Plate-based dormancy culture system for *Mycobacterium smegmatis* and isolation of metronidazole-resistant mutants

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Received 26 March 2001; received in revised form 9 May 2001; accepted 11 May 2001

First published online 5 June 2001

**Abstract**

*Mycobacterium smegmatis* is an obligate aerobe. However, growth analyses in oxygen-limited liquid cultures have shown that the bacillus is able to survive anoxia with a half-life of 4 days by shifting down to a drug-resistant, dormant state. Metronidazole is the first lead against dormant bacilli and shows selective toxicity for this physiological state. Here, we report a plate-based dormancy culture system employing anoxic jars for *M. smegmatis*. Its usefulness for the genetic analysis of dormancy was demonstrated by isolating the first metronidazole-resistant mutants. Highly resistant mutants formed slightly yellow (as opposed to creamy) colonies. Furthermore, high-level metronidazole resistance correlated with an increased half-life of 12 days under anoxic conditions. This suggests a link between metronidazole susceptibility and anaerobic survival. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Dormancy; Metronidazole; Drug resistance

1. **Introduction**

Mycobacteria require oxygen for growth. However, the slow-growing parasitic *Mycobacterium tuberculosis* [1], its attenuated relative *Mycobacterium bovis* BCG [2] and the fast-growing saprophyte *Mycobacterium smegmatis* [3] all have the capability to adapt to anoxia by developing a defined non-replicating or dormant form. For the tubercle bacilli, the dormant state could play a role in the survival of hypoxic environments encountered in the human host. For *M. smegmatis* the ability to shift down to dormancy may be involved in the survival of changing oxygen availability in the soil. Importantly, the dormant state is resistant against conventional anti-mycobacterials. Therefore, dormant tubercle bacilli could, at least in part, be responsible for the observed persistence of infection during chemotherapy [4].

To study the dormancy response we apply the dormancy culture system that was developed by Wayne and Hayes for *M. tuberculosis* [1] on *M. smegmatis* [3]. Wayne and Hayes' dormancy culture system is based on growth of the bacilli in liquid medium under oxygen-limited conditions in sealed, stirred tubes. Initially the cultures grow exponentially and consume the available oxygen. A temporal oxygen gradient is generated and the cultures terminate growth when the oxygen concentration reaches a hypoxic threshold level. The bacilli in the anoxic stationary phase are in a reversible, non-replicating or dormant form. For the tubercle bacilli, the dormant state could play a role in the survival of hypoxic environments encountered in the human host.

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culture system is relatively poor. Exposure of *M. smegmatis* to 100 µg ml⁻¹ MTZ for 10 days results in only 100-fold reduction in the number of viable bacilli (3), see Fig. 3. Increasing the concentration of the drug does not result in a reduction of the number of wild-type bacilli to a level that makes testing of survivors for MTZ resistance feasible (see Section 3.2). Thus, the liquid medium-based dormancy culture system is not suitable for the isolation of MTZ⁺ mycobacteria. The isolation of MTZ⁺ mutants by simply spreading culture on drug-containing agar and picking colonies is not possible because MTZ sensitivity is expressed specifically in the dormant stage. Growing bacilli are not affected by the drug (2,3,5).

To facilitate genetic analyses of dormant mycobacteria, we report here a solid medium-based dormancy culture system for *M. smegmatis* and demonstrate the usefulness of the system by isolating the first MTZ⁺ mutants in mycobacteria.

