were used. A “master strain” G96 (galAl; pyroA4; facA303; sB3; nicB8; riboB2) was used to assign new mutations to linkage groups. Strains resistant to MNNG and derived from L20 were designated RAG, resulting from rag mutations (resistance to alkylating damage). Growth media and standard genetical manipulations were as in Pontecorvo et al. (1953) and Clutterbuck (1974). All cultures were grown at 37°C.

Isolation of resistant mutants

Plates containing 15 ml of minimal medium agar (MM) and paba were poured. Overlays of 5 ml of the same medium containing MNNG in M9 (pH 6) phosphate buffer were then poured to give a final concentration of 4 μg ml⁻¹ in the total volume of 20 ml of agar. Final 5-ml overlays containing L20 conidia were then made and the plates incubated for 4 days. After this period, vigorously growing colonies were subcultured and tested further.

Growth measurement

Freshly prepared MNNG as a solution (200 or 400 μg ml⁻¹) in ethanol was added evenly to the bases of empty petri dishes. Cool (45°C) agar (MM or CM) was then overlaid at a volume of 20 ml per plate to give the required concentration of MNNG.

For accurate measurements of growth rate constants (kᵣ), MM (paba) was prepared in M9 phosphate buffer (pH 6) to improve the stability of MNNG. Two types of inoculum were used. Stab conidial inocula were taken from plates (CM) and slopes (stored at 6°C) and growth was recorded as colony diameter after 24 or 48 h, or calculated as growth rate constants (kᵣ) (Trinci and Gull, 1970) between these two periods of time.

Mycelial plug inocula were taken from non-sporulating lawns on CM less than 20 h after inoculation with spores. Colony growth rates were calculated over 24 h upon transfer to test medium. Each strain/treatment combination was replicated at least 3 times.

Stability characterisation

Single conidial isolates were taken from each of 6 vigorous growths from MNNG plates (RAG1→6) and the stability of the resistance phenotype studied in 2 ways. Firstly, each strain was inoculated centrally on CM and subcultured 7 times over the period of 1 month’s growth, on each occasion taking fresh spore inoculum from the edge of the colony. At the end of this period, each strain was multiply inoculated onto CM containing MNNG, 0, 4 and 6 μg ml⁻¹ for comparison with the original isolates and “wild-type” L20. Secondly, 40 single conidial isolates were taken from each of two resistant mutants (RAG1 and RAG4) and multiply inoculated onto CM containing MNNG, 0, 4 and 6 μg ml⁻¹).

Observations of spore germination and colony establishment

Observations of spore germination were made on conidia taken from CM plates (L20, RAG1, RAG4) inoculated 48 h previously. 100 μl of a spore suspension of 10⁶ conidia ml⁻¹ was spread on cellophanes (BCL, 350 P-00) on plates of CM. Cellophanes were examined at hourly intervals for germination of conidia. The same inoculum source was used to inoculate plates of CM with 50, 100, 500 and 1000 spores and colony establishment scored after 24 and 48 h.

Assays of 3-methyladenine DNA glycosylase ‘in vitro’

The assay was an adaptation of that used for E. coli by Riazuddin and Lindahl (1978). It was based on specific enzymatic release of tritiated 3-methyladenine from calf-thymus DNA (Sigma) previously reacted with [³H]dimethyl sulphate (New England Nuclear). This S₉₂ type reaction (Lindahl, 1979) does not produce O⁶-methylguanine adducts, therefore transferase activities were excluded from the assays. Assays were performed in the presence of 7 mM EDTA (disodium
salt, Sigma) to prevent non-specific nuclease activity. Full details of the technique are given in Swirski et al. (1988).

Responses to other mutagenic agents
The method was slightly modified from Jansen (1970) to test UV response of RAG1 and RAG4. Aliquots of ~250 conidia in Tween 80 water were added to 3-ml portions of CM + 0.04% S.D. and poured onto 15-ml basal layers of the same medium. The medium was allowed to solidify and the plates incubated at 37°C for 7 h to allow spore germination to occur.

