Light inhibits rifampicin inactivation and reduces rifampicin resistance due to a cloned mycobacterial ADP-ribosylation gene

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Abstract

Rifampicin is a principal drug used to combat infections by mycobacteria and related organisms. Most strains of Mycobacterium are able to inactivate this antibiotic by ribosylation via an ADP-ribosylated intermediate. We found that this inactivation was inhibited by light at levels similar to those prevailing in laboratory environments. Rifampicin resistance arising from the cloned ADP-ribosyl transferase was also greatly diminished at these light levels. The cloned Rhodococcus equi monooxygenase which inactivates this antibiotic by a different mechanism was, in contrast, not inhibited by light. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Rifampicin, Mycobacterium, Antibiotic inactivation, ADP-ribosyl transferase, Light inhibition

1. Introduction

Sunlight has long been considered beneficial for individuals suffering from mycobacterial infections such as tuberculosis but studies in this direction have usually focussed on the effects upon the patient’s metabolism, for example elevating serum 25-hydroxy vitamin D [1]. More recently low-intensity laser radiation has been used together with drugs and shown to enhance the efficacy of such chemotherapeutic treatments [2]. We were interested to investigate if such benefits might in part be due to light disabling the bacterial response to antibiotic challenge.

Rifampicin is a principal chemotherapeutic agent used to combat infections by Mycobacterium and related genera [3,4]. Increasingly, clinical isolates show resistance to this and other antibiotics [5,6]. Many clinical isolates are rifampicin resistant but do not have mutations in the known target of this antibiotic, RNA polymerase, suggesting another cellular component is altered [7,8]. We have found that drug inactivation is part of response to rifampicin in these bacteria. Four mechanisms have been identified: decomposition [9], phosphorylation [9], glucosylation [10], or ribosylation [11]. The last of these has been detected only in Mycobacterium and its close relatives [12].

Gene disruption experiments have shown that presence of a functional ribosylation gene product reduces rifampicin susceptibility 12–15 fold in Mycobacterium smegmatis [13] and very recently we have found that the gene product initially ADP-ribosylates rifampicin, the first known instance of such an antibiotic inactivation mechanism [14]. Many bacterial mono (ADP-ribosyl) transferases are toxins, such as those of diphtheria, cholera, Bordetella pertussis or Clostridium botulinum C3 [15]. ADP-ribosylation of the nitrogen fixing enzyme dinitrogenase reductase in the photosynthetic bacterium Rhodospirillum rubrum mediates its reversible inactivation [16]. Bacteriophage T4 possesses two ADP-ribosylating activities targeting the host DNA-dependent RNA polymerase and altering promoter specificity [17]. In all these cases the acceptors are proteins and the ADP-ribosyl moiety is joined to a nitrogen atom. However ADP-ribosylation of rifampicin is on an oxygen atom [14]. Endogenous mono ADP-ribosylation has been demonstrated in several bacteria including M. smegmatis [18], but little is known about the physiological role of this modification process.

M. smegmatis possesses at least two rifampicin inactivation systems [13] and at least one additional rifampicin resistance determinant which is not RNA polymerase [19]. Therefore to separate the effects on individual cellular components interacting with this antibiotic we studied the influence of light on cloned rifampicin inactivation genes in organisms unable themselves to inactivate the drug.
Rhodococcus rhodochrous strain CW25 (closely related to Mycobacterium) and Escherichia coli. We compared the ADP-ribosyl transferase (adr) gene with the rifampicin inactivation gene (iri) cloned from the opportunistic pathogen Rhodococcus equi, which on the basis of sequence is a monoxygenase [20].

2. Materials and methods

2.1. Bacterial strains and plasmids

These are listed in Table 1. E. coli and R. rhodochrous were grown in Luria Bertani medium at 37°C and 28°C, respectively. Plasmids were selected for and maintained in E. coli by the addition of ampicillin, 100 μg ml⁻¹; in nocardioform strains they were selected using 40 μg ml⁻¹ chloramphenicol and maintained using 20 μg ml⁻¹ of this antibiotic. Shuttle vector pDA71 [21] is able to replicate in both E. coli and a number of nocardioform bacteria; it has a positive selection function due to the EcoR endonuclease with expression turned off by the λ repressor so it is maintained in a λ lysogen of E. coli. Plasmid transformations were performed as previously described [13,22,23].

