Methanol and acriflavine resistance in Dictyostelium are caused by loss of catalase

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INTRODUCTION

A number of environmental poisons are not inherently toxic, but are metabolized in vivo to produce toxic products. One example is methanol in Dictyostelium, which is lethal to cells containing the acrA gene, but relatively harmless to acrA mutants. This makes methanol resistance one of the tightest genetic selections in Dictyostelium. Loss of acrA also confers cross-resistance to unrelated compounds such as acriflavine and thiabendazole. We have used insertional mutagenesis to demonstrate that the acrA locus encodes the peroxisomal catalase A enzyme. Disruption of the catA gene results in parallel resistance to acriflavine. Molecular and biochemical studies of several previously characterized methanol-resistant strains reveal that each lacks catalase activity. One allele, acrA2, contains a 13 bp deletion which introduces a frameshift in the middle of the gene. The involvement of catalase in methanol resistance in Dictyostelium compares with its role in methanol metabolism in yeast and rodents. However, this is the first study to show that catalase is required for the toxicity of acriflavine. Our results imply that acriflavine and thiabendazole are precursors which must be oxidized to generate biologically active species. The catA/acrA gene is also a potentially invaluable negative selectable marker for Dictyostelium molecular genetics.

Various chemicals with harmful effects are not themselves toxic, but are metabolized in vivo to produce toxic products. One example is methanol in Dictyostelium, which is lethal to cells containing the acrA gene, but relatively harmless to acrA mutants. This makes methanol resistance one of the tightest genetic selections in Dictyostelium. Loss of acrA also confers cross-resistance to unrelated compounds such as acriflavine and thiabendazole. We have used insertional mutagenesis to demonstrate that the acrA locus encodes the peroxisomal catalase A enzyme. Disruption of the catA gene results in parallel resistance to acriflavine. Molecular and biochemical studies of several previously characterized methanol-resistant strains reveal that each lacks catalase activity. One allele, acrA2, contains a 13 bp deletion which introduces a frameshift in the middle of the gene. The involvement of catalase in methanol resistance in Dictyostelium compares with its role in methanol metabolism in yeast and rodents. However, this is the first study to show that catalase is required for the toxicity of acriflavine. Our results imply that acriflavine and thiabendazole are precursors which must be oxidized to generate biologically active species. The catA/acrA gene is also a potentially invaluable negative selectable marker for Dictyostelium molecular genetics.

Keywords: alcohol metabolism, methanol, xenobiotics, prodrug, isoniazid

INTRODUCTION

A number of environmental poisons are not inherently toxic, but are metabolized in vivo to produce toxic products. Proteins involved in this process cause sensitivity to poisons and when the genes which encode them are mutated, cells become resistant. Mutations of this type can be used as recessive selectable markers for genetic studies. For example, the URA3 gene of Saccharomyces confers sensitivity to 5-fluoroorotic acid. This property has been used in genetic selections for loss of URA3 (Boeke et al., 1987). In Dictyostelium, this type of mutation has been used as a marker for parasexual genetic studies (Williams et al., 1974). One particularly useful class of mutants is resistant to acriflavine, a chemical which has been used as an antiseptic but whose uses are limited by its toxicity. Genetic complementation and linkage analyses revealed that the mutations were recessive and mapped to three different loci – acrA, acrB and acrC – located on linkage groups II, I and III, respectively (Katz & Kao, 1974; Rothman & Alexander, 1975; Williams et al., 1974). This implies that acriflavine requires at least three different enzymes, perhaps steps in a metabolic pathway, for full toxicity. Interestingly, acrA mutations also confer resistance to the unrelated chemicals methanol and thiabendazole (Williams et al., 1974). Conversely, all mutations primarily selected for methanol resistance have been found to map only to acrA (Williams et al., 1974).

These drug-resistance mutations have been essential in genetic mapping in Dictyostelium (Alexander et al., 1986; Welker & Deering, 1976) and in integrating the genetic and physical maps of the Dictyostelium linkage groups (Loomis et al., 1995). However, the structural gene encoded by acrA has remained unknown for the past 25 years. In this study, an insertional mutagenesis screen for the genes involved in methanol resistance has

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Abbreviation: REMI, restriction enzyme mediated integration.