The germlings were then irradiated for various times at a dose rate of 1 J·m⁻²·sec⁻¹. The plates were kept in the dark following irradiation to prevent photoreactivation and incubated at 37°C for 3 days. After this period the number of resulting colonies were counted.

Plates were inoculated and irradiated in duplicate for each strain/dose combination and L20 and L130 were included as controls.

Sensitivity to MMS (methyl methanesulphonate), EMS (ethyl methanesulphonate) and 4 NQO (4-nitroquinoline-1-oxide) of RAG1 and RAG4 was tested in 2 ways. Firstly, aliquots of the inhibitors in ethanol were added to individual 20-ml universals of agar at 48°C, mixed and poured in petri dishes along with ethanol, inhibitor-free controls. Point inoculations with conidia were made and inhibition assessed as for MNNG.

Secondly, filter paper discs impregnated with aliquots of the inhibitors in ethanol were placed on plates of CM "seeded" with conidia of the test strains. Inhibition haloes were scored after 24 and 48 h of incubation.

Results
Isolation of resistant mutants
Putative resistant mutants appeared at a frequency of approximately one colony per 10⁶ spores plated. 6 of these (RAG1 → 6) were subcultured and single spore isolates made for further characterisation.

Resistance to MNNG
Each of the 6 RAG strains showed a marked resistant response when inoculated either as con-}

![Graph](image_url)

Fig. 1. A comparison of hyphal extension rates of resistant (RAG1, RAG4) and wild-type (L20) strains. Colonies were inoculated as 6-mm hyphal plugs from CM plates onto CM + MNNG, and colony extension rates were calculated from diameter measurements taken over a 24-h period. Symbols are as follows: △, L20; ○, RAG1; ●, RAG4.
**Stability of resistance**

Each of 40 single conidial isolates of RAG1 and RAG4 were uniformly resistant and were indistinguishable in MNNG response from their parental isolates. All produced smaller colonies than L20 in the absence of MNNG.

Following prolonged serial subculture in the absence of MNNG, all of the 6 RAG strains were still uniformly resistant to MNNG and were indistinguishable from their parental isolates.

**Spore germination and colony establishment**

When spore germination (in the absence of MNNG) was examined, both RAG1 and RAG4 showed reduced rates of germination relative to L20. Germination of L20 conidia appeared fairly synchronous (nearly 100% after 6 h) and after 7 h the largest germ tubes were up to 15 × the diameter of the conidium in length, and laterals had begun to form. In contrast, RAG1 and RAG4 showed only 35–40% germination after 6 h whilst after 7 h, although germination approached 100%, a range of germling stages from the same size as L20 down to some ungerminated conidia was apparent.

When colony establishment from single conidia of L20 and RAG1 was compared, MNNG was found to markedly stimulate establishment of the resistant mutant, (Fig. 3). In the absence of MNNG, colonies produced from L20 conidia were much larger than those from RAG1 or RAG4.

Whilst a slightly slower germination rate may have allowed RAG strains to establish colonies when MNNG had decayed somewhat, control experiments revealed that CM plates containing MNNG remained highly toxic to wild-type conidia even after 48 h incubation at 37 °C (data not shown).

**3-Methyladenine DNA glycosylase assays**

Using the modified assay procedure, 250 μg of protein extract from both RAG1 and RAG4 each released approximately 10% of the total counts in the reaction mixture in 45 min. These results indicate that RAG1 and RAG4 have similar 3-methyladenine DNA glycosylase activities to those of wild-type L20.

**Responses to other mutagenic agents**

Both RAG1 and RAG4 showed an increase in UV resistance at the “7 h germling” stage, compared with “wild-type” L20. L130, a strain carrying uvsC_{14}, was the most sensitive strain tested (Fig. 4).
Incubation Period (days)

Fig. 3. Establishment of colonies from single conidia of resistant (RAG1) and wild-type (L20) strains on complete medium (Cm) containing MNNG (0, 2 and 4 μg/ml) colony numbers were counted following 24 h and 48 h incubation at 37°C. The total number of conidia plated was determined by counting samples in a Neubauer haemocytometer.