2.2. DNA manipulations

Restriction endonucleases and other enzymes used in DNA manipulations were employed according to the manufacturer’s instructions. All restriction mapping was done using DNA from E. coli dam dcm strain GM2929 (Table 1). Chromosomal and plasmid DNAs were purified by cesium chloride gradient centrifugation as described previously [23]. DNA fragments were purified after electrophoresis through low gelling temperature agarose (SeaPlaque).

2.3. Rifampicin inactivation and susceptibility testing

Rifampicin concentrations were measured by zones of inhibition on plates spread with a lawn Bacillus subtilis strain 1A3-1 [24]. Rifampicin minimum inhibitory concentration (MIC) was found by spotting about 10⁴ cfu on plates with a range of antibiotic concentrations, using a replicator; the highest concentration at which there was confluent growth was taken as the resistance level. Plates were incubated for 5–6 days in the case of nocardioform strains or 14 h for E. coli. Illumination was measured in lx (lumen per square meter) using a Type 3281 Luxmeter (Yokogawa Hokushin Electric Co.).

2.4. SDS-polyacrylamide gels

These were prepared according to the method of Hames and Rockwood [25]. Separating gel was 10% acrylamide and stacking gel 6% prepared from an acrylamide:bisacylamide 30:0.8 stock. E. coli extracts were prepared from 1-ml aliquots of stationary phase culture. Electrophoresis of samples, together with Bio-Rad low molecular mass standards, was at a constant current of 25 mA for 3.5 h. Proteins were visualized by staining with amido black.

3. Results and discussion

3.1. Choice of strain for testing effects of illumination on rifampicin inactivation ability due to cloned genes

Response to rifampicin challenge of M. smegmatis and most related organisms involves two or more other determinants apart from RNA polymerase [13] so to investigate the effects of light upon a cloned individual component of this response strain CW25, a closely related bacterium, was used. CW25 cannot inactivate rifampicin and so is highly susceptible to this antibiotic; it has an MIC of 30 ng ml⁻¹ in contrast to M. smegmatis strain DSM43756 which has an MIC of 20 μg ml⁻¹. The M. smegmatis rifampicin ADP-ribosylation gene (adr), the R. equi rifampicin monoxygenase gene (iri), each carried by E. coli-Rhodococcus shuttle plasmid pDA71, were transformed into CW25 as was vector pDA71 itself.

Cultures of these transformants were challenged with 20 μg ml⁻¹ rifampicin and the residual antibiotic assayed, as previously described [24]. When cultures carrying the ADP-ribosyl transferase clone were illuminated with visible light, the antibiotic inactivation process was completely inhibited at 600 lx, illumination comparable to that measured in laboratory environments; at 200 lx the rate was already substantially reduced (Fig. 1). In contrast inactivation of the drug by the iri-encoded monoxygenase was not inhibited at all even at 1000 lx. As controls,

Fig. 1. Rifampicin inactivation by cultures of strain CW25 carrying plasmid pS3, illuminated by: ●, 0; ○, 200; □, 400; ▲, 600 lx. Strain CW25 carrying plasmid pSAN1, illuminated by: ○, 0; ▲, 1000 lx. Antibiotic is not reduced by a detectable amount when incubated for 25 h in the presence of strain CW25 carrying vector pDA71 only.
the endogenous aminoglycoside or macrolide inactivation activities of strain CW25 were tested but neither was significantly inhibited by light. The growth rate of strain CW25 was not measurably impaired by light at 1000 lx.