2. Materials and methods

All experiments were conducted with *M. smegmatis* mc²155 [8] at 37°C. Dubos Tween–albumin broth (Becton Dickinson) was dispensed in 17-ml aliquots to screw-cap glass tubes, 20 × 125 mm. To grow experimental exponential-phase bacilli, pre-cultures were diluted to OD₆₀₀ = 0.05 and caps were loosely screwed down allowing exchange of air. Cultures were aerated by incubation on a shaker–incubator at 250 rpm. To grow dormant bacilli, pre-cultures were diluted to OD₆₀₀ = 0.005. Magnetic stirrers (and MTZ if appropriate) were added, the caps were tightly screwed down to seal the tubes and the cultures were stirred at 170 rpm [3]. For cultivation of bacilli on solid medium plates (85 mm diameter) with 30 ml Dubos oleic–albumin agar were used. Plates containing a drug gradient from 0 to 500 µg ml⁻¹ MTZ were prepared by firstly pouring 15 ml of drug-free agar into the slightly tilted Petri dish. After the agar had solidified the dish was put on a flat surface and 15 ml of agar containing 500 µg ml⁻¹ MTZ was added. Anoxic atmosphere for solid medium experiments was generated using GasPak 100 anaerobic jars according to the instructions supplied by the manufacturer (Becton Dickinson). This system is based on a hydrogen generator envelope containing sodium borohydride. The addition of water starts a H₂ generating reaction. The reduction of O₂ by H₂ is catalysed by palladium. Anoxia was monitored using the oxygen indicator methylene blue (Sigma). Methylene blue was diluted in the medium (broth or agar) to yield a concentration of 1.5 µg ml⁻¹ [3]. Anoxia in the atmosphere of the GasPak jars was monitored by methylene blue strips supplied by the manufacturer (Becton Dickinson). MTZ was obtained from Sigma. 10 mg ml⁻¹ stock solutions were prepared in water.

3. Results and discussion

3.1. Solid medium dormancy culture system

To establish a dormancy culture system based on solid medium we tested growth and survival of *M. smegmatis* on agar plates under hypoxic conditions. Anoxic atmosphere was generated in jars using the GasPak system in which O₂ is reduced to water in a palladium-catalysed reaction with H₂ as described in Section 2. Diluted culture samples containing 300 cfu were spread on plates and transferred into jars. The hydrogen generating reaction was started and the jars were sealed. The generation of anoxic conditions in the atmosphere of the jars was monitored using an oxygen indicator strip containing methylene blue. Within 30 min the strips turned white, indicating anoxia in the gas phase. Methylene blue-containing agar plates decoloured after 5–6 days. After a total of 10 days, the jars were opened. Inspection of the agar surfaces under a dissection microscope did not show any growth. To determine whether any bacilli on the agar surface had survived the oxygen-starvation treatment the plates were incubated under normal air. Colony count after 3 days showed that 90% of the initially plated cfu had survived the 10 days of hypoxia (Fig. 1). Next we tested a key feature of hypoxic dormant bacilli, the development of sensitivity to MTZ. The anoxic jar experiment was carried out in the same way, but using agar plates containing 100 µg ml⁻¹ MTZ. In contrast to the anoxic survival of the bacilli on drug-free plates, incubation for 10 days in the anoxic jars on plates containing MTZ resulted in a 100% killing of the bacilli (Fig. 1). This result showed that the bacilli in the jar developed sensitivity to MTZ. In contrast to the cidal effect of MTZ on the non-growing bacteria in the anoxic jar the drug did not show any effect on the colony formation of bacilli incubated under normal at-

![Fig. 1. Growth and survival of *M. smegmatis* on agar plates as a function of oxygen and MTZ. 300 cfu (=100%) from exponentially growing cultures were spread on agar plates with and without MTZ and incubated in normal atmosphere (+O₂) for 3 days or under anoxic atmosphere in jars (−O₂). After 10 days incubation under anoxic conditions plates were exposed for 3 days to normal atmosphere. Numbers on the plates shows percentage of plated cfu that was observed to form colonies after each incubation step. The experiment was repeated once with the same results.](https://academic.oup.com/femsle/article-abstract/200/2/215/597574)
mosphere (Fig. 1). Taken together, our experiments suggest that bacilli sitting on the agar surface exposed to the anoxic atmosphere generated in the jars shifted down to a state of MTZ-sensitive dormancy that is similar to the state of bacilli in the anoxic stationary phase generated in the liquid medium-based Wayne and Hayes dormancy culture model.