When tested as conidial stabs, both RAG1 and RAG4 were clearly more resistant to EMS than L20 and at low doses (1 μl ml⁻¹) this agent stimulated colony extension. Again, both RAG1 and RAG4 were more resistant to 4NQO at lower doses (0.5 μg l⁻¹). Scoring resistance at higher doses was confused by delayed germination and the slower growth rate exhibited by RAG1 and RAG4. In contrast, RAG1 and RAG4 appeared to be slightly more sensitive to MMS than control L20.

Measurement of radii of inhibition haloes of impregnated filter discs indicated that RAG1 and RAG4 were slightly more sensitive to both EMS and MMS as compared to L20. This may be an artefact of delayed germination and decreased growth rates. Measurements of 24–48 h growth rates were inconsistent, presumably because of the volatility of EMS and MMS at 37°C.

Inheritance of resistance

Replica plates on MNNG 0, 4 and 6 μg ml⁻¹ were scored for the resistant phenotype 24 h after inoculation. Progeny of crosses RAG1 × L19 and RAG4 × L19 segregated 1:1 into clear wild-type and resistant phenotypes (20:20 and 23:16 respectively). Each cross segregated as expected for spore colour and auxotrophy markers although an imbalance of \text{pyro:} + (27:12, \chi^2 = 5.77) was apparent in RAG4 × L19 progeny. Whilst \text{yA2} and \text{pabaA1} showed clear linkage in each cross no such association was evident between \text{rag} and either \text{wA3}, \text{yA2}, \text{pyroA1} or \text{pabaA1}. In blind tests on a separate occasion, re-scoring master plates of RAG1 × L19 progeny, 95% of isolates were allocated as previously to their correct wild-type, or
resistant phenotype classes. Sexual progeny showed no clear segregation of the small colony size phenotype of RAG1 and RAG4 (delayed spore germination/colony establishment). Each of 40 progeny examined from each cross showed a non-uniform colony establishment rate similar to L20, whilst both RAG and L19 parents produced smaller colonies after 24 h. L19 itself showed poor conidial viability and slow colony establishment.

When a resistant isolate from the progeny of RAG1 × L19 was backcrossed to the resistant parent, all of 48 progeny tested were uniformly resistant.

Diploid strains constructed between G96 and RAG1 or RAG4, when tested on minimal medium + MNNG, showed a wild-type level of response indicating that mutations leading to resistance were functionally recessive.

Haploid sectors were obtained from these diploid strains by plating on CM + 1.5 × 10⁻⁵ mg·ml⁻¹ benzate (Hastie, 1970). These segregants were tested for their nutritional requirements and response to MNNG. Segregants from the G96/RAG4 and G96/RAG1 diploid showed a co-segregation of nicB8⁺ and MNNG resistance. This suggests that the rag4 and rag1 mutations are located on chromosome VII. Haploid sectors showed a clear association between MNNG resistance and slow colony establishment on MNNG-free media. In each case, 18 haploid sectors from the G96/RAG1 diploid and 18 haploid sectors from the G96/RAG4 diploid, showed co-segregation of MNNG resistance and slow colony establishment on MNNG-free media.

Discussion

Strains of A. nidulans resistant to MNNG can be isolated at a relatively high frequency from conidia on plates containing the compound. Such strains show a high though variable level of resistance to MNNG in the growth medium, expressed throughout the growth cycle during conidial germination/colony establishment and vegetative growth. Resistance is stable through prolonged subculture away from the selective agent and is transmitted uniformly through asexual spores. Analysis of sexual progeny of two crosses suggests that resistance behaves as an allele of a single gene, segregating wild-type and resistance isolates in a 1:1 ratio. Two resistant strains and haploid derivatives from mitotic analysis also showed slow conidial germination and colony establishment in the absence of MNNG when compared to wild-type, suggesting the pleiotropic action of a single gene.