3.2. Effect of light upon antibiotic susceptibility in E. coli transformed with rifampicin inactivation genes or carrying an rpoB mutation

Both rifampicin inactivation genes confer a phenotype of increased resistance to the antibiotic upon strain CW25, respectively, 15 and 150 fold for \( \text{adr} \) and \( \text{iri} \) [21]. Light apparently abolished this effect in the case of the former clone but not the latter; however, the prolonged exposure to light necessary for full growth of nocardioform transformants (>5 days) resulted in partial bleaching of the antibiotic. To minimize the effects of bleaching further experiments were performed in the much faster growing E. coli. Like CW25, this organism is unable to inactivate rifampicin. Both strain MM 294-4 and a derivative of MM294-4 with an \( rpoB \) chromosomal mutation conferring rifampicin resistance showed a increase in MIC with increasing illumination (Fig. 2a,b). The \( \text{adr} \) gene carried on plasmid pS3 is apparently expressed from its own promoter in E. coli [13] conferring an approximately three-fold increase in antibiotic MIC but this increase was abolished by >600 lx illumination (Fig. 2d). Expression of \( \text{adr} \) from the \( \text{lac} \) promoter conferred much higher drug resistance [13], to 100 \( \mu \text{g} \cdot \text{ml}^{-1} \), but illumination reduced this and at 800 lx abolished it completely (Fig. 2e): the MIC of 5 \( \mu \text{g} \cdot \text{ml}^{-1} \) was similar to that of E. coli not carrying any resistance determinants (Fig. 1a). The results with the ADP-ribosyl transferase expressed from the \( \text{lac} \) promoter suggested that light did not exert its effect at the promoter level but on later stages of gene expression.

3.3. Contrasting effect of light upon antibiotic inactivation and resistance due to the rifampicin monoxygenase gene

This conclusion was supported by the contrasting effects of light on rifampicin inactivation by the clones in strain CW25 (Fig. 1), though the \( \text{iri} \) and \( \text{adr} \) ORFs show no significant similarity their putative regulatory upstream DNA sequences are strikingly alike, suggesting common features in regulation [13,21]. Plasmid pSAN1 (pDA71 carrying the \( R. \text{equi} \) \( \text{iri} \) gene) was transformed into strain MM294-4, the construct expressing the monoxygenase

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Strains and plasmids used in this work</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>R. rhodochrous</em></td>
<td></td>
</tr>
<tr>
<td>CW25</td>
<td>Unable to inactivate rifampicin</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>MM294-4</td>
<td>( \text{hsdR}17 \text{ endA}1 \text{ gyrA} )</td>
</tr>
<tr>
<td>MM294-41</td>
<td>MM294-4 ( \text{rpoB} )</td>
</tr>
<tr>
<td>MM294-4 (( \lambda ))</td>
<td>MM294-4 lysogenized with ( \lambda \text{B57S7} )</td>
</tr>
<tr>
<td>GM2929</td>
<td>( \text{dcm-6 dam13::Tn9 recF143 hsdR2} )</td>
</tr>
<tr>
<td>B. subtilis 1A3-1</td>
<td>spectinomycin resistant</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pDA71</td>
<td>( E. \text{coli-Rhodococcus} ) shuttle vector</td>
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<tr>
<td>pGEM 3Zf((-))</td>
<td>( \text{lac} ) expression vector</td>
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<tr>
<td>pSAN1</td>
<td>( R. \text{equi} ) \text{ gene on BamHI-BglII fragment in pDA71}</td>
</tr>
<tr>
<td>pS3</td>
<td>( M. \text{smegmatis adr} ) gene on BglII fragment in pDA71</td>
</tr>
<tr>
<td>pGEM3Z-Bst49</td>
<td>( \text{adr} ) gene on BstNI fragment in pGEM3Z((-))</td>
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</table>
3.4. Effect of illumination upon presence of cloned enzyme from the \( \lambda_{pr} \) promoter since previous work had demonstrated it is not significantly expressed from its own promoter in \( E. coli \) and that high level expression from the \( lac \) promoter is toxic to this organism [20]. Increasing illumination did not lower rifampicin MIC but instead moderately increased it (Fig. 2c). In addition, other plasmid-borne determinants were tested in \( E. coli : \) \( L -lactam, \) chloramphenicol, and tetracycline resistance genes (the mid-borne determinants were tested in \( M. smegmatis \): [10]).

Therefore the significance of this parallel remains to be determined.

**References**


