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Antibiotic Resistance Protocols

Second Edition

Edited by

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Preface

Several years have passed since the first edition of our book, and much has changed in the world of microbiology. The trend of increasing antibiotic resistance has continued with, for example, the emergence of extensively resistant *M. tuberculosis*. Our understanding of antibiotic resistance biology has increased. Genomic methods have become more accessible allowing antibiotic researchers to probe not only the sequence of antibiotic resistance determinants but also the mechanism whereby they are expressed and regulated. Array methodology has allowed a large number of genes to be studied simultaneously, while real time PCR has allowed detailed dissection of gene expression. We have also begun to understand the importance of biofilms and the physiological state of the organism in permitting organisms to survive in the face of antibiotics and the act of developing resistance. The molecular revolution has provided the tools that allow us to detect and characterise resistance determinants, and these may be sequence based, nucleic acid amplification methods, or detection of the activity of efflux pumps. The ability of a resistant organism to survive and thrive in the clinical environment is fundamental to the evolution of resistance, and considerable progress has been made in the understanding of the role of fitness in the selection of resistant organisms. This understanding has been aided by an increasing range of physiological characteristics that are tested, and automated methods of testing have improved the reproducibility and robustness of these assays. The need to develop new antibiotic agents means that we have to develop new high throughput methods of evaluating susceptibility. The interaction between bacterial and human populations is an important area of investigation, and we need better techniques to characterise the population genetics of the organisms and better sampling tools to ensure that this data is representative.

The editors have sought to bring together examples of a diverse range of modern techniques applied in antibiotic research, and they hope that you find these useful in planning your own research.

London, UK

Stephen H. Gillespie
Timothy D. McHugh

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Part I

Epidemiology and Population Genetics

Chapter 1

The RM Test for Determining Methicillin-Resistant *Staphylococcus aureus* Lineages

Jodi A. Lindsay and Julia M.-L. Sung

Abstract

Staphylococcus aureus lineages evolve independently and differ in hundreds of genes. Identification of lineages can be useful for epidemiological typing and infection control at the local or global level, and can also be useful when investigating differences in pathogenesis between strains. MLST (multilocus sequence typing) and *spa* typing (polymorphisms in the protein A gene) are useful methods for identifying lineages but can be time-consuming and expensive. Here, we describe a method for identifying lineages using PCR, which is very easy to perform and can generate results within hours. It can also be adapted to commercial or real-time platforms. The RM (restriction modification) test is based on unique sequences found in each lineage that determine the specificity of an RM system, which detects and digests foreign DNA, thereby controlling the independent evolution of the lineages; thus, it is the ideal single gene to target for a rapid lineage test.

Key words: Methicillin-resistant *Staphylococcus aureus*, MRSA, Restriction modification, RM test, Lineages, Typing, PCR

1. Introduction

Staphylococcus aureus is a commensal of the human nose and a common cause of minor skin infections and more serious hospital infections such as wound and catheter site infections, bacteremia and pneumonia (1). Methicillin-Resistant *Staphylococcus aureus* (MRSA) are *S. aureus* that have acquired a novel piece of DNA, a mobile genetic element (MGE) called SCC*mec* that carries resistance to β-lactam antibiotics. There are a variety of SCC*mec* types, and transfer to *S. aureus* has occurred on multiple occasions (2–4). MRSA infections account for 20–80% of all *S. aureus* infections in most hospitals (5), so that we now rely on glycopeptide

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antibiotics such as vancomycin for prophylaxis and treatment. However, nine cases of fully vancomycin-resistant MRSA have now been described in the USA, and they have all acquired the *vanA* gene from enterococci (6). Hospital MRSA can be found in the nose of staff and patients, and in hospital environments, and they can also be carried by people outside of hospitals who have had prior antibiotic exposure or contact with healthcare services (5). Carriage rates and incidence in hospitals vary widely according to country and healthcare systems (5). In addition, community-acquired-MRSA (CA-MRSA) strains that have evolved independently of hospitals are becoming widespread, especially in the USA, and these strains cause severe skin and soft tissue infections in healthy hosts (1). MRSA are also emerging in animals, such as pets, where they act as a reservoir for human and animal infection (7), and in pigs and pig farmers (8).

Epidemiological typing is often performed to track the local spread of strains and identify outbreaks, and this aids in infection control strategies. Typing methods often require specialised equipment and skills, so they are usually performed at national reference centres, and this can delay the timely implementation of appropriate control strategies. Epidemiological typing is also performed to identify regional, national or global trends in strain evolution and spread, and, for this purpose, lineage detection is very important as it is extremely reproducible and comparable. Since lineages carry unique combinations of hundreds of surface and other factors implicated in virulence (9), identification of lineages is also useful for basic researchers wishing to investigate host–pathogen interactions.

Staphylococcus aureus populations are clonal, with about ten major human lineages dominating (9). The major hospital-associated MRSA lineages (10) are CC5 (including USA100 and USA800), CC8 (including USA500 and ST239), CC22 (including EMRSA-15), CC30 (including EMRSA-16, ST36 and USA200) and CC45 (including USA600). Most regions have only a small number of dominant MRSA lineages in their hospitals (2); for example, the UK has predominantly CC22 and CC30, and Japan predominantly CC5. The CA-MRSA lineages include CC1 (including USA400) and CC8 (including USA300). Animal MRSA lineages include CC22. The best methods for determining lineage of a *S. aureus* isolate are MLST (11), *spa* typing (12) or multi-strain microarray (9), but all require specialised equipment and are time-consuming techniques. In this chapter, an inexpensive PCR method for determining lineages that can be performed in a few hours using standard laboratory equipment is described (10). The test can also be adapted for commercial platforms, RT-PCR tests, or any hybridisation technology.

Within lineages, each isolate can carry any number of MGE encoding virulence and resistance genes, which can move into

and out of the strains (2). Carriage of MGE can be useful adjunct information for lineage typing. SCC*mec* typing (4) and antimicrobial resistance profiles can be useful, although detection of other MGE can be complex and many are unstable. Some popular typing methods, such as PFGE, which detect pattern variation that sometimes corresponds to MGE, are much more useful when combined with a lineage test.

The restriction modification (RM) test method works by targeting a specific region of the *S. aureus* genome that varies according to lineage (13). Each *S. aureus* strain encodes two different copies of the *sau1hsdS* gene, which is part of a type I RM system. The *Sau*1 RM system protects the cell from foreign DNA (13). It recognises specific sequences on foreign DNA and digests the DNA. The bacteria's own DNA is protected by recognising the same specific sequences and modifying and protecting them. The *sau1hsdS* genes encode the specificity subunit of the RM complexes that determines which sequences are digested or modified. Bacteria with the same *sau1hsdS* genes exchange DNA at higher frequency than bacteria with differing *sau1hsdS* genes. This system contributes to the independent evolution of lineages and delays the spread of resistance genes between *S. aureus* lineages (13). It also suggests that variation in *sau1hsdS* genes is the first step to the evolution of a new lineage, making it the best candidate for a single gene lineage test (10).

2. Materials

2.1. Bacterial Culture and Lysis

1. Brain Heart Infusion powder from Oxoid (Basingstoke, UK) used to make sterile Broth and Agar Plates.
2. Bacterial Genomic DNA Purification Kit from Edge BioSystems, Gaithersburg, USA, stored 4°C.
3. Lysostaphin from Sigma (L7386), dissolved to 5 mg/mL in 20 mM sodium acetate pH 5.2, aliquoted and stored at −20°C.
4. Isopropanol (Sigma, molecular grade).
5. Ethanol, diluted to 70% (v/v) and stored at −20°C.
6. TE Buffer :10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
7. Benchtop centrifuge that accepts 1.5 mL microcentrifuge tubes and spins at approximately 11,000 × *g*.
8. Vortex (optional).
9. Waterbath at 37°C and 65°C.

2.2. PCR Reaction

1. HotstarTaq kit 203205 from Qiagen, stored –20°C. (Includes HotStar Taq DNA polymerase (5 units per μL), 10× PCR buffer (100 mL Tris–HCl pH 8.3, 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatin).
2. dNTPs: A mixture of A,T, G and C deoxynucleoside triphosphates, diluted to 10 mM (2.5 mM each) in water, aliquoted into small volumes, stored –20°C.
3. Control DNA from reference strains of MRSA lineages (strains available from national reference laboratories or www.narsa.net; DNA extracted as above).
4. Primers, see Table 1. Purchased from a commercial supplier such as Sigma Genosys, and diluted in water to 10 μM.
5. Thermocycler machine. For example, we use a Thermo Scientific Hybaid PxE Thermal Cycler, 96 × 0.2 mL.
6. Molecular biology grade water.

2.3. PCR Detection

1. Agarose, electrophoresis grade, Invitrogen.
2. TAE buffer : 50× stock is made from 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0), made up to 1 L with water, stored room temperature. Working solution diluted 1:20 in water.
3. Ethidium bromide, Sigma, dissolved in water to 10 mg/mL, see Note 7.

Table 1
RM test primers

Test	Forward primer	Reverse primers	Product size (bp)	Lineage
1	AF: AGGGTTTGAAGGCGAATGGG	AR30: CAACAGAATAATTTTTTAGTTC	203	CC30
		AR22: TCAGAGCTCAACAATGATGC	990	CC22
2	AF: AGGGTTTGAAGGCGAATGGG	AR45: GGAGCATTATCTGGTGTTTTCC	722	CC45
		AR1: GGGTTGCTCCTTGCATCATA	1,037	CC1
3	BF: CCCAAAGGTGGAAGTGAAAA	BR8: CCAGTTGCACCATAGTAAGGGTA	680	CC8
		BR5: TCGTCCGACTTTTGAAGATTG	1,071	CC5

All primers are listed 5′–3′

4. Loading dye: dye stock made with 1% bromophenol blue, 1% xylene cyanol FF, 50% sucrose, stored wrapped in foil at 4°C. Working solution, mix 100 µL dye stock, 100 µL glycerol, 200µL water, store at room temperature.
5. 1 kb DNA ladder, Invitrogen, diluted to 0.05 µg/µL in water.
6. Electrophoresis gel tank and powerpack. For example, we use a standard 15 × 15 cm horizontal gel tray that can hold two combs with 16–24 wells each.
7. Waxed sealing film (Parafilm, Pechiney Plastic Packaging, Chicago, Illinois).
8. UV light box (302 nm wavelength) and protective goggles, or UV light/digital camera detection system.

3. Methods

High-quality DNA extraction from *S. aureus* is more difficult than most bacteria due to a thick cell wall that is resistant to lysozyme and cell debris that contaminates the DNA. Most successful methods require lysostaphin, an enzyme that specifically targets the staphylococcal cell wall. We use the Edge BioSystems kit because it is rapid, inexpensive and produces high-quality DNA that is stable and can be stored. Commercial automated bead methods or Qiagen columns may be substituted.

There are many variations on a basic PCR method, and we expect most of them to be successfully adaptable to the RM test. The PCR conditions described here are those we use routinely in our research laboratory. There are three possible RM tests available (Table 1), each requiring separate PCR reactions. The user should choose which of the tests is appropriate according to the major lineages found in their area (10). For example, RM test 1 is most useful in the UK where 95% of hospital MRSA are due to CC22 and CC30. Any strains negative using the RM test can then be tested with RM tests 2 and 3 (see Notes 3, 4, and 5).

3.1. Bacterial Culture and Lysis

1. Inoculate the bacterial strain of interest onto BHI agar and incubate overnight.
2. Choose several well-isolated colonies and use them to inoculate 5 mL of BHI broth in a sterile tube and grow at 37°C with shaking at 150 rpm until the culture reaches stationary phase (overnight is sufficient). (Note 1).
3. Transfer 0.5 mL of the culture to a micro-centrifuge tube and centrifuge for 5 min. Discard the supernatant.

4. Add 100 µL Spheroplast Buffer from the kit and re-suspend pellet using a pippette or by vortexing at the highest speed.
5. Add 2 µL of lysostaphin and incubate for 30 min at 37°C. (Note 2)
6. Add 25 µL of Lysis 1 buffer from the kit.
7. Add 25 µL of Lysis 2 buffer from the kit, mix thoroughly and incubate at 65°C for 5 min.
8. Add 25 µL of Advamax Beads from the kit, followed by 25 µL of Extraction Buffer from the kit, and vortex the mixture for 10 s.
9. Centrifuge in a micro-centrifuge for 3 min.
10. Transfer the supernatant to a new micro-centrifuge tube, ensuring the pellet is left behind.
11. Add an equal volume of isopropanol to the supernatant and gently invert the tube several times to mix.
12. Centrifuge for 10 s on maximum speed to pellet the DNA. Carefully tip off the isopropanol.
13. Add 250 µL of cold 70% ethanol and centrifuge for another 5 min.
14. Carefully tip off the supernatant and pipette off any excess liquid being careful to not disturb the pellet. Dry the pellet at room temperature for approximately 30 min.
15. Dissolve the pellet in 25 µL of TE buffer. Store the DNA at –20°C.

3.2. PCR Reaction

1. Prepare a PCR master-mix with all the PCR components except the template DNA. Calculate the number of PCR reactions to be run, including a control DNA sample and a control with no DNA, and add 10% to give *x*. The master-mix will contain *x* times the following:

   35 µL water

   5 µL 10× PCR buffer

   1 µL dNTPs

   2.5 µL of each primer stock (three primers per reaction, see Table 1)

   0.5 µl HotStar Taq DNA polymerase
2. Mix and aliquot 49 µL into each labelled PCR tube.
3. Add 1 µL of template DNA from Subheading 3.1. Include a control tube with DNA from a known positive strain, and a control tube with no DNA.
4. Place tubes in the thermocycler and run the following conditions: 94°C for 5 min, then 35 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 2 min), hold at 4°C.

3.3. PCR Detection and Data Interpretation

1. Pour the electrophoresis gel. Depending on the size of the tank, make up a 1.5% (w/v) mixture of agarose in TAE. Heat in the microwave until the agarose dissolves and the solution is clear, being careful not to let the mixture boil over. Cool slightly on the bench.
2. Prepare the gel tray by sealing the ends with autoclave tape or supplied rubber ends. Add comb. Pour in gel mixture and allow to set approximately 20 min.
3. Fill electrophoresis gel tank with TAE. Add approximately 20 μL ethidium bromide solution (use gloves), and stir in with pipette tip. Remove comb and tape from gel tray and lower into tank, ensuring the gel wells are at the negative electrode end, and all wells are covered with buffer.
4. On a square of Parafilm place a 3 μL spot of loading buffer, and then space out more spots, one for each sample plus one for the DNA ladder. Take 2 μL of DNA ladder in a pipette tip and mix it gently into the first loading buffer spot, then gently load the mixture into the first well of the gel. Take 5 μL sequentially of each of the PCR reaction products, mix it with the next spot and load into the next well of the gel.
5. Attach lid of electrophoresis gel, and connect to power source. Run gel at 80 V for about 1 h, until the blue dye front is approximately 80% down the gel.
6. Lift out gel tray, slide gel onto a UV light box, and visualise bands and their approximate sizes by comparing to the DNA size ladder (Note 7). Confirm that the positive control has the correct band size and the negative control is blank (Note 8).
7. Interpretation. From Table 1, using the RM test 1 primers, a band of 203 bp is interpreted as a CC30 isolate and a band of 990 bp is a CC22 isolate. Using the RM test 2 primers, a band of 722 bp is a CC45 and a band of 1,037 bp is CC1. Using the RM test 3 primers, a band of 680 bp is CC8 and a band of 1071 bp is CC5. CC1 isolates also give a band of 680bp in the RM3 test. (Note 9).

4. Notes

1. If pressed for time, and the original culture plate has several well-isolated colonies, these can be carefully collected with a sterile swab and re-suspended in 0.5 mL BHI broth. Centrifuge for a few minutes. Proceed directly to DNA extraction step 4.
2. Lysostaphin solution that is allowed to reach room temperature or is repeatedly freeze-thawed will lose activity. Store it in small aliquots, and after use refreeze immediately.

3. As each regional area has its own combination of major lineages, it might be useful to adapt the RM tests accordingly. For example, to combine the CC30 and CC45 tests by using the AF, AR30 and AR45 primers into a single PCR reaction. We encourage users to try unique combinations, provided the new combination is validated using a suitable number of strains when compared to the validated test combinations described here (10).
4. To distinguish between CC8 and ST239 (which are closely related, (14)), the easiest method is to compare capsule types. CC8 is capsule type 5, while ST239 is capsule type 8. A PCR test can be used to distinguish them (15).
5. To distinguish between USA300 and USA500, which are both CC8, the most useful test is to determine the presence of the PV-luk toxin (only in USA300), using PCR primers (16).
6. In the future, the RM test will be extended to cover emerging MRSA lineages CC59, CC80, and CC398, as well as other dominant *S. aureus* lineages such as CC12, CC15, CC25, and CC59.
7. Ethidium bromide is toxic and a suspected carcinogen. Use gloves. If spilled on skin or eyes wash for 15 min with water. Use powder in a fume hood and do not inhale. Read hazard warnings. Only use a UV transilluminator wearing safety goggles that protect your eyes from UV light.
8. Troubleshooting. If the positive control is blank, then there is a problem with the PCR reaction itself, most likely a component of the master mix; fresh reagents should be obtained. If the positive control has a band of the incorrect size and/or the negative control has a band, this could represent DNA contamination of the master mix and the experiment should be repeated.
9. Unexpected results. If any lane on the gel is blank, it could be one of two things. Firstly, the strain could belong to a lineage not covered in that test, and if it is important to identify the novel lineage, RM tests for additional lineages should be performed (Table 1). Secondly, the DNA preparation could be a problem, and this can be determined by testing the prep by PCR with a known positive pair of primers, such as the *mecA* pair (6). If any lane on the gel has two bands, it is likely to be due to bacterial contamination, and the original strains should be re-streaked, DNA freshly extracted and the test repeated.

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Chapter 2

Unravelling the Ecology of Antibiotic Resistant Bacteria in the Nasopharynx

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Abstract

To study the dynamics and diversity of pneumococcal carriage and antibiotic resistance, a more thorough and systematic approach has been employed compared with routine surveillance of serotype and antibiotic resistance. Up to ten pneumococcal isolates from pernasal (nose) and oropharyngeal (throat) sites are isolated and characterised. Our carriage studies have revealed a diverse community of pneumococci with multiple strains colonising the nasopharynx of children. In Tanzanian children less than 6 years of age, up to six serotypes and up to six different antibiotic sensitivities (as distinguished by at least a four-fold difference in the minimum inhibitory concentration) have been found. Serotyping by the Quelling reaction is prone to inaccuracy and requires expensive serological reagents. To improve the accuracy and reduce the costs, an alternative capsular typing DNA-based method has been developed. This chapter will describe the methods we have employed with emphasis on the capsular typing method.

Key words: *Streptococcus pneumoniae*, Capsular typing, Nasopharyngeal colonisation, Antibiotic resistance, Diversity

1. Introduction

The heavy burden of disease in resource-limited developing countries leads to high mortality rates in children with almost two million deaths directly attributable to pneumococcal disease. Acquisition and colonisation of a new serogroup or serotype invariably precedes invasive disease (1, 2). Furthermore, during colonisation organisms can be exposed to sub-lethal levels of antibiotics that can lead to selection of antibiotic non-susceptible strains (3). Survival, colonisation, and transmission depend upon adaptation of serogroup and antibiotic resistance. The mechanisms for adaptation are poorly understood. The mechanisms may include expansion of existing clones by new acquisitions (1),

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or unmasking of minority colonisers (4), or less frequently by genetic recombination of the capsule synthesis locus or genes, or antibiotic resistance determinants such as the penicillin-binding protein genes (5–7). It is postulated that because pneumococci are highly transformable that they are 50 times more likely to undergo genetic changes via horizontal genetic exchange than genetic mutation (8). Thus, pneumococci colonising the nasopharynx have a greater chance for recombination than invasive isolates. This means that carriage isolates may undergo capsule switching more frequently than invasive isolates, particularly if they are carried for longer and are simultaneously colonising with other pneumococci expressing different serotypes (9).

For post-vaccination surveillance and epidemiological studies of nasopharyngeal colonisation by *Streptococcus pneumoniae* it is usually sufficient to characterise a single isolate taken from the nasopharynx, but the finer detail of the diversity of colonisation is missed (10). Mapping the diversity of the colonising community can estimate the potential for unmasking and characterise the genetic pool available for recombination and transformation.

With respect to antibiotic resistance the genetic pool is an important concept in the distributed-genome hypothesis where the diversity of the pneumococcal strains colonising an individual's nasopharynx increases the size of the "supragenome" (11). The size of the supragenome is not only defined by the pneumococcal strains but also closely related Streptococci of the *oralis* group that also reside in the nasopharynx and oral cavity (12, 13). The significance of this theory is emphasised by the work, which showed by nucleotide sequencing of eight carriage strains obtained from single colony methodology from individual children that only 46% of the genome is common to all strains (11). Thus, the presence of multiple strains colonising the naso- and oropharynx simultaneously could significantly increase the size of the supragenome and increase its evolutionary potential to adapt and survive. To enable the diversity to be studied multiple colony methodology on both nose and throat samples is required.

As early as 1933, studies on multiple colonisation revealed that up to five serotypes could colonise the nasopharynx simultaneously (14). In this study, the nasopharyngeal swab samples were passaged sequentially in mice (14). More recent studies characterised from 3 to 50 pneumococcal colonies usually from direct plating out of the primary swab usually from single swab site. These studies revealed that only a minority of the children carried multiple serogroups or serotypes, with a maximum of three different serogroups or serotypes (15–18). In addition, some of these studies also uncovered colonisations by mixed penicillin susceptible and non-susceptible pneumococci (16).

The following chapter describes the methods we employ to study the biology of pneumococcal carriage in the nasopharynx of children.

These techniques enable us to start to understand the complex relationships between organisms of different serotypes and antibiotic susceptibilities. To achieve this we characterise up to 20 pneumococcal isolates from pernasal (nose) and oropharyngeal (throat) sites a diverse community of pneumococci that can multiply colonise the nasopharynx was revealed. In Tanzanian children less than 6 years of age living in a semi-closed community on a sugar plantation up to six serotypes and six different antibiotic sensitivities, as distinguished by at least a fourfold difference in the minimum inhibitory concentration have been found (10). Several groups have employed a PCR approach that uses serotype specific primers (19–21). However, this does not reduce the costs of serotyping significantly. We have developed an alternative genetic approach that involves first a universal PCR amplification of the whole capsulation locus of around 20,000 nucleotide base pairs, and then generating a restriction fragment length polymorphism profile of the amplicon with the enzyme, *Hinf* 1 (22, 23). The RFLP profile of the unknown serotype is compared with RFLP profiles to a database of reference serotypes compiled from in vitro and in silico data (Charalambous et al.; manuscript in preparation). For researchers seeking to undertake effective carriage studies, the conventional sampling, serotyping/serogrouping, and antibiotic susceptibility methods that we are employing for our carriage studies have also been included in the notes section.

2. Materials

2.1. Sample Collection, Processing, and Archiving

1. For details and preparation of the transport and archiving medium, STGG (see Note 1).
2. A selective medium was used to culture *S. pneumoniae* for subsequent identification (see Note 2).
3. Standard reagents were used for the Optichin sensitivity test (see Note 3).
4. Standard reagents were used for bile solubility test (see Note 4).
5. Standard reagents were used for the preparation of blood agar plates made selective for *S. pneumoniae* (see Note 5).
6. Standard reagents were used to prepare blood culture bottles (see Note 6).
7. BHI from oxoid was used to prepare BHI broth (see Note 7).
8. All reagents were quality controlled before use (see Note 8).

2.2. Determination of Antibiotic Sensitivity

Standard materials are used for antibiotic sensitivity testing by disc diffusion and the determination of the Minimum Inhibitory Concentration by E-test (see Notes 9–11).

2.3. Serogrouping and Serotyping

The "checker board"-pooled serum reagents from Statens Serum Institute (Denmark) are used (see Note 12).

2.4. Preparation of Genomic DNA

All reagents are obtained from Sigma unless otherwise stated.

1. Wizard Genomic DNA purification kit that included the lysozyme solution and the protein precipitation solution (Promega).
2. 0.05 M EDTA.
3. RNase (1 μg/mL).
4. Isopropanol.
5. 70% (v/v) ethanol.

2.5. PCR Amplification of Capsulation Locus

All reagents are obtained from Sigma unless otherwise stated.

1. The two PCR primers are HPLC-purified: AliA2: 5′-ATG CAG CTA AAG TAG TCG CC-3′ (forward), DexB2: 5′-GAC CGT CGC TTC CTA GTT GT-3′ (reverse).
2. REDAccuTaq LA DNA polymerase (Sigma).
3. Thermal cyclers used were either the Progene (Techne, UK) or GeneAmp 9700 (Applied Biosystems, Warrington, UK).
4. 0.2-mL thin-walled tubes (Alpha Laboratories, Hampshire, UK or Applied).

2.6. Restriction Fragment Length Polymorphism

1. Bovine Serum Albumin (1 mg/mL BSA, Promega).
2. 3% (w/v) agarose (Bioline, London, UK).
3. Tris-borate electrophoresis buffer.
4. DNA fragment standard hyperladder I (Bioline).
5. Digital camera DC 120 and Kodak Digital Science 2.0 imaging software.

2.7. Analysis of Data

This is performed using BioNumerics software 3.5 (Applied Maths, Kortrjk, Belgium).

3. Methods

3.1. Sample Collection, Processing, and Archiving

For routine studies where characterisation of a single isolate is sufficient, a nose (pernasal) sample should be taken as this is more efficient for capturing colonising *S. pneumoniae* than throat samples. For studies on the dynamics diversity of pneumococcal colonisation both nose and throat swabs should be taken and ten isolates are characterised from each site (see Note 1).

3.2. Determination of Antibiotic Sensitivity

Antibiotic sensitivity testing by disc diffusion and the determination of the Minimum Inhibitory Concentration by E-test were performed using standard methods (see Notes 9 and 11).

3.3. Serogrouping and Serotyping

This is performed using slide agglutination (see Note 12).

3.4. Preparation of Genomic DNA

1. Harvest bacterial cells from a fresh overnight culture on blood agar and extract the DNA using the Wizard Genomic DNA purification kit according to the manufacturer's instructions.
2. Make up a dense suspension of cells in 480 μl EDTA, cell lysis solution (120 μl), and 60 μl lysozyme from the Promega DNA extraction kit and mix by gentle inversion. Incubate at 37°C for 30–60 min.
3. Centrifuge the cell suspension at 13,000 x*g* for 2 min, and discard the supernatant.
4. Resuspend the cells in 600 μl nuclei lysis solution from the Promega DNA extraction kit), by gentle pipetting and incubate at 80°C for 5 min and cool to room temperature.
5. Add 3 μl of RNase solution to the lysing cells and incubate at 37°C for 15–30 min then cool to room temperature.
6. Add 200 μl of the protein precipitation solution (Promega DNA extraction kit), and vortex the tubes for 20 s and incubate on ice for 5 min.
7. Precipitate the cell debris by centrifugation at 13,000 x*g* for 3 min.
8. Transfer the supernatant to a fresh tube containing 0.6 mL isopropanol at room temperature and mix by gentle inversion until a precipitate is seen. Centrifuge the tubes for 10 min and wash the pellet in 70% ethanol (v/v) and dry for 15 min at 37°C.
9. The pellet is then redissolved in 100 μl DNA rehydration solution and incubated at 65°C in a water bath for 60 min, with mixing every 20 min.
10. The genomic DNA should be used immediately after it is prepared as DNA that has been stored at 4°C for more than 24 h can yield multiple PCR amplicons.

3.5. PCR Amplification of the Capsulation Locus

The method of Batt et al., 2005 is used (23).

1. The PCR is performed from the AliA2 and DexB2 primers with the REDAccuTaq LA DNA polymerase using the following reaction mix: 1.5 μl fresh genomic DNA, 2 μl Taq (2 U), 5 μl buffer, 0.5 mM deoxynucleoside triphosphates, and 0.6 mM primers, made up to 50 μl final reaction volume with PCR-grade water in 0.2-mL thin-walled tubes.

2. The PCR cycle used is:
   - 93°C for 2 min 30 cycles:
   - 93°C for 15 s
   - 50°C for 30 s
   - 68°C for 20 min
   - Held at 4°C.
3. Amplicons were kept at 4°C until restriction enzyme digests were performed and should be stable at this temperature for up to 1 month.

3.6. Restriction Endonuclease Digestion

The amplicon is digested using the restriction enzyme, *Hinf*I. Work described by Batt et al., 2005 shows that digestion with this enzyme yields a RFLP pattern unique to each serotype (23). This unique pattern can then be compared to patterns from a set of reference strains using software that can calculate the DNA fragment sizes from a digital image and compare them to other RFLP patterns.

1. Digest a 20 μl aliquot from the PCR with 1 μl of *Hinf*1 restriction enzyme (10 U), 5 μl digestion buffer, and 0.2 μl BSA for 4 h at 37°C according to the manufacturer's instructions.
2. Size-fractionate the restriction digests by horizontal gel electrophoresis at 100 V. Continue the electrophoresis until the loading dye reaches the bottom of the gel.
3. Obtain a digital image of the gel using a stand-mounted digital camera and imaging software. Store the image as a Tiff file.

3.7. Analysis of Restriction Fragment Length Polymorphisms

1. Open the Tiff images in the BioNumerics software as an RFLP experiment, which allows input of the RFLP patterns by a single investigator and save into a single database.
2. Normalise the images by assigning band sizes to the bands of the DNA fragment reference standards.
3. Select all the bands >400 bp that are visible on the gel image. Only select double bands when two distinct peaks are visible on the densitometric curves observed in the analysis window of BioNumerics. Apply these criteria to every gel.

3.8. Comparison of In Vitro RFLP Data with In Silico RFLP Data

The generation of a complete set of reference PCR-RFLP capsular serotype patterns from laboratory experiments is limited to obtaining accurately phenotyped reference strains. Thus, alternative strategies were sought. Studies have shown that in silico data have been used to create databases consisting of virtual PCR-RFLP fingerprints (24, 25) and these in silico fingerprint patterns have been used to validate experimental in vitro data (23, 26).

1. Download the nucleotide sequences of the capsulation locus encoding the different pneumococcal serotypes from the worldwide web.
2. In silico restriction enzyme digests were performed by entering these nucleotide sequences and the restriction enzyme, *Hinf*I, into the Bionumerics programme. This programme identifies the position(s) of the *HinfI* restriction site within the nucleotide sequence of the capsulation locus. The analysis package can be used to generate RFLP patterns with any restriction enzyme of choice.
3. The programme then generates a list of numeric values representing the size in base pairs of each restriction fragment.
4. Using a bespoke computer script written by Dr. Steve Platt, (Health Protection Agency, Colindale, UK) the numerical data is converted into RFLP patterns This script can be obtained by contacting Dr. Platt directly.
5. A comparison was made of the in silico fingerprint patterns and matched with RFLP patterns produced in vitro either to validate the data (Fig. 1) or to identify an unknown capsular serotype (Fig. 2).

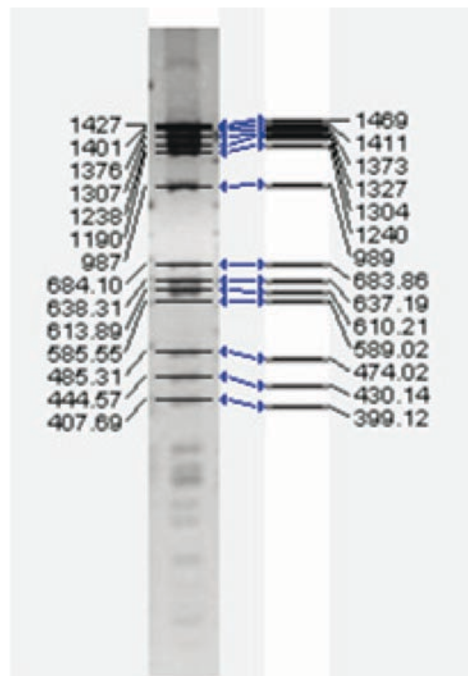

Fig. 1. Comparison of in silico and in vitro RFLP patterns generated by BioNumerics software showing 100% similarity between RFLP fingerprint pattern for serotype 10A.

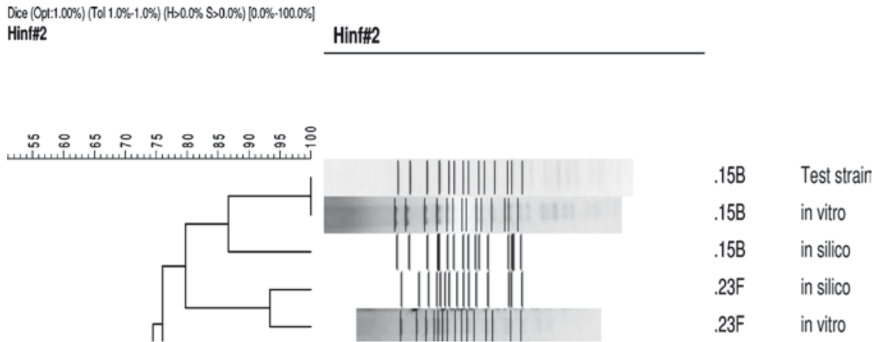

Fig. 2. Test strain serotype 15B clustered with in vitro and in silico strains of serotype 15B in the database.

3.9. Generation of In Silico RFLP Profiles

The generation of an in silico RFLP profile that represents the predicted restriction enzyme digestion of the capsular locus is performed by a new computer programme that runs within the Bionumerics programme. This further development undertaken in collaboration with Dr. Steve Platt (Health protection Agency, Colindale, UK) is to be described in Charalambous et al. (manuscript in preparation). A brief description is given below.

1. Set up a separate experiment in the Bionumerics programme for each of the RFLP profiles together with the migration distances of known molecular mass markers that are associated with each experiment. This effectively creates calibration curves of distance (X-axis) against molecular mass (Y-axis).
2. Each RFLP profile generated as a list of raw digest data is subjected to the following manipulations.
3. The first value in the list of data represents the sample identifier and is removed from the list of digest data and is used to create a new database record if one did not already exist. A new, blank, in silico "gel" record is also associated with the newly created record.
4. Each of the remaining size values in the fragment list are recovered and compared to the calibration curve on the appropriate restriction enzyme to provide a migration distance. A graphical marker is placed on the in silico profile at the position indicated by the migration distance calculated from the calibration curve.
5. Repeat the last two steps until all sets of fragment data are rendered and associated with database entries. The outcome is a series of artificially generated images that illustrate the predicted electrophoresis migration pattern of the fragments generated by the in silico digestion.

3.10. Cluster Analysis of In Vitro and In Silico Data

Comparison of in vitro and in silico data is achieved via a clustering technique that begins with the assessment of the similarity of all possible pairs of samples, i.e. given samples A, B and C, pairs A-B, A-C, and B-C would be assessed. To analyse experimental and/or simulated electrophoresis profiles on the BioNumerics© platform:

1. Select all samples that are to be analysed and create a comparison.
2. Select the appropriate fingerprint experiment for the samples to be analysed. Recommended analysis parameters are: clustering with the Dice algorithm (27), using a position tolerance of approximately 1–2%, and dendrogram generation via the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method (28). Profiles that form groups in the resultant tree are seen to be related. Unknown profiles can be identified by finding the closest known profile in the same group of the tree (Fig. 2). If this software is not available the similarity between two electrophoresis profiles, of the same length, can be calculated manually using the following procedure:
3. Set a constant threshold for the maximum difference between apparently equal bands. If two electrophoresis patterns are compared visually it is easy to see where band migration is almost identical; however, the definition of the point at which two unequal bands continue to be considered to match requires a precise definition. Therefore, it is necessary to specify the limit at which two unequal bands are considered to match. This latitude is termed “position tolerance”; however, care must be taken when specifying this limit as it can have a huge impact on the outcome of the analysis, as illustrated by Fig. 3.
4. Manually align the images of two profiles.
5. Count the bands that match in both profiles (ΣAB), the bands present only in the first profile (ΣAb) and the bands present only in the second profile (ΣaB).

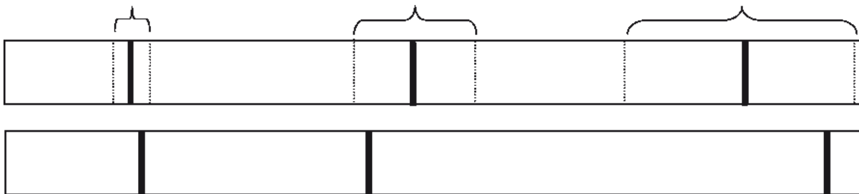

Fig. 3. Two electrophoresis lanes laid horizontally with increasingly large position tolerances indicated by brackets; in all three cases the pairs of bands are assessed as “matching”.

6. Enter the band count values into the Dice coefficient, Eq. 1, to calculate the similarity between the two profiles.

$$\text{Similarity}_D = \frac{2\Sigma AB}{2\Sigma AB + \Sigma Ab + \Sigma aB} \quad (1)$$

The outcome is a value within the range 0–1 where higher values indicate greater similarity between two profiles.

7. To calculate a dendrogram of relationships for more than two profiles repeat the three previous steps for each pair of profiles and apply the UPGMA method to the resultant matrix.

4. Notes

1. Calcium-arginate swabs on a flexible aluminium shaft for nose swabs and cotton tipped swabs for throat swabs should be used (Medical Wire & Equipment Co., Ltd., Corsham, UK). Pernasal samples are taken by inserting the swab in through the individual's nose and sweeping the roof of the nasal cavity. If pernasal samples cannot be taken throat samples can be taken by inserting a swab through the individual's mouth until it touches the back of the throat behind the tongue and the gagging reflex is stimulated. The shaft of the swabs is cut approximately 2.5 cm above the swab by a sterilised scissors and put into a bijoux container with transport media, i.e. skim milk, tryptone soya broth, glucose, and glycerol (STGG). All the STGG components were purchased from Oxoid (Hampshire, UK). Swabs can be transported to the laboratory on ice, or at ambient temperature if this will only take a few hours. Longer term storage until processing is at −70°C (29). Isolates are archived by harvesting a spread plate (Columbia blood agar) into 1.0 mL STGG and stored at −70°C. Several aliquots should be archived. STGG is a mixture of skimmed milk, glucose, tryptone soya broth, glycerol, and water (29).

2. The primary swab samples (10 μl) archived in STGG are plated onto Columbia blood agar plates with 5 μg/mL gentamicin (BAG). Pneumococci are identified from morphology (greyish colonies with characteristic draught-man shape) and an α-haemolytic zone surrounding the colony. *S. pneumoniae* is naturally resistant to gentamicin and α-haemolytic with a greenish zone of clearing in contrast to a colourless zone of clearing of β-haemolytic bacteria. From the primary plate a single colony or up to ten colonies suggestive of pneumococci from each plate are incubated separately on blood agar plates aerobically at 37°C with an Optochin disc for 24 h.

Colonies are confirmed as pneumococci by the presence of a zone of inhibition around an Optochin disc and soluble in a bile (sodium deoxycholate) solution. Pneumococcal colonies are stored at −70°C in 1.0 mL STGG medium in Nunc cryotubes™.

3. The Optochin (ethylhydrocuprein) Sensitivity Test differentiates between *S. pneumoniae* and Viridans Streptococci. The test uses absorbent paper discs impregnated with ethylhydrocuprein (commercially available) which are placed on to inoculated culture plates. After incubation, the zone of inhibition is noted. Pneumococci are sensitive (a zone of clearing); viridans streptococci are resistant (no zone of clearing).
4. The bile Solubility test differentiates between *S. pneumoniae* and Viridans Streptococci. *S. pneumoniae* differ from other streptococci in having an autolytic enzyme. Bile and bile salts activate the autolytic enzyme and thus speed up cell lysis. Two methods can be employed by either the plate or the tube method.
5. Columbia agar (Oxoid) is made up according to the manufacturer's instructions and then autoclaved. Once cooled to 50–60°C (cool enough to hold with your hand for a count of 10) fresh defibrinated sheep's blood is added to 5% v/v, and inverted gently to mix. Media plates can be stored for several weeks in a plastic bag at 4°C. Blood plates can be made selective for pneumococci by supplementing with 5 μg/mL gentamicin sulphate.
6. Brain Heart Infusion Broth (aerobic broth) and Thioglycollate broth (anaerobic broth), or Robertson's cooked meat broth are prepared according to the instructor's manual. The blood bottles are filled with approximately 100 mL of the appropriate broth (aerobic or anaerobic) and fit with screw caps. The top of the cap should be perforated with a hole of 5 mm diameter and the inside of the cap should be covered with a thick rubber diaphragm. The cap should be covered with a square piece of aluminium foil or grease proof paper prior to sterilisation. Sterilise by routine autoclaving and label each bottle appropriately.
7. Prepare the Brain Heart Infusion Broth according to manufacturer's protocol and store at 4–8°C.
8. Each batch of solid or liquid media should be quality controlled with appropriate negative and positive controls.
9. The Kirby–Bauer method is used for antibiotic sensitivity testing. This uses absorbent paper discs impregnated with an antibiotic which are placed on to inoculated culture plates. The precise protocols should be obtained from CLIS.

10. *Muller Hinton media with 5% sheep blood* is used for the purpose of antibiotic susceptibility testing of *S. pneumoniae*. The Muller Hinton is made according to the manufacturer's (Oxoid) protocol (Oxoid) and autoclaved. Cool to 50–60°C (cool enough to hold with your hand for a count of 10) and add 50 mL of fresh defibrinated sheep blood to 5% v/v and mix gently by inversion. Pour 20 mL of the media if the plates are for disc diffusion testing and pour media to a 4 mm depth for E-tests into each plate and allow to solidify. Label and store in a plastic bag at 4–8°C.
11. Determination of the Minimum Inhibitory Concentration (MIC) of Antibiotics using Є-test® strips is done according to standard CLIS guidelines. Isolates are tested for susceptibility to oxacillin, sulphamethoxazole/trimethoprim (cotrimoxazole), tetracycline, chloramphenicol, erythromycin, and amoxicillin using Kirby–Bauer disc diffusion method (30). Colonies resistant to oxacillin are classified as penicillin resistant. The minimum inhibitory concentration (MIC) is determined for resistant isolates by Є-test® (AB Biodisk, Sölna, Sweden). A plastic strip is used for each antibiotic that is impregnated with 15 twofold dilutions of the antibiotic at a defined gradient on the surface opposite to the concentration (μg/mL) scale.