The GenBank accession number for the sequence reported in this paper is AF090443.
identified the Dictyostelium catalase A (catA) gene as acrA. We present genetic and biochemical evidence that a deficiency in CatA enzyme activity causes resistance to both acriflavine and methanol.

Catalases are ubiquitous, highly active enzymes which break down H₂O₂ to water and oxygen, and thus protect against cellular damage caused by peroxides. Dictyostelium has been shown to contain two catalases—one, the product of the catA gene, is active in growing cells, whereas the other, encoded by catB, is expressed late in development (Garcia et al., 2000). CatA would therefore appear to be the standard metabolic enzyme, whereas CatB is predicted to have an as yet unknown role in development. Until the present work, no gene disruptants of catA had been isolated. However, a mutant which coincidentally expresses low levels of catA was found to be highly sensitive to exogenous H₂O₂, though normally resistant to UV light (Garcia et al., 2000).

This work exposes the biochemical pathway underlying the toxic effects of methanol and provides an intriguing insight into the mechanism of acriflavine toxicity. Both pathways are centred on catalase.

METHODS

Strains and growth conditions. Dictyostelium strains AX2 and AX3, the parental strains used for homologous recombination, were grown in HL-5 medium (Cocucci & Sussman, 1970) and passed when they reached a density of 2 x 10⁶–3 x 10⁷ cells ml⁻¹. Gene disruption mutants were grown in HL-5 medium containing 10 µg blasticidin ml⁻¹. Strains NC4, SA31, SA219 and their respective methanol-resistant mutant strains (Table 1) were all grown in association with Klebsiella aerogenes on SM agar plates until they reached mid-exponential phase (approx. 2 x 10⁶ cells per 100 mm plate; Sussman, 1987). Cultures were started from stored stocks on a monthly basis.

Table 1. Cross-resistance of catA mutants to methanol and acriflavine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AX2 (wild-type)</th>
<th>IR29 (catA mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>0.5% Methanol</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>1% Methanol</td>
<td>−</td>
<td>+ +</td>
</tr>
<tr>
<td>2% Methanol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>3% Methanol</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1% Ethanol</td>
<td>+ +</td>
<td>+ + +</td>
</tr>
<tr>
<td>2% Ethanol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25 µM Acriflavine</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>100 µM Acriflavine</td>
<td>−</td>
<td>+ +</td>
</tr>
<tr>
<td>500 µM Acriflavine</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Restriction enzyme mediated integration (REMI) mutagenesis and selection for methanol-resistant mutants. REMI mutagenesis using blasticidin S resistance as a selection marker was performed as described by Kuspa & Loomis (1992). A REMI-mutagenized AX2 library was prepared as described previously (Williams et al., 1999). To obtain methanol-resistant mutants, the library of bsr-resistant mutants (approx. 5000) was selected for growth on SM agar plates containing 2% methanol (Williams et al., 1974). Surviving cells were plated for single colonies on SM plates. Clones were picked and retested for growth on 2% methanol. The disrupted gene flanking the insertional vector in the methanol-resistant mutants was excised and sequenced. The rescued plasmid was used to recapitulate the mutation by homologous recombination in both AX2 and AX3.

Identification of mutations. Genomic DNA was prepared from the acrA2 mutant XP210 as described by Sun & Devreotes (1991). This DNA was then used as a template for PCR reactions using a range of primer pairs covering the entire acrA gene. Initial sequencing identified a deletion at approximately 580 bp (numbered according to the GenBank entry no. AF090443). This was then precisely localized using a second PCR reaction, with primers from 141 to 157 bp and 1099 to 1083 bp and a sequencing primer from 704 to 690 bp (5’-GGCAGCTTCTTCAGC-3’). Sequences were aligned using MacVector (Oxford Molecular).

Assays for resistance to methanol and acriflavine. Plates containing different concentrations of alcohols or acriflavine were prepared by adding appropriate additives to molten SM agar just before it began to set. Methanol plates were stored in separate boxes from others, as methanol vapour was found to cause significant toxicity to cells in surrounding plates. Set plates were spread with lawns of Klebsiella, then 10 µl drops containing approximately 10⁸ Dictyostelium cells were spotted in the middle of each plate. After 5 d incubation at 22 °C, plates were examined and scored for cell growth.

Southern and Northern analyses. Southern and Northern analyses were performed as described previously (Lee et al., 1996). The catA probe was prepared by excising the full-length catA cDNA as a 1.5 kb SalI-NotI fragment from plasmid FC-AH16 (Garcia et al., 2000).