3.2. Isolation and characterisation of spontaneous MTZ-resistant mutants

To determine whether the plate-based dormancy culture system is suitable for the selection of spontaneous Mtz\(^r\) mutants an increased number (10\(^7\) cfu) of bacilli were spread on agar plates containing 100 and 250 \(\mu\)g ml\(^{-1}\) MTZ. Growth was examined after incubation in anoxic jars for 10 days and exposure to normal atmosphere for 3 days. The selection at 100 \(\mu\)g ml\(^{-1}\) MTZ appeared to be somewhat leaky upon plating large numbers of bacilli. However, selection on agar containing 250 \(\mu\)g ml\(^{-1}\) MTZ eliminated the background of wild-type bacilli (from 10\(^7\) cfu plated, only 100 cfu formed colonies; see below). In parallel experiments employing the liquid dormancy culture system, an increase of the MTZ concentration from 100 to up to 500 \(\mu\)g ml\(^{-1}\) did not result in the elimination of wild-type bacilli. Both drug concentrations reduced viability only about 100-fold after 10 days of incubation (from about 10\(^7\) cfu ml\(^{-1}\) to 10\(^5\) cfu ml\(^{-1}\)). Thus, MTZ appears to be more effective in the anoxic jars compared with the liquid dormancy culture system. A possible reason for this difference could be that MTZ exerts its cidal activity only in a strictly anoxic environment, as defined by the complete decolourisation of methylene blue (H.L. Peh and T. Dick, unpublished data). In the Wayne and Hayes dormancy culture system, oxygen is depleted slowly over a period of 7–8 days (see Fig. 4).

In contrast, oxygen is removed rapidly from the gas phase in the solid medium-based culture system and the generation of anoxia in the agar takes only 5–6 days. Therefore, bacilli on the agar surface are exposed to anoxia earlier and (because the total cultivation time (10 days) in both systems is kept constant) longer than bacilli in the sealed liquid culture system. Taking these observations together, it is conceivable that different oxygen depletion kinetics in the two culture systems are responsible for the difference in the cidal effect of MTZ.

To isolate spontaneous Mtz\(^r\) mutants, 5 \(\times\) 10\(^7\) cfu were spread on agar plates containing 250 \(\mu\)g MTZ ml\(^{-1}\), incubated for 10 days in anoxic jars, and then exposed to normal atmosphere to detect surviving, presumably Mtz\(^r\) bacilli. One of 10\(^5\)-plated cfu formed a colony. 3% of the apparent Mtz\(^r\) colonies were found to be slightly yellow (Yeo) colony colour. A typical example of a streak test is shown.
(Yeo) as opposed to the creamy colour of the wild-type. Colony purification of mutants on drug-free plates showed that the yellow colour was independent of MTZ. To determine relative resistance levels of the apparent Mtz\(^r\) mutants, hypoxic survival of 50 mutants (15 Mtz\(^r\)-Yeo, 35 Mtz\(^r\)) was tested on agar plates containing a gradient of the drug. Mutants were streaked out along the gradient and incubated for 10 days in anoxic jars. Then the plates, which did not show any growth after removal from the jars, were exposed to normal atmosphere for 3 days. The rationale behind this experiment was that extent and density of growth of the streak along the MTZ gradient under anoxic conditions should reflect the survival of the non-growing bacilli exposed to increasing MTZ concentrations under hypoxic conditions. Fig. 2 shows that Mtz\(^r\) wild-type bacilli survived hypoxia only along the section of the streak that was exposed to low concentrations of the MTZ gradient. All 15 Mtz\(^r\)-Yeo mutants formed dense streaks up to the highest MTZ concentration of the gradient. Extent and density of growth of the streaks of the 35 Mtz\(^r\) mutants that had retained their wild-type colour varied (i.e. was strain-specific) and covered the whole spectrum from the weak growth, shown by the wild-type, to the strong growth shown by the Mtz\(^r\)-Yeo mutants. The Mtz\(^r\) mutant isolation and characterisation experiment was repeated once in the same way yielding similar results. The frequency of Mtz\(^r\) cfu was found to be about \(10^{-5}\). 6\% of the Mtz\(^r\) colonies showed a Yeo phenotype and were highly MTZ-resistant in the gradient plate assay.