    The Є-test® strips are provided with a technical guide 2B (AB Biodisk, Sweden) that should be followed closely. For pneumococcal sensitivity testing use Muller Hinton agar with 5% sheep blood (see Note 10).
12. When the bacterial cells and the antisera (Statens Serum Insitute, Denmark) are mixed at a ratio of 1:5 agglutination will occur with the corresponding antisera that represents a positive result. The test is performed on a clean plain glass slide and a light microscope is used to visualise the agglutination. Briefly, four drops of cell suspension are placed onto a glass slide using a pipette and 1–2 μl of antisera A, B, D, and H are individually added to the cell suspension and mixed If agglutination is seen with any of the antisera, cross reactions with pooled antisera P, Q, R, S, and T are performed according to Table 1. If no agglutination is seen with A, B, D, or H, repeat the procedure using pooled sera C, E, F, and G and subsequently perform cross reactions on any of the antisera that agglutinates, e.g. if agglutination is seen with antisera A, testing is proceeded using P, Q, R, S, and T or if agglutination is seen with C then testing on the same isolate is done with P and T.

Table 1
Slide agglutination serotyping checkerboard

	A	B	C	D	E	F	G	H
P	1	19	7					14
Q	18	6						23
R	4	3		9	12			
S	5	8			10	17		15
T	2		20	11	33	22		

Agglutination with more than one antiserum or none at all is recorded as non-typable (NT). Agglutination with G means that it maybe any of the following serotypes: 29, 34, 35, 42 or 47

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Part II

Genomics and Gene Expression

Chapter 3

Methods to Determine Antibiotic Resistance Gene Silencing

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Abstract

The occurrence of antibiotic-resistant bacteria is an increasingly serious problem world-wide. In addition, to phenotypically resistant bacteria, a threat may also be posed by isolates with silent, but intact, antibiotic resistance genes. Such isolates, which have recently been described, possess wild-type genes that are not expressed, but may convert to resistance by activating expression of the silent genes. They may therefore compromise the efficacy of antimicrobial treatment, particularly if their presence has not been diagnosed.

This chapter describes the detection of silent resistance genes by PCR and DNA sequencing. A method to detect five potentially silent acquired resistance genes; *aadA*, $bla_{\text{OXA-2}}$, *strAB*, *sul1*, and *tet*(A) is described. First, the susceptibility of the isolates to the relevant antibiotics is determined by an appropriate susceptibility testing method, such as E-test. Then the presence of the genes is investigated by PCR followed by agarose gel electrophoresis of the amplification products. If a resistance gene is detected in a susceptible isolate, the entire open-reading frame and promoter sequence of the gene is amplified by PCR and their DNA sequences obtained. The DNA sequences are then compared to those of known resistant isolates, to detect mutations that may account for susceptibility. If no mutations are detected the expression of the gene is investigated by RT-PCR following RNA extraction. The methods described here can be applied to all acquired resistance genes for which sequence and normal expression data are available.

Key words: Antibiotic resistance, Silent genes, Gene expression, *Escherichia coli*, PCR, DNA sequencing, RT-PCR

1. Introduction

The occurrence of antibiotic-resistant bacteria has become a serious clinical problem. One possible strategy to combat antibiotic resistance is to reduce antibiotic prescribing temporarily, in the hope that resistance prevalence will decrease as resistant bacteria are out-competed and displaced by susceptible organisms (1, 2). This outcome is dependent on the assumption that antibiotic resistance imposes a fitness cost in the absence of antibiotic selection; either

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due to interference of antibiotic resistance with the cell's normal metabolism or due to the requirement for extra resources (1, 2). Antibiotic resistance has often been demonstrated to confer an initial fitness cost on bacteria; however, such a cost can often be reduced or eliminated through the acquisition of compensatory mutations (3, 4). Another way in which bacteria can reduce the fitness cost of resistance is by silencing resistance genes when they are not required. Most studies concerned with bacterial antibiotic resistance screen for the presence of antibiotic resistance genes in resistant isolates only, and many clinical laboratories diagnose antibiotic resistance based on phenotype alone. The possibility that silent or unexpressed resistance genes are present is rarely investigated. Most examples of silent genes reported in the literature fail to confer resistance because they lack the necessary promoter sequences for their expression, e.g. the *mecA* gene of *Staphylococus sciuri* (5) and the *cfiA* gene of *Bacteroides fragilis* (6). However, a recent study demonstrated that plasmid-borne resistance genes were silenced following passage of an *Escherichia coli* strain through the pig gut. These genes were not expressed despite retaining intact, wild-type promoter, and resistance gene sequences (7). Silent resistance genes have also been detected among naturally occurring *E. coli* isolates from farm animals (8).

In this chapter, a method for detecting, by PCR, the five most commonly found silent resistance genes in *E. coli* – *aadA, bla*$_{\text{OXA-2}}$, *strAB, sul1,* and *tet*(A), which normally confer resistance to streptomycin/spectinomycin, beta-lactams, streptomycin, sulphonamides, and tetracycline, respectively, is described. Possible reasons for the lack of expression of any silent genes detected by PCR using DNA sequence analysis and RT-PCR; to establish the possible role or mutations or lack of transcription, is described. A third explanation for the presence of silent genes, whereby a gene is transcribed but the gene product is not produced or is inactive also exists. Transcription of resistance genes in susceptible isolates has been reported for *aadA* (8). However, this chapter will not describe methodologies to investigate such isolates further. Although the detection of five silent acquired resistance genes is described here, in principle the same methodologies can be applied to detect almost any silent acquired resistance genes in any bacterial species that can be cultured.

2. Materials

2.1. Susceptibility Testing

1. Fresh cultures of *E. coli* or other bacterial isolates to be tested, grown on antibiotic-free agar, such as Luria Bertani (LB) agar.

2. 0.5 MacFarland Standard (Add 0.5 mL of 0.048 M $BaCl_2$ to 99.5 mL of 0.18 M H_2SO_4 and store in the dark at room temperature).
3. Suspension medium, 0.85% NaCl in 5 or 10 mL volumes.
4. Mueller Hinton agar plates.
5. Sterile cotton swabs.
6. E-test strips. For the examples described here use ampicillin (0.016–256 μg/mL), streptomycin (0.064–1,024 μg/mL), sulphamethoxazole (0.064–1,024 μg/mL, and tetracycline (0.016–256 μg/mL) (Biomerieux, Basingstoke, UK).
7. Quality control strain *E. coli* ATCC 25922 (ampicillin minimum inhibitory concentration (MIC) 2–8 μg/mL; streptomycin MIC 2–8 μg/mL; sulphamethoxazole MIC 32–128 μg/mL; tetracycline MIC 0.5–2 μg/mL).

2.2. PCR Amplification

1. Fresh cultures of test and control strains (e.g. *E. coli* NCTC 5011 which contains plasmid TP120 (also known as R46) that encodes *aadA*, $bla_{OXA\text{-}2}$, *sul1*, and *tet*(A) and *E. coli* NCTC 50020, which contains plasmid R300B that encodes *strAB*).
2. 1 μL plastic loops.
3. Molecular biology grade water (e.g. Eppendorf, Cambridge, UK).
4. 1.5 mL microcentrifuge tubes.
5. Dry heat block.
6. Extensor Hi-Fidelity master mix (Thermo Scientific, Epsom, UK) (see Note 1).
7. Oligonucleotide primers (Tables 1 and 2). Each primer should be diluted in sterile water to a concentration of 160 μM (master stock) and a 1/10 dilution of this stock made (16 μM) and used as the working stock.
8. Thin-walled 0.2 mL tubes.
9. Thermal cycler.

2.3. Agarose Gel Electrophoresis

1. Loading dye (0.25% w/v bromophenol blue, 25% w/v Ficoll 400 in water).
2. Agarose (e.g. Helena Biosciences, Gateshead, UK).
3. Ethidium bromide stock solution, 10 mg/mL in water. Shield from light (see Note 2).
4. 1× Tris Borate EDTA (TBE) buffer.
5. Electrophoresis unit consisting of tank, gel tray, and comb.
6. Constant voltage power supply.

Table 1
Oligonucleotide primers for initial gene detection (8–11) (see Notes 4, 5)

Gene	Primer name	Sequence (5′ to 3′)	Position in gene	PCR product size (bp)	Annealing temperature (°C)
aadA	AADAF	CATTTGTACGGCTCCGCAGT	93–112	259	55
aadA	AADAR	AGAATGTCATTGCGCTGCCA	352–333		55
bla_{OXA-2}	OXA2F	TTCAAGCCAAAGGCACGATAG	113–133	702	58
bla_{OXA-2}	OXA2R	TCCGAGTTGACTGCCGGGTTG	815–795		58
strA	STRAF	CAACTGGCAGGAGGAACA	207–225	1131	55
strB	STRBR	GGCATTGCTCATCATTTG	472–454		55
sul1	SUL1F	CCGATATTGCTGAGGCGGACT	337–357	266	58
sul1	SUL1R	CCAACGCCGACTTCAGCTT	603–585		58
tet(A)	TETAF	GTAATTCTGAGCACTGTCGC	24–43	937	55
tet(A)	TETAR	CTGCCTGGACAACATTGCTT	980–961		55

7. UV transilluminator or gel imaging system with UV light (see Note 3).
8. Molecular weight marker (e.g. Hyperladder I, Bioline, London, UK).

2.4. DNA Purification and Sequencing

1. QIAquick gel extraction kit (Qiagen, Crawley, UK).
2. 1.5 mL microcentrifuge tubes.
3. Microcentrifuge.
4. Molecular biology grade water.
5. Spectrophotometer capable of reading at 260 nm.
6. Quarz cuvette.
7. Oligonucleotide primers used for PCR amplification.
8. Computer with internet access for sequence analysis.
9. Sequence analysis software (e.g. Lasergene, DNASTAR, Madison, USA, or Chromas, http://www.technelysium.com.au/chromas.html).

2.5. RNA Extraction and RT-PCR

1. Nutrient broth (Oxoid, Basingstoke, UK) 10 mL volumes.
2. RNeasy Protect RNA extraction kit (Qiagen).
3. RNAse-free DNAse (e.g. Promega, Southampton, UK).
4. Dry heat block or water bath.

Table 2
Oligonucleotide primers for amplification of entire open-reading frames of genes and their promoters (see Notes 7, 8) (7, 8)

Gene	Primer name	Sequence (5′ to 3′)	Position in gene	PCR product size (bp)	Annealing temperature (°C)
aadA1	AADA1F	AACATCATGAGGGAAGCGGT	–7 to 13	858	50
aadA1	AADA1R	ACTAACGCTTGAGTTAAGCC	851–832		50
aadA2	AADA2F	CATGAGGGTAGCGGTGACCA	–13 to 7	825	56
aadA2	AADA2R	GTAGCGCGGTCGGCTTGAAC	812–793		56
Class 1 integron promoter	INT1F	GCCGCCAATGCCTGACGATG	253–234 (*intI1*)	373	58
Class 1 integron promoter	INT1R	CTGCCCTGCTGCGTAACATC	20–39 (*attI*)		58
Class 2 integron promoter	INT2F	GTGAAACAGAATAAAACGCTTA	113–92 (*intI2*)	387	48
Class 2 integron promoter	INT2R	CTGATGCTTACCGTTAATTAAT	–34 to –55 (*attI*)		48
bla_{OXA-2}	OXA2WHOLEF	TTGGGCATTAAGGAAAAGTT	–21 to –2	896	48
bla_{OXA-2}	OXA2WHOLER	GTTGAAGTAACCGGCGCTGC	875–859		48
strAB promoter	STRPROF	GCACATTCGGGATATTTCTC	–58 to –39 (*strA*)	281	50
strAB promoter	STRPROR	GTTCCTCCTGCCAGTTGATG	223–204 (*strA*)		50
strA	STRAWHOLEF	TTGAATCGAACTAATATTTTTTT	1–23	827	54
strA	STRAWHOLER	CAGGAAAAACAGGCGGCATG	808–827		54
strB	STRBWHOLEF	ACGCCTTGCCTTCTATCTGC	–45 to –26	909	53
strB	STRBWHOLER	CCAGGGGATAGGAGAAGTCG	864–845		53
sul1	SUL1WHOLEF	CTTTGTAGGTATGGGGCTCA	–81 to –62	997	55

(continued)

Table 2 (continued)

Gene	Primer name	Sequence (5′ to 3′)	Position in gene	PCR product size (bp)	Annealing temperature (°C)
sul1	SUL1WHOLER	TGACGAGCCCAGCATGTCTG	916–897		55
sul1 promoter	SUL1PROF	CGCTGGGTTTGCCGTTTCTC	–694 to –675	761	60
sul1 promoter	SUL1PROR	TCTAGCCGCCGGCTCTCATC	67–48		60
tet(A) and promoter	TETAWHOLE1F	AGTAGAGCGCTGGCTGTTGC	–218 to –199	650	57
tet(A) and promoter	TETAWHOLE1R	CATACCCACGCCGAAACAAG	432–413		57
tet(A)	TETAWHOLE2F	GTTGCTGGCGCCTATATCGC	343–362	833	60
tet(A)	TETAWHOLE2R	TCAGGTCGAGGTGGCCCGAC	1176–1157		60
tetR(A)	TETRF	TTCAGCTAGGTGACTTTGCT	–64 to –45	810	53
tetR(A)	TETRR	GTGCCTGACTGCGTTAGCAA	746–727		53

5. Spectrophotometer capable of reading at 260 nm.
6. Quarz cuvette.
7. Thin-walled 0.2 mL tubes.
8. One-step RT-PCR kit (Qiagen).
9. Oligonucleotide primers (Table 1). The oligonucleotide primers described in Table 1 can also be used for RT-PCR. The exceptions are the *strA* and *strB* genes. In addition, each reaction should be performed as a multiplex reaction combined with that for the *rpsL* house-keeping gene (see Note 6).

3. Methods

3.1. Susceptibility Testing of Bacterial Isolates Using E-Test Strips (See Note 9)

1. Culture bacterial isolate(s) to be tested overnight on appropriate antibiotic-free agar.
2. Emulsify some fresh bacterial growth from the plate into the suspension medium. Adjust the turbidity to a 0.5 McFarland standard.
3. Spread the suspension evenly onto dried Mueller-Hinton agar using a sterile swab.
4. Apply an E-test strip of the test antibiotic on to the centre of the plate using sterile forceps, with the numbers on the strip facing upwards.
5. Incubate plates for 18–20 h at 37°C (or as appropriate if a bacterial species other than *E. coli* is being investigated).
6. Read the MIC value as determined by the zone of inhibition in the vicinity of the E-test strip. The MIC is defined as the lowest concentration of antibiotic at which there is no bacterial growth.
7. Determine whether the bacterial isolate is susceptible or resistant to the antibiotic in question according to established interpretative criteria recommended by organisations such as the CLSI or BSAC (12, 13). Only isolates defined as susceptible to a given antimicrobial should be investigated for the presence of silent antibiotic resistance genes. For *E. coli* and the example antimicrobials described here the most appropriate interpretative criteria for susceptibility are: ampicillin MIC <16 μg/mL, streptomycin MIC <16 μg/mL, sulphamethoxazole MIC <64 μg/mL, and tetracycline MIC <16 μg/mL.

3.2. PCR Amplification of Potentially Silent Resistance Genes and Their Promoter Regions

1. Make the PCR template by suspending one bacterial colony into 100 µL of molecular biology grade water using a 1-µL plastic loop. Make one suspension for each bacterial strain to be tested plus a suspension of the control strain (which carries the resistance gene of interest).
2. Heat the suspension in a heat block at 95°C for 5 min (see Note 10).
3. For each reaction to be carried out pipette 12.5 µL of Extensor Hi-fidelity master mix, 10.5 µL of molecular biology grade water and 0.5 µL of each of the forward and reverse primers into a 0.2 mL thin-walled PCR tube. For larger numbers of reactions a master mix can be prepared and aliquotted into the tubes.
4. Add 1 µL of heated bacterial suspension to the 24 µL PCR reaction. Mix by pipetting up and down. Additionally, for each batch of reactions prepare one reaction containing the positive control template and one reaction containing no template to act as the negative control (see Note 11).
5. Place the reaction tubes in the thermal cycler and run the following programme: 95°C for 5 min, 35 cycles of 95°C for 1 min, annealing temperature for 1 min (see Tables 1 and 2 for appropriate temperature) and 68°C for 1 min and a final extension at 68°C for 10 min (see Notes 12, 13).
6. When the run is complete remove the samples from the thermal cycler. Samples can be analysed immediately by agarose gel electrophoresis or stored until needed. For short-term storage up to 48 h samples can be placed at 4°C, for longer-term storage place at –20°C. If you are unable to remove your samples from the thermal cycler promptly (such as in the case of overnight runs) the instrument should be set to hold the samples at 4°C after the run has been completed.

3.3. Agarose Gel Electrophoresis of PCR Products

1. Prepare a 1% mixture of agarose and 1×TBE buffer of an appropriate volume for the gel tray to be used. For example, for a 100-mL tray weigh out 1 g of agarose into a 250 mL Erlenmeyer flask and add 100 mL of 1×TBE. Dissolve the agarose by heating the mixture in a microwave oven for approximately 2 min at high power. Allow the solution to cool to approximately 50–60°C.
2. Add 10 µL of ethidium bromide solution and mix by swirling gently (see Note 2).
3. Seal the edges of a gel tray with tape and place an appropriate comb in the tray. Gently pour in the warm agarose/ethidium bromide mixture. Remove any trapped air bubbles using the comb. Leave the gel to cool until it is completely set. Carefully

remove the tape and comb and place the gel in an appropriate electrophoresis unit. Completely immerse the gel with 1×TBE buffer.

4. Mix 10 μL of each PCR product with 2 μL of loading dye. Carefully pipette each mixture into a separate well of the submerged gel, taking note of the loading order. Be sure to load molecular weight marker into at least one well per gel row, using the volume recommended by the manufacturer, for example for Hyperladder I this is 5 μL.
5. Close the lid of the electrophoresis unit and connect the power supply, negative at the top. Depending on the size of the gel apply approximately 80–120 V.
6. When the loading dye has run for at least 15 cm (approximately ½ to ¾ of the gel length) and before it has reached the bottom of the gel turn off the power supply. This will take approximately 1 h. Visualise the gel with a UV transilluminator and take a photograph.
7. Interpret the results according to the expected sizes given in Tables 1 or 2. The presence of a band of the expected size, equal to the size of the band obtained for the positive control, indicates a positive result. When no bands are seen or bands are of a different size to those expected the result is negative.

3.4. Purification and DNA Sequencing of PCR Products

1. If only a single band of the correct size is visible on the gel proceed with step 2. If more than one band is apparent or smearing is visible, the band should be cut from the gel for processing. Using a clean, sharp scalpel cut the band from the gel as precisely as possible and place the gel slice in a 1.5-mL microcentrifuge tube.
2. If the PCR reaction has generated a single discrete product, purify the remainder of the PCR product (15 μL) according to the manufacturer's instructions using the "PCR purification using a microcentrifuge" protocol from the Qiaquick gel extraction kit. If purifying a DNA product excised from a gel, purify it according to the manufacturer's instructions using the "gel extraction using a microcentrifuge" protocol from the kit. Perform all optional steps. Elute the product in 35 μL of molecular biology grade water. Store purified PCR products at –20°C until DNA sequencing.
3. Pipette 995 μL of sterile water into a 1 mL quartz cuvette and use it to calibrate a spectrophotometer at 260 nm.
4. Add 5 μL of purified PCR product and mix gently with a stirring rod. Measure the absorbance at 260 nm.

5. Calculate the concentration of your PCR product. An OD_{260} reading of 1.0 is equivalent to a concentration of 50 μg/mL of DNA. The dilution factor used in measurement is 200, i.e. 1,000/5. Therefore, to calculate the concentration of DNA in you sample use the following formula:

$$\text{DNA concentration } (\mu g/mL) = 50 \times (OD_{260} \text{ value} \times 200)$$

For example, the concentration DNA in a sample with an $OD_{260} = 0.002$ (typical value for a small PCR product) would be $50 \times (0.002 \times 200) = 20\,\mu g/mL$.

6. Carefully prepare the sequencing reactions according to the instructions provided by your sequencing provider (see Note 14), or if preferred, perform in-house sequencing according to your standard method. Prepare two sequencing reactions per PCR product, one with the forward primer and with the reverse primer. Sequence at least two independent PCR products per sample.
7. Once you receive the DNA sequencing data align the sequences from the forward and reverse primers using an appropriate sequence analysis programme, such as the Seqman tool in the Lasergene software package. Add all sequences relating to a particular gene from a given isolate to the same file. Check the sequence for any errors such as unread bases (*N*) and correct manually if possible. If disagreement is found between the different sequencing runs, repeat the reaction and take the base that is in the majority. Export the sequence as a FASTA file.
8. Perform a BLASTN search of the sequence against the nr database (http://www.ncbi.nlm.nih.gov/BLAST/). Check that the sequence corresponds to the intended target of the PCR reaction against the sequences present in the database (sequences of the control strains may also be used as comparators). If the results indicate that the PCR product corresponds to the intended gene target and the isolate in question is susceptible, amplifications covering the entire open-reading frame of the gene and its promoter should then be performed to determine whether the isolate is susceptible due to mutation or silencing. Perform PCR and sequencing for the open-reading frames and promoters using the primers described in Table 2, as described above. When a BLAST alignment for the entire gene and its promoter has been obtained carefully check the newly determined sequence for mutations. If no mutations are identified in structural or promoter regions of the gene, but the isolate is susceptible, the resistance gene in question is silent. If mutations are identified, check whether they occur in the promoter region or the structural regions of the gene.

If mutations occur in the structural region of the gene, determine whether they are silent, i.e. change one codon to another that codes for the same amino acid, or result in amino acid changes in the translated protein. This can be done by looking at the protein translation provided by the Seqman programme or by performing a protein translation using one of the many web-based tools available (e.g. http://us.expasy.org/tools/dna.html). The predicted amino acid sequence should then be compared to that of the wild-type sequence from the database and known to confer resistance. If promoter mutations or mutations that result in amino acid changes in the protein are detected, it is likely that these are responsible for the susceptibility of the isolate. If necessary, this can be checked by obtaining revertants that display wild-type levels of resistance to the antibiotic in question and demonstrating that the mutation has been changed back to the wild-type sequence (see Note 15).

3.5. RNA Extraction and RT-PCR

If no mutations in the promoter or coding sequence of the resistance gene are identified, RT-PCR can be used to determine whether mRNA for the resistance gene is transcribed.

1. Inoculate 10 mL of nutrient broth with 100 µL of an overnight nutrient broth culture, for each bacterial isolate to be analysed. If analysing *tet*(A) gene expression, add tetracycline to the broth at a final concentration of 250 ng/mL. Incubate cultures with shaking at 37°C.
2. Regularly measure the absorbance of the culture with a spectrophotometer at 600 nm until the culture reaches mid-logarithmic phase. Normally this corresponds to approximately OD_{600} 0.4–0.5.
3. Harvest the cells from 1.5 mL of broth culture by spinning it in a microcentrifuge at maximum speed for 2 min. Remove the supernatant (see Note 16).
4. Prepare RNA from the cell pellet using the RNeasy Protect Bacteria Mini Kit according to the manufacturer's instructions. This version of the kit is preferred to the standard RNeasy mini kit as it includes the RNeasy Protect Bacteria reagent which stabilises bacterial RNA.
5. Treat the RNA sample with RNAse-free DNAse according to the manufacturer's instructions to remove any contaminating DNA.
6. Run 5 µL of the digested RNA sample on an agarose gel to ensure that the RNA is intact, as indicated by the two major bands for 16S and 23S rRNA.

7. Measure the concentration of the DNAse-treated RNA using a spectrophotometer at 260 nm. Adjust the concentration of RNA to 100 ng/μL. Store the RNA at −20°C for later use or proceed with RT-PCR.
8. Add reagents from the One-step RT-PCR kit to a 0.2 mL thin-walled tube as follows: 1 μL RT-PCR enzyme mix, 1 μL 10 mM deoxynucleoside triphosphate mix, 5 μL 5× RT-PCR buffer, 0.5 μL of each appropriate primer, 15 μL of RNAse-free water, and 1 μL RNA. Each reaction to detect resistance gene mRNA should be run as a multiplex reaction with primers to detect the *rpsL* house-keeping gene to control for the quality and quantity of RNA present. For each batch of RT-PCR add a negative control containing no RNA and a positive control from an isolate known to express the gene (such as those used as positive controls for the PCRs). It is also essential that a control is carried out for the residual presence of DNA in the sample. This is done by performing a normal PCR reaction for the resistance gene using the RNA as template. The result of this reaction should be negative; if a band is present, the RNA must re-digested with DNA.
9. Place the reaction tubes in a thermal cycler and run the following programme: 1 cycle of 50°C for 30 min, 1 cycle at 95°C for 15 min, 35 cycles of 94°C for 1 min, annealing temperature (see Table 1) for 1 min and 72°C for 1 min, followed by 1 cycle at 72°C for 10 min.
10. After the run is complete remove the samples from the thermal cycler. Analyse samples immediately by agarose gel electrophoresis or store. For short-term storage up to 48 h samples, can be placed at 4°C, for longer-term storage place at −20°C. If you are unable to remove your samples from the thermal cycler promptly (such as in the case of overnight runs) the instrument should be set to hold the samples at 4°C after the run has been completed.
11. Analyse 10 μL of your RT-PCR reactions by electrophoresis as described in Subheading 3.3 (see Fig. 1, for example). Presence of an *rpsL*-specific band and a resistance gene-specific band indicates that resistance gene mRNA is expressed. Presence of the *rpsL* band only indicates that no resistance gene mRNA is produced, while presence of neither band indicates the RT-PCR reaction has failed and should be repeated. If necessary, a fresh RNA preparation should be made. If the *rpsL* bands are of equal intensity in the samples to be compared then the resistance genes bands can be compared and their intensity is approximately indicative of the relative levels at which the gene is transcribed in different isolates. For precise quantification however, quantitative RT-PCR should be performed.

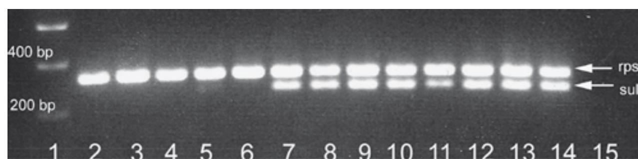

Fig. 1. Example of an agarose gel of multiplex RT-PCR analysis for the *sul1* gene, incorporating RT-PCR for the *rpsL* gene as a control for mRNA quality and quantity. *Lane 1*, Hyperladder I DNA marker; *lanes 2–6*, RT-PCR carried out using RNA from sulfonamide-susceptible *Escherichia coli* isolates with silent, but intact *sul1* genes; *lanes 7–14*, RT-PCR carried out with RNA from sulfonamide-resistant, *sul1*-encoding *E. coli* isolates; *lane 15*, negative control (no template). (Reproduced from ref. 7 with permission from ASM Journals).

4. Notes

1. It is not essential to use the Extensor Hi-fidelity PCR master mix (Thermo Scientific) recommended here. Users may employ a master mix by another manufacturer or use separate PCR reagents (enzyme, buffer, and nucleotides) if preferred. However, please note that if DNA sequencing of the amplification products is intended it is recommended that the polymerase enzyme used has a proof-reading function.
2. Caution, ethidium bromide is a potential carcinogen; therefore, protective gloves should be worn at this and all subsequent steps.
3. Caution, UV light can damage eyes. Protective eyewear should be worn.
4. The primers described here detect five mobile antibiotic resistance genes and their variants; *aadA*, $bla_{\text{OXA-2}}$, *strAB*, *sul1*, and *tet*(A). In principle, any acquired resistance gene can be silenced and PCR can be performed to detect any desired gene. The PCR primers for such genes should be designed such that both primers bind internally within the gene, they recognise molecular variants of the same gene (e.g. different forms of TEM β-lactamases), they do not contain sequences able to form secondary structures such as loops or hairpins and they do not bind non-specific target sequences.
5. The primers described have been designed to detect the named genes as well as their molecular variants. In particular, the AADF and AADR primers detect *aadA1,* 2, 3, 6, 7, 8, 10, 11, 12, 13, and 15, and the OXA2F and OXA2R primers detect $bla_{\text{OXA-2, 3, 15, 21, 32, and 46}}$.
6. To detect the *strA* gene by RT-PCR the STRAF primer should be combined with primer STRARTR 5′ CGCAGATAGAA-GGCAAGG3′ using an annealing temperature of 55°C

resulting in a product size of 572 bp. For *strB* the STRBR primer should be combined with primer STRBRTF 5′ ATGTTCATGCCGCCTGTTTT3′ using an annealing temperature of 55°C generating a product size of 472 bp. The *rpsL* house-keeping gene can be detected using the primers RPSLF, 5′ CTCGCAAAGTTGCGAAAAGC 3′, and RPSLR, 5′ TTCACGCCATACTTGGAACG 3′. The reaction should be multiplexed with that to detect the antimicrobial resistance gene in question, and the annealing temperature used should appropriate for the resistance gene primers. This will produce an *rpsL* product of 321 bp.

7. These primers should only be used in cases where further investigation of potentially silent isolates is required. These will be isolates that are susceptible to the antibiotic concerned and have tested positive for the corresponding resistance gene by PCR (verified by DNA sequencing). Should investigators wish to explore the reasons for these observations, PCR and DNA sequencing can be performed using the described primers to determine whether mutations that lead to susceptibility are present or whether no such mutations occur, suggesting the gene is silent.

8. Depending on whether initial sequencing of an *aadA* PCR product reveals the presence of *aadA1* or *aadA2* the primers AADA1F& R or AADA2F&R should be used, respectively. Primers for amplifying other *aadA* variants are not described here. The primers for $bla_{\text{OXA-2}}$ also amplify $bla_{\text{OXA-15}}$ and $bla_{\text{OXA-32}}$. As *aadA* and bla_{OXA} genes occur as cassettes within integrons, in order to investigate the promoter regions of these genes, the integron promoter must be sequenced. Primers for amplification of the promoter regions of class 1 and class 2 integrons, the two most common types, are given in Table 2, primers for amplifying other integron types are not described. In the case of *tet*(A), the *tetR*(A) regulator of the gene should also be investigated.

9. Other antibiotic susceptibility testing methods, such as agar or broth microdilution can be used instead of E-test strips if these are established in your laboratory. Use of E-test strips is advantageous for laboratories unaccustomed to determining antibiotic susceptibilities as they require no specialised equipment or prior set up and are simple to perform.

10. Once cooled, the heated suspension can be frozen at −20°C and re-used as template for other PCR reactions for up to 3 months.

11. Following agarose gel electrophoresis of PCR products no band should be visible in the negative control reaction. If a band is visible, it indicates that one or more of the PCR components are contaminated and that the results are unreliable

and should be ignored. Accordingly, the reaction should be repeated with fresh reagents. If no product is visible in the positive control lane the procedure may have failed and the analysis of the batch should be repeated.

12. Most modern thermal cyclers have heated lids and do not require the addition of mineral oil to PCR reactions. However, if your instrument does not have a heated lid, reaction mixtures should be overlaid with two drops of mineral oil prior to being placed in the thermal cycler.
13. Note that the polymerase enzyme within the Extensor Hi-Fi master mix works optimally at an extension temperature of 68°C; however, most other polymerases work optimally at 72°C. If you are using a different polymerase consult the manufacturer's instructions to determine the correct extension temperature.
14. In our laboratory, we send DNA away to be sequenced by commercial sequencing service providers. We generally find this to be a fast, reliable and cost-effective way of obtaining DNA sequence and gives considerable savings in terms of the time and resources that would be required to perform sequencing in-house.
15. There are several ways in which revertants may be generated; however, you should be aware that all methods have potential pitfalls. The simplest method is to grow a culture of the putative silent isolate overnight in broth and then culture the growth onto agar supplemented with the appropriate antimicrobial. Any colonies that grow may be potential revertants, which can be checked by re-sequencing the gene and verifying that the mutation thought to be responsible for antimicrobial susceptibility has changed back to wild-type (e.g. reversion of a premature stop-codon to an amino acid). However, not all mutations are readily reversible (particularly if they are not point mutations). Also, mutation at a secondary site in the gene may result in restoration of antimicrobial resistance, as well as mutation in other, unrelated genes. The latter case applies particularly to those antimicrobials that are prone to resistance through chromosomal mechanisms, such as up-regulation of efflux pumps or point mutations in the antimicrobial target. Another method is to modify the putative mutated gene to its wild-type version by site-directed mutagenesis, whereby the original copy of the gene is exchanged for one designed by the investigator in situ. Several different site-directed mutagenesis methods are described in the literature and usually involve introduction of homologous DNA designed by the researcher into a bacterium, followed by replacement of the target gene by one of the researcher's choice by recombination. However, although significantly

more accurate than screening for naturally occurring revertants, such methods are often technically more demanding, particularly when the bacteria being studied are wild-type clinical isolates.

16. In the subsequent steps wear gloves and use filter tips, in order to protect the sample from environmental RNAse enzymes.

Acknowledgments

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Chapter 4

Studying Gene Induction of Glycopeptide Resistance Using Gene Swapping

Hee-Jeon Hong

Abstract

Gene swapping is a simple but effective genetic tool for characterizing the functioning of a gene, where the gene in question is known to fulfil a distinctive biological role in the cell. VanS is a sensor kinase which, in conjunction with its cognate response regulator VanR, triggers resistance to vancomycin. One of the most important questions yet to be answered in the study of vancomycin resistance is the nature of the specific ligand recognized by the VanS sensor. A "VanRS-swap" experiment between two glycopeptide-resistant *Streptomyces* species known to exhibit differing responses to inducer molecules can investigate whether inducer specificity is determined solely by differences between the amino acid sequences of the VanRS two-component systems present, or by inherent differences in cell wall structure and biosynthesis between the strains. Results from such experiments demonstrate that inducer specificity is determined by the origin of the VanRS proteins and provides useful circumstantial evidence that the VanS effector ligand is the drug itself, and not an intermediate in cell wall biosynthesis that may accumulate as a result of drug action.

Key words: Vancomycin resistance, Glycopeptides, *S. coelicolor*, *S. toyocaensis*, VanS/VanR signal transduction system, Gene swap, VanS effector ligand

1. Introduction

The glycopeptide antibiotic vancomycin is used to treat problematic infections caused by MRSA (methicillin-resistant *Staphylococcus aureus*). The spread of vancomycin resistance through bacterial populations poses an acute public health issue, highlighted by the recent emergence of vancomycin-resistant MRSA (VRSA) in hospitals (1–3). One of the most important questions yet to be answered in the study of vancomycin resistance is to address the nature of the specific ligand recognized by the VanS sensor. Two distinct models exist: direct induction, in which VanS is activated

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by direct binding of antibiotic to the sensor domain; and indirect induction, in which the sensor kinase is activated by binding an intermediate in cell wall biosynthesis or degradation that accumulates as a result of antibiotic action. Gene swapping makes it possible to begin to distinguish between these options.

Inducible resistance to vancomycin and other glycopeptide antibiotics was, until recently, thought only to be present in pathogenic or in glycopeptide antibiotic-producing bacterial strains (4–7). A cluster of seven genes (*vanSRJKHAX*) that confer inducible, high-level resistance to vancomycin in *Streptomyces coelicolor* (8), a non-pathogenic, non-glycopeptide-producing soil bacterium have been described. The *S. coelicolor* vancomycin-resistant system possesses a number of interesting novel features (9). On exposure to vancomycin, the *S. coelicolor* VanS protein switches from exhibiting a phosphatase activity to being a kinase, and the resulting accumulation of the phosphorylated form of its partner response regulator VanR activates transcription from the *van* gene promoters which ultimately results in resistance to the antibiotic (Fig. 1). In the absence of drugs, however, VanR is phosphorylated by the small molecule phosphodonor acetyl phosphate, and the phosphatase activity of VanS acts to suppress the level of Phospho-VanR. Thus, VanS negatively regulates VanR function in the absence of antibiotic, and deletion of *vanS* results in the constitutive expression of the *van* resistance genes (10).

In addition to *S. coelicolor*, glycopeptide resistance has been explored in one other streptomycete, *Streptomyces toyocaensis* (11).

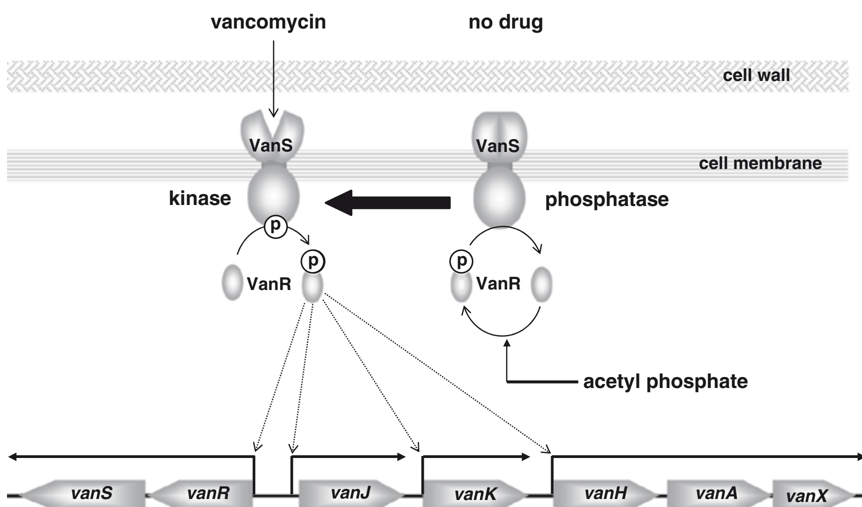

Fig. 1. A model for the function of the vancomycin-resistant VanRS two-component signal transduction system in *S. coelicolor*.

S. toyocaensis produces the "sugarless" glycopeptide A47934 and the resistance genes in this organism are associated with the A47934 biosynthetic cluster. In *S. coelicolor*, expression of the *van* genes is induced by both vancomycin and the glycopeptide A47934, whereas in *S. toyocaensis* resistance is induced only by A47934 but not by vancomycin. This implies that the ligands recognized by the VanS proteins from these two *Streptomyces* species are different, but does not distinguish between the direct versus indirect induction hypotheses. Swapping the VanRS regulatory systems between the species does, however, allow the two hypotheses that the VanS proteins are reacting differently because of the differences in drug structures, or to different cell wall intermediates/metabolites that may accumulate in each strain to be distinguished: the former explanation is favoured if the sensors are induced by the same drugs irrespective of the identity of the host strain. We therefore introduced the *S. toyocaensis vanRS* genes (*vanRSst*) into the *S. coelicolor vanRS* deletion mutant (Δ*vanRSsc*) using the integrative vector pRT801 (12). Significantly, the resulting *S. coelicolor* strain was now resistant to A47934 but sensitive to vancomycin. Consistent with this result, vancomycin did not induce transcription from the *vanH* promoter in this strain, but A47934 did. Thus, switching the VanRS signal transduction system from one species to the other also switched inducer specificity, supporting direct induction as the mechanism by which VanS is controlled. The gene swap analysis presented in detail below is specific for the genes and strains under investigation in this work, but serves as a general illustration that can be readily adapted for undertaking similar studies in other organisms. Gene swapping and related methods (e.g. domain swapping) have found significant use in studies of both eukaryotes and prokaryotes, providing important information on gene (or domain) function (13–18). In a recent eukaryotic study, evidence that isoform 1 of the human gene COQ4 possesses coenzyme Q biosynthetic activity was obtained following expression of the gene in COQ4(null) yeast strains (13). Mutant strains of a number of different bacteria have similarly often been used to demonstrate the activity (or lack of it) of genes derived from other prokaryotic and eukaryotic sources by looking for functional complementation (14–17). In one recent interesting example, a DivIVA-depletion strain of the rod-shaped bacterium *Corynebacterium glutamicum* that exhibits a coccoid morphology, due to a deficiency in polar growth, was used to assess the activity of DivIVA homologues from four different bacterial species (17). The homologues from *Streptomyces* and *Mycobacterium* restored rod-shaped growth and were shown to localize to the cell poles, whereas those from *Bacillus subtilis* and *Streptococcus pneumoniae* localized to the cell septum and failed to alter the coccoid shape of the mutant strain.

2. Materials (see Note 1)

2.1. Bacterial Strains and Culture Media (see Note 2)

1. *Streptomyces* strains: *Streptomyces coelicolor* wild type M600 (*S. coelicolor* M600), *S. coelicolor* Δ*vanRS* null mutant (Δ*vanRSsc*), and *S. coelicolor* Δ*vanRS* null mutant complemented with the *vanRS* genes from *S. toyocaensis* (Δ*vanRSsc* + *vanRSst*).
2. *Escherichia coli* strains: *E. coli* DH5α (competent cells purchased from Invitrogen), *E. coli* ET12567 (ATCC BAA-525) containing the helper plasmid pUZ8002 (19).
3. Mannitol Soya flour (MS) agar medium consisting of 2% (w/v) mannitol, 2% (w/v) soya flour (use soya flour from a health food shop such as "Holland & Barrett") and 2% (w/v) agar. Autoclave the medium twice at 121°C (1.05 bar) and store at room temperature as 100 mL aliquots in 250-mL flasks. Re-melt the medium using a microwave prior to use.
4. NMMP minimal liquid medium consisting of 0.2% (w/v) $(NH_4)_2SO_4$, 0.5% (w/v) casamino acids (Difco), 0.06% (w/v) $MgSO_4 \cdot 7H_2O$, 5% (w/v) PEG6000, 0.1% (v/v) minor elements solution. Dispense in 80 mL aliquots and autoclave. At time of use, add 15 mL of NaH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 6.8) (see Note 3), 2.5 mL of 20% carbon source, 2.5 mL of any required growth factors. For this experiment, glucose should be used as carbon source and no growth factors are necessary.
5. Minor elements solution consisting of 0.1% (w/v) $ZnSO_4 \cdot 7H_2O$, 0.1% (w/v) $FeSO_4 \cdot 7H_2O$, 0.1% (w/v) $MnCl_2 \cdot 4H_2O$, 0.1% (w/v) $CaCl_2$ anhydrous. Make a fresh solution every 2–4 weeks and store at 4°C.
6. Lennox Broth (LB) consisting of 1% (w/v) Bacto tryptone (Difco), 0.5% (w/v) Bacto yeast extract (Difco), 0.5% (w/v) NaCl, 0.1% (w/v) glucose.
7. L agar consisting of 1% (w/v) agar, 1% (w/v) Bacto tryptone, 0.5% (w/v) NaCl, 0.1% (w/v) glucose.
8. SOB-Mg consisting of 2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 0.0584% (w/v) NaCl, 0.0186% (w/v) KCl. Adjust pH to 7.0 with 10 N NaOH before autoclaving.
9. 2× YT consisting of 1% (w/v) Bacto yeast extract, 1.6% (w/v) Bacto tryptone, 0.5% (w/v) NaCl.
10. 0.05 M Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer (pH 8).
11. Double strength germination medium (2× GM) consisting of 1% (w/v) Bacto yeast extract, 1% (w/v) casamino acids. Add $CaCl_2$ to 0.01 M before use.