Catalase activity assays. Vegetatively growing cells were harvested, washed with LPS buffer (20 mM KCl, 2.5 mM MgCl₂, 40 mM potassium phosphate, pH 6.5, containing 0.5 mg streptomycin sulfate ml⁻¹) and pelleted. The cell pellets were lysed in lysis buffer (10 mM potassium phosphate, pH 7.0, 0.1% Triton X-100, 1x protease inhibitor cocktail [100× = 20 mM AEBSF, 100 µg pepstatin A ml⁻¹, 10 mg leupeptin ml⁻¹]) and centrifuged for 3 min at 4 °C. Samples of 1.25 and 2.5 µl of the supernatants were then assayed for catalase activity by mixing with 10 mM H₂O₂ in 50 mM potassium phosphate, pH 7.0, and monitoring the degradation of H₂O₂ for 90 s (Garcia et al., 2000). Specific catalase activity was calculated as µmol H₂O₂ degraded min⁻¹ (mg protein)⁻¹. Catalase activities of the mutants were expressed as the percentage of the activities of the parental strains. Protein concentrations were determined using BCA reagent (Pierce).

RESULTS

The catA gene is disrupted in methanol-resistant mutants

We selected methanol-resistant Dictyostelium mutants from a library of REMI insertional mutants by growth on 2% methanol, using the same method as previous
genetic studies. A single methanol-resistant clone, named IR29, was isolated from a library of approximately 5000 clones. Genomic DNA from IR29 was cut with BsrGI, circularized and used to transform Escherichia coli. The resulting clones contain two fragments of genomic DNA flanking the insertion site, one of 242 bp and one of approximately 1-4 kb (Fig. 1). Sequencing of these fragments reveals that the methanol-resistant mutant contains a single insertion at the DpnII site at position 409 of the catA gene (Fig. 1), which encodes the growth-specific catalase (Garcia et al., 2000). This suggests that loss of catA function causes methanol resistance in Dictyostelium.

To confirm that the catA disruption was specific for the methanol resistance phenotype, the rescued plasmid was used to redisrupt the catA gene by homologous recombination in strains AX2 and AX3. These catA disruption mutants, IR40 and IR41, were both found to be methanol-resistant like IR29, while both parents were fully methanol-sensitive. Southern analysis confirmed the disruption in the catA loci of both mutant strains (Fig. 2). The small difference in the size of the high molecular mass bands observed between the two strains indicates the presence of an EcoRI restriction fragment length polymorphism between AX2 and AX3.

Catalase disruption mutants show cross-resistance to acriflavine

Previous work has shown that methanol resistance mutations in Dictyostelium universally map to a single locus, implying that methanol sensitivity is mediated by a single gene product. All these mutants were also found to be allelic to the acriflavine resistance gene acrA (Rothman & Alexander, 1975; Williams et al., 1974). We therefore predicted that the catA gene is required for both methanol and acriflavine sensitivity in wild-type cells. Tests of this prediction are described in the legends to Figs 4 and 5 and in Table 1.

We first tested whether the disruption mutant was resistant to acriflavine. Wild-type (AX2) and catA mutant (IR29) cells were plated on SM agar plates containing increasing concentrations of methanol, ethanol or acriflavine. The results in Table 1 clearly show that the catA mutant indeed exhibits cross-resistance to acriflavine. This suggests, unexpectedly, that acriflavine must be oxidized by a catalase-dependent pathway for it to show significant toxicity to Dictyostelium.

In some organisms, in particular the mouse, catalases can play a major part in the breakdown of ethanol as well as methanol. There is only a very slight difference in the sensitivity of wild-type and catA mutant to ethanol (Table 1). This could imply either that the CatA catalase has very little activity against ethanol or that the products of ethanol oxidation are far less toxic to Dictyostelium than those from methanol.

Phenotypes of catA disruption mutants

None of the catA-deficient strains described in this work, in particular the disruptants IR29, IR40 and IR41, showed any obvious mutant phenotype during normal
laboratory growth and development. Each strain grew at approximately wild-type rates and to wild-type cell densities. When starved, each strain developed at a normal rate, eventually producing apparently normal fruiting bodies. It therefore appears that catalase is not important for growth or development under normal laboratory conditions.