The physiological basis of the resistant phenotypes may be the result of changes in cell membrane permeability, blocks in the normal metabolism of MNNG after entry or modification of the target of inhibition (presumably DNA). In mutants of E. coli resistant to alkylating agents, two groups were described by Sedgwick and Robins (1980). Some resistant strains had a low intra-cellular thiol content, thiols being active in the conversion of MNNG to methylthionitrosamine. Preliminary experiments using the method of Ellman (1959) to measure the thiol content of L20, RAG1 and RAG4 did not reveal any changes in the RAG strains compared with L20 (S.G. Shawcross, unpublished data). A second group of resistant E. coli mutants displayed constitutive expression of a normally inducible response to alkylating agents. Thus, adaptation in E. coli to alkylating agent resistance can involve the induction of a DNA glycosylase. In A. nidulans 3-methyladenine DNA glycosylase appears to be constitutively expressed under normal conditions (Swirski et al., 1988): over-production of this enzyme was not found in RAG1 or RAG4. TLC analysis showed that the major DNA glycosylase activity detected in the assay released 3-methyladenine from DNA (Swirski et al., 1988).

Cross-resistance of rag mutants to another alkylating agent in addition to increases in resistance to both UV and 4NQO, make mechanisms of resistance based upon changed membrane permeability or specific cytoplasmic intermediates unlikely. By analogy with other systems (reviewed by Sedgwick, 1986) alterations in DNA-repair functions are implicated. The lack of increased resistance to MMS by RAG1 and RAG4 suggests that it is unlikely that these strains have increased 3-methyladenine DNA glycosylase activity and direct assays (Swirski et al., 1988) support this supposition. It also seems unlikely that RAG1 and RAG4 have increased O⁶-alkylguanine DNA transferase activities because both demonstrated
increased UV and 4NQO resistance. The data imply that a more general aspect of their repair systems may be affected, perhaps involving recombination repair of bulky lesions and UV damage. However, the possibility of an increased methyltransferase activity cannot be ruled out for RAG3. Preliminary experiments showed that this strain is cross-tolerant only to EMS. This agent produces a small quantity of O6-ethylguanine that perhaps could be repaired by a transferase acting on the O6-methylguanine produced by MNNG.

One possible explanation for the sensitivity of RAG1 and RAG4 to MMS at high concentrations and also for the reduced growth rates in the absence of MNNG is that of mis-directed mismatch repair. All of the RAG strains (except RAG5) show that low doses of MNNG have a stimulatory effect on growth. This suggests that these strains may have under-methylated DNA in the absence of an alkylating agent. If, at high doses of MMS, a mis-match type of repair forms a large portion of the repair process, then because of the lack of ‘direction’ provided by methylation, the repair may be directed in the wrong way to produce lethal/mutagenic lesions (Lien-Lu et al., 1983). The mutagenicity of the effect could be monitored by comparing reversion of methG in double mutant (rag;methG) strains with those carrying methG alone. Further experiments using suitably marked strains are in progress.

An alternative explanation for the MNNG stimulation effect found in the RAG mutants could be that MNNG methylates cellular component(s) other than DNA. In these strains this may act as a signal to induce growth (and perhaps DNA repair, cf. Sedgwick, 1987). Work is in progress to understand the basis of the MNNG stimulation effect.

In other systems involving mutants resistant to toxic compounds, amplification of relevant genes improves responses by dosage effects as, for example, in pesticide resistance in insects (Hyrien and Buttin, 1986) and methotrexate resistance in mammalian cells (Shields, 1978). Resistance to alkylating agents in yeast following transformation with gene bank DNA has been attributed to a similar dosage effect of a high-copy number vector carrying repair sequences (Ruhland et al., 1986). However, if our RAG mutants do show amplification of specific genes, this is a very stable process throughout prolonged vegetative subculture in the absence of selection and through meiosis to allow the normal segregation of resistance as a single gene marker.

The development of alkylating agents as anti-tumour drugs dependent upon increased sensitivity of tumour versus normal cells (Stevens et al., 1984), may make the study of resistance in eukaryotic model systems of some clinical interest. “Resistance” to MNNG has been reported in human cell lines (RSb), previously sensitive to this agent (Suzuki, 1987). However, the difficulty of maintaining suitable controls (i.e. equivalent, normal cell lines), makes it difficult to show that such derived mutants are not mere “revertants” to a “wild-type” level of resistance. In this respect, A. nidulans may provide a robust model system for general studies of DNA repair in eukaryotes.

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