12. Antibiotic stock solutions used for this experiment: apramycin (50 mg/mL), kanamycin (50 mg/mL), chloramphenicol (25 mg/mL), nalidixic acid (50 mg/mL), vancomycin (10 mg/mL), A47934 (10 mg/mL; a kind gift from Professor Gerry Wright, McMaster University, Canada). Chloramphenicol is dissolved in ethanol and the other antibiotics in water. Filter-sterilize all antibiotic solutions and store in aliquots at −20°C.

2.2. Construction and Preparation of Plasmid DNA

1. Conjugative and integrative plasmid vector pRT801 (*see* Note 4).
2. Blunt-ending of protruding ends of DNA fragments: DNA polymerase I and supplied 10× reaction buffer (New England Biolabs). Store at −20°C.
3. Ligation: T4 DNA ligase and supplied 10× reaction buffer (New England Biolabs). Store at −20°C.
4. Dephosphorylation: Calf intestinal alkaline phosphatase (CIAP) and supplied 10× reaction buffer (New England Biolabs). Store at −20°C.
5. Restriction enzymes: *Sac*I and supplied 10× reaction buffer (New England Biolabs), *Pvu*II and supplied 10× reaction buffer (New England Biolabs). Store at −20°C.
6. Plasmid purification: QIAprep Spin Miniprep Kit (QIAGEN).

2.3. Agarose Gel Electrophoresis

1. TBE running buffer (10× stock solution) consisting of 10.8% (w/v) Trizma base, 5.5% (w/v) boric acid, 4% (v/v) ethylenediaminetetraacetic acid (EDTA, 0.5 M, pH 8). Dilute 1/10 in water to make 1× TBE for gel running.
2. Agarose gel: Melt 1% (w/v) agarose (Melford) in 1× TBE using a microwave, and pour the hot gel solution into a gel casting tray to set, creating wells for sample loading using a suitable comb.
3. Gel electrophoresis unit with power supply (Bio-Rad).
4. Ethidium bromide (EtBr): DNA separations on agarose gels were visualised using a 0.5–1 μg/mL staining solution of EtBr in 1× TBE (see Note 5).
5. DNA loading dye consisting of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol. Store in aliquots at −20°C.
6. DNA size marker: 1-kb DNA ladder (New England Biolabs). Store at −20°C.
7. UVIdoc gel documentation system (UVItec, Cambridge, UK).

2.4. Electroporation

1. Electroporation unit GenePulser II (Bio-Rad).
2. Electroporation cuvettes 4-mm gap (Bio-Rad).

2.5. RNA Preparation

1. Modified Kirby mix consisting of 1% (w/v) sodium-triisopropylnaphthalene sulphonate (TPNS; Phase Separations Ltd, Deeside, UK), 6% (w/v) sodium 4-amino salicylate, 6% (v/v) phenol mix in 50 mM Tris–HCl (pH 8.3). Store in 50 mL aliquots at –20°C.
2. Phenol mix consisting of 0.1% (w/v) 8-hydroxyquinoline in phenol (see Note 6). Equilibrate with 50 mM Tris–HCl (pH 8.3). Store at 4°C.
3. Phenol/chloroform consisting of 50% (v/v) phenol mix, 50% (v/v) chloroform, 1% (v/v) isoamyl alcohol. Store at 4°C.
4. RNase-free DNaseI and supplied 10× reaction buffer (Roche). Store at –20°C.
5. RNA precipitation agents: 0.3 M sodium acetate (pH 6.0); isopropanol.
6. RNA washing: 70% (v/v) ethanol in water.
7. Ultrasonic disintegrator (MSE Soniprep 150, SANYO) for quick cell lysis.
8. ND-1000 UV–Vis Spectrophotometer (NanoDrop).

2.6. DNA Labelling and Polymerase Chain Reaction

1. DNA probe labelling reagent: (γ-^{32}P)-ATP (3,000 Ci/mmol; DuPont-NEN: see Note 7).
2. T4 polynucleotide kinase and supplied 10× reaction buffer (Roche). Store at –20°C.
3. *Taq* DNA polymerase and supplied 10× reaction buffer (Roche). Store at –20°C.
4. dNTP nucleotides (Roche): Make a 100-mM mix and store in aliquots at –20°C.
5. Dimethyl Sulphoxide (DMSO, BDH).
6. Oligonucleotides for PCR primers (Genosys). Dissolve in water to 100 nM and store at –20°C.
7. Thermal PCR cycler: PTC-200 Peltier Thermal Cycler (MJ Research).

2.7. Hybridization and S1 Nuclease Mapping

1. Hybridization buffer consisting of 3 M sodium trichloroacetate (NaTCA), 50 mM 1,4-Piperazinediethanesulfonic acid Piperazine-1,4-bis(2-ethanesulfonic acid) Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 5 mM EDTA, pH 7.0. Dissolve 2.53 g PIPES in 90 mL water and add 1.67 mL 0.5 M EDTA. Adjust to pH 7 with 5 M NaOH, then autoclave. Dissolve 93.1 g NaTCA in the PIPES/EDTA solution,

then make up to a final volume of 167.4 mL with sterile distilled water. Store in 100 μL aliquots for single use at −80°C.

2. 5× S1 digestion buffer consisting of 1.4 M NaCl, 150 mM sodium acetate pH 4.4, 22.5 mM zinc acetate, 100 μg/mL partially cleaved, denatured, non-homologous DNA (Calf thymus DNA for this experiment). Store in 1 mL aliquots at −20°C.
3. S1 nuclease (Roche).
4. S1 termination solution consisting of 2.5 M ammonium acetate, 0.05 M EDTA.
5. S1 loading buffer consisting of 80% (v/v) formamide (see Note 8), 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.
6. Molecular biology grade glycogen (Roche).
7. 6% (w/v) Polyacrylamide–urea sequencing gel (Severn Biotech Ltd), ammonium persulfate, *N*,*N*,*N*,*N'*-Tetramethylethylenediamine (TEMED, Bio-Rad).
8. 3 MM paper (Whatman).
9. X-ray film: Kodak X-OMAT film.

3. Methods

3.1. Construction and Preparation of an Integrative, Conjugative Plasmid Vector Carrying the vanRSst Genes

1. These instructions are specific for cloning a piece of DNA carrying the *S. toyocaensis vanRS* operon into a vector suitable for use in *S. coelicolor*, and assume the availability of a 3-kb *Sac*I fragment of *S. toyocaensis* genomic DNA containing the *vanRS* operon.
2. Prepare blunt-ended and dephosphorylated pRT801 vector DNA ready for cloning by cutting with *Pvu*II restriction enzyme at 37°C for 1 h then removing terminal phosphate groups by adding 0.5 U CIAP and incubating for a further 30 min at 37°C (see Note 9). Purify the enzyme-treated DNA by first extracting twice with double the volume of phenol/chloroform, then once with double the volume of chloroform (see Note 10). Ethanol precipitate the DNA in the aqueous layer by adding one-tenth the volume of 3 M sodium acetate, then double the volume of ethanol and incubating at −80°C for 30 min. Collect the pellet by centrifugation, wash with 70% ethanol and allow to dry by exposure to the air for 10 min (see Note 11). Dissolve the pellet in sterile water.
3. Prepare the insert DNA by blunt-ending of the 3-kb *Sac*I *S. toyocaensis* genomic DNA fragment carrying the *vanRS*

genes using DNA polymerase I at 37°C for 30 min. Purify the enzyme-treated DNA as above, redissolving the ethanol precipitated pellet in water.

4. Ligate the blunt-ended dephosphorylated pRT801 vector DNA to the blunt-ended insert by incubating with T4 DNA ligase at 18°C for 4–24 h (see Note 12).
5. Transform the ligated DNA mixture into *E. coli* DH5α. Immediately before they are required, quickly thaw a frozen 50 μL aliquot of *E. coli* DH5α competent cells and maintain on ice. Add DNA ligation mixture to the competent cells, mix by tapping and incubate on ice for 20 min. Heat shock the cells at 42°C for 90 s, cool on ice for 2 min, then add 1 mL of LB. Allow the cells to recover by incubating for 1 h at 37°C with shaking at 250 rpm, then plate out aliquots for selection onto L agar containing 50 μg/mL apramycin. Incubate the transformation plates at 37°C for 12–16 h (see Note 13).
6. Select 5–10 apramycin-resistant transformant colonies for plasmid isolation and verification. Pick each colony individually into 5 mL LB containing 50 μg/mL apramycin using a sterile toothpick and incubate for 12–16 h at 37°C. Harvest cells from 1.5 mL of each culture broth by centrifugation, and isolate the plasmid DNA using the Qiaquick Miniprep kit according to the manufacturer's instructions. To verify that at least one of the transformant candidates contains the desired plasmid construct, digest the isolated plasmid DNAs with an appropriate restriction endonuclease and check the size of the resultant fragments agrees with predictions from the sequence using agarose gel electrophoresis.
7. The resulting plasmid with the expected 3 kb of insert DNA in pRT801 is referred to in the following text as pIJ10277.

3.2. Agarose Gel Electrophoresis

1. Mix 2–5 μg of the digested DNA samples with 1/6 volume of 6× loading dye, and load into the sample wells of an agarose gel (prepared as detailed in Subheading 2.3, step 2). For reference, the DNA size marker is also loaded alongside samples.
2. Run the gel using a power supply set to provide a constant voltage of 100 V, until the fastest moving blue dye has migrated 90% down the gel (about 30–60 min).
3. Stain the gel for approximately 10 min in EtBr stain, and record the result by photographing while illuminating at a wavelength of 254 nm on the UVIdoc (or equivalent).

3.3. Conjugal Plasmid Transfer from E. coli to Streptomyces

1. This procedure is for the introduction of the *vanRS* genes from *S. toyocaensis* (*vanRSst*), carried in the conjugative plasmid pIJ10277 constructed in Subheading 3.1 above, into the

S. coelicolor vanRS null mutant strain (Δ*vanRSsc*). The resulting *S. coelicolor* strain carries *vanRSst* instead of *vanRSsc*. *S. coelicolor* possesses a potent methyl-specific restriction system, and for successful introduction vector DNA must first be passaged through a methylation deficient *E. coli* host. In addition, the *oriT*-containing plasmid pIJ10277 is mobilizable *in trans* from *E. coli* only in the presence of a helper plasmid. For these reasons *E. coli* strain ET12567/pUZ8002 is used, which contains kanamycin and chloramphenicol resistance genes as selectable markers.

2. Plasmid pIJ10277 is introduced into ET12567/pUZ8002 by electroporation. To prepare electrocompetent cells, grow ET12567/pUZ8002 in LB under chloramphenicol (25 μg/mL) and kanamycin (50 μg/mL) selection at 37°C overnight. Use 100 μL of the overnight culture to inoculate 10 mL SOB-Mg medium containing chloramphenicol and kanamycin as above. Incubate for 3–4 h at 37°C with shaking until the culture reaches an OD_{600} of approximately 0.6. Remove the culture and place on ice for 1 h. Harvest the cells by centrifugation for 5 min at 3,000 × *g* at 4°C and wash by gently resuspending in 10 mL ice-cold 10% glycerol. Repeat the centrifugation and washing steps, and finally suspend the cell pellet in about 100 μL 10% glycerol (see Note 14). For electroporation, add 100 ng of pIJ10277 plasmid DNA to the cell suspension on ice and transfer immediately to a pre-chilled ice-cold electroporation cuvette. Electroporate using a GenePulser II set to 200 Ω, 25 μF and 2.5 kV, and immediately add 1 mL ice-cold LB medium to the shocked cells. Incubate with shaking at 37°C for 1 h, then spread onto L agar plates containing 50 μg/mL apramycin, 50 μg/mL kanamycin, and 25 μg/mL chloramphenicol. Successful transformants are resistant to all three antibiotics and will appear as single colonies after overnight incubation of the plates at 37°C.
3. For conjugation, pick a single colony of *E. coli* ET12567/pUZ8002 carrying the conjugative plasmid pIJ10277 into 5 mL LB containing 50 μg/mL apramycin, 50 μg/mL kanamycin and 25 μg/mL chloramphenicol using a toothpick, and grow at 37°C to an OD_{600} of 0.4–0.6. Harvest the cells by centrifugation at room temperature (3,000 × *g*, 5 min) and remove all traces of the antibiotics used by washing the pelleted cells twice with 5 mL of LB, centrifuging as before to collect the washed cells. Finally, resuspend the washed pellet in 500 μL of LB. While washing the *E. coli* cells, prepare *S. coelicolor* Δ*vanRSsc* spores for conjugation by activating them for rapid germination using a heat shock treatment. Add approximately 10^8 spores (usually equivalent to 10 μL of a spore preparation) to 500 μL 2× YT medium and heat at 50°C for 10 min. Cool the spores briefly on ice, then mix

with the washed *E. coli* cell suspension prepared above. Plate out on MS agar containing 10 mM $MgCl_2$ and incubate at 30°C for 16–20 h (see Note 15). Overlay the plate by flooding with 1 mL distilled H_2O containing 0.5 mg nalidixic acid (to kill off any *E. coli* cells) and 1 mg apramycin (to select for introduction of pIJ10277 into *S. coelicolor* Δ*vanRSsc*), and continue incubation at 30°C for 4–5 days.

4. Check the exconjugant candidates that have grown for apramycin resistance by streaking for single colonies on MS agar plates containing 25 μg/mL nalidixic acid and 50 μg/mL apramycin. Prepare spore stocks of at least two of the genuine exconjugants.

3.4. Phenotypic Analysis of the Gene Swapped Strain Using a Replica Plate Assay for Drug Resistance/Sensitivity

1. To compare and assess the resistance/sensitivity of the gene swap strain *S. coelicolor* Δ*vanRSsc* + *vanRSst* with the wild type, and *S. coelicolor* Δ*vanRSsc* strains, first make a master plate by streaking out a spore preparation of each on an MS agar plate that has been divided into three sectors. Plate one strain per sector (as shown in Fig. 2) and grow the strains until they have achieved confluent sporulation by incubating at 30°C for about 6 days.

2. For replica plating, wash and sterilize a sterile square of cotton velvet, and place over a cylindrical block (80 mm diameter) which fits comfortably inside a standard Petri dish. The cloth is held tightly in place by a metal or plastic ring (85 mm internal diameter). Also prepare two MS agar plates, one containing 10 μg/mL vancomycin and the other 10 μg/mL A47934.

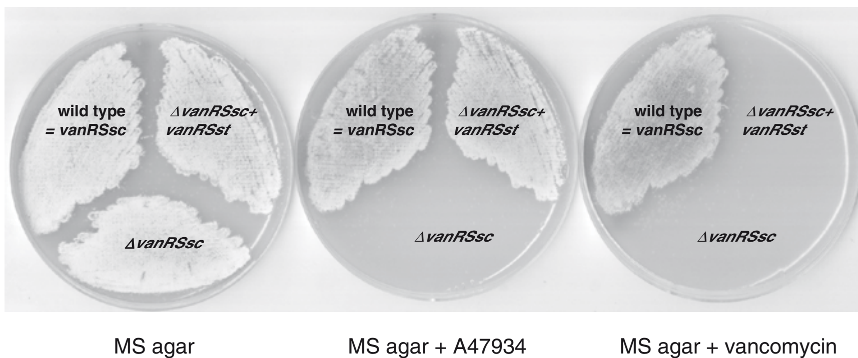

Fig. 2. Wild-type *S. coelicolor* is resistant to both vancomycin and A47934. However, when the *vanRS* genes of *S. coelicolor* are deleted and replaced with the *vanRS* genes from *S. toyocaensis* (Δ*vanRSsc* + *vanRSst*), the strain is resistant to A47934 but not to vancomycin, the same phenotype displayed by *S. toyocaensis* itself. The *vanRS* deletion mutant in *S. coelicolor* (Δ*vanRSsc*) is sensitive to both vancomycin and A47934.

3. Print the spores from the surface of the master plate onto the velvet by pressing it gently but firmly onto the cloth. From this, transfer the printed spores onto the MS agar plates containing antibiotics by pressing each plate in turn onto the velvet. Assess the ability of each strain to grow on the antibiotic plates by incubating at 30°C for 4–5 days. Examples of the drug resistance and sensitivity of the constructed strain compared with the other strains are shown in Fig. 2.

3.5. Analysis of the Expression of van Genes in the Gene Swapped Strain Using S1 Nuclease Protection

To assess the patterns of transcription of the *van* genes in the gene swap strain *S. coelicolor* Δ*vanRSsc*+*vanRSst* in response to exposure to vancomycin or A47934, it is necessary to isolate RNA from drug-treated cultures and quantify abundance of the *van* gene transcripts in the isolated samples (see Note 16). We routinely use S1 nuclease protection experiments for quantification, although qRT-PCR would also be suitable.

3.5.1. Isolation of RNA from Streptomyces Cultures Before and After Treatment with a Sub-inhibitory Concentration of Vancomycin or A47934

1. To culture the strains, first germinate aliquots of spores by heat shock treatment in 5 mL TES buffer (0.05 M, pH 8) at 50°C for 10 min, and then incubate (with shaking) at 37°C for 2–3 h following dilution with an equal volume of 2× GM. Inoculate NMMP broth with the germinated spore preparation to produce an initial $OD_{450} \approx 0.025$. Incubate at 30°C with 250 rpm shaking until an OD_{450} of 0.3–0.6 is reached (approximately 10–12 h), then treat by addition of a sub-inhibitory concentration (10 μg/mL) of vancomycin or A47934.
2. Cultures are sampled at times immediately before and after drug treatment by rapidly harvesting cells from 10 mL aliquots by centrifugation for 1 min at 3,000×*g*. Immediately resuspend the pelleted cells in 1 mL of ice-cold Kirby mix, and transfer to a 2-mL centrifuge tube.
3. Disrupt the cells by sonication of the mycelial suspension using two bursts of 5 s each, resting for 30 s on ice between bursts. Extract the lysed cells with 0.8 mL phenol/chloroform (pH 8.0), by vortex mixing for 2 min and centrifuging to separate the layers.
4. Perform a second phenol–chloroform extraction as before, and finally precipitate nucleic acids by adding one-tenth the volume of 0.3 M sodium acetate (pH 6.0), an equal volume of isopropanol, and incubating at −20°C for 30 min. Collect the precipitate by centrifugation at 4°C for 30 min, and wash with 70% (v/v) ethanol.
5. Redissolve the nucleic acid pellet in DNase I buffer, and treat with 5 units DNase I at 37°C for 30 min. Extract with phenol/chloroform (pH 8.0) and ethanol precipitate the RNA.

6. Dissolve the RNA pellets in RNase-free distilled water, and quantify using UV spectrophotometry. Store at –80°C until required. This procedure typically yielded ≈0.5 mg RNA.
7. RNA quality can be tested by RNase-free agarose gel electrophoresis (see Note 17).

3.5.2. Preparing a Radiolabelled Probe Specific for Assessing van Gene Transcription

1. To create a DNA probe for transcriptional analysis using S1 nuclease mapping, PCR amplify a 0.27-kb DNA fragment carrying the *vanH* promoter (*vanHp*) region from *S. coelicolor* M600 chromosomal DNA using a 5′end-labelled (see step 2 below) reverse primer internal to *vanH* (vanHp II; 5′-TGAGAGTCGCCTCGACGCCGAAA-3′) and an unlabelled upstream primer (vanHp I; 5′-TTCGACCTCTATATGAAGCGACGT-3′). The PCR reaction mixture contained 1× reaction buffer, 200 μM final concentration of each of the four dNTPs, 2.5 U *Taq* polymerase, 100 pmol each primer, 5% (v/v) DMSO and 50 ng template DNA in a final volume of 100 μL. After denaturation at 95°C for 5 min, the samples were subjected to 25 cycles of denaturation (95°C, 1 min), annealing (55°C, 45 s), and extension (72°C, 45 s) and then incubated for 5 min at 72°C.
2. Prior to the PCR in step 1, 5′ end-label the vanHp II reverse primer by incubating 100 ng of the vanHp II oligo DNA for 1 h at 37°C in 1× kinase reaction buffer with 10 units of T4 polynucleotide kinase and (γ-^{32}P)-ATP (3000 Ci/mmol; DuPont-NEN) in a total volume of 40 μL. Ethanol precipitate the radiolabelled primer DNA with one-tenth volume 3 M sodium acetate (pH 6) and double the volume of ethanol. Dissolve the pellet in sterile water.

3.5.3. S1 Nuclease Protection Analysis

1. For each sample to be analyzed, thoroughly dissolve 30 μg RNA and 25 pmol labelled probe in 20 μL NaTCA buffer in a 1.5-mL tube (see Note 18). Denature the nucleic acids by heating the tubes to 65°C for 15 min in water bath, then allow the labelled DNA probe to hybridize its complementary RNA transcript in a 45°C water bath for 4–18 h (see Note 19).
2. After hybridization, remove the samples onto ice and dispense 300 μL of 5× S1 digestion buffer containing 100 units of S1 nuclease into each. Mix immediately by vortexing, and incubate at 37°C for 1 h. Add 75 μL S1 termination solution to stop the S1 nuclease reaction. This step will digest single-stranded DNA probe, but not the DNA where it is hybridized to a transcript. It therefore digests back the labelled strand of the DNA from the 5′ end to the base where the transcript begins.
3. To precipitate the samples, add 1 μL glycogen and 400 μL isopropanol then leave at –20°C for 1 h. Harvest the precipitate by centrifugation for 30 min at 5°C and full speed (13,000 rpm)

in an Eppendorf centrifuge, and rinse the pellets gently with 1 mL 70% ethanol.

4. Resuspend the pelleted samples in 6 μL S1 loading buffer and denature by heating to 90–100°C for 1–2 min. The samples are now ready for separating using polyacrylamide gel electrophoresis.

3.5.4. Polyacrylamide Gel Electrophoresis

1. The samples are separated on a standard polyacrylamide DNA sequencing gel. The gel is cast between two glass sequencing plates (20 × 40 cm) which should be cleaned thoroughly before use. To clean, wash with soapy water, rinse thoroughly with distilled water, and dry. Wipe vigorously with a tissue soaked in ethanol before assembling the plates, using bulldog clips to firmly hold the plates together for casting the gel.
2. Prepare the gel by mixing 100 μL 25% (w/v) ammonium persulfate, 100 μL TEMED and 50 mL of 6% (w/v) polyacrylamide–urea sequencing gel. Immediately inject the gel mix into the gap between the two clean glass sequencing plates using a syringe until completely filled. Take care not to introduce any air bubbles. Insert the comb to form the sample wells. The gel should set in 10–20 min.
3. Once the gel has set, insert the sequencing gel into an electrophoresis tank and add 1× TBE running buffer to the upper and lower chambers of the unit. Carefully remove the comb and wash the wells with 1× TBE running buffer using syringe to remove any debris. Equilibrate the gel to a suitable running temperature (about 50–55°C) by pre-running for 30–45 min at 1,000 V.
4. Wash the wells with running buffer once again as before, and load 3 μL of the heat-denatured S1 hybrid sample in each well. Also load an appropriate radiolabelled molecular weight ladder alongside the samples.
5. Run the gel at 40 W constant power (which should provide 1,000–1,300 V) until the front blue dye reaches the bottom of the gel. This usually takes about 100 min.
6. Disconnect the gel unit from the power supply. Carefully separate the glass plates leaving the gel adhering to one (see Note 20). Remove both spacers and overlay the gel with Whatman 3 MM paper, pressing evenly so that the gel will adhere to the paper. Gently peel the paper and gel away from the plate and cover the gel surface with cling film. Place on a gel dryer, and dry at 80°C for 1 h.
7. Transfer the dried gel (still stuck to the paper and covered in film) to an X-ray film cassette and expose to film for a suitable length of time to produce a good image, normally overnight (see Note 21).
8. An example of the results produced is shown in Fig. 3.

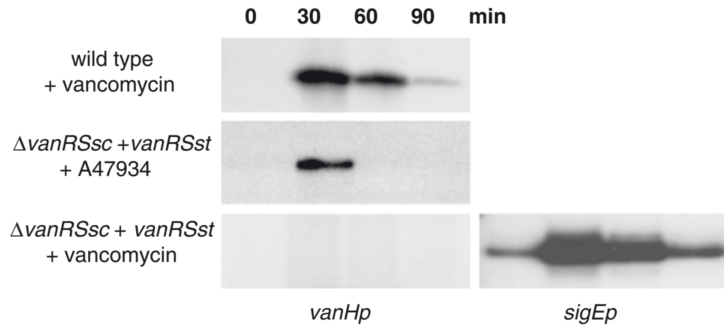

Fig. 3. S1 nuclease protection analysis of *vanH* transcription in *S. coelicolor* M600 wild-type or *S. coelicolor* Δ*vanRS* carrying the *S. toyocaensis vanRS* genes (Δ*vanRSsc* + *vanRSst*). Vancomycin did not induce transcription from the *vanH* promoter in this strain, but A47934 did. The *sigE* promoter was used as a positive control to ensure that the absence of signals in the vancomycin-treated Δ*vanRSsc* + *vanRSst* strain was not due to cell death. Thus, switching the VanRS signal transduction system from one species to the other also switched inducer specificity. The three *vanHp* panels are equivalent exposures and are therefore directly comparable.

4. Notes

1. All chemicals were purchased from Sigma-Aldrich unless there is a specific description for suppliers. The manufacturer and model for items of equipment used in this study are listed, but equivalent pieces of equipment from alternative suppliers can equally well be used.
2. Due to the slow growth of *Streptomyces* strains, there is a greater risk of contamination than when using other, more rapidly growing bacteria such as *E. coli*. Manipulation of *Streptomyces* strains and cultures should therefore be done in a suitable laminar flow hood, and extra attention given to aseptic technique.
3. Prepare 0.1 M solutions of NaH_2PO_4 and K_2HPO_4 separately, then mix together in equal volumes. Adjust pH to 6.8.
4. Ideally, to prevent abnormal levels of gene expression complicating interpretation of the analysis, the genes at the centre of the swapping study should be introduced into the new host in single copy and under the control of their native promoter (or the promoter of the gene being replaced). In the case of *S. coelicolor*, this is most conveniently achieved using a plasmid vector that integrates site specifically into the chromosome in single copy following introduction by conjugation from *E. coli*. Plasmid pRT801 is therefore ideal, and carries an apramycin resistance cassette for selection.

5. EtBr is known to be a powerful mutagen and is moderately toxic. Observe your local laboratory safety rules for handling and disposing of this compound. Liquid waste solutions should usually be decontaminated by passing through activated charcoal columns or by using commercially available destaining bags.
6. Phenol is extremely toxic and should be handled in a suitable fume hood. Gloves and safety spectacles should be worn, and waste disposed of carefully in accordance with your local laboratory safety rules.
7. Radioactive material should be handled with appropriate care in accordance with your local radiation protection rules.
8. Animal studies have shown formamide to be a teratogen, and women in the early stages of pregnancy should be made aware of the risks of potential abnormal foetal development following exposure to formamide. Local laboratory safety rules should be observed when handling and disposing of formamide, and particular care should be taken to avoid inhalation of formamide vapour. Containers which have been opened must be carefully resealed and kept upright to prevent leakage.
9. It is possible to overdo the dephosphorylation of linearized plasmid vectors, resulting in DNA ends that ligate inefficiently with their intended insert DNA sequences. Use the amount of CIAP recommended by the manufacturer, and do not incubate for longer than is necessary.
10. When extracting DNA-containing solutions with phenol/chloroform it is important that the phenol/chloroform has been equilibrated to the correct pH. Above pH 8, extraction will remove contaminating protein into the lower organic layer, leaving DNA in the upper aqueous phase. Acidic phenol/chloroform however, will extract both DNA and protein into the organic layer. A chloroform extraction step is included after extracting with phenol containing solutions to help remove all traces of the phenol that would inhibit enzyme activity in any subsequent use of the DNA, e.g. ligation, PCR. Ethanol precipitation of the DNA also assists in this.
11. The precipitated DNA pellet may be very difficult to see by eye, and care should be taken not to dislodge and lose the pellet while removing the supernatant, or when washing with 70% ethanol. The 70% ethanol wash is important for removing salt from the DNA preparation that may interfere with the efficiency of the subsequent ligation reactions.
12. The optimal ratio of vector/insert DNA used for cloning can be determined experimentally, but a ratio between 3:1 and 1:3 is usually effective. When using linearized vector DNA

that can potentially self-ligate, as is the case here, it is preferable to provide an excess of insert DNA e.g. by using a 1:3 ratio. Ligation temperature is a compromise between the optimal conditions for the enzyme (usually 25°C) and the temperature required to ensure annealing of the DNA ends (which can vary with the length and base composition of any overhanging DNA sequences). Blunt-end ligations are generally optimal at 15–20°C for 4–18 h. It is good practice to include a positive control for the ligation, which is usually self-ligation of cut vector DNA that has not been dephosphorylated, and also a negative control containing the cut and dephosphorylated vector but no insert.

13. To ensure that transformation plates containing distinct single colonies are obtained, plate a range of volumes of each transformation mix e.g. 20, 50, and 100 μL. Include a positive control to assess the efficiency of the transformation process by using an equivalent amount of uncut vector DNA in one transformation. Because of the time required for the transformants to appear on the plates, it is usually convenient to set up transformations late in the afternoon and incubate the plates overnight.
14. The glycerol washing steps are essential to desalt the cells ready for electroporation. The presence of any salt will lead to arcing, and failure to electroporate.
15. To help guarantee obtaining exconjugant candidates growing as distinct single colonies, prepare a 10^{-1} to 10^{-4} dilution series of the conjugation mixture in a total of 100 μL water, and plate out each dilution separately.
16. RNase enzymes are a significant problem when handling RNA under laboratory conditions. All reagents, tubes and pipette tips, etc., used in RNA work should be RNase-free, and suitable powder-free gloves should be worn at all times. Care should be taken not to contaminate the gloves by touching skin or hair. RNase-free aqueous solutions and 1.5-mL tubes are most conveniently prepared by double-autoclaving.
17. As RNases are abundant and very stable, RNase contamination of gel tanks can often occur. Degradation of RNA during gel electrophoresis is therefore a common problem, leading to a much more pessimistic assessment of the quality of RNA samples than is actually warranted. To avoid these problems, the agarose, 1× TBE buffer and loading dye used for RNA work should be autoclaved and kept separate from stocks used for routine DNA analysis. It is also most important that the gel tank, the plate on which the gel is poured and the comb used to form the sample loading wells are thoroughly decontaminated. This can be achieved by soaking them in

100 mM NaOH for at least 2 h, then rinsing in RNase-free water immediately before use. Another way to avoid degradation of RNA during electrophoresis is to incorporate sodium iodoacetate (RNase inhibitor) in the agarose to a final concentration of 10 mM.

18. Carefully evaporate the RNA sample solution to dryness using a vacuum dryer, dissolve the residue completely in the NaTCA with vortexing, then add the radiolabelled probe DNA.
19. Ensure that the 1.5-mL tubes are submerged up to their necks while hybridizing. If not completely submerged, the sample solution will evaporate from the bottom of the tube and condense on the insides of the tube higher up above the water line. Any sample in this location will be at a significantly lower temperature than desired.
20. The gel is very thin and delicate. Take great care not to rip the gel when separating the plates. It is useful to treat one of the glass plates with a siliconizing agent before casting the gel: this acts as a non-stick surface and encourages the gel to adhere only to the non-treated plate.
21. The exposure time needs to be determined largely by trial and error. As a first attempt, it is usual to expose the film overnight (about 16 h) before developing. If the image proves too strong or too weak, a second exposure with a new piece of film can be undertaken, increasing or decreasing the time as appropriate.

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Chapter 5

Real-Time PCR Methods to Study Expression of Genes Related to Hypermutability

Denise M. O'Sullivan

Abstract

Pathogenic bacteria can have sub-populations of hypermutable bacteria. This sub-population has a higher spontaneous mutation rate than the majority of the population which can be attributed to defects in proofreading and repair mechanisms. This leads to the evolution of drug-resistant strains of bacteria through genetic change. It is important to study the expression of genes involved in, for example, mismatch repair and the SOS system by real-time PCR to determine hypermutability and therefore provide an indicator of the mutagenic ability of certain strains of pathogenic bacteria.

Key words: Hypermutability, DNA repair, Mutation

1. Introduction

Hypermutable bacteria or hypermutators occur when there are defects in proofreading and DNA repair mechanisms (1). These defects may be found in the methyl directed mismatch repair system encoded by *mutS*, *mutL*, and *uvrD* (2). This leads to an increase in mutation frequency up to a 1,000-fold (3). Hypermutators were firstly identified in pathogenic *Escherichia coli* (4) and subsequently in cystic fibrosis patients colonized with *Pseudomonas aeruginosa* causing chronic infection (5). The ability of a strain to mutate at an elevated frequency can be advantageous, resulting in enhanced survival through antibiotic resistance and increased virulence.

Comparative genomics and gene expression profiling has been used to investigate the effect of antibiotics on bacteria including the identification of novel targets for new therapeutic agents, the investigation of the mechanisms of resistance and bacterial evolution (6–9). Recently, it has been shown by microarray

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analysis that in an isogenic *E. coli* population, antibiotic resistance can evolve through changes in gene expression, specifically through an over-expression in genes involved in resistance (10). This resistance occurs at a higher frequency than can be explained by spontaneous DNA mutation, and it is highly unstable with a high reversion rate which disappears as the time under antibiotic selection and the concentration of antibiotic increases. The impact of genetic evolution on *Salmonella enterica* serotypes has been studied using microarrays to investigate small evolutionary events that are occurring in sub-populations within an infection (11).

In addition to microarray analysis, quantitative reverse transcriptase real-time PCR (qRT-PCR) can be used for the study of small-scale gene expression studies in response to antibiotic exposure (12). By identifying the expression by qRT-PCR of genes involved in proofreading and SOS repair, the potential of a bacterial population to be a hypermutator can be determined. This could have important clinical significance by indicating the patients who are likely to develop resistance throughout the course of infection. We have shown that sub-inhibitory quinolone treatment of *Mycobacterium tuberculosis* is hypermutable through the SOS response mediated through the expression of *recA* and *lexA*. This observation could be extended to other bacteria by investigating genes involved in damage reversal and excision repair, e.g. *mutS, mutL, mutY* and *uvrD*.

2. Materials

2.1. Growth of Mycobacterium tuberculosis

1. *M. tuberculosis* H37Rv (NCTC 7416) (see Note 1).
2. Middlebrook 7H9 broth (BD, Le Pont de Claix, France) (see Note 2).
3. Distilled water.
4. Tween 80 [polyoxyethylene (20) sorbitan mono-oleat] (BDH, VWR International Ltd., Poole, England).
5. Middlebrook albumin dextrose catalase (ADC) enrichment (BD) (see Note 3).

2.2. RNA Extraction

1. Guanidine thiocyanate (Promega, Wisconsin, USA) (see Note 4).
2. Sodium N-lauroyl sarcosine (Sigma Aldrich, Steinheim, Germany).
3. β-mercaptoethanol (Sigma Aldrich) (see Note 5).
4. 1 M sodium citrate pH 7.0 (Sigma Aldrich).
5. Tween 80.

6. Q-Biogene Fast RNA Pro-Blue kit (Q-Biogene Inc. CA, USA).
7. Ribolyser (Hybaid, Ashford, UK).
8. Chloroform (BDH) (see Note 6).
9. Ethanol (BDH).
10. 75% Ethanol: Prepare with diethyl pyrocarbonate (DEPC) water and keep at −20°C.
11. DEPC water (Q-Biogene) (see Note 7).
12. Cold absolute ethanol.
13. Sterile 50 mL Apex centrifuge tubes.
14. Sterile screw-capped 1.5 mL micro-centrifuge tubes with 0-rings.

2.3. RNA Clean Up

1. R Neasy MiniElute Cleanup Kit for bacteria (Qiagen Ltd., West Sussex, UK).
2. β-mercaptoethanol.
3. RNase-free DNase set (Qiagen).

2.4. Quantitative Reverse Transcriptase Real-Time PCR (qRT-PCR)

1. QuantiTect Probe RT-PCR Master Mix (Qiagen, Hilden, Germany).
2. Primers and dual-labelled probe (Sigma Genosys, Suffolk, UK).
3. RNase free water (Qiagen).
4. Rotorgene™ 3000 thermal cycler (Corbett Research, Mortlake, Australia).

3. Methods

3.1. Culture of M. tuberculosis

1. Treat 10 mL aliquots of exponentially growing *M. tuberculosis* H37Rv (approximately 10^6 cfu/mL) in Middlebrook 7H9 media with one-quarter or one-half MIC of ciprofloxacin for 24 h (see Note 8).

3.2. RNA Extraction

1. Extract RNA from each culture at time points 0, 4, 12 and 24 h using an adaptation of the method of Mangan (13) and the Q-Biogene Fast RNA Pro-Blue kit (see Note 9).
2. Aliquot 10 mL of culture into apex centrifuge tubes and add 4 volumes (40 mL) of 5 M GTC solution to each culture (see Note 10).
3. Harvest by centrifugation (Centaur 2) at 2,000 × *g* for 30 min.

4. Discard spent GTC leaving the residual volume of GTC into 1 apex centrifuge tube (<1 mL).
5. To this aliquot, add 1 mL RNA Pro solution and re-suspend the cells by pipetting (see Note 11).
6. Transfer this suspension in 1 mL aliquots to a Fast RNA Pro blue cap tube containing Lysing Matrix B and agitate the tube in the ribolyser for 40 s at a setting of 6.0 (see Note 12).
7. Incubate the tube at –20°C for 2 min and repeat the agitation step (see Note 13).
8. Centrifuge the tube at 13,000 × *g* for 5 min.
9. Transfer the supernatant to a new micro-centrifuge tube, taking care to avoid transferring the debris pellet and lysing matrix.
10. Incubate the sample at room temperature for 5 min to increase RNA yield.
11. Add 300 μL of chloroform and vortex for 10 s.
12. Incubate the tube for 5 min at room temperature to permit nucleoprotein dissociation and increase RNA purity.
13. Centrifuge the tube at 13,000 × *g* for 5 min.
14. Transfer the upper phase to a new micro-centrifuge tube.
15. Add 500 μL of cold absolute ethanol; invert the tube 5 times to mix and then store at –20°C overnight.
16. The following day, centrifuge the tube at 13,000 × *g* for 20 min at 4°C and then remove the supernatant.
17. Wash the pellet in 500 μL of ice cold 75% ethanol, made with DEPC water.
18. Centrifuge the tube at 13,000 × *g* for 15 min at 4°C and decant the ethanol.
19. Air-dry the RNA pellet for 30 min on the bench (see Note 14).
20. Re-suspend the pellet in 100 μL of DEPC water (see Note 15).
21. Store the tubes at –80°C or perform the RNA clean-up immediately (see Note 16).

3.3. RNA Clean-Up

1. Clean up RNA samples using buffers from Qiagen RNeasy MiniElute Cleanup Kit for bacteria following manufacturer's instructions (see Note 17).
2. Warm buffer RLT to 37°C for 30 min prior to clean up (see Note 18).
3. Add 10 μL of β-mercaptoethanol per 1 mL buffer RLT and 4 volumes of ethanol to Buffer RPE.
4. Add 350 μL of buffer RLT to the tube, followed by a further 250 μL. Mix the solution thoroughly by pipetting.

5. Apply the sample (~700 μL) to an RNeasy mini column supported in a 2 mL collection tube.
6. Centrifuge the tube for 15 s at 10,000 × *g*. Discard the flow through and the collection tube.
7. Transfer the RNeasy mini column to a new tube and add 350 μL of buffer RW1. Centrifuge the tube at 10,000 × *g* for 15 s.
8. Add 10 μL of DNase stock solution to 70 μL buffer RDD and mix by gently inverting the tube (see Note 19).
9. Add the DNase:buffer RDD mix (80 μL) directly onto the RNeasy silica gel membrane and incubate at room temperature for 15 min.
10. Wash the column through with 350 μL buffer RW1 and centrifuge for 15 s at 10,000 × *g*.
11. Discard the collection tube and flow through and transfer the RNeasy column to a new 2 mL collection tube.
12. Apply 500 μL of buffer RPE to the top of the column and centrifuge for 15 s at 10,000 × *g*.
13. Add a further 500 μL buffer RPE to the column and centrifuge for 2 min at 10,000 × *g*.
14. Discard the flow through and centrifuge the tube for a further minute at 13,000 × *g* to remove residual ethanol.
15. Transfer the column to a new collection tube and add 30 μL of RNase free water directly onto the membrane to elute the RNA.
16. Incubate the tubes at room temperature for 5 min and centrifuge for 1 min at 10,000 × *g*.
17. Aliquot the eluate 7 × 4 μL and store at −80°C.