Previous work (Garcia et al., 2000) showed that strain X9 coincidentally expresses unusually low (~3%), but measurable, levels of catalase A (Madigan & Katz, 1989). X9 cells were also found to be hypersensitive to H₂O₂. As shown in Fig. 3, the catA null strain IR29 is extremely sensitive to H₂O₂ – even more so than Garcia et al. (2000) observed for X9 – consistent with a more severe phenotype. At low concentrations of H₂O₂, which barely affect the parental strain, IR29 cells are nearly all killed (~90% at 0·1 mM; ~99·9% at 0·3 mM). However, a significant but small number of IR29 cells survived treatment with 1 mM H₂O₂, which killed 99·9% of parental cells (Fig. 3). This appears to be due to the stochastic nature of death by oxidative damage, rather than reversion, as the survivors are still sensitive to H₂O₂ when regrown (data not shown).

Independently isolated methanol-resistant mutants lack CatA activity

To confirm that a loss in catA function was responsible for the methanol-resistant phenotype, lysates from the methanol-resistant mutants IR40 and IR41 and their respective parent strains were assayed for catalase activity. Additionally, other independently isolated methanol-resistant mutant strains and their parents (see Table 2 for details) were assayed for their catalase activities. Spectrophotometric assays showed that all the methanol-resistant mutants had no detectable catalase activity or, in the case of strain X9, 3% of parental activity (Fig. 4a). Our results therefore fully agree with the prediction that all methanol-resistant strains lack catalase A and therefore that the catA gene is the site of previously identified acrA mutations.

Northern analyses of catA gene expression in independently isolated methanol-resistant mutants revealed that different kinds of mutations in the catA gene have resulted in methanol resistance (Fig. 4b). IR40 and IR41 are catA disruption mutants. As predicted, no catA mRNA was observed in these strains. X9 carries theacrA1 allele which was the first acriflavine (and methanol)-resistant allele isolated (Williams et al., 1974). This strain had previously been found to express unusually low, but measurable, levels of catalase (Madigan & Katz, 1989). This mutant expresses less than 1% of catA mRNA (Fig. 3; see also Garcia et al., 2000) and a band of the appropriate size is just barely visible. The low level of enzyme activity in X9 therefore appears to

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**Table 2. Dictyostelium methanol-resistant strains used in this study**

<table>
<thead>
<tr>
<th>Methanol-resistant strain</th>
<th>Parental strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>X9</td>
<td>NC4</td>
<td>acrA1; spontaneous mutation; selection on 100 µg acriflavine ml⁻¹; cross-resistant to 2% methanol</td>
<td>Williams et al. (1974)</td>
</tr>
<tr>
<td>IR29</td>
<td>AX3</td>
<td>catA gene disrupted by REMI mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>IR40</td>
<td>AX2</td>
<td>catA gene disrupted by homologous recombination</td>
<td>This study</td>
</tr>
<tr>
<td>IR41</td>
<td>AX3</td>
<td>catA gene disrupted by homologous recombination</td>
<td>This study</td>
</tr>
<tr>
<td>XP210</td>
<td>XP95</td>
<td>acrA2; spontaneous mutation</td>
<td>Williams et al. (1974)</td>
</tr>
<tr>
<td>SA32</td>
<td>SA31</td>
<td>acrA50; spontaneous mutation; selection on 2% methanol</td>
<td>Alexander et al. (1986)</td>
</tr>
<tr>
<td>SA220</td>
<td>SA219</td>
<td>acrA51; spontaneous mutation; selection on 2% methanol</td>
<td>Alexander et al. (1986)</td>
</tr>
</tbody>
</table>
be caused by a diminished expression level of a normal mRNA (see below). Two other strains containing spontaneous acrA mutations were analysed. SA32 (acrA50) contains no detectable catA mRNA. This is also likely to be due to a mutation in the cis-regulating region, since PCR analyses showed that the catA coding sequence is intact in this mutant (data not shown). On the other hand, SA220 (acrA51) possesses no detectable catalase activity (see Fig. 4a), but shows a normal level of catA mRNA. We would therefore predict that SA220 contains a point mutation, somewhere within the structural gene for catA, which causes loss of catalase activity.