One mutant, mtz-1, that displayed the Mtz\(^r\)-Yeo phenotype was selected for further analysis in the liquid medium-based dormancy culture system employing sealed tubes. To confirm the high MTZ resistance level of the mutant that was indicated by the MTZ gradient plate assay, sealed cultures were grown in the presence of various MTZ concentrations. After 10 days, survival was determined by plating and colony count. Fig. 3 shows that the wild-type was killed 100-fold by 100 \(\mu\)g ml\(^{-1}\) MTZ. In contrast mtz-1 was hardly affected by the drug, thus validating the results from the drug gradient plate assay. To determine whether mtz-1 was affected in the survival under anoxic conditions, long-term drug-free sealed cultures were grown and viability was determined at various time points by colony count. Fig. 4 shows that the half-life of anoxic culture of mtz-1 was increased three-fold (\(t_{0.5} = 12\) days) compared to that of the wild-type (\(t_{0.5} = 4\) days). Thus, the high level of MTZ resistance of mtz-1 appears to be associated with increased viability of dormant culture under drug-free conditions.

Upon plating of anoxic cultures of mtz-1 for cfu determination, a number of apparent revertants were observed that had lost their Yeo phenotype. This high reversion frequency (\(10^{-2}\)) was observed in both MTZ-containing and MTZ-free cultures and was thus independent of the drug. To determine whether the high frequency of reversion of the Yeo phenotype was specific to anoxic cultures of mtz-1, the frequencies of Yeo reversion in growing and anoxic culture of mtz-1 were compared. A pre-culture was split and used to generate aerated exponentially growing cultures (\(OD_{600} = 0.2\)) and sealed 10-day-old anoxic cultures. Culture samples from both growth phases were spread on plates and the frequencies of appearance of creamy colonies were observed. No Yeo revertant colonies were detected in \(10^4\) cfu plated from growing culture. Thus, the reversion frequency was less than \(10^{-3}\) and the Yeo phenotype appeared to be stable in the exponential growth phase. As before, about one in 100 colonies from anoxic culture were observed to have reverted to creamy colony colour. This experiment was repeated once, yielding the same results. Thus, the frequency of Yeo reversion appears to be at least 100-fold higher in non-growing anoxic culture compared to growing culture. It is important to note that yellow colonies were not observed upon plating of dormant wild-type or dormant Yeo revertant cultures. Thus, dormancy-dependent instability appears to be specific to mtz-1. Recently, it was shown that brief exposure of \(M.\) smegmatis to hypoxic conditions stimulates the transposition of the \(M.\) tuberculosis IS6110 [9]. Furthermore, it was demonstrated that the stationary growth phase of \(M.\) smegmatis is associated with hypermutability [10]. Whether dormancy is associated with genetic instability has not been determined and the mechanism responsible for the apparent dormancy-specific instability of mtz-1 remains to be elucidated. Furthermore, it remains to be established whether the reversion frequency of mtz-1 is increased only in anoxic dormant culture or generally in the stationary phase.

To confirm that the three observed phenotypes of mtz-1 (Mtz\(^r\), Yeo and increased anoxic survival) were genetically linked, we determined MTZ susceptibility and half-life of anoxic culture of one Yeo revertant, mtz-1rev, in sealed liquid culture experiments. mtz-1rev was found to display wild-type sensitivity to MTZ and wild-type survival of anoxic culture. The concurrent reversion of all three phenotypes suggests that the traits were indeed due to the same mutation.

In conclusion, we report a culture system for dormancy in \(M.\) smegmatis that is based on solid medium and thus facilitates genetic analysis of dormancy. We demonstrated its usefulness by isolating the first mutants that are resistant against MTZ. High-level MTZ resistance was associated with yellow colony colour and with an increase in viability of anoxic culture. This suggests a link between MTZ susceptibility, metabolism (accumulation of a yellow metabolite?) and dormancy survival. It is interesting to note that in a parallel effort to isolate Mtz\(^r\) mutants by Tn5-based [11] transposon mutagenesis 35 000 insertion mutants were generated and subjected to selection for MTZ resistance. However, no Mtz\(^r\) colonies were obtained. This could suggest that the genes conferring MTZ susceptibility are essential for viability. Experiments are now under way to identify the gene(s) involved in
spontaneous MTZ resistance. This work will reveal the molecular nature of MTZ susceptibility, shed light on its linkage with dormancy viability, and should lead to an understanding of the mechanism underlying the dormancy-dependent high reversion frequency of the Mtzr-Yeo mutant mtz-1.

Acknowledgements

We would like to thank Pamela Thayalan for help with the transposon mutagenesis and Bernadette Murugasu-Oei for comments on the manuscript. This study was supported by the Institute of Molecular and Cell Biology.

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