3.4. Quantitative Reverse Transcriptase Real-Time PCR (qRT-PCR)

1. You need to include a positive control for hypermutability. One method of achieving this is to use mitomycin C treatment. Treat a sample with 0.2 μg/mL mitomycin C (Sigma Aldrich) for 24 h and extract and clean up RNA as described in Subheadings 3.2 and 3.3.
2. Perform RT-PCR using primers and dual-labelled probes to target *lexA*, *recA*, *dnaE2* and *sigA* from Table 1 (see Note 20).
3. Add 5 μL of RNA (approximately 1 pg/reaction) to 1× QuantiTect Probe RT-PCR Master Mix including 4 mM $MgCl_2$, 0.4 μM primers, 0.2 μM probe, 0.5 μL/reaction QuantiTect RT mix and make up to a final volume of 25 μL with RNase-free water (see Note 21).
4. Perform the reaction on a Rotorgene™ 3000 thermal cycler (see Note 22) with the following conditions; a reverse

Table 1
Probes and primers used for qRT-PCR

Target	Primer/probe	Sequence	Reference
lexA	Probe	tcttcccgctgccgcgtgagc	This experiment
	Forward primer	atccttgccgaggaagcc	(14)
	Reverse primer	ctgctcaaggtgatcggtga	
recA	Probe	ttcgggcaccacggcgatcttcat	This experiment
	Forward primer	aatgaccggcgcgctga	This experiment
	Reverse primer	cgttgaagttctacgcgtc	This experiment
dnaE2	Probe	cagcgtcctgcaatgggacaaaga	(15)
	Forward primer	ccggtggaatgggcg	
	Reverse primer	gcaatcggcttggtgaaatt	
sigA	Probe	cctgcgcctggtggtttcgc	(16)
	Forward primer	aaaccatctgctggaagcca	
	Reverse primer	ttctcgacctgatccaggaag	This experiment

transcription step 50°C for 30 min, PCR activation step 95°C for 15 min, and 45 repeats of 94°C for 15 s and 60°C for 60 s. Acquire data during the combined annealing/extension step of 60°C on the FAM channel (excitation 470 nm, detection 510 nm, gain 10) or the JOE channel (excitation 530 nm, detection 555 nm, gain 10). The reaction components and the cycling conditions were as outlined in manufacturer's instructions in the QuantiTect™ Probe RT-PCR Handbook.

5. Process each sample in triplicate, including a non-RT control for each sample, also in triplicate.
6. Include a positive control of RNA from *M. tuberculosis* H37Rv.
7. Set the threshold level to a value which is above the background fluorescence of the "no template" control in the exponential phase of the curve (see Note 23).
8. Calculate the relative gene expression using the equation $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between the ΔCt for the tested genes *dnaE2*, *recA* or *lexA* from the ΔCt of the housekeeping gene, *sigA* (17). This equation can only be used when the efficiency of each probe and primer pair are greater than 99% (see Note 24).

4. Notes

1. *M. tuberculosis* is a Hazard Group 3 infectious biological agent that can cause severe human disease and requires handling in a Containment Level 3 laboratory.
2. Prepare Middlebrook 7H9 media by dissolving 4.7 g Middlebrook 7H9 powder in 900 mL distilled water in a litre bottle. Add 2 mL Tween 80 and incubate the bottle in a 55°C water bath for 20 min to dissolve the powder and Tween. The detergent Tween 80 is necessary to prevent clumping. Aliquot into 5×180 mL and autoclave at 121°C, 15psi for 15 min. Store the media at room temperature. Before use, add 20 mL Middlebrook ADC enrichment to each aliquot.
3. ADC enrichment can be prepared by adding 50 g Bovine Albumin fraction V, 20 g glucose and 0.03 g catalase to 1 L distilled water. Mix thoroughly and filter-sterilise before use.
4. Guanidine thiocyanate is harmful if inhaled. Use within a fume hood and wear protective clothing.
5. β-mercaptoethanol must be used inside a fume hood as it is toxic. Protective clothing including laboratory coat and gloves must be worn.
6. Chloroform must be used inside a fume hood as it is toxic. Protective clothing must be worn.
7. DEPC water is best purchased commercially, but it can be prepared by adding 1 mL DEPC (0.1%) to 1 L distilled water. This is incubated overnight at 37°C and then autoclaved. DEPC must be handled in a fume hood with appropriate protective equipment (laboratory coat and gloves).
8. Culture *M. tuberculosis* in Middlebrook 7H9 broth to exponential phase of growth (turbidity corresponding to a 0.5 McFarland growth standard which is approximately 10 days following inoculation). Cultures were maintained by dilution; pass the broth culture through ~6 times a 1 mL fine needle syringe to get a single cell suspension prior to dilution. Sonication can also be used to disperse clumps.
9. RNase and DNase contamination can be reduced by treating all pipettes, glassware and the work area with RNase Away (Molecular Bioproducts Inc., San Diego, CA), using filtered pipette tips and DEPC-H_2O or RNase free water. If possible, it is helpful to designate a specific bench for RNA work.
10. Prepare 5 M Guanidine thiocyanate (GTC) solution by dissolving 295.4 g guanidine thiocyanate in 200 mL distilled water. Care must be taken to prevent inhalation and skin contact by wearing gloves, laboratory coat and safety glasses. Mix

well and incubate at 37°C overnight. Mix the solution again the next day and incubate at 37°C for another 30 min. Add 2.5 g sodium N-lauroyl sarcosine, 12.5 mL 1 M Sodium citrate pH 7.0 and 5 mL Tween 80 to the solution. Make the solution up to a final volume of 500 mL with distilled water. Add 3.5 mL β-mercaptoethanol immediately prior to use in a fume hood. The solution minus the β-mercaptoethanol can be stored at room temperature for ~3 months. The GTC solution must be added quickly to the sample to initiate RNase inhibition. Samples can be stored in GTC at 4°C overnight.

11. The RNA*pro*™ is light sensitive and must be kept in a closed box. As with GTC, care must be taken to prevent skin contact and inhalation. TRIzol® reagent (Invitrogen) can be used as an alternative.

12. Adding 1 mL of re-suspended cells will provide adequate air-space in the tube containing the matrix and prevent sample leakage. If a greater number of cells need to be processed, then a second matrix tube can be used instead of using the Q-Biogene.

13. An additional processing step in the ribolyser is required for bacterial samples that are difficult to lyse, e.g. *M. tuberculosis*. If the additional step is required, then the sample must be incubated on ice for 2 min to prevent possible RNA degradation.

14. Tubes are left open to air-dry, but do not dry the sample completely as it will reduce the solubility of the pellet.

15. For subsequent time points, two tubes were pooled for each sample. 50 μL DEPC water was added to each tube and combined. This can be performed if expected RNA yield is low or if samples had to be spilt for initial processing.

16. RNA is stable at –80°C, freeze-thaw cycles should be avoided.

17. An alternative to the column-based Qiagen clean-up system involves aqueous separation by centrifugation of RNA mixed with phenol/chloroform/isoamyl alcohol, followed by a further extraction with chloroform to remove the phenol and an ethanol precipitation to recover the RNA. The DNase digestion must be performed prior to the clean-up.

18. It is necessary to warm buffer RLT to redissolve the precipitate formed during storage and then place at room temperature.

19. The extra on column DNase digestion is required for procedures which are sensitive to DNA contamination, e.g. qRT-PCR. Dissolve DNase I in 550 μL RNase-free water, aliquot

and store at −20°C for 9 months or at 4°C for 6 weeks. Thawed aliquots cannot be re-frozen.

20. Sequence specific probes will ensure the highest efficiency in real-time RT-PCR to target products of 70–150 bp in length. Certain conditions should be considered when designing primers and probes. The melting temperature (Tm) of the probe should be ≥10°C than the Tm of the primers. The probe should be 20–30 bp in length, contain more cytosine than guanine bases (because guanine bases have a quencher effect) and ideally ≤10 bp downstream of the forward primer. Ideally, although difficult to adhere to with GC rich mycobacteria and streptomyces, the primers and probe should have 50–60% GC content, to reduce the occurrence of non-specific interactions. Also avoid a run of guanine and cytosine bases at the end of the forward primer. The *dnaE2, recA* and *lexA* probes were fluorescently labelled with 6-carboxyfluorescein (FAM) on the 5′ end and Black Hole Quencher® I dye on the 3′ end. The *sigA* probe was labelled with 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE) on the 5′ end and Black Hole Quencher® I dye on the 3′ end. The reaction can be multiplexed using recommended dyes; FAM, JOE, ROX (6-Carboxyl-X-Rhodamine) and Cyanine 5. For multiplexing, each primer and probe set must be optimised individually.

21. The QuantiTect Probe RT-PCR master mix should be stored at −20°C, thawed on ice when required and returned immediately to −20°C. All samples and solutions should be kept on ice.

22. There are other options for real-time PCR machines which include ABI Sequence Detection Systems (Applied Biosystems), Smart Cycler (Cepheid), Light Cycler (Roche Molecular Biochemicals), Mx400 (Stratagene), iCycler (Bio-Rad) and the updated Rotor-gene 6000 system. Each system uses different size and type sample formats.

23. The crossing cycle threshold value (Ct) was the cycle at which there was a significant increase in fluorescence above the specified threshold.

24. It is important to determine the efficiency of the RT-PCR for each target. Prepare a dilution series for each target and amplify by RT-PCR. Calculate the difference in the Ct values between both targets and plot these differences against the logarithm of the template amount. Amplification efficiencies are comparable if the slope of the straight line is <0.1. If this is not the case, then the Pfaffl method can be applied (18). See Table 2 for calculations with and without the Pfaffl correction.

Table 2
Example calculation of relative gene expression

Condition	Reference gene	Target gene	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$	Pfaffl equation[a]	Target gene treated: Control
Control	23.15	27.25	4.1	0	1	14.57	1
Test	22.18	24.23	2.05	–2.05	4.1	3.89	3.74

[a]If efficiency of target gene = 92.2%, so $1.922^{4.1}$ and efficiency of reference gene/internal standard = 94%, so $1.940^{2.05}$ is calculated. Then, the ratio treated to control of target gene is 14.57/3.89

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Chapter 6

Use of DNA Arrays to Study Transcriptional Responses to Antimycobacterial Compounds

Simon J. Waddell and Philip D. Butcher

Abstract

Analysis of the transcriptional profiles of *Mycobacterium tuberculosis* after treatment with antimycobacterial compounds has improved our understanding of the ways mycobacteria respond to antibiotic stress, and revealed new insights into the mode of action of different antimycobacterial compound classes. RNA profiling of drug-induced changes has become an important tool in multiple stages of the antibacterial drug development process from target elucidation, to identifying target drift, and ultimately to revealing drug resistance mechanisms. The transcriptional response of *M. tuberculosis* to antimycobacterial compounds may be determined in isolation, in comparison with other compound classes, or between drug-sensitive and drug-resistant mycobacterial isolates. Additional information confirming the growth state of mycobacteria on addition of the antibacterial compound, and the effect that this compound has on mycobacterial growth, is essential for interpreting the transcriptional signatures acquired. This chapter describes the methods required for the extraction of representative total mycobacterial RNA, the subsequent hybridisation of this RNA to an *M. tuberculosis* complex microarray, and the analysis strategies employed to interpret the transcriptional data generated.

Key words: Microarray, Mycobacterium, Transcriptome, Antibacterial compound, Drug response

1. Introduction

Genome-wide expression profiling technologies have become important tools in the search for improved drug candidates for the treatment of *Mycobacterium tuberculosis*, and the definition of mycobacterial drug resistance mechanisms. The transcriptional response of mycobacteria to antibacterial compounds may be used to further understand the mechanism of drug action (1), to determine common and drug-specific gene expression signatures (2), and to reveal potential detoxification and efflux systems (3). Expression profiling of antibacterial compound-treated *M. tuberculosis* by microarray

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analysis plays an important role at multiple points in the drug discovery process from discriminating antibacterial compounds to identifying target drift (4). Microarray analyses may also be useful in identifying differences between drug-sensitive and drug-resistant mycobacteria at both the DNA and RNA levels (5, 6), thus uncovering novel mechanisms of mycobacterial drug resistance.

This chapter details the extraction of *M. tuberculosis* total RNA from mid-log phase culture, and the hybridisation of this RNA-derived labelled-cDNA to a spotted *M. tuberculosis* complex DNA microarray against a DNA reference (in a common reference/type II experimental design). The choices of compound concentration and timepoints to assay are clearly related and will vary depending on the aims of each study; a range in final concentration from 0.1× to 10× MIC and exposure times from 20 min to 24 h have all been described in the literature. Treatment of 4–6 h with 1–5× MIC antimycobacterial compound, however, is likely to be a reasonable starting point, where a drug-induced transcriptional response of *M. tuberculosis* is detectable but without the complications of multiple secondary effects and extensive mycobacterial killing that will accompany higher compound concentrations or longer exposures.

Transcriptional data exploring the response of mycobacteria to antibacterial compounds may be utilised in two distinct ways, and the system chosen may influence the experimental design. The first strategy focuses on the potential mechanism of action of (or resistance to) a single antimicrobial compound (1). This approach may require multiple timepoints and/or multiple compound concentrations (and/or several mycobacterial strains) to probe the mycobacterial response to a single drug. In this instance, experiments should be designed with a minimum of three biological replicates, as statistical testing will be applied to the microarray data to identify significantly differentially expressed genes.

A second strategy compares the gene expression profiles of *M. tuberculosis* treated with a range of different antibacterial compounds to identify specific and common transcriptional patterns (2). This analysis may require the selection of fixed MIC and timepoint parameters but exposure to multiple antimycobacterial agents. The application of statistical testing using this approach may not be imperative, where robust clustering algorithms will be more useful to distinguish compound class-specific responses.

The sensitivity of mycobacteria to antibiotics has been demonstrated to vary with growth conditions (7), and this may affect the gene expression pattern on exposure to different antimycobacterial compounds. Although the methods below describe the treatment and microarray analysis of *M. tuberculosis* in log phase aerobic growth, the protocols are applicable to any *in vitro* model.

Additionally, the methods detailed here may be used to compare the RNA (or DNA) profiles of susceptible and resistant mycobacterial strains in the absence of drug exposure. The RNA extraction protocol detailed herein is developed from the method described by Mangan *et al.* (8); and the microarray hybridisation protocols are based on methods described by Stewart *et al.* (9) and Hinds *et al.* (10).

2. Materials

2.1. Culture Conditions

1. Log phase mycobacterial broth culture (Notes 1 and 2).
2. Middlebrook 7H9 Broth (BD, Franklin Lakes, USA).
3. Middlebrook ADC Enrichment (BD).
4. Antimycobacterial compounds under test (Sigma, Saint Louis, USA).
5. Spectrophotometer.

2.2. RNA Extraction

1. 30 mL plastic V-bottom universal tubes (VWR, West Chester, USA).
2. GTC solution – 5 M guanidine thiocyanate (Promega, Madison, USA), 0.5% sodium N-lauroyl sarcosine (Sigma), 25 mM sodium citrate (Sigma), 1% Tween-80 (Sigma), 0.1 M β-mercaptoethanol (Sigma) (Note 3).
3. TRIzol Reagent (Invitrogen, Carlsbad, USA) or TRI Reagent (Sigma).
4. 2 mL screw top tubes containing 0.1 mm silica beads (Lysing matrix B, MP Biomedicals, Irvine, California).
5. Reciprocal shaker (FastPrep FP120, MP Biomedicals).
6. Chloroform (VWR).
7. Propan-2-ol (VWR).
8. 70% ethanol (RNase-free).
9. RNase-free water (Sigma).
10. RNeasy Mini Columns (Qiagen, Hilden, Germany).
11. RNase-free DNase kit (Qiagen).

2.3. Microarray Hybridisation

1. Random Primers (3 μg/μL) (Invitrogen).
2. dNTPs (5 mM dATP/dGTP/dTTP, 2 mM dCTP) (Invitrogen).
3. Cy3-dCTP 25 nmol (GE Healthcare, Chalfont St. Giles, UK), light-sensitive.

4. Cy5-dCTP 25 nmol (GE Healthcare), light-sensitive.
5. 5× First Strand Buffer (Invitrogen).
6. Dithiothreitol (100 mM) (Invitrogen).
7. SuperScript II Reverse Transcriptase (200 U/μL) (Invitrogen).
8. 10× REact 2 Buffer (Invitrogen).
9. DNA polymerase I Large Fragment (Klenow) (3–9 U/μL) (Invitrogen).
10. Nuclease-free water (Sigma).
11. Mycobacterial RNA (DNase-treated from 2.2) 5 μg test RNA/hybridisation.
12. Mycobacterial DNA (RNase-treated, phenol/chloroform-free) 2 μg DNA/hybridisation for use as a common reference.
13. MinElute PCR Purification Kit (Qiagen).
14. SSC (saline sodium citrate) 20× (Sigma) 0.2 μm filter-sterilised.
15. SDS (sodium dodecyl sulphate) 20% (w/v) (Flowgen Bioscience, Wilford, UK) 0.2 μm filter-sterilised.
16. Bovine serum albumin (BSA) fraction V 96–99%: 100 mg/mL, 0.2 μm filter-sterilised (Sigma).
17. Pre-hybridisation buffer: 3.5× SSC, 0.1% SDS, 10 mg/mL BSA.
18. Coplin staining jar (Fisher Scientific, Pittsburgh, USA).
19. Slide-staining troughs (Raymond A. Lamb, London, UK).
20. Slide-staining racks (Raymond A. Lamb).
21. 50 mL Falcon tubes (Fisher Scientific).
22. Propan-2-ol (VWR).
23. Hybridisation chamber II (Corning, Corning, USA), Hybridisation cassette (TeleChem International, Sunnyvale, USA), or suitable alternative.
24. 22 × 22 mm LifterSlips (Erie Scientific, Portsmouth, USA).
25. Water bath set at 65°C.
26. Wash A: 1× SSC, 0.05% SDS.
27. Wash B: 0.06× SSC.

2.4. Data Acquisition and Analysis

1. Dual laser microarray scanner such as Affymetrix 418/428 (MWG Biotech, Ebersberg, Germany), Axon GenePix (Molecular Devices, Sunnyvale, USA), or Scanarray (Perkin Elmer, Waltham, USA).
2. Image analysis software such as ImaGene (BioDiscovery, El Segundo, USA), GenePix Pro (Molecular Devices), Quantarray

(Perkin Elmer), or BlueFuse for Microarrays (BlueGnome, Cambridge, UK).

3. Microarray analysis packages such as GeneSpring GX (Agilent Technologies, Santa Clara, USA), GeneSight (BioDiscovery), Rosetta Resolver (Agilent Technologies), BlueFuse for Microarrays (BlueGnome), Cluster and TreeView (11), Significance Analysis of Microarrays (SAM) (12).

3. Methods

3.1. Culture Conditions

1. Culture mycobacteria to mid-log phase in 50–100 mL volumes in Middlebrook 7H9 liquid medium (with added ADC supplement) under aerobic conditions, by either stirring at approximately 280 rpm with a magnetic stirrer or in roller bottles (Notes 1, 2 and 4).
2. Add antimycobacterial compound to required final concentration (Note 5), in addition add an equal volume of compound carrier (Note 6) to a control RNA culture. Ensure good mixing.
3. Incubate for chosen time interval before extracting RNA from mycobacterial cultures.

3.2. RNA Extraction

1. Add the mycobacterial liquid culture to 4 volumes of 5 M GTC solution and mix (Note 7). Aliquot into 30 mL plastic V-bottom universal tubes and centrifuge at 1,500 × *g* for 20 min to pellet the bacteria (Note 8).
2. Remove and discard the supernatant and combine the bacterial pellets from multiple universals, washing each universal with a few milliliters 4 M GTC solution to ensure all bacilli are recovered. Re-centrifuge bacilli in a single universal tube at 1,500 × *g* for 20 min.
3. Remove excess GTC solution, and add 1.2 mL TRIzol reagent (Note 9). Transfer to 2 mL screw-top tube containing 0.5 mL of 0.1 mm silica beads (Lysing matrix B) (Note 10). Lyse bacterial cells using a reciprocal shaker (FastPrep FP120) for 45 s at a speed of 6.5 (Note 1).
4. Incubate disrupted cells at room temperature for 10 min, before adding 200 μL chloroform. Vortex for 30 s and incubate at room temperature for a further 10 min to partition the aqueous and phenolic phases before centrifuging at 13,000 × *g* for 15 min at 4°C.
5. Transfer the aqueous phase to a fresh 1.5 mL microcentrifuge tube and re-extract with an equal volume of chloroform

(centrifuging at 13,000 × *g* for 15 min at 4°C). Precipitate the nucleic acid from aqueous phase in a fresh microcentrifuge tube, adding 0.8 volumes isopropanol, mixing and incubating overnight at –20°C (Note 11).

6. Centrifuge the nucleic acid extractions at 13,000 × *g* for 20 min at 4°C. Remove the supernatant (by pipetting) and discard, before washing the pellets with 500 µL cold 70% ethanol, re-centrifuging at 13,000 × *g* for 15 min at 4°C and removing the ethanol by pipetting. Re-spin the tubes briefly and remove any excess 70% ethanol (Note 12).
7. Air-dry the nucleic acid pellets at room temperature for 5–10 min and then resuspend in 100 µL RNase-free water (Note 13). Store briefly on ice before continuing with the RNA clean-up.
8. DNase I treat and purify the RNA samples using the RNeasy Mini Columns (Qiagen) with reagents provided by the manufacturer (detailed here in steps 8–12). Add 350 µL RLT buffer to each 100 µL RNA sample and mix thoroughly by pipetting (Note 14). Then add 250 µL ethanol, mix, and apply immediately to an RNeasy Mini column placed in a 2 mL collection tube.
9. Centrifuge the columns for 15 s at 8,000 × *g*, then discard the flow-through and collection tubes. Transfer the RNeasy mini columns to new 2 mL tubes, and add 350 µL RWI buffer. Centrifuge for 15 s at 8,000 × *g* and discard the wash solution.
10. DNase I treat the RNA samples on the columns using the Qiagen RNase-free DNase kit. Pipette 80 µL DNase I/buffer RDD mix directly onto the column matrix, and incubate at room temperature for 20 min to remove contaminating DNA (Note 15).
11. Wash the columns by adding 350 µL buffer RWI and centrifuge for 15 s at 8,000 × *g*. Then, apply 500 µL of RPE buffer before centrifuging for 15 s at 8,000 × *g* and discarding the flow through. Add a further 500 µL buffer RPE to each column, and centrifuge for 2 min at 8,000 × *g*. Place the columns into new 2 mL collection tubes and centrifuge for an additional 1 min at 13,000 × *g* to prevent carry-over of ethanol.
12. Transfer the columns to new 1.5 mL microcentrifuge tubes, pipette 50 µL RNase-free water directly onto the column membrane and incubate at room temperature for 2 min, before centrifuging for 1 min at 8,000 × *g* to elute the RNA. Repeat with a second 50 µL elution, combine and store on ice before quantification (Note 16).

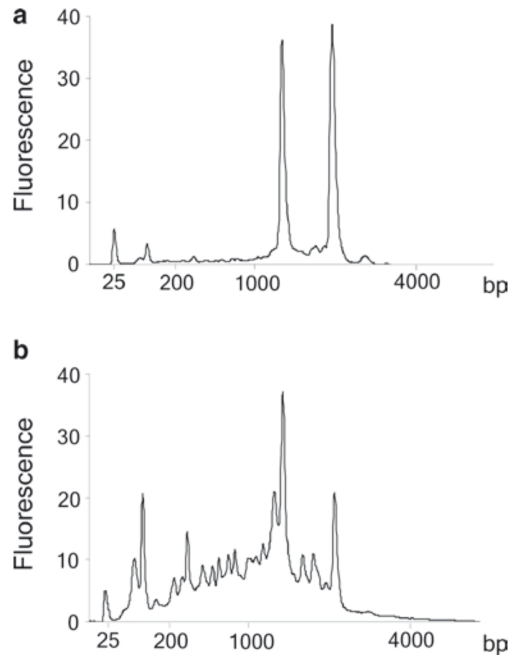

Fig. 1. The size distribution of (**a**) total *M. tuberculosis* RNA extracted using the method described herein showing clearly defined 16s and 23s ribosomal RNA peaks; (**b**) partially degraded total mycobacterial RNA. The peaks at 25 bp represent a marker added to both samples. Abundance units are detailed in relative fluorescence and plotted against migration time that has been converted into a base pair (bp) estimate of product size as measured using the Agilent Bioanalyser.

13. Analyse the RNA extraction yield and the size distribution of nucleic acid using the Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and Agilent 2100 Bioanalyzer NanoChip (Agilent Technologies, Santa Clara, USA) systems according to manufacturers' instructions. The size distributions of representative mycobacterial total RNA preparations are depicted in Fig. 1. Store the RNA at –70°C.

3.3. Microarray Hybridisation

1. Prepare one Cy3 and one Cy5 sample per microarray hybridisation (Notes 17 and 18). For RNA labelling, add 3 μg random primers to 2–10 μg total mycobacterial RNA in a final volume of 11 μL (made up to the correct volume with RNase-free water) (Note 19). Heat to 95°C for 5 min, snap cool on ice and centrifuge briefly.
2. Add 5 μL 5× First Strand buffer, 2.5 μL DTT, 2.3 μL dNTPs, 1.7 μL Cy3-dCTP OR Cy5-dCTP, and 2.5 μL Superscript II Reverse Transcriptase. Incubate at 25°C for 10 min, followed by 42°C for 90 min (Notes 20 and 21).
3. For DNA labelling, add 3 μg random primers to 1–5 μg DNA in a final volume of 41.5 μL (made up to the correct volume

with DNase-free water) (Note 19). Heat to 95°C for 5 min, snap cool on ice and centrifuge briefly.

4. Add 8.5 μL master mix containing 5 μL 10× REact 2 buffer, 1 μL dNTPs, 1.5 μL Cy3-dCTP OR Cy5-dCTP, and 1 μL DNA polymerase I large fragment (Klenow) (Notes 20 and 21). Mix and incubate at 37°C for 90 min.
5. Centrifuge briefly (15 s at 13,000 × *g*), combine Cy3- and Cy5-labelled samples in a single tube (Note 22), before adding 500 μL Buffer PB (from MinElute PCR Purification Kit, Qiagen).
6. Mix and apply to MinElute column and centrifuge at 10,000 × *g* for 1 min (Note 23).
7. Discard flow-through and place column back into the same collection tube.
8. To wash, add 500 μL Buffer PE to MinElute column and centrifuge for 1 min (at 10,000 × *g*), discard flow-through and place column back into the same collection tube.
9. Add 250 μL Buffer PE to the column and centrifuge at 10,000 × *g* for 1 min, discard flow-through and place MinElute column back into the same collection tube.
10. Centrifuge at 10,000 × *g* for an additional minute to remove residual wash buffer, place MinElute column into a fresh 1.5 mL tube.
11. Add 40 μL Nuclease-free water to the column membrane; incubate at room temperature for 1 min, then centrifuge at 10,000 × *g* for 1 min (Note 24).
12. Add 12 μL 20× SSC and 9 μL 2% SDS to the labelled nucleic acid samples (final concentration of 4× SSC and 0.3% SDS), and mix thoroughly (Notes 24 and 25).
13. Heat samples at 95°C for 2 min in a heat block or thermocycler. Allow to cool slowly (approximately 2–5 min, do not place on ice), centrifuge briefly, and store in the dark while the hybridisation chambers are set up (Note 21).
14. Mix 50 mL of pre-hybridisation solution in a Coplin jar and incubate at 65°C for 20–30 min to equilibrate (this can be performed while the nucleic acid samples are labelling).
15. Incubate the microarray slide in the pre-heated pre-hybridisation buffer at 65°C for 20 min.
16. Place the slide in a slide rack and rinse in 500 mL water in a staining trough for 1 min with agitation.
17. Rinse the slide in 500 mL propan-2-ol for 1 minute with agitation (Note 26).
18. Place the slide into 50 mL Falcon tube and centrifuge at 400 × *g* for 5 min to dry (Note 27). Store in a dark, dust-free box until hybridisation.

19. Place the pre-hybridised microarray slides into the hybridisation cassettes; add two 15 μL aliquots of 0.2 μm filtered Nuclease-free water to the wells in each cassette.
20. Carefully place LifterSlip(s) over the printed area of the microarray slide, ensuring that the LifterSlip is the correct way up and is not scratched or dusty (Note 28).
21. Slowly pipette the hybridisation sample (from Subheading 3.3, item 13) underneath the LifterSlip, the solution should be drawn evenly under the LifterSlip by capillary action, pipette any excess to top and bottom of LifterSlip edges.
22. Seal the hybridisation cassette, submerge immediately in water bath at 65°C; incubate overnight (16–20 h) (Note 29).
23. Pre-heat 500 mL of Wash A buffer and staining trough to 65°C (Note 30).
24. Remove the slide from hybridisation cassette, immediately dip the slide into Wash A, allow the coverslip(s) to fall off in the buffer, then place the slide into a slide rack submerged in Wash A in the staining trough.
25. Agitate in Wash A for 2 min (Notes 31 and 32).
26. Agitate in 500 mL of Wash B (at room temperature) for 2 min. Transfer into a second staining trough of 500 mL Wash B for a further 2 min.
27. Centrifuge in 50 mL Falcon tubes at $400 \times g$ for 5 min (Note 27).
28. Carefully place the slides into a dust-free slide box, store in the dark; scan immediately (Note 21).

3.4. Data Acquisition and Analysis

1. Scan microarray sequentially at 532 nm and 635 nm corresponding to Cy3 and Cy5 excitation maxima using a dual laser microarray scanner. Microarrays should be scanned to achieve the best dynamic range (Note 33).
2. Derive comparative spot intensities and apply flagging algorithms using image analysis software such as ImaGene, GenePix Pro, Quantarray or BlueFuse for Microarrays (Note 34).
3. Import into further analysis packages such as GeneSpring GX, SAM (12), or Cluster (11). Determine significantly differentially expressed genes by comparison of compound-treated to carrier control-treated cultures using, for example, ANOVA or *t*-test ($p < 0.05$), and applying a multiple testing correction (*e.g.* Benjamani and Hochberg (13)). Significant genelists may be further queried against functional classifications, as defined by Cole *et al.* (14), or genelists derived from other relevant treatments/conditions using the hypergeometric function (3). Figure 2 displays the genes significantly

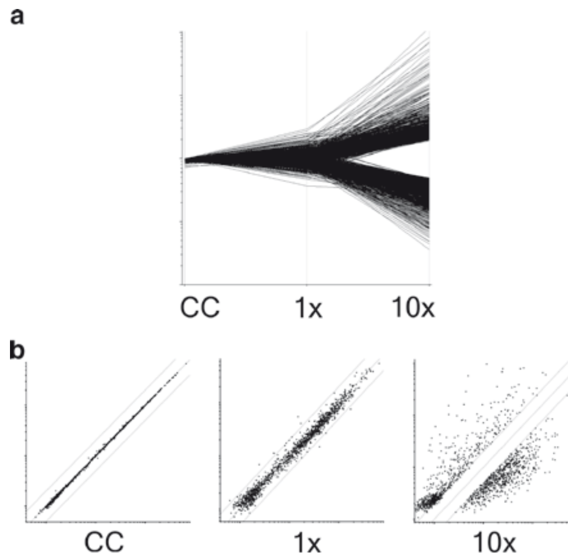

Fig. 2. Graphs plotting the expression ratio of *M. tuberculosis* genes shown to be significantly differentially expressed after 4 h treatment with 10× MIC of an antimycobacterial compound. (**a**) A line graph showing the expression of these genes in three conditions: carrier control (CC), 1× MIC and 10× MIC. The *y*-axis indicating fold change relative to carrier control. (**b**) The same data plotted as three scatterplots, one for each condition: carrier control (CC), 1× MIC and 10× MIC. *x* and *y*-axis plotting the adjusted signal intensity for each channel after normalisation to CC. The diagonal lines mark genes >2-fold induced, with a ratio of 1:1 (unchanged), and >2-fold repressed compared to carrier control.

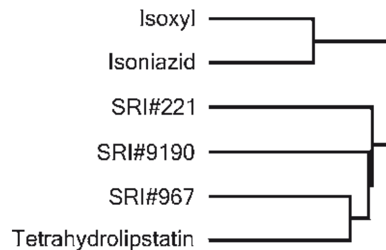

Fig. 3. A cluster diagram describing the relationship between *M. tuberculosis* responses to six compounds with antimycobacterial activity. This Pearson correlation was generated in Cluster and displayed in TreeView (11) using genes identified to be significantly differentially expressed by one or more of these treatments (3).

induced or repressed after addition of an antibacterial compound at 1× and 10× MIC in comparison with the control mycobacterial cultures treated with carrier alone. Figure 3 illustrates the clustering of *M. tuberculosis* responses to a range of antimycobacterial agents. Table 1 lists the genes identified by several groups to be induced by isoniazid treatment.

Table 1
Mycobacterial genes induced by isoniazid treatment, implicating the FAS-II mycolic acid elongation cycle as a potential metabolic pathway perturbed by isoniazid (and as such a possible target for drug action), and genes putatively involved in drug resistance (Note 35). These genes were demonstrated to be induced from the microarray analysis of *M. tuberculosis* exposed to isoniazid in three independent studies (1, 3, 17)

Rv no.	Name	Product	Function
Rv0341	*iniB*	Isoniazid-inducible protein	Unknown
Rv1772	*Rv1772*	Hypothetical protein	Unknown
Rv2243	*fabD*	Malonyl CoA-ACP transacylase	Malonyl-CoA-ACP transacylase
Rv2244	*acpM*	Acyl carrier protein	Mycolic acid biosynthesis (FAS- II)
Rv2245	*kasA*	β-Ketoacyl synthase	Mycolic acid biosynthesis (FAS- II)
Rv2246	*kasB*	β-Ketoacyl synthase	Mycolic acid biosynthesis (FAS- II)
Rv2846c	*efpA*	Efflux protein	Export of undetermined substrate

4. Notes

1. All cultures with Hazard Group III pathogens should be handled in accordance with Health and Safety Legislation current in the country in which experiments are being performed, and according to local Health and Safety regulations. It is essential to perform all the work with live organism up to the addition of TRIzol Reagent (which contains phenol) in a Class I biological safety cabinet in a Category III laboratory according to local Health and Safety regulations.
2. 50 mL mid-log phase culture (OD^{580} of approx. 0.3–0.5) should yield 50–100 μg total RNA. It is advisable to set-up an additional control culture to monitor OD^{580} readings though the growth curve to aid reproducibility and ensure the cultures are in log phase when the antibacterial compound is added. Differences in growth phase between multiple conditions/strains may contribute significantly to variation in mycobacterial transcriptional responses, and therefore confound microarray comparisons. If statistical testing is to be applied to the transcriptional data to identify significantly differentially expressed genes a minimum of three biological replicates should be performed.

3. 500 mL 5 M GTC Solution – 295.4 g Guanidine thiocyanate, 2.5 g N-lauroyl-sarcosine, 12.5 mL 1 M sodium citrate (pH 7.0), 5 mL Tween 80, 3.5 mL β-mercaptoethanol. Add GTC powder to graduated 500 mL flask. Add approximately 200 mL dH_2O, mix and leave in warm room overnight (reaction is endothermic, GTC dissolves slowly, shake occasionally). Do not microwave to heat. When GTC powder has dissolved, add remaining constituents (except β-mercaptoethanol). Adjust volume to 500 mL by adding dH_2O. Store at room temperature. Add β-mercaptoethanol before use. Discard if GTC Solution develops a yellow colour.
4. To make 400 mL Middlebrook 7H9 liquid medium (with added ADC supplement) dissolve 1.88 g 7H9 broth base in 360 mL water, add 200 μL Tween 80 or 800 μL glycerol, autoclave at 120°C for 15 min before aseptically adding 40 mL ADC Enrichment supplement, leave at 37°C for 24 h to test for sterility (15).
5. A final antibacterial compound concentration of 1–5× MIC and an exposure of 4–6 h would be a recommended starting point. The MIC of each compound should be determined in the same culture system used for the RNA extractions. Additional information on the solubility and stability of the compound will aid the selection of suitable experimental parameters. Filter sterilisation of the antimycobacterial compound should be considered if extended treatments are required.
6. The transcriptional response to antimycobacterial compounds should be determined by comparing the profile of drug-treated to the mycobacterial response to compound carrier alone, *i.e.* the same volume of the solvent the compound was resuspended in, often DMSO, dH_2O or methanol. In this way gene expression changes due to addition of the solvent alone are removed from the analysis. It is advisable to set up carrier control cultures alongside compound-treated cultures to help reduce experimental variation.
7. Pre-aliquot the GTC solution into suitable containers, then add the mycobacterial culture directly to the flask as soon as it is removed from the incubator. Transcription ceases on addition of GTC solution; do not centrifuge bacterial cultures or place on ice before adding GTC solution, as the transcriptional profile of bacilli will change. Mycobacteria should not lyse on addition of GTC solution; an increase in viscosity on addition of GTC solution may indicate contamination with other bacteria.
8. Plastic universals with V-bottoms rather than falcon tubes should be used, as the bacilli centrifuge into tighter pellets

using universal tubes and it is easier to pour off the supernatant without dislodging the bacterial pellet.

9. If RNA extraction is to be completed at a different location, add 1 mL TRIzol Reagent to each bacterial pellet, mix and store at –80°C. Transport as hazardous substance, preferably on dry ice. Otherwise, avoid freezing RNA samples by continuing immediately with RNA purification.
10. Use approximately 1 ribolyser tube/50 mL mid-log phase mycobacterial culture.
11. Nucleic acids may be precipitated for several hours at –70°C or on dry ice; however, overnight at –20°C is recommended. It is not necessary to add additional salt to increase precipitation efficiency, and the use of coloured precipitation reagents is not recommended.
12. A white nucleic acid pellet should be visible from mid-log phase culture volumes of >30 mL, the pellet may be invisible with lower numbers of bacilli.
13. 100 μL RNase-free water is used to resuspend the nucleic acids extracted from every 50 mL of mid-log phase culture volume (and cleaned up using a single RNeasy column). RNA preparations may be stored at –70°C at this point, although it is recommended to continue immediately with RNA clean up to avoid freeze-thaw cycles.
14. A final concentration of 10 μl/mL β-mercaptoethanol was added to buffer RLT immediately before use.
15. Combine 10 μL DNase I with 70 μL RDD buffer before adding to the columns. A minimum incubation time of 15 min is recommended, 30 min maximum. If RNA samples are to be used for applications that are particularly sensitive to genomic DNA such as quantitative RT-PCR, DNase I treatment may be repeated several times after washing with RW1 buffer.
16. Elution volumes should be a minimum of 30 μL, maximum 100 μL. A second elution is recommended to increase RNA yield. Eluate may be re-applied to the column to reduce the final volume. Quantitate RNA sample yields before freezing, or remove two 2 μL aliquots of each RNA preparation for Nanodrop and Bioanalyser analyses before freezing to avoid freeze–thaw cycles.
17. In many microarray studies where multiple samples are to be compared, a common reference is used (type II experimental design), and the samples compared indirectly (Cy3 reference *vs.* Cy5 RNA1 or RNA2) rather than performing multiple direct comparisons (Cy3 RNA1 *vs.* Cy5 RNA2). The common reference should hybridise to all (or at least most) of the elements on the microarray (so accurate Cy3/5 ratios can be cal-

culated). We therefore use a common reference of H37Rv genomic DNA. Stocks of *M. tuberculosis* genomic DNA may be requested from Colorado State University, TB Vaccine Testing and Research Materials (HHSN266200400091C), to preserve DNA quality standards across multiple microarray hybridisations. DNA amplification may be required where the same common reference DNA is required for many microarray analyses (for example, the REPLI-g system, Qiagen).

18. Cy3 or Cy5 channels may be used as the reference channel (if using), we commonly use Cy3 for the DNA reference. A dye swap experiment (where the same samples are labelled with the alternative Cy-dye) may also be useful to ensure that any differences identified are not related to dye-dependent effects.
19. We generally use random primers to prime the labelling reactions; however, genome-directed primers designed against the mycobacterial genome are an alternative (16).
20. This protocol describes the direct labelling of nucleic acid with Cy3/5-dCTP, dCTP was chosen as mycobacterial genomes are particularly GC rich. Labelling efficiency may be checked by spectrophotometer (NanoDrop ND-1000) if problems arise.
21. The Cy-dyes are sensitive to light; therefore incubate and store tubes or hybridised slides in the dark. We also recommend using amber-coloured tubes to help reduce this problem.
22. If using a common reference combine the reference labelling reactions before re-distributing into Cy3/5 reaction mixes, this will help to reduce array/array variation.
23. We use Qiagen MinElute columns for purification of the labelled samples; however, a number of similar commercially available columns are also suitable. Ethanol precipitation is also effective at removing unincorporated Cy-dye, with the addition of 1/10 volume 3 M sodium acetate and 0.8% volume 100% isopropanol for 30 min at –20°C, followed by a 70% ethanol wash.
24. The hybridisation volumes detailed in this protocol are for use with a 22 × 50 mm LifterSlip area; if using a single 22 × 22 mm LifterSlip a final hybridisation volume of around 23 μL is recommended (elute in 15.9 μL RNase-free water, add 3.5 μL SDS, 4.6 μL SSC). This volume should be adjusted down if flat coverslips are used. We have found that hybridisations using LifterSlips are more even, and there is less array/array and user/user variation compared to conventional flat coverslips.

25. Do not create an SSC/SDS hybridisation master mix, as the SDS will begin to precipitate out. Pipette the SSC and SDS separately, mixing thoroughly between additions.
26. It is possible to re-cycle the propan-2-ol used to rinse the pre-hybridised slides, change periodically depending on the frequency of use. Discard water used to wash slides after 8 slides.
27. Slides should be centrifuged immediately after washing, to prevent any drying of the wash buffers onto the microarray surface. Slides should be placed microarray side down and label side down in the Falcon tubes for centrifugation to help prevent scratching of the array surface.
28. Compressed air may be used to remove dust from slides, coverslips, and slide boxes. Take care not to expel vapour from the pressurised canister.
29. The hybridisation temperature using this method is 65°C. Lower temperatures may be required for different microarray formats using formamide-based systems.
30. Prepare Wash A buffer while labelling nucleic acid samples, and incubate overnight at 65°C with staining trough.
31. Performing the first wash at 65°C helps to remove unwanted background from the microarrays, and retains the hybridisation stringency through the first wash. When washing, repeatedly raise and lower slide rack in wash buffer keeping the slides immersed in buffer as far as possible. Do not splash Wash A into Wash B containers, and clean or change gloves between washes to limit the transfer of SDS from Wash A to Wash B. If washing multiple microarray slides, leave the slides in the slide rack in Wash A while others are unpacked. We wash 4 microarrays at a time, and recommend changing the Wash B buffers every 8 slides.
32. High background in the Cy3 channel is likely due to excess salt, or drying of the slides between wash steps; high background in the Cy5 channel may be due to SDS coming though the washes. If Cy3 or Cy5 background remains consistently high, review washing process changing gloves and/or slide racks between washes, and/or adding an additional Wash B step.
33. A pre-scan may often be helpful to check correct slide orientation and define scan area. When scanning adjust PMT (voltage of the photomultiplier tube) or laser settings to scan just below saturation (excluding control spots) in each channel to maximise the dynamic range. Multiple scans using different PMT/laser settings may be useful if image analysis packages such as MAVI Pro.2.6.0 (MWG Biotech) are to be used. Avoid multiple test scans if possible as photobleaching may occur.