**A previously described acrA mutant contains a lesion in catA**

As reported previously, sequencing of the catA gene in an acrA1 strain revealed no changes in the coding sequence. Together with the low level of expression, this suggests that the defect in acrA1 cells affects the regulation of expression (Garcia et al., 2000). We therefore isolated and sequenced various fragments of the catA gene from strain XP210, which contains the acrA2 allele. As shown in Fig. 5, the sequencing data show that catA has a 13 bp deletion within the coding sequence, between positions 573 and 585 (numbered according to the GenBank entry for catA, accession no. AF090443). The effect of this mutation will be to introduce a frameshift into the coding sequence, in addition to the removal of 4 aa. The identification of this lesion independently confirms the connection between catA and the original acrA gene. Since all known mutations which confer methanol resistance have mapped to the same locus, this suggests that the catalase which is the product of catA is necessary and sufficient for cellular toxicity of methanol.

**DISCUSSION**

Methanol resistance has been used extensively as a genetic marker in Dictyostelium because methanol resistance maps to the single acrA gene and because selection for methanol resistance is easy (Rothman & Alexander, 1975; Williams et al., 1974). Forward mutations to methanol resistance have also been used as a simple mutation assay for testing the effects of different DNA-damaging agents (Podgorski & Deering, 1980). Although many methanol-resistant Dictyostelium strains have been generated previously (Alexander et al., 1986; Rothman & Alexander, 1975; Williams et al., 1974), the molecular basis for resistance to methanol in Dictyostelium cells remained unknown. In this study we used REMI to identify mutations that were responsible for methanol resistance and demonstrated that the acrA locus contains the catA gene.

**Fig. 4. catA expression and enzyme activity in methanol-resistant mutants.** (a) Cell lysates of each strain were assayed for catalase activity as described in Methods. Catalase activities of the methanol-resistant mutants (indicated by *) were expressed as a percentage of the activities of their parents (bar to left of mutants). (b) Total RNA (7.2 µg) from each strain was separated on a 10% agarose-formamide gel, blotted onto a nitrocellulose membrane and hybridized with a catA cDNA probe. Lanes: 1, NC4; 2, X9; 3, AX2; 4, IR40; 5, AX3; 6, IR41; 7, SA31; 8, SA32; 9, SA219; 10, SA220.

**Fig. 5. Identification of the genetic lesion in acrA2 mutants.** Genomic DNA was prepared from the acrA2 mutant XP210. The catA gene was isolated by PCR using this DNA as a template and two catA-specific primers (see Methods). This fragment was purified and sequenced, and the resulting sequence aligned with wild-type catA. A 13 bp deletion is apparent.
**Fig. 6.** Biochemical pathways for methanol metabolism. In all species studied, methanol is metabolized to CO₂ via the same intermediates although the enzymic pathways involved may vary in different species. Step 1. Methanol is oxidized to formaldehyde by alcohol dehydrogenase, alcohol oxidase or the catalase peroxidative pathway. Step 2. Formaldehyde is rapidly oxidized to formic acid mainly by formaldehyde dehydrogenase. Other enzymic systems such as catalase and aldehyde dehydrogenases can also mediate this step. Step 3. Formic acid, which is the principal toxic agent in methanol poisoning, is detoxified in mammals mainly by tetrahydrofolate (THF)-dependent enzymic activities. In yeasts, formic acid is metabolized by formate dehydrogenase. The catalase peroxidative pathway can also oxidize formic acid to CO₂ in yeast and mammalian cells. In Dictyostelium, steps 1 and/or 2 are clearly mediated by catalase and thus the absence of CatA activity in the methanol-resistant mutants hinders the formation of the toxic formic acid.

**catA** levels to treatment with exogenous H₂O₂ (Garcia et al., 2000; Madigan & Katz, 1989).

Methanol itself is essentially non-toxic but gets oxidized to formaldehyde and then to formic acid (Fig. 6) which are both highly toxic and reactive metabolites (Kruse, 1992). In mammals, most of the toxicity of methanol is attributed to formic acid, which is a potent inhibitor of cytochrome oxidase, thereby causing histotoxic hypoxia and metabolic acidosis (Liesivuori & Savolainen, 1991; Tephly, 1991). Indeed, administering formic acid alone can mimic methanol toxicity in animal models (Martin-Amat et al., 1978). Thus, the pathways that regulate the relative rates of formic acid generation and formic acid oxidation to CO₂ greatly determine the toxic effects of methanol in different species. Our work shows that this is also the case for Dictyostelium.