34. Microarray data should also be deposited into an MIAME-compliant (minimum information about a microarray experiment) database such as ArrayExpress (http://www.ebi.ac.uk/arrayexpress), or other accessible databases such as GEO (http://www.ncbi.nlm.nih.gov/geo) or CIBEX (http://cibex.nig.ac.jp).
35. Several genes in the same pathway that are consistently differentially expressed after drug treatment may represent the metabolic process directly targeted by the antimycobacterial compound; however, there will also be potentially confounding secondary effects of drug treatment due to the interruption of this critical pathway and cell death. The induction of genes involved in export or degradation on drug exposure may highlight functionally significant mycobacterial drug resistance mechanisms. Similarly, the identification of genes differentially expressed between sensitive and resistant mycobacterial strains may reveal potential drug target pathways, or genes directly involved in drug resistance.

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Part III

Fitness Mutation and Physiology

Chapter 7

Assays of Sensitivity of Antibiotic-Resistant Bacteria to Hydrogen Peroxide and Measurement of Catalase Activity

Mirjana Macvanin and Diarmaid Hughes

Abstract

Bacteria, in common with other organisms that take advantage of aerobic respiration, generate and accumulate reactive oxygen species (ROS) that damage DNA, fatty acids, and proteins. In addition, intracellular pathogens like *Salmonella enterica* are exposed to an oxidate burst produced by host macrophages. The relative ability of aerobically growing bacteria to withstand oxidative stress and eliminate ROS has a large impact of their fitness in vitro and in vivo.

Methods are described here to measure the viability and relative fitness of bacteria in the presence of hydrogen peroxide. A protocol for the determination of catalase activity, an important part of the ROS detoxification process, is also described.

Key words: Antibiotic resistance, Fitness cost, Reactive oxygen species, Hydrogen peroxide, Catalase, *Salmonella typhimurium*

1. Introduction

Aerobic respiration, with molecular oxygen (O_2) as the electron acceptor, is the most efficient means of cellular energy production. However, the incomplete reduction of O_2 during respiration results in the production of several major reactive oxygen species (ROS) such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). Superoxide anions are generated as a by-product of electron transport in respiration and can be converted to H_2O_2 either enzymatically or non-enzymatically (1). The rate of H_2O_2 generation increases tenfold during the exponential phase of aerobic growth in *Escherichia coli* (2). Hydroxyl radicals are generated via the Fenton reaction when an electron from ferrous iron is transferred to hydrogen peroxide.

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The hydroxyl radical is the most reactive of these three species, but all ROS are toxic to bacteria. The harmful effects of ROS include damage to DNA; oxidation of fatty acids in lipids; and oxidation of amino acids in proteins. Together, these effects of ROS on the cell are referred to as oxidative stress.

Bacteria, in common with other organisms that take advantage of aerobic respiration, must be able to defend themselves against the accumulation of the ROS that they generate. Responses to oxidative stress involve complex mechanisms which include alterations in gene expression patterns and the activation of a cascade of enzymes which prevent ROS accumulation. The exposure of enteric bacteria to ROS results in extensive alterations in protein expression patterns. Thus, in *E. coli* more than 100 genes were observed to have altered levels of expression upon exposure to superoxide (3), or to hydrogen peroxide (4). The relative ability of aerobically growing bacteria to withstand oxidative stress can have a large impact on their fitness. However, the relationship between effectiveness of stress responses and fitness is complex and involves trade-offs between stress resistance and the ability to compete for growth substrates, elevation of mutation rates, and virulence (5–7).

There is no single metabolic pathway that is able to eliminate the ROS in one step, and aerobically growing bacteria produce two types of enzymes to combat these agents: superoxide dismutase, which reduces O_2^- to H_2O_2, and catalase, which converts H_2O_2 to water. Note that both catalase and peroxidase catalyse the breakdown of H_2O_2 to water and oxygen. The difference between catalase and peroxidise activity is in the actual sequence of steps of chemical conversion of H_2O_2. In the catalase reaction, in the first step of catalysis, H_2O_2 is added to the ferric enzyme and converted to water and oxygen bound to iron. This compound (compound I) can react with a second molecule of H_2O_2 reducing it back to the ferric state. In contrast, in a peroxidise reaction, compound I is reduced in a one-electron reduction to compound II. Compound II has a spectrum very similar to that of the ferric form, so it may possess an amino acid radical. Finally, compound II is reduced back to the ferric enzyme in a second one-electron reduction. The molecular details of catalase versus peroxidise activity are not discussed further in this review or in the literature referenced.

The process of detoxification of ROS is very efficient, which is reflected in the finding that in wild-type *E. coli* intracellular H_2O_2 concentration is kept at a steady-state value of around 0.2 μM over a broad range of cell densities in a rich medium (2). Enteric bacteria such as *Salmonella enterica* and *E. coli* possess two catalases. One is the bifunctional catalase hydroperoxidase I (HPI), encoded by the *katG* gene, which is active as tetramer with subunits of 81 kDa (8). The other catalase is the monofunctional HPII, encoded by the *katE* gene, which is also active as a tetramer,

with subunits of 78 kDa (9). HPI is transcriptionally induced by OxyR as part of a genetic response to H_2O_2 (10). HPI activity is observed in the periplasm and in cytoplasmic membrane fractions (11). HPII is under the control of *rpoS* (12) and is localized in the cytoplasm (11).

The magnitude of the fitness cost of some antibiotic resistance mechanisms may depend on how they affect the ability of the resistant strains to withstand oxidative stress. For instance, strains of *Salmonella* resistant to the antibiotic fusidic acid have decreased levels of both HPI and HPII catalases, which results in their increased sensitivity to hydrogen peroxide in vitro, in laboratory media, and in vivo, in experimental animals. The fitness of these strains is greatly improved under anoxic conditions of growth, suggesting that hypersensitivity to oxidative stress contributes to the fitness cost of fusidic acid resistance during aerobiosis (13).

The purpose of this chapter is to describe assays useful for the measurement of sensitivity of antibiotic-resistant bacteria to oxidative stress. The protocols listed below detail procedures for the determination of bacterial viability and relative fitness in the presence of hydrogen peroxide. The details are primarily derived from papers published by the authors (13, 14). The protocol for the determination of catalase activity, based on the method by Winquist et al. (15), is given here with minor modifications, as described in Ref. (13).

2. Materials

2.1. Growth of Bacteria

1. Luria Bertani growth medium.
2. Minimal M9 medium containing 0.2% glucose.
3. Spectrophotometer.
4. LA plates (LB supplemented with 1.5% agar; Oxoid, Basingstoke, England).
5. 0.9% NaCl.

2.2. Hydrogen Peroxide Sensitivity Assay

1. Hydrogen peroxide (30% stock in water, Merck).
2. Minimal M9 glucose medium supplemented with 0.2% glucose.
3. Luria Bertani medium.
4. 0.9% NaCl.
5. LA plates.
6. LA plates with appropriate antibiotic (see Note 1).

2.3. Catalase Activity Assay

1. Dicarboxidine (γγ′-(4,4′-diamino-3,3-biphenylylenedioxy) dibutiric acid, Sigma-Aldrich).
2. Lactoperoxidase (EC 1.11.1.7) (170 U/mg of protein; Fluka/Sigma-Aldrich).
3. Hydrogen peroxide (30% stock, Merck).
4. Minimal M9 medium supplemented with 0.2% glucose.
5. Minimal M9 medium supplemented with 0.2% glycerol.
6. Shaking water bath set at 37°C.
7. Spectrophotometer.
8. Cuvettes for spectrophotometer.
9. Centrifuge capable of supporting sample formats of at least 30 mL.
10. 30 mL centrifuge “Corex” tubes.

3. Methods

3.1. Hydrogen Peroxide Sensitivity Assays

Three methods, which can be used for testing bacterial sensitivity to hydrogen peroxide, are described: the first two methods listed below are used to estimate the number of viable cells after exposure to hydrogen peroxide. The third method is used to measure the relative fitness of an antibiotic-resistant strain in the presence of hydrogen peroxide.

3.1.1. Viability of Bacteria Grown in LB Medium in the Presence of Hydrogen Peroxide

1. Cultivate the bacteria in Luria Bertani medium until mid-exponential phase, to an optical density of 0.2–0.4 at 600 nm.
2. To determine the viable count of the culture prior to treatment with hydrogen peroxide, make serial dilutions of the culture sample in 0.9% NaCl and plate them onto LA.
3. Add hydrogen peroxide to the culture to a final concentration of 2.5 M (see Note 2).
4. To determine viability, take 100 μL aliquots at indicated time points (usually 15, 30, and 45 min). Make serial dilutions of these samples in 0.9% NaCl and plate them onto LA. Incubate the plates on 37°C overnight.
5. Count colony forming units (CFU) after an overnight incubation at 37°C. Plot the number of viable cells (CFU/mL) as a function of time of incubation in the presence of hydrogen peroxide. The number of viable cells prior to exposure to hydrogen peroxide is expressed as 100%. From the figure, estimate the percentage of viable cells remaining after the treatment with hydrogen peroxide.

3.1.2. Measurement of Bacterial Viability in M9 Minimal Medium in the Presence of Hydrogen Peroxide

1. Grow an overnight culture of bacterial strain in M9 minimal glucose medium.
2. From an overnight culture, inoculate 1×10^6 to 2×10^6 cells/mL in minimal glucose medium containing 70 µM H_2O_2 (see Note 3).
3. Incubate the culture at 37°C without shaking.
4. Take 100 µL samples over the course of 24 h (see Note 4).
5. Make serial dilutions (10^{-1} to 10^{-7}) of the samples in 0.9% NaCl and spread them onto LA. CFU after an overnight incubation at 37°C (see Note 5).
6. To monitor the extent of growth inhibition by hydrogen peroxide, plot CFU/mL as a function of number of hours of incubation in the presence of hydrogen peroxide.

3.1.3. Competitions in M9 Minimal Medium Supplemented with Hydrogen Peroxide

1. Start 3–4 mL volume overnight cultures of a wild-type strain (as a standard control) and the mutant strain(s) of interest in minimal M9 medium with glucose.
2. Inoculate a total of 1×10^6 cells of the wild type and the mutant strain (1:1 ratio) per mL into M9 minimal medium containing 0.2% glucose supplemented with 70 µM H_2O_2.
3. Incubate the mixed cultures at 37°C for 18 h with shaking.
4. Counts of viable wild-type and mutant cells are made by taking aliquots after the incubation period, diluting them, and plating them onto both LA and LA with the appropriate antibiotic (see Note 6).
5. The competition index (C.I.) is expressed as the ratio of mutant/wild-type cells measured in CFU at the end of one cycle of growth competition, normalized to the ratio of mutant to wild-type at start of the experiment (time zero).

Thus, C.I. = [CFU mutant t1/CFU wild-type t1]/[CFU mutant t0/CFU wild-type t0].

3.2. Catalase Activity

Relative catalase activity can be quantified by a colorimetric assay based on the use of dicarboxidine, a sensitive substrate for the detection of peroxidase activity (16). Dicarboxidine is converted into a coloured product in a reaction catalysed by the activity of lactoperoxidase. The amount of colour developed is directly proportional to the amount of H_2O_2 present in the medium (13, 15).

1. Grow a culture of the wild-type (standard control strain) and the bacterial strain(s) of interest in M9 glucose minimal medium to mid-exponential phase (OD_{460} 0.2–0.4). See Note 7 regarding assays on other bacterial species.

2. Centrifuge 30 mL volumes of each culture. Wash the pellet in fresh minimal M9 medium without a carbon source.
3. Resuspend the cell pellet in M9 medium with 0.2% glycerol as a carbon source, pre-warmed at 37°C (see Note 8). The concentration of cells at the start of the assay should be adjusted to 2×10^8 cells/mL.
4. Immediately before each experiment, mix a solution of 50 μg of lactoperoxidase/mL with an equal volume of 1 mM dicarboxidine solution in water.
5. To begin the reaction, add H_2O_2 to the bacterial culture to a final concentration of 100 μM. Incubate the culture at 37°C with agitation. Take a 1-mL sample immediately after the addition of H_2O_2. Then take 1 mL samples at regular time intervals (usually each 5 min) up to 45 min.
6. Add each sample immediately to a 200-μL aliquot of the dicarboxidine–lactoperoxidase mixture at room temperature (see Note 9).
7. Measure the absorbance of the samples in colorimeter at 450 nm (see Note 10).
8. The decrease in OD_{450} as function of time is plotted for the wild-type sample and for the sample of the mutant of interest. Catalase activity for the wild-type is expressed as 100%. Relative catalase activity is estimated by comparing the slope obtained for the mutant with the slope for the wild-type sample.

4. Notes

1. LA plates with antibiotic are used to score the number of viable cells of the assayed antibiotic-resistant mutant. In our experiments with fusidic acid-resistant (FusR) *Salmonella enterica* serovar Typhimurium, we used LA containing 15 μg/mL tetracycline, since FusR strains with mutations in the *fusA* gene were "tagged" with Tn10 (*fusA zhb-736*::*Tn*10). Although it is advisable to "tag" the antibiotic-resistant mutant of interest with a neutral marker, it is also possible to use the actual antibiotic resistance phenotype of the mutant for the analysis of growth in this experiment.
2. 2.5 M hydrogen peroxide in LB medium is a commonly used concentration in assays of determination of bacterial sensitivity to oxidative stress (17, 18). However, the concentration of hydrogen peroxide in LB medium can be varied in the range from micromolar to millimolar concentrations. Previous studies have shown that bacterial killing by micromolar

concentrations of H_2O_2 (mode I bacterial killing) is mediated by iron-dependent DNA damage, while killing by millimolar H_2O_2 concentrations (mode II killing) is caused by oxidative damage to multiple cellular targets (19). To test mode I killing, H_2O_2 concentration can be decreased to 750 μM H_2O_2 whereas for mode II killing, a concentration of 4 mM H_2O_2 is commonly used (20).

To obtain an accurate concentration of hydrogen peroxide in the micromolar range, a commercially available stock (30%) could be diluted in water prior to addition to the growth medium.

3. Sensitivity to H_2O_2 is more pronounced in M9 minimal medium than in LB. We assayed bacterial survival in various H_2O_2 concentrations ranging from 1 μM to 10 mM in M9 medium supplemented with glucose and found that survival of *Salmonella* strains in the presence of 70 μm H_2O_2 corresponds to their survival in macrophages and in the mouse infection model (14). This H_2O_2 concentration in minimal medium is recommended if in vivo conditions of survival are to be mimicked, because it approximates the concentration generated during the respiratory burst of macrophages (14, 21–23).

4. We found that the viability of *Salmonella* strains upon exposure to 70 μm H_2O_2 in M9 minimal medium with glucose does not change significantly over the course of the first 6 h of incubation (14). Therefore, it is sufficient to take a sample for measurement of the number of viable cells 6 h after the beginning of incubation.

   We observed, however, that a decrease in the number of viable cells in the presence of 70 μm H_2O_2 in minimal medium occurs after 8 h of incubation and continues decreasing up to 24 h of incubation. For the wild-type *Salmonella* strain LT2, a 100-fold decrease in the number of viable cells is measured after 22 h of incubation in 70 μm H_2O_2 in minimal medium. For the hydrogen peroxide hypersensitive fusidic acid-resistant mutants, 4–6 logs of killing are observed after 22 h of incubation in the presence of 70 μm H_2O_2 (14). For this reason, during this period, it is advisable to take samples for the determination of viable count each hour, or as frequently as possible.

5. It is advised that the remainder of each culture is incubated for several additional days (usually 2–3 days). The estimation of viable counts as described above should be carried out to determine whether any living cells remained after prolonged H_2O_2 treatment.

6. For competition assays, the mutant strain should be tagged with an antibiotic resistance marker (see Note 1). The total number

of cells (wild-type + mutant) is scored as CFU on LA, whereas the number of mutant cells is scored as CFU on antibiotic-containing plates.

7. M9 minimal medium with either glucose or glycerol as a carbon source is suitable for *E. coli* and *S. enterica* species. The fitness assay described here is relevant for intracellular pathogens like *S. enterica* that are exposed to ROS produced during the oxidative burst in macrophages. Those wishing to make similar measurements on other bacterial species will first have to test the suitability of the available growth media for this assay. Possible minimal media that could be used have been described for *Mycobacteria* (24) and for *Listeria monocytogenes* (25, 26).
8. We found that the presence of glucose in the medium interferes with the sensitivity of the reaction; thus, glucose is replaced with glycerol which does not exert any influence on this reaction. We do not know the reason for this. Is it possible that glucose binds to dicarboxidine whereas glycerol does not?
9. Within 1 min a stable light-yellow colour develops.
10. The decrease in the amount of developed colour as a function of time is proportional to the amount of H_2O_2 degraded by catalase and is thus inversely proportional to catalase activity.

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Chapter 8

Measurements of Heme Levels and Respiration Rate in *Salmonella enterica* Serovar Typhimurium

Diarmaid Hughes and Mirjana Macvanin

Abstract

This chapter describes assays for the measurement of heme levels and the rate of respiration in bacteria. An assay of ALA supplementation is described, in which the effect of exogenous ALA in reversing sensitivity to hydrogen peroxide is an indication of a reduced flow through the heme biosynthesis pathway. A protocol for measurement of the relative amount of heme by a fluorescence assay, based on stripping the iron from the heme moiety, leaving a protoporphyrin molecule which fluoresces following excitation at 400 nm, is also provided. Finally, a method for the measurement of respiration (oxygen consumption) rate is provided. In this method, the respiration of the cell population is expressed as the specific respiration rate during one doubling time of the population.

Key words: Antibiotic resistance, Fitness costs, Heme, Respiration, *Salmonella typhimurium*, Aminolevulinic acid (ALA)

1. Introduction

Proteins carrying a prosthetic heme group are integral components of energy-conserving electron transport chains and of enzymes involved in bacterial metabolic and stress responses. In *Salmonella* and *Escherichia coli*, heme *b* (Fe protoporphyrin IX or protoheme) and various modified hemes are cofactors for a number of cytochromes as well as two catalases (1). Cytochromes of the respiratory chain are an essential part of the process of generation of a proton motive force across the bacterial membrane, with oxygen as a terminal electron acceptor. This process drives the efficient synthesis of ATP (2–4). Catalases are enzymes which convert hydrogen peroxide to water and thus protect cells from the harmful accumulation of reactive oxygen species (ROS). In addition, the heme biosynthetic pathway branches to produce siroheme, the

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cofactor for sulphite and nitrite reductases (5), and cobalamin (vitamin B12), which is de novo synthesized in *Salmonella* under anaerobic or low-oxygen growth conditions (6). Thus, the products of the branched heme biosynthetic pathway have a variety of functions related to oxygen, respiration and electron transfer (7).

Heme consists of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin. The biochemistry of heme synthesis is well established, and, with the exception of the initial reactions leading to 5-aminolevulinic acid (ALA), the pathway is conserved among all organisms that make heme (8). Two different mechanisms have been found for the synthesis of ALA in nature: one, by a C5 route from glutamate, and the other, by a C4 route from succinyl coenzyme A and glycine (9). *S. enterica* and *E. coli* use the C5 route (10, 11). The key C5 enzyme glutamyl-tRNA reductase converts charged glutamyl-$tRNA^{Glu}$ to glutamate-1-semialdehyde (GSA) or its cyclic form. GSA is then converted to ALA by the *hemL*-encoded enzyme, glutamate-1-semialdehyde aminotransferase (8). Since only a small fraction of the charged $tRNA^{Glu}$ of the cell is used to make heme, the reductase reaction is considered to be the first committed step in heme and tetrapyrrole biosynthesis. The final step of heme biosynthesis is catalysed by the *hemH* gene product, and involves the insertion of ferrous iron into protoporphyrin IX; reviewed in reference (8).

Heme homeostasis is an important determinant of bacterial fitness since perturbed levels of heme are associated with the generation of harmful, highly reactive oxygen species (ROS). The accumulation of either porphyrins or iron is toxic to *E. coli* and *Salmonella* because these compounds stimulate the generation of ROS (12, 13). Also, a reduced production of heme was found to increase the rate of generation of hydroxyl radicals due to the respiratory defect which results in an increased amount of cytosolic reductants, such as NADH, available to reduce free ferric iron. In the presence of hydrogen peroxide, ferrous ion thus formed will donate an electron and generate harmful hydroxyl radicals (14). Thus, a heme deficiency in *Salmonella* is associated with hypersensitivity to hydrogen peroxide, a reduced respiration rate, and a reduced growth rate (14–16).

The purpose of this chapter is to describe assays for the measurement of heme levels and the rate of respiration. An assay of ALA supplementation is described, in which the effect of exogenous ALA in reversing sensitivity to hydrogen peroxide is an indication of a reduced flow through the heme biosynthesis pathway (17). A protocol for measurement of the relative amount of heme by a fluorescence heme assay is also provided (18). This assay is based on stripping the iron from the heme moiety, which leaves a protoporphyrin molecule that fluoresces following excitation at 400 nm. The oxalic acid method described here is simple, rapid, and well suited for assaying multiple samples. Finally, a method

for the measurement of respiration (oxygen consumption) rate is provided. In this method (see Subheading 3.3), the respiration of the cell population is expressed as the specific respiration rate (q) during one doubling time of the population (the time for the culture's OD_{600} to increase from 0.2 to 0.4).

2. Materials

2.1. δ-Aminolevulinic Acid (ALA) Supplementation Experiments

1. ALA (Sigma Aldrich).
2. Nutrient broth (NB) medium (8 g of Difco nutrient broth and 5 g of NaCl per liter) (19).
3. Hydrogen peroxide (30% stock, Merck).
4. 0.9% NaCl.
5. Nutrient broth plates (8 g of Difco nutrient broth, 5 g of NaCl, and 15 g of Difco Bacto Agar per liter).

2.2. Fluorescence Assays of Heme Levels

1. LB medium.
2. Dulbecco phosphate-buffered saline (D-PBS) (Sigma-Aldrich).
3. Centrifuge.
4. Spectrophotometer.
5. Oxalic acid (Sigma-Aldrich).
6. Spectrofluorometer.
7. Fluorescence cuvettes (Sigma Aldrich).

2.3. Respiration Assay

1. LB with 0.2% glucose.
2. LB with 2% glucose.
3. Polypropylene glycol (BDH, Poole, United Kingdom).
4. Bioreactor equipped with stirrer, oxygen electrode and pH-meter (see Note 1).
5. Cuvettes.
6. Spectrophotometer.

3. Methods

3.1. δ-Aminolevulinic Acid Supplementation

1. Grow two cultures of the strain of interest overnight at 37°C; one in NB medium with 50 μg/mL ALA and the other in NB medium alone.
2. Dilute the overnight cultures 100-fold in fresh medium of the same composition (NB medium with or without 50 μg of ALA/mL).

3. Grow cultures until the OD_{600} is between 0.2 and 0.4 (in exponential growth phase).
4. Add hydrogen peroxide to cultures to make a final concentration of 2.5 mM. Take 0.1 mL sample of culture immediately prior to exposure to hydrogen peroxide (time zero sample) and add it to 0.9 mL 0.9% NaCl (tenfold dilution).

   Take 0.1 mL samples every 15 min, up to 1 h after addition of hydrogen peroxide.
5. Assay bacterial viability by making tenfold serial dilutions of culture samples (from 10^{-1} down to 10^{-7}) starting from the tenfold diluted sample described above. Inoculate the dilutions onto NB plates.
6. Count the number of colony forming units (CFU) after an overnight incubation at 37°C. Plot CFU/mL as a function of time of incubation in the presence of hydrogen peroxide. The CFU/mL in the time zero sample (prior to treatment with hydrogen peroxide) is expressed as 100%. Compare the percentage of viable cells remaining after exposure to hydrogen peroxide between cultures grown in NB medium supplemented with ALA and cultures grown in NB medium alone (Fig. 1).

3.2. Heme Measurements

1. Grow cultures of the wild type and the mutant of interest overnight with shaking in LB. Centrifuge the cultures and wash in Dulbecco phosphate-buffered saline.

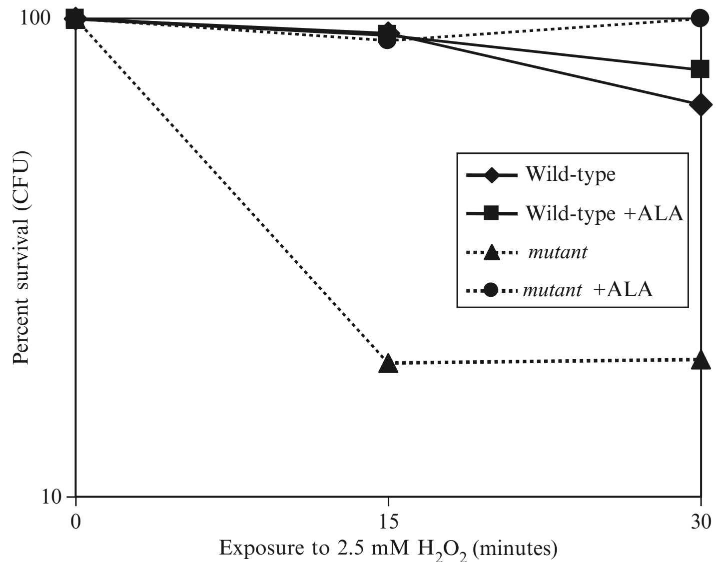

Fig. 1. The effect of H_2O_2 on bacterial viability illustrated for wild-type *S. enterica* and an isogenic fusidic acid-resistant mutant, as a function of the presence or absence of ALA, a precursor in the heme biosynthetic pathway. Note that the presence of ALA has no effect on the viability of the wild-type but that it significantly improves the viability of the mutant strain.

2. Resuspend the pellet in Dulbecco phosphate-buffered saline. Measure OD_{600} of the cultures, and make appropriate dilutions so that different 50-μL aliquots contain cell numbers corresponding to 0.05–1.0 OD_{600} units (1 OD_{600} unit is defined as the number of cells that give an OD_{600} of 1 in 1 mL) (see Note 2).
3. Each 50-μL aliquot of cells is mixed with 500 μL of heated 2 M oxalic acid (Sigma-Aldrich) and incubated in an oven at 100°C for 30 min. This treatment strips iron from heme, and the resultant protoporphyrin can be measured by fluorescence (Note 3).
4. After 30 min at 100°C, 500 μL of Dulbecco phosphate-buffered saline (or water) is added to the cell-oxalic acid mixture, and the new mixture is allowed to cool to room temperature.
5. Measure fluorescence on a spectrofluorometer using fluorescence cuvettes (see Note 4).
6. Fluorescence spectra of samples are recorded at room temperature using 400-nm excitation, whereas emission is scanned from 200 to 800 nm (see Notes 5 and 6).

3.3. Respiration Assay

1. Grow an overnight culture of wild-type and strain of interest in a shaking flask at 37°C in LB medium supplemented with 0.2% glucose.
2. Measure the OD_{600} of the overnight cultures. This is so that one can inoculate equal numbers of cells into fresh medium to start new cultures for growth in the bioreactor.
3. Determine the bioreactor oxygen transfer rate (OTR) under the same value parameters (volume, temperature, aeration, stirrer speed, and pressure) which will be applied to the actual culture.

   The overall respiration rate of the population, qX, is in equilibrium with the oxygen transfer rate (OTR) of the bioreactor, i.e.

$$\text{OTR} = qX$$

   where X is the cell concentration.

   The bioreactor OTR is calculated from the formula:

$$\text{OTR} = kLa\,(c^{*} -- c)$$

   where kLa is the volumetric mass transfer coefficient, c^{*} is the saturated oxygen concentration, and c is the measured oxygen concentration at a certain time point. Both kLA and c^{*} are determined by the sulfite oxidation method (20) under the same value parameters (volume, temperature, aeration, stirrer speed, and pressure) which will be applied to the actual culture (Note 7).

4. Fill the bioreactor with 10 L of LB supplemented with 2% glucose and 3 mL of polypropylene glycol (Note 8).
5. Adjust the temperature and pH of bioreactor to 37°C and 7, respectively.
6. Inoculate the bioreactor with an appropriate amount of overnight culture to achieve an OD_{600} of 0.08.
7. During the respiration experiment, the OD_{600} is recorded every 20 min by taking 3–5 mL samples of culture from the bioreactor.
8. Dissolved oxygen and pH should be registered in short, regular time intervals or, if possible, continuously (Note 9).
9. Measurement of dissolved oxygen is stopped when the cell concentration (X) reaches an OD_{600} of 0.4. If the monitoring of parameters was continuous, the record of dissolved oxygen concentration is read. If monitoring was done in intervals, dissolved oxygen concentration is plotted as a function of time.
10. The specific respiration rate of the culture (q), expressed in micromoles of O_2 per liter per min per OD_{600}, is determined by calculating OTR/X.

4. Notes

1. Definition of bioreactor: Metal container or fermentation chamber for growing large volumes of bacterial cultures which can be used in the biotechnological production of substances such as pharmaceuticals, antibodies, or vaccines, or for the bioconversion of organic waste.
2. For each strain, assay 10 independent cultures, using 1 OD_{600} unit of cells for each measurement. Fluorescence should be normalized to biomass, measured as either optical density of the starting culture or dry weight of the cells; in our experiments the two methods gave the same result for tested strains.
3. 2 M concentration of oxalic acid was prepared by dissolving 10.08 g of oxalic acid with heating and stirring in 30 mL of water and then bringing the final volume to 40 mL.
4. We used an old spectrofluorometer available in the building (an SPF-500 corrected-spectrum spectrofluorometer from Aminco-Bowman, Silver Spring, Md.) with fluorescence cuvettes from Sigma-Aldrich (Hellma cuvette, fluorescence, 10 mm, semi micro, quartz, type 101-QS or 104F-QS).
5. Two emission peaks for porphyrin fluorescence are observed, at 608 and 662 nm.

6. To verify the results obtained on whole cell extracts, it is possible to make, in parallel, fluorescence measurements with crude membrane preparations prepared by French pressing and ultracentrifugation.

7. A method of measuring *kLa* involves carrying out a sulphite oxidation in the fermentation apparatus. In the presence of copper or cobalt salts, which act as catalysts, the reaction with oxygen or air proceeded rapidly and irreversibly to completion in the liquid phase. The reaction rate is not only much more rapid than the absorption rate, but it is independent of sulphite ion concentration at molarities above 0.015 (21, 22).

8. Polypropylene glycol is included in the medium as an anti-foaming agent.

9. In our experiments, respiration assays were carried out in a 10-L (working volume) stirred-tank bioreactor equipped with Wonderware 2000-based software (Belach Bioteknik, Stockholm, Sweden) which enabled continuous monitoring of dissolved oxygen, carbon dioxide content, and pH.

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Chapter 9

Methods to Determine Fitness in Bacteria

Cassie F. Pope, Timothy D. McHugh, and Stephen H. Gillespie

Abstract

Acquisition of antibiotic resistance may be associated with a physiological cost for the bacterium. Determination of growth rate and generation time is often used to measure fitness costs associated with antibiotic resistance. However, fitness costs may be small and difficult to quantify and multiple models are required. Available in vitro models that can be used to measure fitness include quantification of biofilm growth, survival in water, resistance to drying, and measurement of planktonic growth rates. The use of growth curve techniques to determine generation time is laborious, time-consuming, and can introduce sampling error. We have described the use of a semi-automated liquid culture system to estimate generation time in *Burkholderia cepacia* complex bacteria. We have also used the BacT/ALERT system to determine generation time and enumerate bacterial numbers in *Mycobacterium tuberculosis.* We describe methods for measuring biofilm growth and environmental survival in *Burkholderia cepacia* complex bacteria. These methods can be adapted for use with other organisms.

Key words: Fitness, Pseudomonas, Evolution antibiotic resistance

1. Introduction

Antibiotic resistance in a bacterial population occurs due to selection of resistant mutants in the presence of antibiotics. The existence of a continuing antibiotic selective pressure is responsible for high numbers of resistant organisms. When bacteria are exposed to antibiotics, a mutation conferring resistance to that antibiotic gives the bacterium an advantage. It is an accepted dogma that a resistant organism pays a physiological price for resistance, particularly where resistance is mediated by chromosomal mutations (1, 2). Although studies have shown that acquisition of antibiotic resistance can incur a biological cost (1, 3), there is evidence that some mutations conferring resistance may result in a low or no fitness deficit (4–6). It is widely accepted that with rational use of

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antibiotics, resistant mutants will be outcompeted by their susceptible counterparts and will be lost from the population. To test this assumption, in vitro models of fitness have been used in order to investigate the evolution of antibiotic resistance and to assess the physiological price associated with acquisition of resistance. Furthermore, quantification of fitness costs is important when determining the stability of antibiotic resistance in a population.

The growth rate of bacteria in culture medium is a commonly used model for evaluating fitness as a measure of reproductive potential (7–10). However, fitness is a complex characteristic that encompasses the ability of a genotype to reproduce within a host, be transmitted, and to resist host defences. Thus, multiple models are required to build up a complete picture of the fitness deficit of a resistant mutant as compared to the susceptible parent.

The major factors that influence the frequency of antibiotic resistance in a population of bacteria are the extent of antibiotic use, the physiological cost of resistance and the ability of the bacteria to compensate for this cost. Within a population of bacteria, different genotypes must compete with each other to reproduce. Therefore, incidence of resistance should be reduced by the rational use of antibiotics as resistant bacteria can be selected against in the absence of antibiotics due to a fitness cost. Resistant organisms will not disappear from the population if mutations conferring resistance have a low or no fitness cost.

Defining fitness cost can be difficult due to variations in measurements in experimental procedures. No one method is likely to be sufficient in isolation since "fitness" is a characteristic based on multiple bacteriological properties, and so multiple models are required. The models selected will depend on the organism, its natural lifestyle, and its mode of growth. For example, biofilms have a role in many infectious diseases and a suitable biofilm model should be included for bacteria that are known to form biofilms within the human body, e.g. *Pseudomonas aeruginosa* within the cystic fibrosis lung (11, 13). This is because a change in the ability to form a biofilm is likely to have a significant impact on the capacity of the organism to survive in the environment or to cause infection. Environmental survival, e.g., resistance to drying or survival in water, is likely to be critically important for nosocomial pathogens that can be transmitted via contaminated surfaces and may be subject to drying stresses. To address this, environmental survival could be assessed by measuring the ability of an organism to survive in water or survive drying on a surface (12, 13).

Fitness deficits will vary depending on the resistance mutation, the organism, and the model used to quantify the cost. For example, Sanchez et al. assessed the fitness costs associated with overproduction of multidrug efflux pumps, encoded by *nalB* and

nfxB, in *P. aeruginosa* using survival in water, maintenance on dry surfaces, biofilm formation, nematode killing, production of pyocyanin and pyoverdin, and quantification of proteases (12). These mutants have been shown to have fitness costs in terms of resistance to desiccation, survival in water, loss of quorum-sensing response, and loss of virulence in the nematode-killing model. However, the *nalB* mutant exhibited greater biofilm formation than the wild type. Hence, the overall fitness cost may need to reflect the sum of the deficits identified in all of the model systems used and should be chosen to reflect the pathogenic mechanisms of the organism.

Fitness costs are measured in a number of ways, and a variety of in vitro and animal models are available. These include comparison of growth rate in monocultures (4). For example, we have used paired competition assays to assess fitness costs of fluoroquinolone resistance in *Streptococcus pneumoniae* (5) and *Burkholderia cepacia* and rifampicin resistance in *M. tuberculosis* (13, 14).

Relative fitness is often determined by competition assay between isogenic antibiotic-susceptible and antibiotic-resistant bacteria in culture or in animal models. These models can be adapted for use in many bacterial species. For *Burkholderia cepacia*, we have used competitive pairwise cultures, planktonic growth rate, biofilm formation, and environmental survival models to assess the fitness cost of acquisition of fluoroquinolone resistance (13).

Viable cell count estimation can be subjective and dependent on the accurate enumeration of colonies that grow under the conditions provided, introducing significant sampling error. By using an automated system, there is reduced manipulation of the bacterium and the readout is not subject to observer error and, importantly, makes it possible to determine growth rate for large numbers of strains. Youmans and Youmans used the difference in time to positivity, measured as time to a defined turbidity, of small inoculums of *M. tuberculosis* to determine generation time (15). We have adapted this method to determine generation times in a semiautomated liquid culture system for *B. cepacia* and *M. tuberculosis*, using the difference in time to positivity of diluted inoculums. These methods may minimize observed variation and allow fitness costs to be calculated in terms of generations. Laurent et al. have also used an automated liquid culture system (MS2 Research System, Abbott Laboratories, Dallas, Tx, USA) and paired competitive cultures to determine growth rate, as a measure of fitness in MRSA (16). This chapter describes use of a semiautomated liquid culture system for measurement of generation time in *Burkholderia cepacia*.

We have used the Bactec 9240 continuous, blood culture system with standard aerobic medium was used to determine growth rate in *B. cepacia*. The Bactec 9000 (9240/9120/9050) series of

automated blood culture systems are used to rapidly detect viable microorganisms in clinical specimens and is used in most clinical laboratories with in the UK. Bactec Plus Aerobic vials, contain 25 mL of enriched soybean–caesin digest broth, 0.05% sodium polyanetholesulfonate (SPS), resins, CO_2, O_2 and a sensor. This sensor, within each vial, responds to changes in oxygen and carbon dioxide levels as a result of bacterial metabolism. These changes are measured by an increase in the fluorescence of the sensor which is monitored every 10 min. A positive fluorescence reading indicates an increase in CO_2 or decrease in oxygen and the presence of microorganisms in that vial.

There are numerous models that have been described to measure biofilm growth. No single model is ideal for all experimental scenarios as each has been designed for a specific purpose. For use as a fitness assay, the model should be simple and reproducible with sufficient replicate biofilms to allow statistical analysis. The crystal violet microtitre plate assay is a simple and rapid method which quantifies adherence of bacteria to sides of a microtitre plate (17). It is useful as a biofilm fitness assay as it is easy to create a large number of replicate biofilms.

2. Materials

2.1. Planktonic Growth

1. Test organisms.
2. Muller Hinton Broth (BDH, Leicestershire, UK).
3. Orbital incubator (Barloworld Scientific, Staffordshire, UK).
4. Phosphate buffered saline (PBS) (BDH, Leicestershire, UK).
5. Spectrophotometer (Pharmacia Biotech Ultraspec).
6. 0.5 mL syringe (Western Laboratory Supplies, Hampshire, UK).
7. 0.5 mm gauge needle (Western Laboratory Supplies, Hampshire, UK).
8. Bactec automated blood culture system (Bactec 9000 series).
9. Steret alcohol wipes (70% alcohol) (Western Laboratory Supplies, Hampshire, UK).
10. Aerobic Bactec bottles (Becton Dickinson, Oxford, UK).
11. Blood agar plates (Oxoid, Hampshire, UK).
12. Light Microscope (Zeiss, Germany).
13. Gram stain reagents (Oxoid, Hampshire, UK).
14. Glass slides (Western Laboratory Supplies, Hampshire, UK).

2.2. Biofilm Growth

1. Test organisms.
2. Luria Bertani Broth (BD, Le Pont de Claix, France).
3. Crystal Violet (Sigma Chemical Co., St. Louis., USA).
4. Orbital incubator (Barloworld Scientific, Staffordshire, UK).
5. Casamino acids (BD, Le Pont de Claix, France).
6. Spectrophotometer (Ultraspec 2000, Pharmacia Biotech AB, Uppsala, Sweden).
7. Multichannel pipette (Eppendorf, Cambridge, UK).
8. Flexible polyvinylchloride microtitre plates (Falcon 3911 Microtest III flexible assay plate, Becton Dickinson, UK).

2.3. Survival in Water

1. Test organisms.
2. Blood Agar Plates (Oxoid, Hampshire, UK).
3. Phosphate buffered saline (PBS) (BDH, Leicestershire, UK).
4. Microcentrifuge (Sigma, Poole, UK).
5. Autoclaved tap water.
6. Orbital Incubator (Barloworld Scientific, Staffordshire, UK).
7. 1 litre Duran bottles (Western Laboratory Supplies, Hampshire, UK).

2.4. Survival During Dessication

1. Test organism.
2. Flat bottomed Microtitre Plates (Becton Dickinson, UK).
3. Phosphate buffered saline (PBS) (BDH, Leicestershire, UK).
4. Muller Hinton Broth (BDH, Leicestershire, UK).
5. Microcentrifuge (Sigma, Poole, UK).
6. Triton X-100 (Sigma, Poole, UK).
7. Blood Agar Plates (Oxoid, Hampshire, UK).

3. Methods (Please Also Refer to Notes Below)

3.1. Automated Measurement of Fitness by a Modification of the Youmans and Youmans Method

1. Inoculate *B. cepacia* on Columbia blood gar plates and incubate at 37°C for 18 h aerobically.
2. Using a sterile loop, inoculate one colony of *B. cepacia* in Muller Hinton broth (5 mL) and incubate using an orbital incubator (200 rpm) for 4 h to obtain an exponentially growing culture (see Note 1). Dilute the culture to standard optical density using PBS.
3. Make a dilution series from (10^{-1} to 10^{-6}) in PBS.

4. Aseptically inoculate triplicate Bactec bottles with 0.5 mL of the 10^{-2} and 10^{-4} dilutions using a steret alcohol wipe, needle, and syringe. Invert bottles to mix (see Notes 2 and 3).
5. Load into system using the manufacturer's instructions.
6. Incubate the bottles until they "flag" as positive.
7. Print out the growth curve and time to positivity. Then remove bottles and discard (see Notes 4–8).
8. Confirm absence of contaminants by Gram stain and spreading of one drop of bottle content onto Columbia blood agar.
9. Calculate the growth rate constant and generation time can be determined by the following equations

$$K = \frac{\log a - \log b}{t}$$

$$G = \frac{\log 2}{K}$$

where K is the growth rate constant, a is the largest inoculum (1:10), b is the smallest inoculum (1:1000), t is the difference in time (h) taken for each of the sets of bottles to signal positive and G is the generation time (see Note 9).

3.2. Biofilm Growth

1. Briefly, 100 μL of a 1:100 overnight LB broth culture containing 0.5% casamino acids was used to inoculate 8 independent wells of a 96 well polyvinylchloride microtitre plate. Negative control wells contained broth only (see Note 10).
2. Incubated for 24 h at 30°C in a humid atmosphere (see Note 11).
3. Gently remove medium and wash wells three times with 200 μL SDW using a multi-channel pipette.
4. Stain microtitre plate wells with 200 μL of 1% (w/v) crystal violet for 15 min. at room temperature.
5. Remove unbound crystal violet by repeated washing with water (see Note 12).
6. Solubilize bound crystal violet with 2 × 200 μL of 95% ethanol, transfer to a microcentrifuge and make up the volume to 1 mL.
7. The resulting absorbance can be determined at wavelength 590 nm using a spectrophotometer (see Note 13). Each experiment was repeated in triplicate.

3.3. Survival in Water

1. Pellet overnight Muller Hinton 1 mL broth cultures by centrifugation (13,000 × g, 3 min), remove the supernatant with

a fine tipped pipette and wash the pellet three times in PBS (1 mL).

2. Add an inoculum of approximately 1×10^8 CFU to duplicate duran bottles, each containing 19 mL of autoclaved tap water.
3. Aseptically remove 1 mL aliquots at time intervals (Day 0, 1, 2, 5, 7, 8, 9) and determine CFU/mL, using the Miles and Misra technique (see Note 14) (18).
4. Each experiment is repeated in triplicate (see Notes 15 and 16).

3.4. Survival in Dry Conditions

1. Pellet overnight Muller Hinton 1 mL broth cultures by centrifugation ($13{,}000 \times g$, 3 min), remove supernatant with a fine-tipped pipette and wash three times in 1 mL PBS.
2. Concentrate tenfold with PBS to a final cell concentration of approximately 1×10^9.
3. Using a pipette spot aliquots (10 μL) of the concentrated culture into duplicate wells of a 96 well flat bottomed microtitre plate and allowed to dry at room temperature.
4. Sample at hourly intervals for 7 h by the addition of 100 μL of PBS containing 0.25% v/v Triton x 10 to the wells, followed by thorough mixing to form a bacterial suspension (see Note 17).
5. Add 100 μL of suspension to 900 μL of PBS, vortex, perform a dilution series and determine numbers of viable bacteria by the Miles and Misra technique (see Notes 15 and 18) (18).
6. Repeat each experiment in triplicate (see Note 16).

4. Notes

1. Cells must be added to bottles while in exponential phase to limit the effect of lag time differences on time to positivity. An initial growth curve experiment should be performed to determine incubation conditions required for bacterial cells to be in exponential phase.
2. The Bactec 9000 series of instruments are used by most clinical microbiology laboratories and are therefore available for translational research following discussion with the service or laboratory manager.
3. Bactec bottles should be allowed to equilibrate at room temperature before inoculation.

4. Bottles should be left in the system until time to positivity data can be printed, as the system only stores data temporarily. If the local laboratory practice is to remove bottles immediately after flagging as positive, then the growth plot should be printed at the same time.
5. Bottles may flag as positive overnight therefore ensure that the duty biomedical scientist is aware of the experiment and understands the importance of printing the growth plot or leaving bottles in the system. Print out of growth curve can be integrated into clinical service if required.
6. Bottles must to be booked into the laboratory computer system because otherwise sample data will not be recorded.
7. Bottle bar code stickers must be retained in order to identify bottles.
8. Any bottles that do not become positive must be removed from the system.
9. This method can be adapted for other fast growing bacteria. We have also used the BacT/ALERT system to determine generation time in *M. tuberculosis*.
10. Blank controls should be included and consist of broth and casamino acids only.
11. Microtitre plates should incubated in a container with damp paper towels to provide a humid atmosphere.
12. Care should be taken not to leave crystal violet around the rim of wells as this can affect absorbance values.
13. Mean values of absorbance can be used to determine if differences in extent of biofilm formation are significant by ANOVA.
14. Ensure aliquots are removed aseptically.
15. Each dilution was vortexed briefly and three replicate 20 μL volumes of diluted water were spotted onto three segments of blood agar plates from approximately 1 cm above the surface of the plate. Plates were then incubated at room temperature for 30 min to allow the drops to soak into the agar and incubated at 37°C overnight. Colonies were counted using the dilution that yielded between approximately 20 and 40 colonies. The mean number of colonies is used to calculate the number of colony-forming units per millilitre of the neat water or per spot.
16. To detect significant differences between isolates during survival in water or survival during drying by plotting average CFU/mL against time. Correlation coefficient (r^2) should be calculated as an estimate of goodness of fit to determine if there are significant differences in survival. Two-way ANOVA

can be used to determine if survival between isolates is different at different time points.

17. When adding the PBS that contains detergent to each well to solubilize cells ensure mixing occurs by pipetting up and down.
18. Perform Miles and Misra plate count immediately after sampling.

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Chapter 10

Application of Continuous Culture for Measuring the Effect of Environmental Stress on Mutation Frequency in *Mycobacterium tuberculosis*

Joanna Bacon, Kim A. Hatch, and Jon Allnutt

Abstract

The ability of all pathogens to survive within the host is key to their success in establishing disease. Environmental conditions that affect the growth of a pathogen in the host include nutrient status, environmental pH, oxygen availability, and host defences. Studying the response of *Mycobacterium tuberculosis* (*M. tuberculosis*) exposed to these relevant host conditions *in vitro* will further increase our understanding of how these environments have an impact on the molecular mechanisms *M. tuberculosis* adopts to combat the effects of external influences such as antimycobacterials. The methods presented here are used to investigate the effect of environmental factors on the development of drug-resistant *M. tuberculosis*. Cultures grown under controlled conditions in continuous culture are sampled and the frequency with which resistant mutants develop are determined. These studies provide data that aid our understanding of the complex interaction between the host environment and invading bacterium that allow resistant strains to develop and continue to cause disease.

Key words: Mycobacteria, Continuous culture, Chemostat, Mutation frequency, Antibiotic resistance

1. Introduction

Tuberculosis (TB), caused by the agent *Mycobacterium tuberculosis* (*M. tuberculosis*), is one of the most serious bacterial infections, infecting 7–8 million people world-wide and causing death in 2–3 million per year. Hotspots of multi-drug-resistant TB are found in clusters around the world and have become a particular problem in areas where there are high levels of TB and HIV. Treatment is about 80% successful but it is prolonged and can last up to 6–8 months. The most important aim in improving treatment of TB is

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to shorten this period. Novel TB drugs are being designed, developed, and evaluated in clinical trials. The development of new drugs can be informed by *in vivo* and *in vitro* models to determine the efficacy of drugs and the emergence of antibiotic resistance.

The ability of Mycobacterium to survive within the human body is fundamental to their success in causing disease. The pathogenesis of *M. tuberculosis* is a complex process, involving extensive interactions with the host's immune system that determine the outcome of infection. On entry into a macrophage *M. tuberculosis* initially resides in an endocytic vacuole termed a phagosome. During phagosome maturation, the organism encounters a hostile environment that includes acidic pH, restricted iron availability, reactive oxygen intermediates, lysosomal enzymes, toxic peptides, and reactive nitrogen intermediates. Intracellular pathogens such as *M. tuberculosis* have evolved mechanisms for counteracting the effects of this hostile environment by, for example, arresting acidification of the phagosome. Other pathogens have adopted different mechanisms by which they overcome the effects of low pH in the host. *Vibrio cholera*, for example, overcomes an acidic pH by inactivating the porins responsible for the flow of hydrophilic compounds to the periplasm from the environment.

Studying the response of *M. tuberculosis* exposed to relevant host conditions *in vitro* will further increase our understanding of how these environments have an impact on the molecular mechanisms *M. tuberculosis* adopts to establish disease (1). This pathogen will also be adopting strategies to combat the effects of other, external influences such as antimycobacterials. The clinical pathology of the disease can contribute to the development of antibiotic resistance (2, 3). For example, infected patients may harbour the bacteria in pulmonary cavities, where antibiotics cannot penetrate. Environmental factors such as low pH may also inhibit the activity of antibiotics (2, 4).

The level of acidity is an important environmental cue for intracellular pathogens in the host. During the early stages of infection mycobacterial cells limit acidification of phagosomes by inhibiting the proton-ATPase, thereby resisting fusion of the phagosome with the lysosome (5, 6). If bacilli are viable, the pH in the phagosome does not drop below 6; however, dead mycobacteria do not block phagosomal acidification and the pH drops below 5.5 (7). A pH drop may be a signal for the organism to induce the synthesis of proteins that enable it to survive and replicate within the phagosome.

Maintenance of an acidic pH in a batch culture of *M. tuberculosis* is technically very challenging. That is because, as the organism grows, an initial imposed acidic pH will gradually reach neutrality through the metabolic process of the mycobacteria. However, continuous culture is an ideal system for growing bacteria under

defined and controlled conditions (8–10). During steady-state growth in continuous culture the bacteria are in equilibrium with their environment and growing at a constant generation time. Whilst the organisms are in this state, individual growth parameters can be varied independently so that the direct effect of a single stimulus on the physiology and molecular genetics of an organism can be established. Continuous culture can also be used to ascertain whether an organism can survive and grow under a particular growth condition.

Attempts to maintain *M. tuberculosis* growth in continuous culture at pH 5.5 using the chemostat have shown that the organism cannot adapt and survive at this level of acidity (unpublished data). In this system, the minimum pH level that *M. tuberculosis* can grow at is pH 6.2, which reflects the pH level in the phagosome.

Antibiotics impose an additional stress on the organism. In order to survive, the bacterium has to overcome these threats by adapting to the environment and developing resistance or tolerance to antibiotics. Bacteria develop resistance to antibiotics by a variety of mechanisms which can be organised into three main catagories, natural resistance, mutational resistance, and extrachromosomal or acquired resistance. Antibiotic-resistant bacteria grow more slowly than their susceptible parents (11, 12), but this fitness deficit is lost in subsequent generations and mutants can adapt to the growing conditions in culture (13). Newly resistant strains in patients also rapidly adapt and survive to cause disease.

The methods presented here describe the effects of pH stress on the development of mutations conferring resistance to antibiotics, using rifampicin as an example. The majority of *in vitro* studies investigating *rpo*B gene mutations conferring rifampicin resistance have been based on poorly defined batch culture techniques which expose bacteria to ill-defined and constantly changing conditions. The technique of continuous culture in the chemostat coupled with pyrosequencing, facilitates the analysis of the accumulation of resistance mutations under specific environmental conditions, providing an insight into the molecular mechanisms involved in the development of rifampicin resistance in *M. tuberculosis* in the patient (14).

Here, the mutation frequency and distribution of point mutations in the RRDR of the *rpoB* gene have been measured in a steady-state *M. tuberculosis* chemostat culture for an extended period at pH 7.0. The pH of the culture has then been reduced to pH 6.2 in order to reflect the *in vivo* environment. The mutation frequency and distribution of point mutations were measured throughout to gather more information about the effects of relevant host stimuli on the development of antibiotic resistance in *M. tuberculosis.* Other conditions which are associated with latent disease could be tested in this system such as low oxygen or carbon-starvation.

In summary, the methods presented here are used to investigate the effect of environmental factors on the development and persistence of drug-resistant *M. tuberculosis*. Cultures grown under controlled conditions in continuous culture are sampled and the rates at which resistant mutants develop are determined. These studies provide data that aid our understanding of the complex interaction between the host environment and invading bacterium that allow resistant strains to evolve and cause disease.

2. Materials

2.1. Components of the Chemostat (See Fig. 1)

1. 1 L Glass vessel (D.J. Lee & Co., Ferndown, UK. Made to order).
2. Stopper, silicone, SZ4 (Cole-Parmer, Hanwell, London, UK. 06298-08).
3. Stainless steel A4 form B washers (RS components Ltd, Corby, UK. 183-9051).
4. pH probe (220 mm) plus leads (Mettler Toledo – gel filled) (Brighton Systems, New Haven, UK. 104054481).
5. Phoenix Dissolved Oxygen (DO) probe (220 mm) plus leads (Brighton Systems, New Haven, UK. 027NG15.220.DL).
6. Phoenix membrane kit (for DO probe) (Brighton Systems, New Haven, UK. M05000T1-K5).
7. Phoenix filling solution (DO probe electrolyte) (Brighton Systems, New Haven, UK. 692-542).
8. Silicone rubber compound – flowable fluid (RS components Ltd, Corby, UK. R001069).
9. Dual-wire PFA sheathed stainless steel RT probe plus leads (temperature probe) (Brighton Systems, New Haven, UK. Made to order).
10. Titanium chemostat top plate plus collars and screw fittings (CEPR, HPA, Porton Down. Made to order).
11. Silicone rubber gasket (CEPR, HPA, Porton Down. Made to order).
12. Non-melting silicone grease (RS components Ltd, Corby, UK. 6026).
13. Esco silicone tubing – 4 × 1.6 mm (Bore × wall) (Scientific Laboratory Supplies Ltd. (SLS), Nottingham, UK. TUB7042).
14. Portexsil silicone tubing – 6 × 2 mm (Bore × wall) (SLS, Nottingham, UK. 435464-1).

15. Esco silicone tubing – 1×2 mm (Bore×wall) (SLS, Nottingham, UK. TUB7012).
16. Ty-Fast cable ties – 186 mm (RS components Ltd, Corby, UK. 178-484).
17. Ty-Fast cable ties – 141 mm (RS components Ltd, Corby, UK. TY125-40-100).
18. 6 mL glass tubing (D.J. Lee & Co., Ferndown, UK. Made to order).
19. Tubing connectors, Y shaped for 4–5 mm tubing ID (VWR International, Lutterworth, UK. 229-3442).
20. Tubing connectors, Y shaped for 6–7 mm tubing ID (VWR International, Lutterworth, UK. 229-3444).
21. Tubing connectors, T shaped for 6–7 mm tubing ID (VWR International, Lutterworth, UK. 229-3424).
22. Glass media addition anti-grow back device (D.J. Lee & Co., Ferndown, UK. Made to order).
23. Glass side arms (D.J. Lee & Co., Ferndown, UK. Made to order).
24. Quickfit PTFE washer (QW 18/7) (Fischer Scientific, Loughborough, UK. QAK-235-v).
25. Quickfit silicone rubber ring (QR 18/7) (Fischer Scientific, Loughborough, UK. QAK-175-B).
26. Quickfit plastic screw cap (QC 18/11) (Fischer Scientific, Loughborough, UK. QAK115-U).
27. Titanium sample port connector (20 mm ID) (CEPR, HPA, Porton Down. Made to order).
28. In-line connectors (fitting, ¼″) (Cole-Palmer, Hanwell, London, UK. 06360-90).
29. Acro 37 TF vent device with 0.2 μm PTFE membrane (Pall Corporation, Michigan, US. 4464).
30. Nalgene 2 L, 2125 Heavy-duty wide neck round bottles, HDPE (Jencons, Forest Row, UK. 172-622).
31. 10 L or 5 L glass media duran bottle (SLS, Nottingham, UK. BOT5210).
32. Anglicon magnetic stirrer unit (Brighton Systems, New Haven, UK. MS01).
33. Watson Marlow Bredel 101U/R auto/manual control variable speed pump (0.06–2 rpm) (Watson Marlow Limited, Falmouth, UK. 010.4002.00U).
34. Watson Marlow Bredel 101U/R auto/manual control variable speed pump (1.0–32 rpm) (Watson Marlow Limited, Falmouth, UK. 010.4202.00U).

35. 1 L glass media duran bottles (SLS, Nottingham, UK. BOT5216).
36. Stirring bars, PTFE, wheel – 45 mm (VWR International, Lutterworth, UK. 442-0080).
37. Tape heater 5″ diameter (24 v/50 w) (Brighton Systems, New Haven, UK. Made to order).
38. Anglicon microlab fermenter control panels plus leads (Brighton Systems, New Haven, UK. Made to order).
39. Soloview Data logging software package (Brighton Systems, New Haven, UK. Made to order).
40. Buffer, reference standard, pH 4.0 ± 0.01.
41. Buffer, reference standard, pH 7.0 ± 0.01.
42. Hydrochloric acid.
43. Sodium hydroxide pellets.
44. Glass universals for sampling (20 mm ID) (SLS, Nottingham, UK. BOT 5006).
45. Titanium cabinet tubing connectors (CEPR, HPA, Porton Down. Made to order).
46. Digital Thermometer (Tempcon Instrumentation limited, Ford, UK. 2046T).
47. Clips, tubing – 30 mm (VWR International, Lutterworth, UK. 229-0592).
48. Clips, tubing – 40 mm (VWR International, Lutterworth, UK. 229-0593)
49. Keck ramp clamp tubing clamps – ⅜″ (Cole-Palmer, Hanwell, London, UK. KH-06835-07).
50. Keck ramp clamp tubing clamps – ¼″ (Cole-Palmer, Hanwell, London, UK. KH-06835-03).
51. Bulb hand-pump (blowing ball with reservoir) (VWR International, Lutterworth, UK. 612-9953).
52. Nitrogen gas (BOC Medical, Worsley, UK).
53. Air pump (Brighton Systems, New Haven, UK. Made to order).
54. Silica gel (Merck, Nottingham, UK. 94098).
55. Carbon dioxide analyser (Brighton Systems, New Haven. Made to order).
56. Middlebrook 7H10 agar per litre, 15.0 g agar, 1.5 g Na_2HPO_4, 1.5 g KH_2PO_4, 0.5 g $(NH4)_2SO_4$, 0.5 g L-glutamic acid, 0.4 g sodium citrate, 0.04 g ferric ammonium citrate, 0.025 g $MgSO_4 \cdot 7H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg pyridoxine, 0.5 mg biotin, 0.5 mg $CaCl_2 \cdot 2H_2O$, 0.25 mg malachite green, 100.0 mL OADC enrichment, and 5.0 mL glycerol.

57. OADC enrichment per 100 mL, 5.0 g bovine serum albumin fraction V, 2.0 g glucose, 0.85 g NaCl, 0.05 g oleic acid, and 4.0 mg catalase.
58. CAMR Mycobacterium Medium (CMM) per litre, 10.0 g ACES buffer, 0.22 g KH_2PO_4, 500 mL distilled water, 10 mL CMYCO solution 1, 10 mL CMYCO solution 2, 100 mL CMYCO solution 3, 10 mL CMYCO solution 4, 10 mL biotin (10 μg/ml solution), 0.042 g $NaHCO_3$, 0.75 g glycerol 10 mL CMYCO solution 5, and 2 mL Tween 80.
59. CMYCO solution 1 per litre, 0.055 g $CaCl_2 \cdot 2H_2O$, 21.40 g $MgSO_4 \cdot 7H_2O$, 2.88 g $ZnSO_4 \cdot 7H_2O$, and 1.0 L distilled water.
60. CMYCO solution 2 per litre, 0.048 g $CoCl_2 \cdot 6H_2O$, 0.0025 g $CuSO_4 \cdot 5H_2O$, 0.002 g $MnCl \cdot 4H_2O$, 0.5 mL concentrated HCL, and 1.0 L distilled water.
61. CMYCO solution 3 per litre, 1.0 g L-serine, 1.0 g L-alanine, 1.0 g L-arginine, 20.0 g L-asparagine, 1.0 g L-aspartic acid, 1.0 g L-glycine, 1.0 g L-glutamic acid, 1.0 g L-isoleucine, 1.0 g L-leucine, and 1.0 L distilled water.
62. CMYCO solution 4 per litre, 100.0 g pyruvic acid sodium salt, 1.0 L distilled water.
63. CYMCO solution 5 per litre, 1.0 g $FeSO_4 \cdot 7H_20$, 0.5 mL concentrated HCL, and 1.0 L distilled water.

2.2. Establishing Steady-State Growth

1. Chemostat (set up as in Subheading 3.1).
2. CMM medium (see Subheading 2.1 for the recipe).
3. Hand-held pH meter.

2.3. Alteration of pH Levels in Chemostat Culture

1. Chemostat (set up as in Subheading 3.1).
2. Spare 1 L Glass vessel: (D.J. Lee & Co., Ferndown, UK. Made to order).
3. CMM medium (see Subheading 2.1 for the recipe).
4. Hydrochloric acid (1 M).
5. Sodium hydroxide (1 M).

2.4. Sampling and Plating *M. tuberculosis* to Determine Mutation Frequency

1. Antibiotics to be tested.
2. CMM medium (see Subheading 2.1 for the recipe).
3. Middlebrook 7H10 agar plates with OADC enrichment (see Subheading 2.1 for the recipe) and the addition of MIC levels of rifampicin.

2.5. Preparation of Colonies for Determining the Distribution of Mutation within the RRDR

1. TE buffer (10 mM Tris–HCl, pH 7.5 1 mM EDTA) made from1 M stock of Tris–HCl (pH 7.5), and 500 mM stock of EDTA (pH 8.0).

2.6. Monitoring Chemostat Parameters

1. CMM medium (see Subheading 2.1 described for the recipe).
2. 40% v/v Formaldehyde.
3. Columbia blood agar plates (Biomerieux, Basingstoke, UK. 43050).
4. Middlebrook 7H10 agar plates with OADC enrichment (see Subheading 2.1 for the recipe).
5. Hand-held pH meter.

3. Methods

3.1. Assembling the Chemostat

Assemble the chemostat as shown in Fig. 1.

1. Rinse the 1 L glass chemostat vessel with distilled water. Check that the probes are intact and that the oxygen probe contains electrolyte (see Note 1).
2. Insert the oxygen, pH, and temperature probes through the ports in the titanium top plate (Fig. 1), which is achieved by pushing the probes through foam bungs, which are firmly screwed into the ports in the top plate.
3. Assemble effluent and medium lines using silicon tubing and connect them to the vessel (see Note 2).
4. Assemble and connect the air inlet, the off-gas condensate bottle (duran bottle), the dessicator bottle containing silica gel, acid and alkali addition lines, media addition lines, and sample port to the vessel. Add vent filters (0.2 µm) to air inlets, air outlets, sample port side arms, waste bottles, collection bottles, and medium bottles to maintain sterility and to prevent the build up of pressure in the vessel (see Note 3).
5. Prepare 1 M HCL and 1 M NaOH in 1 L duran bottles and autoclave them.
6. Place essential equipment in the safety cabinet, which will house the chemostat. This includes the electronic stirrer, two peristaltic pumps (medium and effluent), the CO_2 analyser, two autoclaved waste pots, and the medium source.

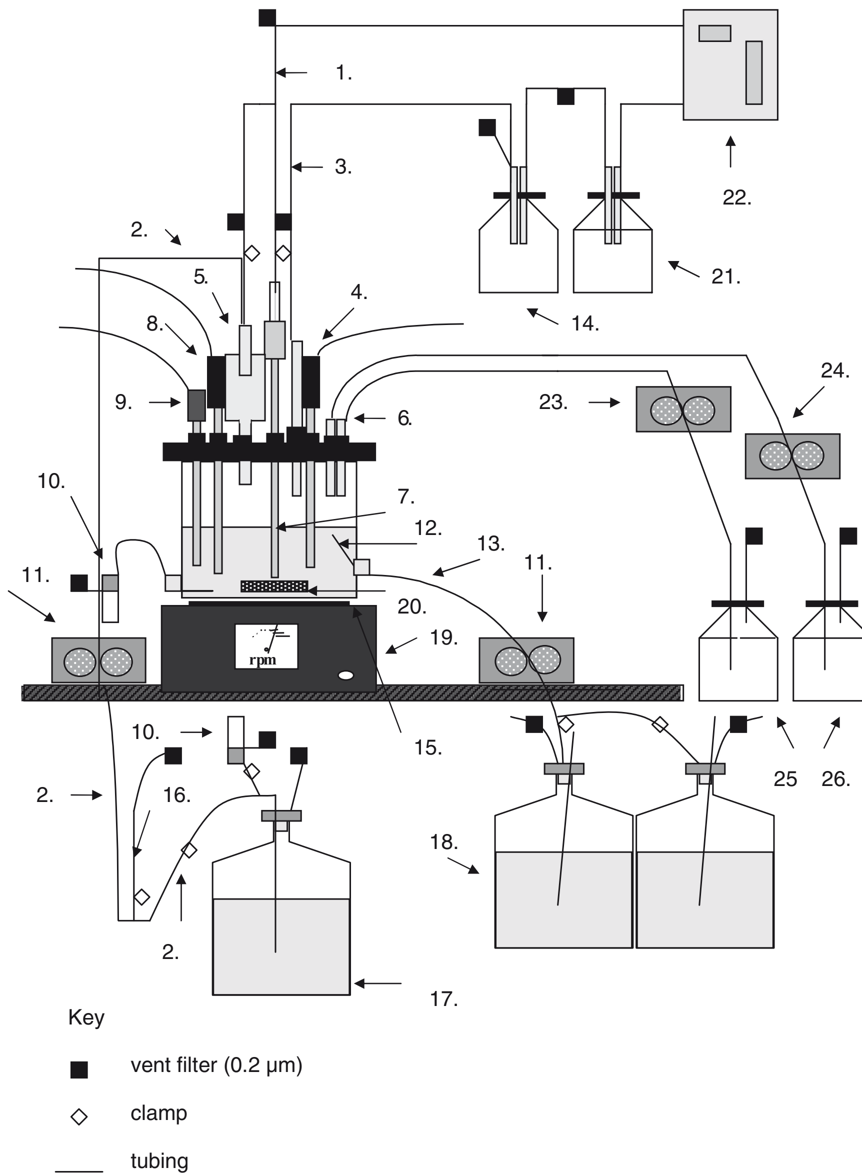

Fig. 1. The chemostat system developed at The Health Protection Agency, CEPR, Porton Down. (1) Air inlet/off-gas return, (2) Medium supply, (3) Air outlet/off-gas, (4) pH probe, (5) Anti-grow back device as part of medium inlet, (6) Spare ports for addition of acid and alkali, (7) Air inlet tube, (8) Temperature probe, (9) Oxygen probe, (10) Sample port, (11) Peristaltic pumps, (12) Effluent port, (13) Effluent line, (14) Off-gas condensate, (15) Heat pad, (16) Glass pipette for flow rate measurement, (17) Medium supply, (18) Effluent waste pots or cell collection pots, (19) Stirrer, (20) Magnetic flea, (21) Dessicator containing silica gel, (22) CO_2 analyser, (23) Peristaltic pump for acid addition, (24) Peristaltic pump for alkali addition, (25) Acid supply, (26) Alkali supply.

7. Add a 45 mm magnetic flea and 600 mL of water to the chemostat vessel.
8. Place the vessel on top of the stirrer and on the heat pad.
9. Connect the probes to the correct controller, which will maintain growth parameters at set values within the culture vessel.

10. Check the oxygen probe has a signal and a rough span between 0% dissolved air saturation (via nitrogen addition) and 100% dissolved air saturation (via air addition) (see Note 4).
11. Calibrate the pH probe with pH 4 and pH 7 buffers. To control the pH, the acid (1 M HCl) and alkali (1 M NaOH) should be delivered in response to a fluctuation in acidity levels via peristaltic pumps, which will also respond to the controller via a negative feedback loop (see Note 5).
12. Heat the water in the vessel to 37°C using the heat pad. To calibrate the temperature probe use a Tempcon hand-held digital thermometer to monitor the temperature in the vessel until it reaches 37°C. Set the temperature reading on the controller to 37°C (see Note 6).
13. Apply grease to the rubber gasket and seal the vessel with the top plate. Pressure-test the vessel prior to autoclaving to check for air leaks. Submerge the vessel in a sink of water with the probe fittings just under the water level. Vent filters should be above the water line so that they do not get wet (see Note 3). Leave all clamps in place apart from the air inlet and air outlet clamps, which should be removed. Extend the air outlet tubing past the vent filter using an additional length of tubing and place the end of the tubing below the water surface. Place a bulb hand-pump onto the air inlet line and pump air in. Air bubbles should be seen coming out of the air outlet. Clamp the air outlet (between the vessel and the vent filter) and pump gently a couple of times. Observe whether air bubbles rise out of the vessel, particularly from the probe fittings on the top plate, side arms etc. Repair and re-test any leaks that appear. Release the clamp on the air outlet and re-clamp the air inlets before autoclaving.
14. Autoclave the vessel at 121°C for 30 min to achieve sterility.
15. Ensure waste, medium, and acid/alkali bottles are connected to the chemostat. Connect tubing to the peristaltic pumps. Connect all probes, stirrer, and CO_2 analyser to the controller unit. Connect the controller output to the computer for data logging.
16. Calibrate the oxygen probe by warming up the vessel to 37°C whilst stirring, and pump in nitrogen and air alternately until calibrated between 0 and 100% dissolved air saturation, which is equivalent to between 0 and 20% dissolved oxygen tension (DOT) (see Note 7).
17. Switch off heater. Drain the water from the chemostat.
18. Fill the vessel with 400 mL of medium via the medium line. Warm the medium to 37°C. Ensure that the stirrer unit is responding to the dissolved air saturation setting on the

controller by seeing an increase in stirrer speed as the DOT level decreases. Set maximum and minimum stirrer speeds.

19. Make up the inoculum by taking approximately five confluent plates of mycobacterial colonies, which have been incubated for 3 weeks (see Note 8), and scrape them into 10 mL of autoclaved, distilled water. Add the inoculum through the sample port.
20. Switch on the CO_2 analyser to measure CO_2 given off by the culture, to be logged by the computer.
21. To prepare middlebrook 7H10 plates add the glycerol to 900 mL of distilled water and add the remaining components, except for the OADC enrichment. Mix thoroughly. Gently heat and bring to the boil. Autoclave the mixture for 15 min at 121°C. Cool to 50°C and asceptically add 100 mL of OADC enrichment. Mix thoroughly and pour into petri dishes.
22. To prepare OADC enrichment add the components to the distilled water and bring the volume to 100 mL. Mix thoroughly and then filter sterilise the solution. OADC enrichment is available as a premixed powder from BBL Microbiology Systems and Difco Laboratories.
23. To prepare CAMR mycobacterium medium add the ACES buffer and KH_2PO_4 to the first volume of distilled water (500 mL). Add the remaining ingredients and solutions in the order listed. Stir the solution to dissolve all the ingredients. Adjust the pH to 6.5 with 20% KOH. Filter sterilise the medium using a 0.2 μm filter. Store the medium between 2°C and 8°C, in the dark and use within 2 months of the production date.
24. To prepare CMYCO solution 1 add the ingredients to the water and stir to dissolve. Store it at 2–8°C. Use it within a 6-month period from the date of production.
25. To prepare CMYCO solution 2 add the ingredients to the water and stir to dissolve. Store it at 2–8°C. Use it within a 6-month period from the date of production.
26. To prepare CMYCO solution 3 add the ingredients to the water and stir to dissolve. Store it at 2–8°C. Use it within a week of production.
27. Prepare CMYCO solution 4 fresh each time CMM is prepared. To prepare add the pyruvic acid to the water and stir to dissolve the ingredients.
28. Prepare CMYCO solution 5 fresh every time CMM is made. To prepare add the ingredients to the water and stir to dissolve the ingredients.

29. To prepare Middlebrook 7H10 agar plates containing antibiotic, melt 170 mL of Middlebrook 7H10 agar. Place the bottle of molten agar in a water bath at 50°C to cool it. Add 20 mL of OADC enrichment. Mix thoroughly. Add 10 mL of the antibiotic, diluted in water, at a concentration that will achieve a final concentration in the agar of 2× minimum inhibitory concentration (MIC). Pour 25 mL of molten agar into each agar plate on a level bench top.

3.2. Establishing Steady-State Growth

1. Leave in batch mode for approximately 48 h. For the first 24 h the air inlet should be closed at the cabinet connection to allow the DOT set point of 10% DOT to be reached (see Note 9). Following this, open the air inlet as the culture will now require additional oxygen.
2. Switch the culture to fed-batch mode by starting the medium pump at a flow rate of 5 mL/h. Set the medium pump by calibrating it to the required flow rate. Keep the culture at 5 mL/h for 24 h to increase the culture volume to 500 mL (see Note 10).
3. Start the culture in continuous mode at 5 mL/h by switching on the effluent pump at a speed that is higher than the medium pump in order to maintain the culture volume at 500 mL. This flow rate is maintained for 2 days to establish the culture in continuous mode prior to an increase in flow rate (see Note 11).
4. Increase the flow rate to 10 mL/h for 2 days.
5. Increase the flow rate to 15 mL/h and monitor the culture daily (see Notes 12 and 13).
6. Establish the culture in steady-state and measure the pH of a culture sample daily using a hand-held pH meter. The DOT level and optical density (OD) should be stable for at least 7–8 days.
7. Sample the culture for mutation frequency measurements (see Subheading 3.4).

3.3. Alteration of pH Levels in Chemostat Culture from pH 7.0 to pH 6.2

1. Establish steady-state cultures at 10% DOT, pH 7.0 (see Subheading 3.2).
2. Remove samples from the chemostat for mutation frequency analysis (see Subheading 3.4).
3. Change the vessel at this stage if wall growth has started to appear (see Note 14).
4. Prime the acid/alkali lines.
5. To achieve a pH level of 6.8, set the acid set point on the controller to 6.82 and the alkali set point to 6.78. Set the

acid/alkali pumps to automatic on the controller (see Note 15).

6. Allow the culture to adjust to the new pH setting over a 5-day period.
7. Adjust the acid set point on the controller to 6.52 and the alkali set point to 6.48.
8. Allow the culture to adjust over a 5-day period.
9. Set the acid set point on the controller to 6.22 and the alkali set-point to 6.18.
10. Allow the culture to re-adjust to a pH of 6.2 and establish steady-state over a 5-day period at a constant pH of 6.2.
11. Sample the culture for mutation frequency analysis (see Subheading 3.4).

3.4. Sampling and Plating M. tuberculosis to Determine Mutation Frequency

1. Sample the culture in steady-state over the time-period required (see Notes 16 and 17).
2. Spin the cell sample in a bench top centrifuge at 1,600 g for 10 min.
3. Remove the supernatant and re-suspend cells in 300 μL of fresh CMM medium.
4. Spread 100 μL onto three 7H10 agar plates containing antibiotic at a concentration of twice the minimum inhibitory concentration (see Note 18) and incubate them for 3–4 weeks at 37°C.
5. Sample the culture and perform a dilution series in water. Spread 100 μL of diluted cells onto 7H10 agar plates in triplicate for each dilution. Incubate the plates for 3 weeks at 37°C to determine the total viable count (TVC) of the culture.
6. Count the colonies that have grown on the antibiotic plates and the TVC plates.
7. Calculate the mutation frequency by dividing the average number of antibiotic-resistant colonies per mL of original sample by the TVC (cfu/mL).

3.5. Preparation of Colonies for Determining the Distribution of Mutation Within the RRDR

1. Pick 100 colonies from antibiotic-containing plates for each day sampled and re-suspend them in 100 μL of TE buffer.
2. Heat-kill the cells at 80°C for 20 min. The DNA from these cells can now be sequenced.

3.6. Monitoring Chemostat Parameters

3.6.1. Daily

1. Check that the volume of liquid in the chemostat vessel is constant.
2. Check medium is entering the vessel and effluent is going into the waste pot.

3. Check that medium and waste volumes are at the expected levels and that pumps, stirrer, and magnetic flea are all working correctly.
4. Check the waste level and swap the waste to an empty pot containing neat disinfectant if required.
5. Fill in the chemostat run sheet for temperature level, pH level, DOT level, and stirrer speed (rpm).
6. Sample 5–10 mL of the culture for optical density. Kill the cells by the addition of 1/10 volume of 40% formaldehyde (v/v). Shake the sample vigorously and leave for 16 h before the sample can be measured for optical density. Dilute each sample fivefold in sterile, distilled water and place the resulting cell suspension in a plastic cuvette. Read the optical density at 540 nm against water (these readings are important for determining when the culture has passed into mid-logarithmic growth and then when it reaches steady-state).
7. Take a sample for independent pH measurement using a hand-held pH probe.

3.6.2. Weekly

1. Monday: Carry out purity checks on agar (2× blood agar and 2× Middlebrook agar plates) and measure the optical density.
2. Friday: Check the waste levels and if necessary divert the waste line to an empty waste bottle. Check that there is sufficient medium supply available for the culture to use over the weekend.
3. Weekend: Check the chemostat once during the weekend either on a Saturday evening or Sunday morning to make sure that all parameters are correct, all equipment is working correctly and that there are no leaks.

3.6.3. Occasionally

1. Move the tubing through the pumps approximately every 2 weeks in order to maintain elasticity of the tubing and to reduce the likelihood of splits developing.
2. Check flow rate (see Note 11) and temperature as required.

4. Notes

1. Store the pH probes in 3 M KCl and rinse with distilled water before use. Oxygen probes are stored dry. Change the membrane on the oxygen probe and refill with fresh electrolyte before every culture. Leak test oxygen probes in a beaker of water 24 h after changing the membrane. If leaks occur, change the membrane and re-test.

2. There are three thicknesses of tubing. To achieve a slow flow rate the internal diameter of the tubing that runs through the medium pump head should have a bore width of no more than 1 mm. However, thicker tubing with a wider bore width of 4 mm is used for the medium and effluent lines, and the thickest tubing with a bore width of 6 mm is used for all gaseous inlets and outlets.
3. Dead ends (some of which will be used for drawing off medium or cell samples) – such as air outlets, sample port side arms, waste bottles, collection bottles, medium bottles, and flow rate measurement devices – also need to be fitted with vent filters to ensure that gas can be released continuously from the culture during growth and pressure does not build up in the vessel. The air outlet/off-gas line can be fed into a CO_2 analyser for measurement of CO_2 levels. If for any reason these filters become wet they will be blocked, a vacuum will build up, and the culture will be sucked up through the air inlet.
4. Do not calibrate oxygen probes before autoclaving because the electrolyte is affected by the heat. Oxygen is only transferred evenly throughout the culture if it is stirred or shaken effectively. Standing cultures of *M. tuberculosis* will result in microenvironments in which the oxygen levels will be very low reaching micro-aerophillic or anaerobic levels.
5. There is a pH/temperature compensation mode on the controller to compensate for temperature differences because pH calibration is done at room temperature. Fittings that are exposed to the acid or alkali should be made of inert metal such as titanium to prevent corrosion caused particularly by the acid.
6. The heat pad is electronically controlled by the controller unit to maintain temperature at 37°C.
7. The dissolved oxygen tension (DOT) is maintained by an immediate response of the controller to a drop in oxygen level, which in turn alters the stirrer speed to draw more air into the medium. The controller unit automatically controls the extent to which the culture is stirred via a magnetic stirring device and a flea. A DOT of 10% is equivalent to 50% dissolved air saturation. The controller displays the dissolved air saturation not the DOT.
8. It is not advisable to use plates that are more than 4 weeks old because growth in the chemostat will be slow and cells will be more clumped in culture.
9. The DOT level in the culture could be above 10% DOT and will need to drop to 10% DOT as soon as possible. It is also important for the stirrer speed to increase to disperse the

cells. Once the DOT has dropped to the set point (10%), the controller will inform the stirrer to increase its speed to maintain a DOT of 10%.

10. DOT levels may fluctuate during a transition from batch to fed-batch.

11. A high flow rate too early on in continuous mode may lead to culture "wash-out." The flow rate is measured using a device that is constructed using a glass pipette, which has been inserted into the tubing between the medium bottle and the medium pump via a connector with a T junction in it. The pipette is capped with a piece of tubing and a vent filter (Fig. 1). The bottom of the pipette is normally clamped off. The clamp is removed and medium is drawn up into the pipette using a syringe attached to the vent filter at the top of the pipette. The medium bottle is then clamped off so that the culture subsequently draws the medium from the pipette and not from the medium bottle. The speed at which the culture uses the medium from the pipette is then measured. The flow rate and dilution rates can then be calculated (see Note 12). Remember to remove the clamp from the medium bottle and replace the clamp at the bottom of the pipette once flow rate determinations have been completed.

12. A flow rate of 15 mL/h will give a dilution rate of 0.03/h and a mean generation time of 23 h. The flow of medium into the vessel (F) is related to the culture volume (V) by the dilution rate (D) where $D = F/V$. The volume is expressed in mL, the flow rate is expressed in mL/h, so that the dilution rate is therefore expressed as per h. Under steady-state conditions the biomass remains constant, therefore the specific growth rate (μ) must equal the dilution rate, i.e. $\mu = D$. The dilution rate is related to the doubling time (T_d) by the equation $T_d = \log_e 2/D$. The equation, ($X = \Upsilon_{x/s}\ (S_o - S)$), has been derived from the material balance equation of the limiting nutrient across the system. The relationship between the yield of cells using substrate, $\Upsilon_{x/s}$ (grams of biomass per gram of substrate), and the limiting substrate can be calculated using $X = \Upsilon_{x/s}\ (S_o - S)$, where X is the biomass at steady state (g/L) and S_o and S are the concentrations of the limiting substrate in the feed and residual substrate in the outflow, respectively.

13. Run sheets and Soloview software are used to record parameters on a daily basis.

14. Under certain growth conditions mycobacteria will adhere to the walls of the vessels and the probes. Once this starts to occur, the optical density is likely to fall and the DOT and pH levels will fluctuate. The culture will no longer be in steady-state and will need to be transferred to another vessel.

15. pH levels should not be dropped rapidly as the culture will wash-out.
16. The sample size is dictated by the likelihood of a resistance mutation arising. For rifampicin this is likely to occur at a frequency of 10^{-9}. The sample will have to contain at least 10^{9} cells to achieve a single mutation that can be visualised by colony counting.
17. The sampling regimen that has been used in previous experiments was to sample daily in steady-state at each pH level for 6 days followed by weekly samples for 3–4 weeks.
18. The antibiotics that have been used in previous studies were rifampicin and streptomycin. These antibiotics were used at 2× MIC, 2 μg/mL and 4 μg/mL, respectively.

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Part IV

Detection of Resistance

Chapter 11

Identification of Efflux-Mediated Multi-drug Resistance in Bacterial Clinical Isolates by Two Simple Methods

Marta Martins, Isabel Couto, Miguel Viveiros, and Leonard Amaral

Abstract

Two simple, instrument-free, user-friendly methods that can readily be implemented by a routine microbiology laboratory are described for the detection of multi-drug-resistant (MDR) isolates that overexpress efflux pump (EP) systems responsible for the MDR phenotype.