Methanol metabolism yields identical products in different organisms but is mediated through different enzymatic pathways (Fig. 6). In the first step methanol is oxidized to formaldehyde. In primates this is mediated primarily by alcohol dehydrogenase (Kavet & Nauss, 1990; Valentine, 1990), while in Drosophila larvae and rodents, this step is mainly performed by the H₂O₂-dependent peroxidase activity of catalase (Karinje & Ogata, 1990; van der Zel et al., 1991). It is thought that primates do not oxidize methanol via the catalase-dependent pathway because of the low enzymic activities of their peroxide-generating oxidases (Goodman & Tephly, 1970). Notably, this initial metabolic step proceeds at similar rates in both rodents and primates despite their use of different enzymic pathways to oxidize methanol (Kavet & Nauss, 1990).

The next metabolic step is the rapid oxidation of formaldehyde to formic acid, which is catalysed primarily by formaldehyde dehydrogenase in all species studied (Liesivuori & Savolainen, 1991), although there is evidence that catalase can also catalyse this reaction (Tephly, 1991; van Dijken et al., 1982; Veenhuis et al., 1983). Formaldehyde itself is potentially toxic, but because there are multiple pathways for its oxidation, it does not accumulate and directly exert a toxic effect in mammals (Kavet & Nauss, 1990).

The identification of the Dictyostelium catA gene as the acrA locus indicates that CatA enzyme activity is overwhelmingly the major enzyme in the metabolism of methanol in this organism. Despite many years of screening, no methanol-resistant mutants have been found outside the acrA locus and deletion of catA allows cells to survive about fivefold higher levels of methanol than wild-type, so it is unlikely that any other enzymes are contributing significantly to methanol breakdown. Thus, the lack of CatA activity in the methanol-resistant mutants enables them to survive the presence of methanol by preventing the formation of formaldehyde and/or formic acid (Fig. 6).

While the importance of catalase peroxidase activity in methanol metabolism has been previously recognized in other systems, the present study is the first to demonstrate a link between catalase activity and acriflavine resistance. Genes associated with acriflavine resistance have been identified in different organisms. Molecular cloning of these genes revealed that many of them encode multidrug resistance pumps that transport acrif-
flavine out of the cell (De Rossi et al., 1998; Masaoka et al., 2000; Nakaune et al., 1998; Pereira et al., 1998). Inactivating mutations in these transporter genes lead to acriflavine sensitivity (Ma et al., 1994). Conversely, mutations that lead to their overexpression or to an increased efflux activity lead to acriflavine resistance (Andrade et al., 2000; Klyachko & Neyfakh, 1998; Nakaune et al., 1998). The observation that mutations in Dictyostelium catA lead to acriflavine resistance represents a novel mechanism for resistance to this drug. It is possible that the peroxidase activity of CatA is involved in a pathway which modifies acriflavine to produce a cytotoxic form. Such a role for catalase has been demonstrated for the antituberculosis drug isoniazid, an antibiotic used against Mycobacterium tuberculosis infections. Recent work has indicated that isoniazid is a produg that has to be activated in vivo by the mycobacterial catalase-peroxidase KatG to form a reactive intermediate that can interact with its cellular targets (Johnsson & Schultz, 1994; Rozwarski et al., 1998). Loss-of-function mutations in KatG are thus sufficient to confer isoniazid resistance and are the most common mechanism for resistance in clinical isolates (Miesel et al., 1998; Zhang et al., 1992).

We predict that the other loci involved in acriflavine resistance, acrB and acrC, also encode enzymes and that together with catA they are responsible for the metabolism of acriflavine to its final, biologically active form. It will be interesting to test this hypothesis by isolating REMI mutations in acrB and acrC and cloning their genes. This should allow the complete metabolic pathway to be mapped out. It is also worth noting that acrA allows a strong negative selection, which is sufficiently powerful to allow isolation of mutants without the use of mutagens and measurement of basal mutation levels during normal growth (Loomis, 1987). We therefore anticipate that the molecular identification of the gene will allow far more versatile genetic selections than are currently available. For example, an extrachromosomal plasmid containing an antibiotic selection marker and catA could be selected positively with antibiotic and negatively by growth on methanol. Such experimental tools will become invaluable as the sequencing projects reveal the full complexity of the Dictyostelium genome.

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