The first method employs the universal EP substrate ethidium bromide (EB) at varying concentrations in agar-containing plates upon which the contents of an overnight culture are swabbed as spokes of a wheel. In this method, named the EB–agar cartwheel method, it is assumed that the smallest concentration of EB that produces fluorescence of the bacterial mass represents the highest concentration of EB that the bacteria can exclude. Consequently, as the efflux system(s) of a given MDR clinical bacterial isolate is overexpressed relative to that of a reference strain, the minimal concentration of EB producing fluorescence is significantly greater. A simple formula is provided which affords the ranking of MDR clinical isolates with respect to the degree of their efflux capacity.

The second method, which can be used after the first one, determines whether the MDR phenotype is based upon an overexpressed efflux system. This method employs a 24-well microplate with separate wells containing or lacking an efflux pump inhibitor (EPI) and Kirby–Bauer discs that correspond to the antibiotics to which the MDR strain is resistant. After the wells are inoculated with the MDR clinical isolate, the plate is incubated overnight and each well is evaluated by eye for evidence of growth. Comparison of growth to the relevant control enables the observer to determine the following outcomes: no growth produced by the EPI–antibiotic combination (i.e., reversal of antibiotic resistance); reduced growth produced by the EPI–antibiotic combination; no difference in growth, i.e., EPI does not affect the resistance to the given antibiotic. If the first method showed that there was a significant difference between the minimum concentrations of EB in agar that produced fluorescence for the clinical isolate and its reference strain, then one can conclude that if the EPI had no effect on reducing antibiotic resistance, the differences in the EB concentrations that produced fluorescence are probably due to differences in the permeability of the strain to EB, reflecting a downregulation of porins if the clinical isolate is a Gram-negative bacterium.

Key words: Multi-drug resistance, Efflux pumps, Efflux pump inhibitors, Ethidium bromide, Screening methods

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1. Introduction

The permeability of bacteria to antibiotics is controlled by the organism's cell envelope, which differs significantly between Gram-negative and Gram-positive bacteria, accounting for the difference in their Gram-staining properties. This structure provides a barrier that cannot be transverse readily by hydrophilic antibiotics. With respect to Gram-negative bacteria, the outer membrane of the cell envelope contains channels that allow a large variety of hydrophilic compounds to pass through and reach the periplasm. These channels are called porins and exist in large number per bacterium (1). Porins are structures that consist of three outer membrane proteins (OMP) that form a barrel-like structure and the tunnel created between these units is used by hydrophilic compounds to enter and reach the periplasm. The cell can respond to the presence of noxious agents that can pass through the porins by downregulating its number (2, 3); hence, the permeability of the cell to hydrophilic agents can be reduced. Gram-positive bacteria do not have porins but do have a large number of lipid-containing molecules on the surface of the envelope that render the cell relatively impermeable to hydrophilic agents. Both Gram-negative and Gram-positive bacteria contain a second line of defense against noxious agents that manage to reach the periplasm or the cytoplasm of the bacteria. These are efflux pumps (EPs), which recognize the noxious agent and rapidly extrude the agent to the medium in which the bacterium resides. The structure and classification of these EP is beyond the scope of this treatise and many excellent reviews that cover these topics have been recently published (4–7). However, at this time, one must distinguish the two types of EP systems that exist in bacteria and these are essentially differentiated by the source of energy that is used to drive the EP itself. Efflux pumps that derive their energy from the hydrolysis of ATP are part of the ATP-binding cassette (ABC) superfamily. These EP can be inhibited by agents that inhibit ATPases (kinases, phosphorylases, etc.). The other types of EP, the ones that derive their energy from the proton motive force, are not immediately affected by agents that inhibit the EP of the ABC superfamily. Given the significance of efflux mechanisms, particularly the ones known to contribute to intrinsic or acquired resistance associated with the MDR phenotype in bacteria (4, 6, 8–10); there is the need to develop new and effective efflux pump inhibitors (EPIs). The main goal has been the discovery and/or creation of new agents which have the capacity to inhibit MDR EP and, hence, render the organism susceptible to the antibiotics to which it was once resistant (2, 11). Over the past decades, many EPIs have been

identified, including phenylalanyl arginyl-β-naphthylamide (PAβN), verapamil, phenothiazines (for example: thioridazine (TZ), chlorpromazine (CPZ)), and compounds isolated from plants.

PAβN is known to be active against multiple EP of the Resistance Nodulation Cell Division (RND) family in Gram-negative bacteria, being active against MexAB-OprM, MexCD-OprJ and MexEFOprN EP, which contribute to fluoroquinolones resistance in *Pseudomonas aeruginosa* (12). The calcium channel antagonist verapamil, a well-known inhibitor of the *p*-glycoprotein (P-gp), also inhibits several bacterial EP of the ABC superfamily (13). Phenothiazines have been shown to inhibit the transport of Ca^{2+} and K^{+} since they inhibit the binding of Ca^{2+} to calmodulin, the Ca^{2+} binding protein. The inhibition of calcium access to Ca^{2+} -dependent ATPase inhibits transport processes such as those performed by influx and efflux pumps. Because phenothiazines inhibit access to calcium, they inhibit the activity of Ca^{2+}-dependent ATPase and, hence, the transport processes (14, 15). Other inhibitors, such as verapamil, are voltage-dependent Ca^{2+} channel blockers that inhibit several bacterial EP (13). EPIs isolated from plant extracts, such as ouabain and reserpine, have been intensively exploited for their ability to inhibit EP. Ouabain is known to block the Na^{+} pump (Na^{+}/K^{+} ATPase) (16) and reserpine is a plant alkaloid known to inhibit P-gp and also to potentiate the activity of fluoroquinolones on MDR Gram-positive bacteria (13). Natural products isolated from *Berberis* plants have also been identified as inhibitors of bacterial EP (17). Most EP systems, except for the ABC family, which utilizes ATP hydrolysis, utilize the proton motive force as an energy source to drive the export of substrates. Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is well known to dissipate the proton motive force, thereby inhibiting efflux (18, 19). EPIs clearly are shown to be promising for developing combination therapies with existing antibacterials to restore their antibacterial activity against MDR bacteria.

The basis for the expression of a MDR phenotype by bacteria is now known to be the result of overexpressed EP system(s), which extrude two or more unrelated antibiotics prior to their reaching their intended targets (4, 7). The ability of a laboratory to identify and report these efflux-driven mediated MDR bacteria is likely to become an important component of a bacteriological laboratory and its results reporting. Inasmuch, the presence of an overexpressed efflux system may either result in the need for a significant increase of antibiotic dose or its periodicity to overcome the capacity of the efflux system, or, at the very least, provide a warning that current therapy if continued as administered, may result in further escalation of resistance possibly including additional classes of antibiotics (20).

Current methods for the demonstration of an efflux system of a clinical isolate usually involve the assessment of ethidium bromide (EB),

a common substrate of EP of bacteria (21), accumulated by a given strain of bacteria in liquid medium subsequent to the addition of an EPI (22). Although this method is useful for identifying bacterial strains that overexpress an EP system, it requires instrumentation that is usually not present in the clinical laboratory. Furthermore, the method suffers from a low sensitivity and hence the assessment of intrinsic efflux systems of wild type strains is not readily possible because it lacks precision. For this reason, the method does not lend itself to interlaboratory comparison. Other methods such as the use of radioactively labeled antibiotics (21, 23) or the use of fluorescent probes (21) have also been developed for assessment of EP activity. However, these methods require specialized instrumentation and employ expertise not usually present in a clinical microbiology laboratory. If a method that assesses EP activity is to be readily implemented in a clinical microbiology laboratory, it would be best that the method employs procedures and instrumentation that are routinely used.

In this chapter, we describe two simple, user-friendly methods for the rapid and easy detection of efflux activity in bacterial isolates of clinical or environmental origin. The methodologies to be described combine simple microbiological routine procedures with some of the concepts described above, in particular, on the role of EB as a common substrate of bacterial efflux pumps and the sensitivity of many of the known efflux systems to a restricted battery of inhibitors.

2. Materials

2.1. EB-Agar Cartwheel Method

2.1.1. Bacterial Culture and Preparation of Plates

1. Tryptic Soy Broth (TSB) or Luria Bertani (LB) media from Oxoid (Basingstoke, Hampshire, UK).
2. Agar from Oxoid.
3. Phosphate Buffered Solution (PBS) from Sigma Aldrich (Madrid, Spain).
4. Antibiotics in powder from Sigma Aldrich.
5. EB solution (Sigma Aldrich) is prepared in distilled water at a stock concentration of 50 mg/mL, protected from light, and stored at 4°C. Working solutions are prepared by dilution in distilled water, protected from light and stored at 4°C (see Chapter 12 for precautions in handling EB).
6. Sterile swabs.
7. Sterilized Petri dishes.
8. TSB plates containing EB at a concentration of 0, 0.5, 1, 1.5, 2, and 2.5 mg/L are prepared by serial dilution of the media containing EB.

2.2. EPI-Based Microplate Assay

2.2.1. Bacterial Culture and EPIs

1. Mueller-Hinton (MH) broth and MH agar from Oxoid.
2. PBS from Sigma Aldrich.
3. Antibiotics discs from Oxoid.
4. Chlorpromazine (CPZ), thioridazine (TZ), phenylalanyl arginyl-β-naphthylamide (PAβN), and carbonyl cyanide m-chlorophenylhydrazone (CCCP) from Sigma Aldrich (see Note 1).
5. EPIs prepared in the appropriate solvent (see Note 2).
6. 24-wells microplate (Nalge Nunc International, USA).

3. Methods

3.1. EB-Agar Cartwheel Method

The EB-agar cartwheel method is used for the identification of presumptive overexpressed efflux system(s) responsible for an MDR phenotype in bacteria. This method employs the fluorochrome EB in agar as the substrate that identifies the existence of an overexpressed efflux system relative to the intrinsic efflux system of the corresponding wild-type strain, which is used as reference. The rationale of the method is based on the concept that there is a maximum concentration of EB which is effectively extruded by the cells and that higher EB concentrations will be retained, and hence when the bacterial mass is exposed to UV light, fluorescence will be detected. As the MDR phenotype may be due to an overexpressed efflux system, the concentration of EB needed to produce fluorescence of the bacterial mass is considerably higher than that concentration which produces fluorescence of the wild-type strain. A formula that affords the ranking of MDR clinical isolates with respect to EP activity is presented as part of the described method.

3.1.1. Preparation of Cultures and Performance of the Method

1. Reference and MDR clinical strains are grown in suitable liquid broth (volumes of 4–10 mL based upon preference) until they reach an optical density of 0.6 at 600 nm.
2. Twenty milliliters of agar-containing growth media supplemented with concentrations of EB ranging from 0 to 2.5 mg/L are dispersed to individual plates and allowed to cool (see Note 3).
3. Make a cartwheel pattern (see Fig. 1a).
4. Dip a single different swab into each culture; press the swab against the inside of the culture tube to reduce volume and streak the swab from the central circle (see Fig. 1a and Note 4) to the margin of the plate taking care not to deviate from the underlying line. The identification of the strain may be made on the side of the bottom plate corresponding to the end of the swabbed line.

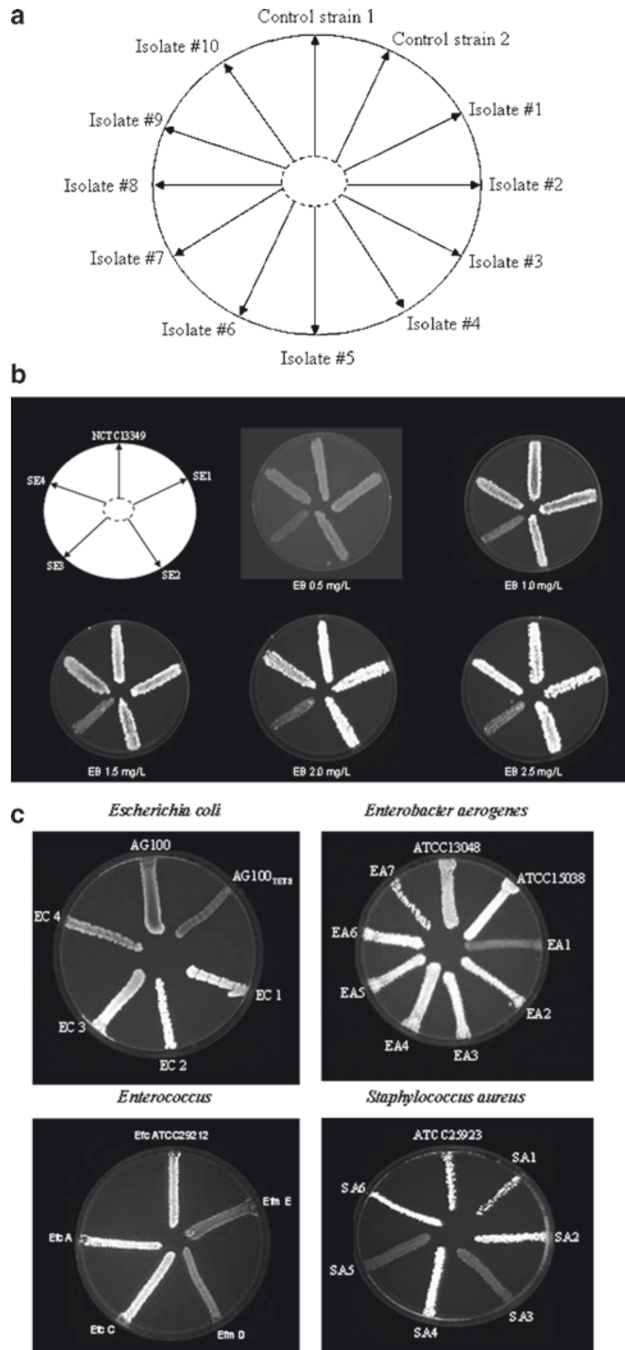

Fig. 1. (**a**) Cartwheel pattern for the swabbing of the strains to be tested by the EB-agar method. The pattern may be made on the bottom side of the agar plate with a water-removable marking pen. One or more control strains can be used, according to the specific design. If possible, we recommend the use of two control strains; one that shows no efflux activity (to be used as the reference strain) and one that has an overexpressed efflux system. The reference strain will begin to fluoresce at low EB concentrations, whereas the strain with the overexpressed efflux system will fluoresce at higher EB concentrations, thus providing an EB concentration range within which these

5. The arrangement of the swabbed lines is suggested as follows: reference strain at 12 o'clock. Swabbed MDR clinical strains at 1, 2, 3, etc., o'clock positions (see Fig. 1a). Depending upon the dexterity of the technologist, as many as 12 strains can be swabbed on a single EB-containing plate.
6. The EB–agar plates are incubated for 16–18 h at 37°C (see Note 5).
7. Each EB plate is examined under a suitable source of UV light (see Note 6). The minimal concentration of EB that produces fluorescence of the bacterial swabbed mass is recorded for the reference and MDR clinical strains. The higher the concentration of EB required for the presentation of fluorescence the greater the presumptive EP activity of the organism (see Fig. 1b and Note 7).
8. If desired, the EB efflux capacity of each MDR clinical strain can be ranked relative to the reference strain by the following formula (see Note 8).

Presumptive efflux-driven MDR phenotype of clinical strains may now be confirmed by the second method that follows.

3.2. Confirmation of EP Activity by a Microplate-Based Assay

Because MDR Gram-negative bacteria may owe their MDR phenotype to the down-regulation of their porins, and hence the permeability of the bacterium to EB may be highly decreased, the difference between the MDR clinical isolate and its reference strain with respect to the minimal concentrations of EB that produce fluorescence may not be at this time positive proof that the MDR is due to an overexpressed efflux system. The distinction between downregulation of porins and an overexpressed EP system can be made by the use of this second simple method that shows whether the EPI reduces or reverses the resistance of the MDR clinical isolate to individual antibiotics to which it was initially resistant.

Fig.1. (continued) two levels of efflux can be distinguished by this method. (**b**) The EB–agar cartwheel method applied to a set of five *Salmonella enteritidis* strains. The four isolates and NCTC13349 reference strain were swabbed as described in Fig. 1a in MH plates containing 0.5–2.5 mg/L of EB. As can be seen in the figure, strain SE3 does not fluoresce even at the highest concentration of EB tested (2.5 mg/L). The SE3 has been made resistant to ciprofloxacin and nalidixic acid by serial exposure to increasing concentrations of ciprofloxacin. This induced MDR phenotype results from the overexpression of *acrB*, which codes for the AcrB transporter of the AcrAB EP. (**c**) The EB–agar cartwheel method applied to different bacterial species. For simplicity, only one concentration of EB is shown. For *Escherichia coli* and *Enterobacter aerogenes*, the agar plate contains 1.0 mg/L of EB, while for *Enterococcus* and *Staphylococcus aureus* strains, the concentration shown is 2.5 mg/L. As can be seen, the $AG100_{TET,}$ EC4, EA1, Efc D, Efc E, SA3, and SA5 strains show lower fluorescence than the control(s) (reference strains) at the mentioned concentrations. These strains are all of an MDR phenotype that overexpress their main EPs.

MDR clinical strains that require significantly higher concentrations of EB for the presentation of fluorescence as evident by the use of the EB–agar cartwheel method can readily be confirmed for their overexpressed efflux system that mediates their MDR phenotype by a simple, instrument-free method that employs at its base the Kirby-Bauer antibiotic containing disc and commonly used EPIs. The method is based on the fact that if an EPI reduces significantly the resistance of the MDR strain to antibiotics to which it was initially resistant or renders the MDR clinical strain as susceptible to the antibiotics as the wild-type strain, then it is highly probable that the MDR phenotype of that strain is due to an overexpressed EP system.

The method employs a 24-well microplate with each well having a volume capacity of 1.0 mL. The antibiotics and EPIs to be tested may be distributed in the microplate as follows (Fig. 2a): the first column will be used to control bacterial growth with/without the EPIs; the wells in the second column will receive discs from the first antibiotic to be tested (AB1); the wells in the third column will receive antibiotics discs (AB2), etc. (until the sixth column). The different EPIs will then be distributed by each lane, so that the effect of each one will be tested against the five antibiotics being assayed.

All the wells will be inoculated with 0.1 mL of bacterial suspension. The plates will be incubated at the desired temperature for 16–18 h, and after this incubation, the contents of each well read by eye. The determination of the effect of an EPI is made by comparing the growth of the bacterium in the well containing a given antibiotic disc to the corresponding well containing that antibiotic disc plus an EPI. The results obtained can be indicative of no effect of the EPI (full growth), reversal of resistance to the antibiotics tested (absence of growth), or reduction of resistance to the antibiotic (less growth than the control).

3.2.1. Preparation of Cultures and Performance of the Method

1. Reference and MDR clinical strains are grown in suitable liquid broth (volume of 4–10 mL based upon preference) until they reach an optical density of 0.6 at 545 nm.
2. One milliliter of MH broth is to be distributed to the wells of a 24-well microplate (control wells).
3. Antibiotic discs will be distributed to the wells of the plate and the plate incubated at 37°C during 1 h, to allow the diffusion of the antibiotic to the medium.
4. After the incubation, the EPIs are to be distributed to the corresponding wells of the microplate at ½ their MIC (see Notes 9 and 10).
5. The wells are to be inoculated with 0.1 mL of the bacterial suspension (see Note 11).
6. The microplate should be incubated for 16–18 h at the appropriate temperature for the species tested (see Note 5).

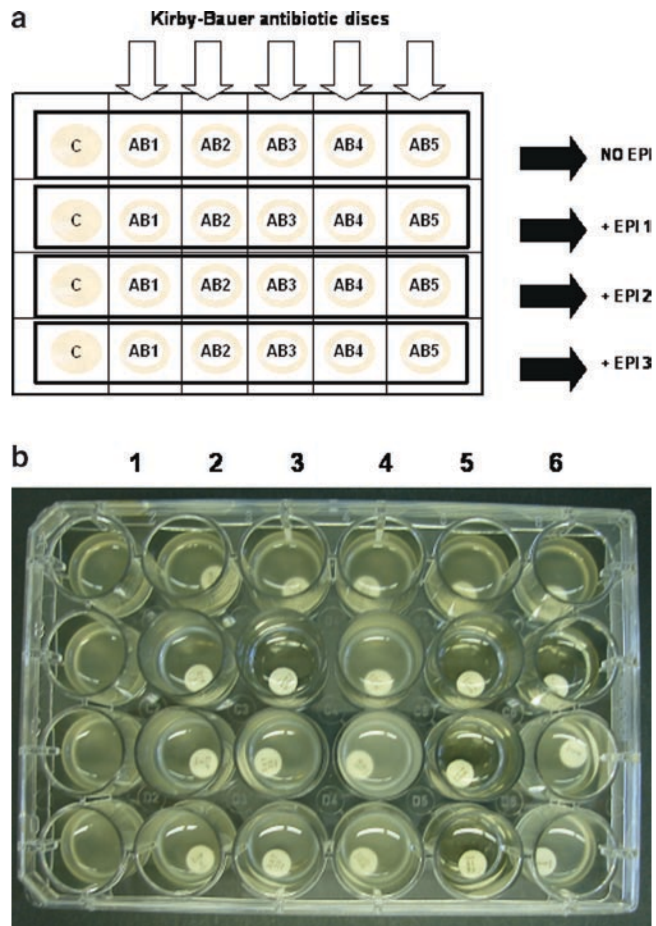

Fig. 2. (**a**) Schematic distribution of the antibiotics discs and the EPIs using the EPI-based microplate assay to evaluate the effect of three EPIs on the resistance to five antibiotics of an MDR isolate. *C* control wells, containing the bacterial culture, with or without the several EPIs tested, *AB* discs of different antibiotic (1–5), *EPI* efflux pump inhibitor. All wells received the same inoculums as the control wells. (**b**) Example of the EPI-based microplate assay applied to a MDR *E. coli* clinical isolate. The first column contains the control wells (see Fig. 2a) and the others contain antibiotics discs for ciprofloxacin (CIP), sulphamethoxazole/trimethoprim (SXT), gentamicin (GEN), tetracycline (TET), and erythromycin (ERY). EPIs distribution was as follows: first row – no EPI; second row – thioridazine (TZ); third row – chlorpromazine (CPZ); and fourth row – verapamil (VP) (see Fig. 2a). As can be seen, the strain showed reversal of resistance to SXT and ERY (rows 3 and 6 respectively) in the presence of TZ and to TET in the presence of all the EPIs tested (column 5). The strain also showed reduction in the resistance to CIP in the presence of TZ (second column, second row), which was confirmed by CFU counting.

7. After the incubation period the wells are to be observed and the results recorded and classified as: reduction, reversal, or no effect (according to the turbidity of the medium) (see Notes 12 and 13).
8. The contents of the wells demonstrating no growth or poorer growth, when compared to the controls, are to be plated in MH-agar plates for CFU counting (see Notes 14 and 15).

Table 1
Effect of the EPIs PAN and TZ on the resistance to tetracycline and ofloxacin of *E. coli* MDR strains

	Antibiotics and EPIs												
	TET		TET + PAN			TET + TZ			OFX		OFX + PAN		
***E. coli* strain**	CFU (×10^4)	MIC	CFU (×10^4)	MIC	EPI activity	CFU (×10^4)	MIC	EPI activity	CFU (×10^4)	MIC	CFU (×10^4)	MIC	EPI activity
EC 1	160,000	480	0	15	*RED*	FG	NA	NE	240000	80	0	2.5	*REV*
EC 2	450,000	240	0.96	15	*RED*	38	30	*RED*	NA	NA	NA	NA	NA

Antibiotic discs: tetracycline (TET) (10 µg); ofloxacin (OFX) (5 µg). EPIs: Phe-Arg-β-naphthylamide (PAβN), and thioridazine (TZ)
NA not applicable (strain susceptible to the antibiotic), *FG* Full growth (same CFUs as control without EPI), *NE* no effect, *RED* reduction, *REV* reversal (25, 26), *MIC* minimum inhibitory concentration (mg/L).

9. In parallel, new MIC determinations for the antibiotics for which reversal or reduction was observed may be conducted in the presence and absence of the EPI if confidence in the method needs to be established (see Notes 12 and 13).

An example of the type of results obtained from the use of this methodology is presented by Fig. 2b and Table 1.

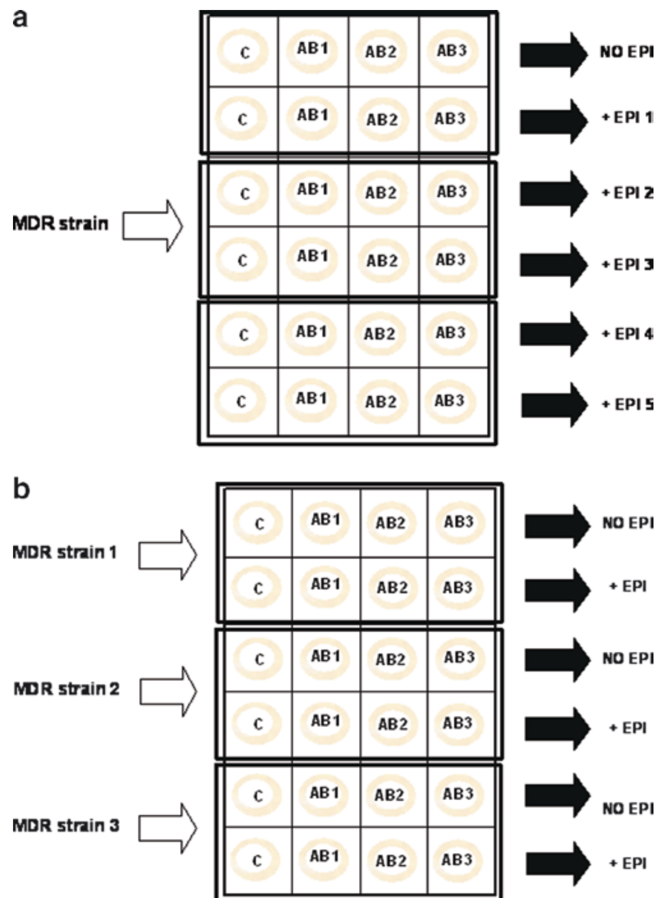

Fig. 3. (**a**) Schematic distribution of antibiotics discs and EPIs using the EPI-based microplate assay to evaluate several EPIs for activity. The method employs a control MDR strain that overexpresses EP activity. This method can be used to screen large collections of EPIs using previously characterized MDR species. *AB* Antibiotic discs (1–3), *EPI* efflux pump inhibitor. All wells containing antibiotic discs received the same inoculum as the controls wells (C) (which contain the bacterial culture and no antibiotic). (**b**) Schematic distribution of the antibiotics discs and the EPIs using the EPI-based microplate assay to evaluate the effect of one EPI on the resistance to antibiotics of several MDR isolates. This method can be used in a different format to assay the effect of one EPI on the resistance of three MDR strains previously characterized for their MDR phenotype. *AB* Antibiotic discs (1–3), *EPI* efflux pump inhibitor. All wells containing antibiotic discs received the same inoculum as the controls wells (C) (which contain the bacterial culture and no antibiotic).

This methodology can be further explored to accommodate other types of screening, namely, the evaluation of large collections of potential EPIs, by testing a single MDR strain against a panel of such compounds (Fig. 3a) or by testing one EPI against several (up to three) MDR strains (Fig. 3b). Other combinations can also be pursued, according to the desired protocol to be implemented in each laboratory.

Thus, besides its simplicity and easiness to perform, the EPI-based microplate assay shows high versatility, which is extremely attractable, when testing large collections of bacterial isolates or potentially new EPIs. Additionally, it requires small quantities of potential EPIs, which is a major achievement, since many of such compounds are either difficult to obtain or only available in minute quantities, both of which increase drastically their costs.

4. Notes

1. Unless stated otherwise, all solutions of EPIs should be freshly prepared in water on the day of any given experiment (see also Note 9).
2. Phenothiazines solutions (TZ, CPZ) should be protected from light and stored in 1 mL aliquots at −20°C (see also Note 9).
3. Concentrations of EB to be used is dependent upon empirical demonstration of ranges expected for a given reference and MDR clinical strains. As an example, we have found that for *E. coli* the selection of the following concentrations was adequate for identifying wild-type (intrinsic EP system) and MDR clinical strains that overexpressed an EP system: 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/L.
4. Recommended diameter of the central circle is 1.0 cm. This will ensure that no contamination between swabbed materials occurs.
5. The optimum incubation temperature depends upon a given species. For MDR strains obtained from poultry sources, a temperature corresponding to the body temperature of the bird may be more appropriate (example: *Campylobacter jejuni* isolated from poultry expresses a greater degree of impermeability at 40°C than at 37°C (24)).
6. Illumination with UV is best from the underside of the EB agar plate. Source of UV illumination may be a handheld UV lamp with option of short and long UV wavelength or any UV transilluminator or semiautomated instruments, which can also photograph the EB fluorescing plates if a permanent record is desired.

7. The term "presumptive" EP activity is used since the reduction in the number of porins due to downregulation of the OMP can reduce the permeability of the Gram-negative bacteria to EB. MDR clinical strains presumptive of overexpressed EP activity are then selected for evaluation by the second method that confirms or contradicts EP overexpression.
8. Formula for ranking MDR clinical strains on the basis of EB efflux capacity:

$$\text{Index} = \frac{MC_{EB}(MDR) - MC_{EB}(REF)}{MC_{EB}(REF)}$$

   where MC_{EB} (REF) and MC_{EB} (MDR) represent the minimal concentration of EB that produces fluorescence of the swabbed bacterial mass of the reference and MDR clinical strains, respectively.
9. The EPIs that we test routinely are chlorpromazine (CPZ), thioridazine (TZ), verapamil (VP), and Phe-Arg-β-naphthylamide (PAβN) (Sigma Aldrich). Stock solutions of each of these EPIs are prepared in distilled, sterile water and stored at −20°C. The phenothiazine solutions need to be kept protected from light. Cyanide m-chlorophenyl-hydrazone (CCCP) (Sigma Aldrich) is dissolved in methanol/water (1:1, v/v) and stored at −20°C. The solution may need to be heated to dissolve. Working solutions of each EPI are prepared in the day of the experiment by dilution in distilled, sterile water.
10. The MIC for each EPI should be determined for each set of strains, by the broth microdilution method. The determination of the MIC is essential to establish the concentration of the compound to be used in the screening assays, in order to ensure that reductions observed in the cell number are due to the EP inhibitory activity of the EPI and not to cell death. In our experience, only by using EPI concentrations at half the MIC or less can cell viability be assured.
11. A bacterial suspension is prepared by dilution of 0.01 mL obtained from a culture (see note 1) diluted in 10 mL of a saline solution (such as phosphate buffered solution – PBS).
12. The effect of each EPI on the resistance to a given antibiotic can be classified as (1) reversal; corresponding to no growth, which implies that bacteria became fully susceptible to the antibiotic; (2) reduction (poorer growth compared to the control, indicating that efflux contributed partially to the resistance) or (3) no effect (no change in the growth in the presence or absence of the EPI) – see Table 1.

13. In terms of antibiotics MICs, these terms should correlate with the susceptibility category, as follows: (1) reversal: in the presence of the EPI, the MIC for the antibiotic decreases to the susceptibility range; (2) reduction: in the presence of the EPI, the MIC for the antibiotic decreases but remains within the resistance/intermediate range; (3) no effect: no change in the MIC value in the presence or absence of the EPI – see also Table 1.
14. Serial dilutions of the well contents should be prepared in a saline solution and plated in MH agar plates for colony forming units (CFU) counts. This will complement the result obtained by visual evaluation of the results.
15. One should bear in mind that the absence of effect by the EPI(s) does not exclude the presence of efflux system(s), which may not be sensitive to the action of the particular EPIs used in the assay.

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Chapter 12

Evaluation of Efflux Activity of Bacteria by a Semi-automated Fluorometric System

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Abstract

A semi-automated method that uses the common efflux pump (EP) substrate ethidium bromide (EB) is described for the assessment of EP systems of bacteria. The method employs the Rotor-Gene™ 3000 thermocycler (Corbett Research) for the real-time assessment of accumulation and efflux of EB in Phosphate-Buffered Solution (PBS) under varying physiological conditions, such as temperature, pH, presence and absence of the energy source, and presence of efflux pumps inhibitors (EPIs). The method is sufficiently sensitive to characterize intrinsic EP systems of reference strains, a prime necessity if there is a need for assessment of EP-mediated multi-drug resistance (MDR). The method has been successfully applied by us to characterize intrinsic and over-expressed EP systems of *Escherichia coli*, *Salmonella* Enteritidis, *Enterobacter aerogenes*, *Enterococcus faecalis* and *Enterococcus faecium*, *Staphylococcus aureus*, and *Mycobacterium smegmatis* and *Mycobacterium avium*, suggesting that if the organism can be maintained in PBS, the system described may suffice for the evaluation and assessment of its EP system.

Key words: Efflux pumps, Efflux pump inhibitors, Permeability, Ethidium bromide, Semi-automated fluorometric method

1. Introduction

Resistance mechanisms to antimicrobials fall into three major categories: (1) Antimicrobial target and receptor alteration; (2) Antimicrobial modification and/or destruction; and (3) Prevention of the antimicrobial from reaching its intended target by either a decrease of permeability due to the outer membrane (lipopolysaccharide) or decrease of porins (restricted to Gram-negatives), or over-expressed EPs that recognize the antimicrobial and extrude it (1).

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During the antibiotic era (from the 1950s to the present), the first two mechanisms have been overcome by chemically designing more potent receptor agonists, inactivation of modifying enzymes and creating antimicrobials that cannot be modified by the bacteria, through the introduction of changes in their chemical structure. The component of the third mechanism that prevents the antimicrobial from reaching its target, namely increased efflux, contributes to the multi-drug resistant (MDR) phenotypes of important clinical isolates. This has recently been accepted as a viable target for the control of MDR bacterial infections although antibacterial efflux was first hypothesized in 1978 with the description of membrane bound antibiotic transport proteins in Gram-negative bacteria (2–5). Since then the role of permeability changed and efflux-mediated resistance in bacteria has been further explored, showing that EPs that extrude noxious agents such as unrelated antibiotics, antimicrobials, toxins, and biocides are common to all pathogenic bacteria and ensure their survival in an environment where such substances are present (6–8).

The genetic and physiological basis for the MDR phenotype of clinical isolates has been associated with porin deficiencies and over-expression of EPs which, when present in the same organism, decrease the permeability of the bacteria to two or more unrelated antibiotics (9–12). Infections by efflux-mediated MDR bacteria cause major therapeutic problems and if these problems are to be avoided, they must be recognized as early as possible. The selection of appropriate therapeutic modality should consider the dosage of drugs, the periodicity and days of therapy. Failure to recognize these bacterial pathogens will also contribute to the selection of chromosomally based resistant variants (13–16).

The ability of a laboratory to identify and report these MDR bacteria has already been stressed in Chap. 11, where the combination of two simple instrument-free and user-friendly methods are described for the detection and confirmation of MDR clinical isolates that over-express EPs systems. These methods allow the rapid detection of MDR bacteria. However, if there is a need to characterize the type of EP system that is responsible for the MDR phenotype of the clinical isolate, the described rapid methods may be followed by other methods recently developed by us (17–19) which allow: (1) the determination of whether the efflux activity is due to over-expressed EPs that derive their energy from the hydrolysis of ATP, which can be inhibited by EPIs that affect ATPases or from the proton motive force, which can be uncoupled by compounds like carbonyl cyanide m-chlorophenylhydrazone (CCCP) (7, 9); and (2) the identification of distinct EPIs that may be used in combination with conventional antibiotic therapy and which, due to their ability to inhibit the type of EP system responsible for the MDR phenotype of the clinical isolate, will lead to successful therapy. Because it will be the clinical

microbiology laboratory that will provide the information required for the therapy of an EP-mediated MDR infection, the methods that are described in this chapter have been designed to accommodate the needs of that laboratory, namely: (1) simplicity of the method; (2) a user-friendly, computer-assisted technology; (3) precision and reproducibility, and (4) provide the basis for inter-laboratory comparison of data.

1.1. Rationale for the Semi-automated Fluorometric Method

The use of fluorometry provides a powerful tool to continuous monitor the transport of fluorescent substrates through the cell envelope of living bacterial cells, which can be correlated with increased efflux activity and decreased permeability of the bacteria in response to a selective environment where the presence of antimicrobials is constant (20–25). For many years, fluorometry has been used and improved to detect and quantify, accurately, the transport of fluorescent substrates across the cell wall of bacteria and the membranes of eukaryotic cells and was the basis upon which flow cytometry became a world-wide recognized technique for cell counting and analysis (26–29). As shown in Chap. 11, EP fluorescent substrates, such as ethidium bromide (EB), have been shown to be particularly suitable to be used as a probe for these studies because they emit weak fluorescence in aqueous solution (outside cells) and become strongly fluorescent in nonpolar and hydrophobic environments, especially as they penetrate the bacterial cell wall and accumulate in the periplasmic space of Gram-negative bacteria or cytoplasm adjacent to the plasma membrane of the cell envelope of Gram-positive bacteria.

Thus, the time-dependent fluorescence intensity of EB has been used for real-time monitoring of the accumulation and efflux of substrates in bacterial cell suspensions (21–23, 26–29). This approach provides the sum of transport kinetics reflecting the balance between accumulation of substrate via passive diffusion through the membrane permeability and extrusion via efflux. The desired aim is to easily and accurately detect and quantify the transport of EB through the bacterial cell wall, at working concentrations that will not affect cell viability nor perturb cellular function, using a methodology that allows the distinction between these two transports across the cell. For this purpose, the use of flow cytometry, real-time single-cell microscopy, and spectroscopy are not appropriate since they reflect single-cell events and the intent is the monitoring of the efflux of substrates that are accumulated by a large population of bacterial cells (24, 27–29).

1.2. General Overview and Applications of the Semi-automated Fluorometric Method

The instrument employed for the method to be described is the Rotor-Gene™ 3000 real-time thermocycler (Corbett Research, Sydney, Australia), which provides among many available wavelengths, the appropriate excitation and emission filters (530 nm bandpass and 585 nm highpass, respectively) for following, on a

real-time basis, the accumulation of the EP substrate EB and its extrusion under a selected temperature that may range from 25 to 44°C or even higher. Higher temperatures may be useful if the MDR clinical isolate to be studied has been obtained from poultry sources (i.e. *Campylobacter pylori* which have different temperature-responsive properties to a given antibiotic/antimicrobial) (30), or if environmental studies with thermophilic bacteria are of interest since the instrument can be programmed to up to 99°C. The data generated from the accumulation and/or efflux of EB by the instrument can be exported to Excel and reflects the degree of fluorescence generated per unit period of time. The user can select and programme for temperature, the unit of time for each measurement, the total number of cycles and the total period of time for measurements to be taken.

Gram-positive and Gram-negative bacteria that over-express efflux pumps, as defined by the methods described in Chap. 11, are grown in suitable broth until they reach a mid-log phase. After these cells are centrifuged and the medium replaced with phosphate-buffered solution (PBS) at a desired pH. Aliquots are transferred to microtubes to which EB is added and the tubes placed into the instrument that has been programmed (as indicated above). The data points representing degree of fluorescence are presented by the monitor as they are determined (real-time data points). This provides the user control over the system such that he may stop the measurements at will if and when data limits are reached, or if there is a need to add a reagent that will affect accumulation and or efflux. Examples are provided through this section that will illustrate the versatility of the system with respect to the characterization of the over-all efflux system of the bacterium.

The accumulation assay quantifies the ability of the strain to handle increasing concentrations of EB. The concept behind this assay is that when the ability of the EP systems to extrude EB is exceeded, EB will accumulate with time. Conditions which are known to optimize efflux by EPs are an energy source (glucose), a physiologically relevant pH of 7.0–7.4 and an incubation temperature of 37°C. An example selected for the demonstration of this type of assay is described by Fig. 1a with the characterization of the intrinsic efflux system of *Escherichia coli* K-12 AG100 by the use of distinct EPIs. As shown by Fig. 1a, accumulation of EB by *E. coli* K-12 AG100 is relatively stable (no increase over time) when EB is at the maximum concentration of 1 mg/L in PBS containing glucose, over a period of 30 min. When EPIs are present in the medium from the start of incubation, increasing accumulation of EB takes place with concentrations of the EPIs (used one-half the MIC or lower) that do not affect replication of the organism (Fig. 1a). That accumulation is due to the inhibition of efflux, which is demonstrated using an efflux assay as shown by Fig. 1b.

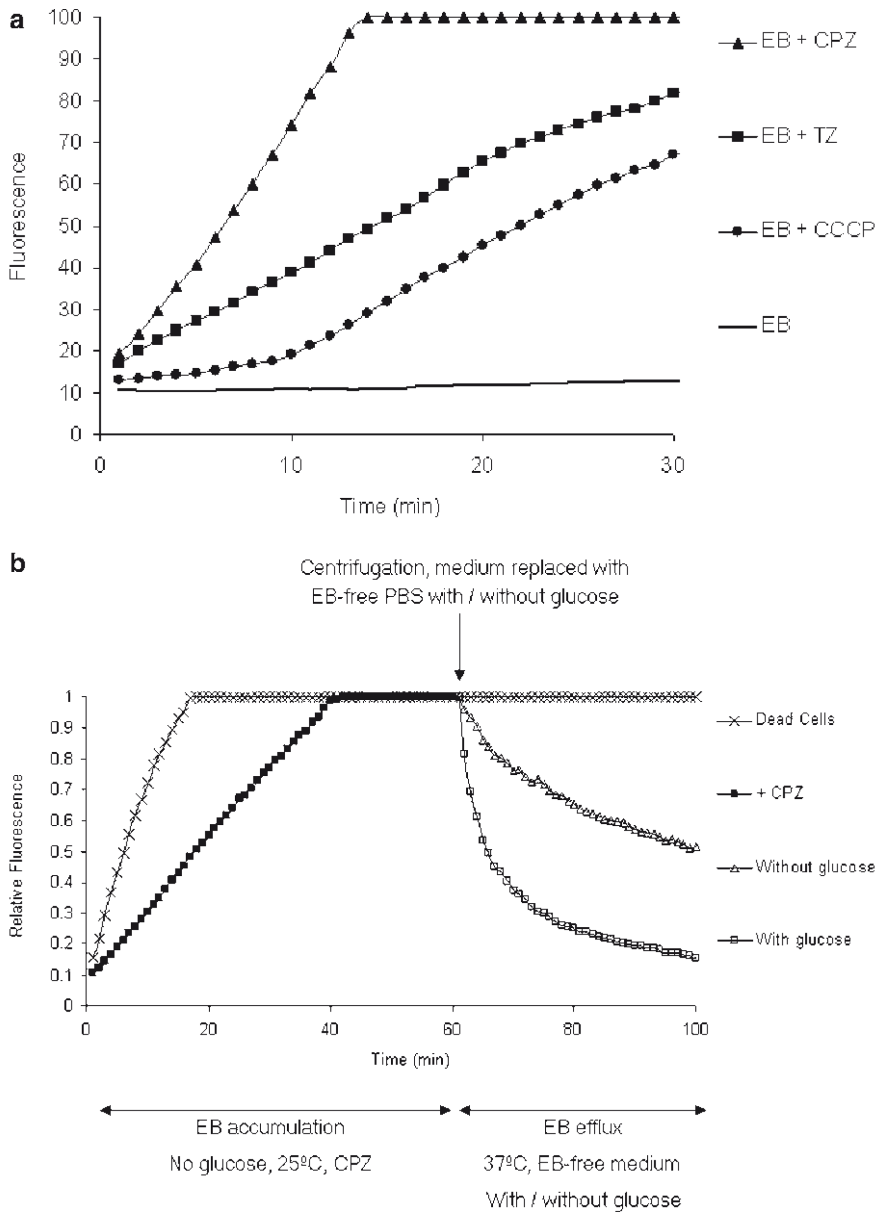

Fig. 1. (**a**) *EB accumulation assay in E. coli K-12 AG100*. The assay was conducted at 37°C with 1 mg/L of EB in the presence of glucose. The EPIs chlorpromazine (CPZ), thioridazine (TZ), and carbonyl cyanide m-chlorophenylhydrazone (CCCP), used at one-half of the MIC, increased the EB accumulation inside the bacterial cells. (**b**) *Accumulation and efflux of EB in E. coli K-12 AG100*. EB efflux takes place in the presence of glucose (in this assay used at 0.4%) and is reduced in the absence of glucose. No efflux is observed when working with KCN-killed cells.

In this assay, the bacterial cells that accumulated EB due to the presence of an EPI (in this case, chlorpromazine (CPZ)) are centrifuged and the medium replaced with EB-free PBS containing or lacking glucose (demonstration of the need for energy).

As evident from Fig. 1b, in the presence of glucose, the amount of fluorescence due to EB decreases with time (efflux). In the absence of glucose, efflux is reduced. In addition, *E. coli* AG100 that had been killed with potassium cyanide (KCN) was incubated in PBS containing EB and CPZ for 60 min, centrifuged and the medium replaced with EB-free PBS containing glucose. Because dead cells that had accumulated EB do not efflux in EB-free medium that contains glucose (Fig. 1b), efflux of EB must be considered to involve the generation of energy.

The semi-automated fluorometric method described above can be employed for the comparison of intrinsic and over-expressed efflux systems of bacteria. As shown by Fig. 2a, in *Salmonella* Enteritidis NCTC13349 reference strain the maximum concentration of EB that this organism can extrude and which represents the steady state (influx = efflux) is 1 mg/L. Above this concentration, EB tends to accumulate. In contrast, *Salmonella* Enteritidis 5408_{CIP}, which has been made resistant to ciprofloxacin (CIP) by serial exposure to this antibiotic, and which has a fivefold over-expressed AcrAB EP does not begin to accumulate EB until a concentration of 5 mg/L of EB is exceeded (Fig. 2b). That the absence of increasing accumulation of EB by the above strain in PBS that contains 5 mg/L of EB is due to an efflux system, is demonstrated when the uncoupler of the proton motive force CCCP is added and, within a few minutes, accumulation of EB begins to take place (Fig. 2c).

The assay described in the protocols below has also been applied to reference and MDR strains of *Enterobacter aerogenes, Mycobacterium avium*, *Enterococcus faecalis*, *Enterococcus faecium,* and *Staphylococcus aureus* (18, 19). It may also be noted that the assay lends itself to studies that investigate physiological conditions that may affect efflux of EB. As an example (Fig. 3), incubating *Salmonella* Enteritidis 104 in PBS at pH 8.0 in the presence of glucose and the EPI (TZ), immediate accumulation of EB takes place, soon followed by efflux of EB. Interestingly, if palmitic acid (PA) is present from the on-set of the incubation, there is little accumulation produced by TZ and, hence, no efflux is visible in the presence of the EPI. These results suggest that if glucose metabolism is blocked, as would be the case with the presence of TZ, an alternative energy source such as a fatty acid can be used for the driving of the EP of this organism. Similar results have been obtained with MDR *E. coli* veterinary strains obtained from poultry sources.

Lastly, the reader is encouraged to manipulate the semi-automated fluorometric method for the characterization of intrinsic and over-expressed EPs relevant to the physiology of efflux for different species of bacteria as new information is presented in the literature.

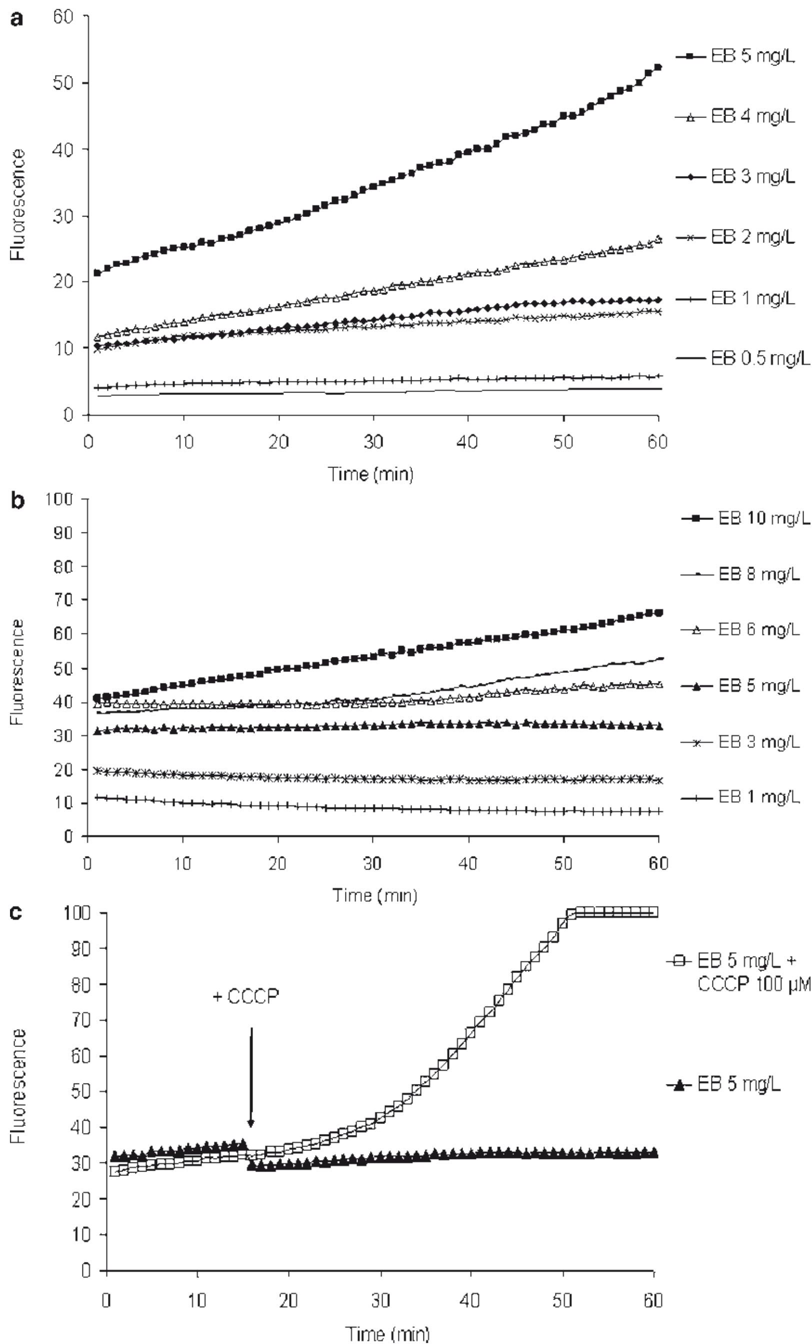

Fig. 2. (**a**) *Accumulation of EB (0.5–5 mg/L) at pH 8.0 in the presence of glucose at 0.6% by Salmonella* Enteritidis *NCTC13349 strain.* Above the concentration of 1 mg/L of EB, the slope of accumulation begins to increase indicating that the strain is beginning to experience difficulty in the extrusion of EB. (**b**) *Accumulation of EB (1–10 mg/L) at pH 8.0 in the presence of glucose at 0.6% by Salmonella* Enteritidis *5408*$_{CIP}$ Above the concentration of EB of 5 mg/L, fluorescence begins to increase indicating that the strain is losing its capacity to extrude EB. (**c**) *Effect of CCCP (100 μM) on accumulation of EB (5 mg/L) by Salmonella* Enteritidis *5408* $_{CIP}$ The assay was conducted at pH 8.0 and in the presence of glucose at 0.6%. Accumulation of EB begins after the addition of CCCP.

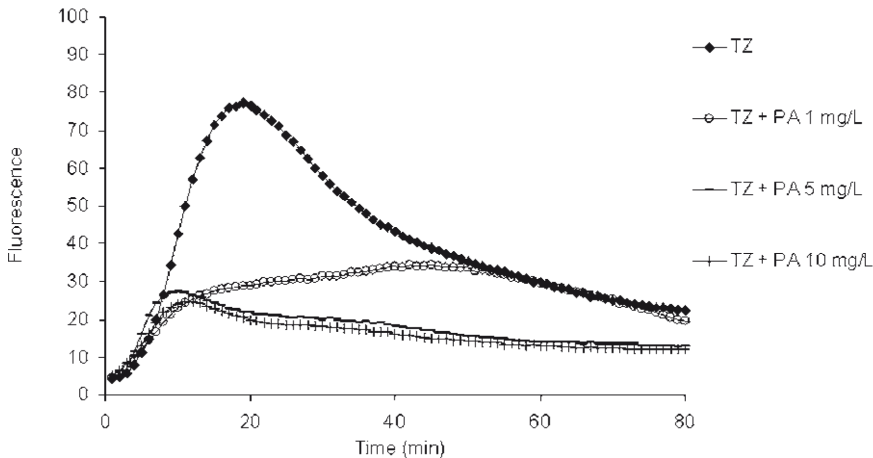

Fig. 3. Accumulation of EB (5 mg/L) by *Salmonella* Enteritidis 104 in the presence of TZ (50 mg/L) at pH 8.0 in PBS containing glucose at 0.6% or increasing concentrations of PA (palmitic acid) at 1, 5, and 10 mg/L.

2. Materials

2.1. Culture Media and Reagents

1. Luria Bertani (LB) and Tryptic Soy Broth (TSB) media from Oxoid (Basingstoke, Hampshire, UK) (see Note 1).
2. PBS in tablets (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) from Sigma Aldrich (Madrid, Spain) is dissolved in distilled, sterile water and autoclaved.
3. Glucose (Sigma Aldrich) is dissolved in distilled, sterile water, and stored in aliquots at 4°C.

2.2. EB and EPIs

1. Stock solutions of EB (Sigma Aldrich) at 50 mg/L are prepared in distilled, sterile water (see Note 2 *for precautions that must be taken when handling EB and the preparation of EB solutions*).
2. CPZ and TZ (Sigma Aldrich) are dissolved in distilled, sterile water and stored at –20°C. The working solutions of CPZ and TZ need to be kept protected from light.
3. CCCP (Sigma Aldrich) is dissolved in methanol/water (1:1, v/v). The solution may need to be heated to dissolve. Aliquot and store at –20°C. Working solutions are prepared on the day of the experiment by dilution in distilled, sterile water.

2.3. Specific Equipment Needed to Perform the Method

1. Single Beam Spectrophotometer (optical density measurements at 600 nm). Suggest visit to website of Direct Industry for complete listing of appropriate, inexpensive single

beam spectrophotometers. (http://www.directindustry.com/industrial-manufacturer/photometers-colorimeters-523/spectrophotometer-64077.html).

2. Microcentrifuge BioFuge Pico (Heraeus); Kendro Laboratory Products.
3. Rotor-Gene™ 3000 real-time thermocycler (Corbett Research, Sydney, Australia).

3. Methods

3.1. Accumulation Assay

3.1.1. Preparation of Cultures

1. Reference and MDR strains are grown in 10 mL of suitable liquid broth until they reach an optical density at 600 nm ($O.D._{600nm}$) of 0.6.
2. Centrifuge 1 mL aliquots at 16,060 × *g* for 3 min, discard the supernatant, wash the pellet 2× with PBS and re-suspend in 1 mL of the same buffer. Adjust the bacterial suspension to a final $O.D._{600nm}$ of 0.3 with PBS.
3. Add glucose to yield a final concentration of 0.4–0.6%.
4. Add EB to yield the desired final concentration (see Note 3).
5. Transfer 0.095 mL aliquots of the bacterial suspension containing EB to 0.2 mL PCR microtubes.

3.1.2. Demonstration of the Effect of EPIs on EB Accumulation

To test the activity of EPIs CPZ, TZ and CCCP 0.005 mL of the compound is added to the above microtubes to yield a final concentration that should not exceed one-half of the minimum inhibitory concentration (MIC) (see Note 4).

3.1.3. Controls for the Assay

The following controls should be included in the first series of assays conducted (see Note 5). Bacterial controls: (1) Bacteria in PBS without EB; and (2) Bacteria in PBS with EB. Controls for EPIs: (1) PBS + EB at different concentrations; (2) PBS + EPI; and (3) PBS + EPI + EB.

3.1.4. Setting the Programme in the Rotor-Gene™ 3000 and Measurement of Fluorescence

1. Programme the instrument with the following settings: a temperature (see Note 6), a unit of time for each measurement (cycle) of 60 s and the number of cycles necessary to obtain the intended total period of time (i.e. 30 cycles to obtain a 30-min assay).
2. Select the appropriate wavelengths for excitation and detection of fluorescence: 530 nm band-pass and the 585 nm high-pass filters, respectively, for EB.

3. Place the 0.2 mL microtubes into the instrument's 36-well rotor, close the protection lid and start the machine. During the assay the microtubes are subject to a centrifugation at 500 rpm and the fluorescence is measured at the bottom of each tube. As data is acquired, a graphic representation of the increase of fluorescence over time is presented in the monitor, allowing the real-time visualization of the results.

3.2. Efflux Assay

3.2.1. Preparation of Cultures

1. Reference and MDR strains are grown in 10 mL of suitable liquid broth until they reach an O.D.$_{600\ nm}$ of 0.6. The bacteria are collected by centrifugation at 16,060 × *g* for 3 min. The supernatant is discarded, the pellet washed once and re-suspended in PBS (1×).
2. EB at a final concentration of 1 mg/L and the EPI that caused the highest level of EB accumulation at one-half the MIC are added to the bacterial suspension (see Note 7).
3. Incubation is at 25°C over a period of 60 min (see Note 8).

3.2.2. Demonstration of Efflux of EB

1. The EB-loaded bacterial suspension is centrifuged at 16,060 × *g* for 3 min, the supernatant discarded and the pellet re-suspended in cold PBS (1×). The tubes are then placed on ice (see Note 9).
2. Aliquots of 0.095 mL of the bacterial suspension are distributed to 0.2 mL PCR microtubes.
3. Glucose to yield a final concentration of 0.4–0.6% and the EPIs at one-half the MIC are added in order to obtain microtubes containing: (1) Bacteria without glucose + the EPI used in the loading of the cells with EB (control for conditions of no efflux); (2) Bacteria without glucose; (3) Bacteria with glucose (control for optimal conditions of efflux); and (4) Bacteria with glucose + EPI.

3.2.3. Setting the Programme in the Rotor-Gene™ 3000 and Measurement of Fluorescence

Proceed as already described for the EB accumulation assay: programme the instrument with a temperature of 37°C, a unit of time for each measurement (cycle) of 60 s and the number of cycles necessary to obtain the intended total period of time (i.e. 30 cycles to obtain a 30-min assay) (see Note 10). Select the excitation (530 nm bandpass) and detection (585 nm highpass) wavelengths of fluorescence, place the 0.2-mL microtubes into the instrument's 36-well rotor and start the assay.

3.2.4. Normalization of Data and Presentation of Results

The results are presented in terms of relative fluorescence, calculated relatively to the control in which the bacteria are under conditions of restricted efflux (see Note 11).

4. Notes

1. The media should be selected according to the bacteria studied. For example, if working with *E. coli* use LB medium, for mycobacteria use Middlebrook 7H9 broth medium supplemented with 10% of oleic acid–albumin–dextrose–catalase (OADC) (Difco, Detroit, Mi, USA).
2. *Caution*: Ethidium bromide (EB) is a powerful mutagen. Preparation of solutions of EB should be under a fume hood. When preparing and working with EB solutions, non-absorbent gloves should be worn; the surface of the bench top must be covered with an absorbent type of paper with the plastic side facing the surface of the bench. Care should be taken to avoid contaminating the working area. All the disposable materials used for EB preparation or manipulation should be identified as contaminated with this dye and disposed in an appropriate container for subsequent incineration (31). EB stock solution is prepared as follows: tare a 25-ml flask containing a magnetic stirrer; carefully weigh out 0.5 g and add 10 mL of distilled water. This solution should be heated in a hot stirring magnetic plate under a fume hood until the solution is completely dissolved. During all of this procedure the operator should be using a mask, two pair of gloves and the laboratory material used to prepare the solution should be identified. After preparation, the solution should be stored at 4°C and protected from light.
3. For each bacterial strain the EB concentration to be used must be determined before the study of the effect of the EPIs. If it is the case that this determination cannot be performed, EB can be used at the empirical concentration of 1 mg/L.
4. Other EPIs such as verapamil and Phe-Arg-β-naphthylamide (PAβN) may be used. Because some EPIs do have antibacterial activity, MICs for the EPIs to be tested on each bacterial strain must be conducted prior to their use. It is also possible to determine the effect of EPIs on efflux of EB if they are added in a concentration-dependent manner to replicate tubes.
5. Bacterial controls for the assay:
   (a) Bacteria in PBS without EB. This will ascertain that the bacteria do not contribute to the emission of fluorescence.
   (b) Bacteria in PBS with EB. This will allow following the accumulation of EB inside the cell that will be reflected by the increase of fluorescence.

Controls for EPIs:

(a) PBS + EB at different concentrations. This will indicate when the concentration of EB produces significant fluorescence. This baseline of fluorescence should be compared to that emitted by EB during the accumulation assay involving bacteria and EB steady-state (influx = efflux). The baseline of emission must be less than one-half of that produced by EB in steady-state.

(b) PBS + EPI. This will insure that the EPI does not contribute to the baseline fluorescence of EB.

(c) PBS + EPI + EB. This will insure that interaction of the EPI with EB does not contribute to baseline fluorescence of EB.

6. Other temperatures can be selected according to biological requirements of the strain used or conditions to be tested.
7. According to the bacterial strain tested and from our experience with MDR strains it might be necessary to use higher concentrations of EB, for example 3 mg/L (18).
8. In order to measure EB efflux, it is necessary to promote the maximum accumulation of EB. This is accomplished by exposing the bacteria to EB under conditions that restrict efflux to its minimal activity, namely: a temperature of 25°C, absence of glucose, and the presence of the most active EPI (17, 18).
9. We have found that in *E. coli*, EB efflux takes place within the first 10–15 min of the assay at 37°C. This period of time is even shorter in the case of MDR. Therefore, in order to avoid the loss of efflux data, it is crucial to restrict any efflux activity from the time that EB and EPI are removed from the medium and the medium replaced with EB-free PBS until the measurement of fluorescence. For this reason, and since the EP activity is diminished at lower temperatures, the bacteria are re-suspended in cold PBS (1×) and the microtubes kept on ice until they are inserted into the Rotor-Gene™ 3000 for fluorescence assessment.
10. In the case of MDR strains it might be necessary to adjust the measurement time, performing shorter acquisition cycles due to the increased efflux activity.
11. Efflux activity is quantified by comparing the data obtained for the bacterial population under conditions that allow maximum efflux (bacteria in the presence of glucose and absence of EPI) against the data obtained from the control tube that contains the EB-loaded cells under conditions that restrict efflux (presence of EPI and no energy source). The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relative to the EB-loaded cells.

Acknowledgments

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Chapter 13

Measuring the Activity of Active Efflux in Gram-Negative Bacteria

Mark A. Webber and Nick G. Coldham

Abstract

Resistance to clinically useful therapeutic antibiotics is an ever-increasing phenomenon seen in a range of bacterial species including those pathogenic to man. There are diverse mechanisms which contribute to inherent and acquired resistance to antibiotics. Gram-negative bacteria are commonly intrinsically more resistant to many drugs as a result of their cell structure and the activity of multidrug efflux pumps. Measurement of the accumulation of antibiotics and the contribution of active efflux has proved important in understanding the mechanisms of resistance to many antibiotics and how bacteria can become multidrug-resistant. Multidrug efflux pumps often have broad substrate ranges allowing detection of their activity by measurement of the accumulation of antibiotic substrates or a range of fluorescent substrates, which can be easily used as markers of efflux activity. This chapter describes methods for the detection of efflux pump activity on Gram-negative bacteria.

Key words: Efflux, Multidrug resistance, Antibiotic, Accumulation, Fluorescence

1. Introduction

For an antibiotic to be effective, it must be able to access its target site(s); for many drugs, this requires penetration of the cell envelope. Gram-negative bacteria pose a particular challenge for antibiotic accumulation due to their double membrane structure (1). To gain access to an intracellular, cytoplasmic target, an antibiotic must cross the outer membrane, rich in lipopolysaccharide, often via water-filled porins for hydrophilic drugs, traverse the periplasmic space filled with peptidoglycan, and penetrate the inner phosholipid bilayer before entering the cell. The greater intrinsic resistance to many antimicrobial compounds of Gram-negative bacteria when compared to Gram-positive bacteria is largely attributable to the lower permeability of Gram-negative bacteria,

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which is due to the double membrane structure and the activity of multidrug efflux pumps (2).

There are five classes of multidrug efflux pump found in bacteria and all are found in Gram-negative bacteria (3). These classes are the MFS (major facilitator superfamily), SMR (small multidrug resistance), ABC (ATP-binding cassette), MATE (multidrug and toxic efflux), and RND (resistance nodulation division) families. Whilst some are single-component systems, which simply export substrates across the inner membrane, other efflux systems are complex three-component assemblies, which consist of an inner-membrane pump and an outer-membrane channel linked by a membrane fusion protein that helps assemble the functional unit. These three component systems allow export of substrates from inside the cell or the inner membrane to the extracellular milieu in one step. The RND family of efflux pumps are the most clinically relevant in Gram-negative bacteria and export an extremely wide range of substrates including antibiotics, biocides, dyes, detergents, and organic solvents (4). The AcrAB-TolC system of *Escherichia coli* and Salmonellae, the CmeABC system of *Campylobacter jejuni*, and Mex systems of *Pseudomonas aeruginosa* are all homologous members of the RND family and dictate intrinsic resistance to antibiotics in these organisms. Derepression of these systems can lead to increased resistance to various drugs and this is commonly observed amongst clinical isolates with multiple drug resistance. Inhibition of efflux systems can increase the potency of antibiotics that are subject to active efflux, and the use of efflux pump inhibitor (EPI) molecules in susceptibility testing or measurement of antibiotic accumulation can reveal the extent of the contribution efflux plays to the level of resistance of a strain to a particular antibiotic (5).

There are many methods to measure efflux activity which have been developed over time. These include methods where cells are incubated with the drug of choice before samples are removed, cells harvested by centrifugation, and lysed before the concentration of antibiotic accumulated within the cells is estimated (6). Early methods to estimate drug concentration relied on microbial plate assays where zones of inhibition around a well containing a defined volume of sample were compared to those resulting from antibiotic stocks of known concentration to allow an estimation of the amount of antibiotic present in the sample. Later analysis using analytical techniques including HPLC or fluorescence for certain antibiotics improved this quantification (6, 7). As knowledge regarding the substrate profiles and action of bacterial multidrug efflux pumps has increased, it has been realized that many of these systems have very wide substrate ranges, which include fluorescent molecules such as ethidium bromide and bis-benzimide (8). These molecules are of interest as they intercalate with DNA and emit much more

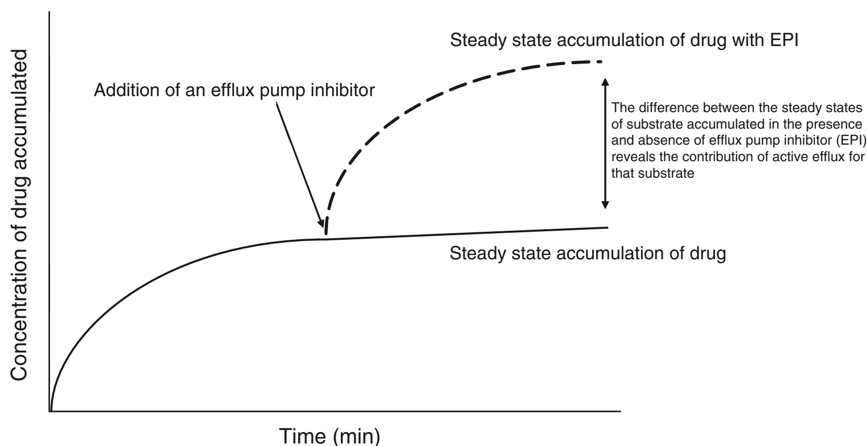

Fig. 1. Model accumulation curve showing the theoretical accumulation of a drug to a steady state and the subsequent addition of an EPI resulting in a new, higher steady state, which reveals the role of efflux in extrusion of this drug.

fluorescence when bound to DNA than in free solution. Consequently, this property can be used to monitor accumulation of these substrates in live cells in real time without the need for sampling, cell disruption, and antibiotic quantification as with other methods. In this chapter, a high-throughput method for measurement of cell permeability and efflux activity will be described which makes use of the fluorescent molecule bis-benzimide, a substrate of RND family efflux pumps in conjunction with EPIs to determine the efflux activity of Gram-negative bacterial strains. This technique can be used to easily identify strains with altered permeability, and via the use of EPIs in conjunction with bis-benzimide can identify the contribution of active efflux to the steady state of bis-benzimide accumulated by a strain of interest (Fig. 1 shows a model accumulation curve and indicates how addition of an EPI can demonstrate the contribution of active efflux to drug accumulation). The technique is high-throughput and assays can be used to identify efflux mutants, investigate the effect of compounds on the permeability of Gram-negative cells, and identify compounds that modulate the activity of the major efflux pumps of Gram-negative bacteria.

2. Materials

2.1. Strains and Culture

1. Luria Bertani broth (Cat # L3022, SIGMA, Poole, UK), prepare from powder and autoclave 250 mL aliquots in sterile glassware.
2. Test strains and appropriate controls for that organism (e.g., NCTC type strains) to give a “wild-type” reference for

Example of a loading plan for a Bis-benzimide accumulation assay with one test and control strain.

Column	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS (blank)	PBS (blank)	PBS (blank)	PBS (blank) + Bis Benz 2.5 µM	PBS (blank) + Bis Benz 2.5 µM	PBS (blank) + Bis Benz 2.5 µM	Boiled control (DNA)	Boiled control (DNA)	Boiled control (DNA)			
B	Control (Cells only)	Control (Cells only)	Control (Cells only)	Control + Bis Benz 2.5 µM	Control + Bis Benz 2.5 µM	Control + Bis Benz 2.5 µM	Test A (Cells only)	Test A (Cells only)	Test A (Cells only)	Test A + Bis Benz 2.5 µM	Test A + Bis Benz 2.5 µM	Test A + Bis Benz 2.5 µM

Fig. 2. Example loading template based on a 96-well plate (NB empty wells are not shown).

comparison with test strains. If available, known mutants with increased or decreased permeability (for example, mutants lacking porins) or efflux activity (see Fig. 2.) are also useful controls. A fresh culture on an appropriate agar plate for the species is needed (see Note 1).

2.2. Bis-benzimide Accumulation Assay

1. Phosphate buffered saline (PBS), 0.1 M, pH 7.2. (Cat # P5119, SIGMA, Poole, UK).
2. Bis-benzimide (Hoescht 33342 – see Note 2) is dissolved in sterile water to give 25 mL of 1 mM H33342 (13.35 mg/mL) in a volumetric flask. This stock is then diluted further 1 in 40–25 µM (0.25 mL stock made up to 10 mL with sterile water in a volumetric flask).
3. EPI solution. A variety of EPIs exist with activity against Gram-negative bacteria (3), amongst which the most commonly used are CCCP (carbonyl cyanide m-chlorophenylhydrazone, Cat # C2759, SIGMA, Poole, UK) and PAβN (phenyl-arginine-β-naphthylamide, SIGMA, Poole, UK). The choice of EPI to be used depends on whether the activity of all pumps relying on the proton motive force is to be inhibited or just RND pump (see Note 3). A stock of the EPI to be used (see Note 3) is needed at 50× the final required concentration, different organisms show different susceptibilities to EPIs, a final concentration of CCCP of 100 µM is effective against *E. coli* and *Salmonella* spp. and a final concentration of 100 µg/mL of PAβN is effective against the same organisms. If using this assay for the first time in a new species, a concentration range of EPI is required in order to identify a concentration of EPI to be used which has activity against efflux in the species of interest. In parallel, the susceptibility of the species of interest to the EPI to be used should also be determined in order to ensure a final concentration is used, which has no inhibitory effect in itself as these compounds are in themselves antimicrobial at high concentrations.

CCCP stocks should be made up by dissolving powder in three parts methanol to two parts sterile water to give a stock with a final concentration of 5 mM in 10 mL, and PAβN should be made up to 5 mg/mL in sterile water (10 mL).

4. Black (see Note 4), flat-bottomed 96-well microtitre plates (Cat # 655076, Greiner, Stonehouse, UK).
5. A fluorescent plate reader (see Note 5), such as a FLUOstar Optima (BMG Labtech, Offenberg, Germany), which will be used as an example in this chapter.

3. Method

3.1. Day 1: Preparation of Cell Cultures

1. Inoculate overnight cultures of test and control organisms in LB or appropriate media by adding one colony from a fresh plate to 5 mL of sterile broth in a sterile 10 mL universal tube under sterile conditions. Prepare triplicate cultures for each strain and then incubate overnight with shaking (150 rpm) at 37°C in an incubator or as appropriate for the species.

3.2. Day 2: Bis-benzimide Accumulation Assay

1. From the overnight cultures add a 4% inoculum of each to 3 mL of fresh, prewarmed (37°C), LB broth in sterile universal tubes and incubate with shaking (150 rpm) at 37°C until mid-logarithmic phase is achieved. This will typically take approximately 2 h to 2 h 30 min for *E. coli* and *S.* Typhimurium (see Note 1). Growth conditions will vary for other species and should be adjusted as appropriate. To ensure whether the correct growth stage has been reached, check the optical density of a 1-mL aliquot of the cultures in a spectrophotometer at 600 nm (for *E. coli* and *S.* Typhimurium mid-logarithmic phase will correspond to an absorbance between 0.5 and 0.7). NB retain one overnight culture for the type or control strain(s) to be used.
2. Harvest the cells by centrifugation at 6,000 × *g* for 10 m at room temperature. Discard the supernatant and resuspend the cell pellet in 3 mL of PBS (0.1 M pH 7.2).
3. Take a 100-μl aliquot and dilute to 1 mL with PBS sterile water, read the absorbance (at 600 nm using a 1-mL aliquot of PBS as a blank).
4. Use this reading to adjust the approximate OD of each sample to 0.1 using PBS.
5. For a control with no efflux activity (consisting of killed cells), boil 500 μl of overnight culture of type/control strain for 5 min. This sample will provide a high level of fluorescence due to the absence of efflux activity and consequent

high concentrations of dye, which will accumulate within the cell, see Note 2.

6. Add diluted suspensions of each strain to a black plastic 96-well plate. A total final volume of 200 μl is needed for each well. For each strain, inoculate three wells with 200 μl of cell suspension and three more with 176 μl of cell suspension (to allow for the volume of EPI to add). Add 4 μl of the 50× stock of EPI to the wells with 176 μl of cell suspension.
7. As a control, add 200 μl of PBS to three wells to act as a blank for the cells only wells and add 180 μl of PBS to three more wells to which Bis-benzimide will be added (see point 8) and will act as a blank for samples with Bis-benzimide.
8. If using a fluorescent plate reader equipped with an injector, add the 25 μM stock solution of Bis-benzimide to the reservoir and set the machine to inject 20 μl of stock to all the wells containing 180 μl of sample at cycle number 3 (see next point). If an injector is not available, then the Bis-benzimide stock can be added to the appropriate wells *immediately* before the assay is started.
9. Measure fluorescence with excitation and emission wavelengths of 355 and 465, respectively, every min for 30 cycles. If possible, set the plate reader to maintain a temperature of 37°C for the duration of the experiment (assays can be conducted at room temperature but efflux is less active at lower temperatures).

3.3. Data Analysis

1. Once the data are recorded, it should be analyzed by averaging each set of triplicate repeats and subtracting the appropriate average blank values, e.g., for test strain "A" average the values from each well for each time point and subtract the average value from the three PBS with bis-benzimide blank wells for each time point to give a corrected value.
2. Calculate the standard error of the mean for each sample at each data point.
3. Plot the data on a graph (including the standard deviation), which should resemble that in Fig. 3.

4. Notes

1. The appropriate media to give best growth in liquid culture will differ amongst species of Gram-negative bacteria. The requirement for the assay is to have cells in mid-logarithmic growth, i.e., growing and dividing rapidly when they are at their most metabolically active. Using mid-logarithmic cultures

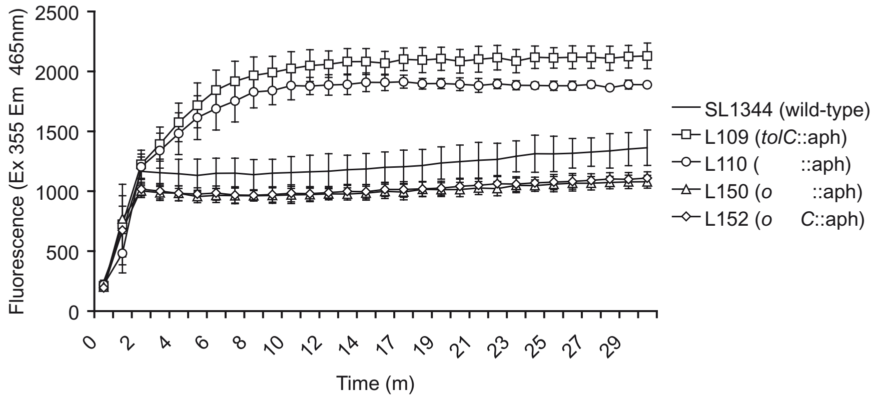

Fig. 3. Accumulation of bis-benzimide by strains of *Salmonella enterica* serovar Typhimurium. SL1344 is a wild-type strain, mutants lacking components of the major AcrAB-TolC efflux system accumulate more bis-benzimide (L109, *tolC::aph* and L110, *acrB::aph*) due to their lack of efflux activity and mutants lacking porins OmpF (L150) and OmpC (L152) accumulate less bis-benzimide than SL1344 due to their lower permeability to the dye.

gives better results than use of cultures at other stages of growth. If in doubt as to what time and growth conditions are required to obtain cells in this state, perform tests of the growth kinetics of your strains by incubating and measuring optical density over time, plotting the results will give a growth curve, which can be used to identify the time period that represents mid-logarithmic phase.

2. In this protocol, we describe the use of Bis-benzimide as a fluorescent marker; there are a variety of compounds that can act as substitutes for Bis-benzimide, notably ethidium bromide, which may be cheaper but potentially more hazardous. The principle of the assay relies on the fact that these dyes accumulate within cells, intercalate with DNA, and change fluorescence wavelength optima (9) when they do so – therefore, this fluorescence is a marker of intracellular accumulation. Both Bis-benzimide and ethidium bromide are substrates for multidrug efflux pumps so differences in pump activity between strains are reflected in higher or lower intracellular accumulation of these drugs and altered fluorescence activity as a result.

3. Various EPIs have been described with activity against Gram-negative bacteria, those most commonly used are CCCP, which acts to dissipate the proton motive force (the majority of efflux pumps use proton antiport as an energy source) and PAβN, which is thought to act as a competitive inhibitor with specificity for RND family efflux pumps. As a result, the two compounds have different uses; CCCP is a broad spectrum inhibitor of efflux by removing the energy source of the

pumps but is nonspecific, whereas PAβN is more selective and targets RND family pumps rather than the cell as a whole. The choice of EPI to use depends on whether the activity of RND pumps alone or efflux as a whole is to be inhibited. It should be noted that for many species the differences seen between the two compounds activity is small as RND pumps are most important in determining intrinsic efflux activity.

4. Black trays must be used to prevent interference in fluorescence signal between adjacent wells.
5. Any plate reader that can measure fluorescence from the top of a plate can be used in this assay; it is preferable to use an instrument with the capacity to maintain a specified temperature and the ability to inject the Bis-benzimide once the assay has equilibrated but not essential.

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Chapter 14

Rapid Characterization of β-Lactamases by Multiplex PCR

Neil Woodford

Abstract

The rising prevalence of the members of the Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter baumannii* that produce extended-spectrum β-lactamases (ESBLs) and carbapenem-hydrolysing β-lactamases (carbapenemases) represents one of the largest "new" resistance problems faced by clinicians and microbiologists during the last 10 years. These diverse enzymes have emerged globally and represent serious health challenges, compromising therapeutic choice and complicating patient management. The rapid detection of strains that produce these β-lactamases in clinical bacteriology laboratories allows appropriate therapy to be implemented promptly, which reduces patient mortality. This chapter describes three multiplex PCR assays, which may be used to detect genes that encode five families of CTX-M-type ESBLs (groups 1, 2, 8, 9, and 25), five families of metallo-carbapenemases (IMP, VIM, SPM, GIM, and SIM enzymes), and four families of OXA-carbapenemases (OXA-23-like, OXA-40-like, OXA-51-like, and OXA-58-like enzymes). The CTX-M ESBLs are the most prevalent of these enzyme groups, particularly, though not exclusively, in isolates of *Escherichia coli* and in *Klebsiella* spp.; metallo-carbapenemases are often found in *Pseudomonas* spp. and other "non-fermenters," but are also emerging problems in members of the Enterobacteriaceae in some countries and locales; with a few exceptions, the OXA-carbapenemases detected by the assay described are limited to isolates of *Acinetobacter* spp. These assays are suitable for deployment in national reference laboratories, but should also be considered for use in regional centres and in tertiary referral hospitals.

Key words: Antibiotic resistance, β-Lactam, Carbapenem, Multiplex PCR, Molecular diagnostics

1. Introduction

The acquisition of genes that encode β-lactamases is the commonest route by which most Gram-negative bacteria become resistant to β-lactam antibiotics. This antibiotic class remains the backbone of antibacterial chemotherapy, and its many members (e.g., penicillins, cephalosporins, monobactams, and carbapenems) are used extensively both in hospitals and in the community (1). The first "penicillinase"-producing isolate of *Escherichia coli* was isolated

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by Edward Abraham and Ernst Chain even before the introduction of penicillin into clinical practice in the early 1940s (2). Fortunately, acquisition of resistance to β-lactams only rarely results in resistance to the entire β-lactam class. This has enabled drug companies to bring successive "generations" of β-lactams to market, with each designed to retain activity against bacteria that had developed resistance to earlier compounds.

This successful drug development strategy is, however, continually being undermined by the emergence of bacterial strains that produce wider spectrum β-lactamases. The TEM-1 and TEM-2 penicillinases emerged in *E. coli* in the 1960s and 1970s, respectively, and have spread (particularly TEM-1) to be produced by >50% of clinical strains. Producers of these enzymes can be countered by subsequent generations of cephalosporins, but now we know of over 160 TEM sequence variants, many of which have expanded the spectrum of β-lactams that are susceptible to degradation and are known as extended-spectrum β-lactamases (ESBLs). These enzymes are capable of hydrolysing all available cephalosporins, though not the cephamycins or carbapenems. Evolution of another penicillinase, SHV-1, has similarly given rise to >100 variants, many of which are ESBLs. These TEM- and SHV-derived ESBLs have never become widely prevalent in *E. coli* and are a cause of concern in nosocomial opportunists, particularly in *Klebsiella* spp. and *Enterobacter* spp. (3). Their importance has, however, been overshadowed almost completely by the rapid rise to prominence of a third group of ESBLs, the CTX-M enzymes.

The history and epidemiology of the CTX-M ESBL class has been documented extensively elsewhere (4–8). These enzymes, originally encoded by chromosomal genes of *Kluyvera* spp. (9–12), now represent the commonest mechanism of cephalosporin resistance in the Enterobacteriaceae in many countries, including the United Kingdom (13). *E. coli* is the primary host species, and enzyme producers are frequently isolated as the cause of both hospital-acquired and community-onset urinary tract infections in elderly patients; carriage is increasingly recognised in nursing homes and in the general population. This epidemiological success reflects multiple factors, including clonal expansion of CTX-M producers, and also interstrain and interspecies spread of the corresponding $bla_{\text{CTX-M}}$ resistance genes, which has been made possible by frequent location of $bla_{\text{CTX-M}}$ genes on plasmids, integrons, and transposable elements. More than 90 CTX-M variants are currently recognised (http://www.lahey.org/studies/webt.html), with these variants falling into five main sequence-based phylogenetic groups; the prototypes for these groups are CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25.

The increasing prevalence of strains that produce ESBLs and other acquired non-carbapenem-hydrolysing β-lactamases, such

as plasmid-mediated AmpC types (14), undermines the efficacy of many empirical β-lactam regimens. In consequence, optimum treatment choice for serious gram-negative infections increasingly relies on carbapenems, and brings with it the certainty that increased usage will increase the selection pressure for the emergence of carbapenem-resistant isolates. Non-carbapenem-hydrolysing enzymes (such as CTX-M ESBLs or AmpC enzymes) can mediate low levels of carbapenem resistance (or reduced susceptibility) when combined with the loss of porins to reduce the permeability of the bacterial outer membrane (this reduces the rate of carbapenem entry). This complex, combinatorial type of resistance is increasingly seen in clinical isolates of *Klebsiella* spp. and *Enterobacter* spp. (15), but has also been reported in occasional isolates of *E. coli* (16). The emergence and spread of genuine carbapenem-hydrolysing β-lactamases (carbapenemases) poses greater concern to clinical microbiologists.

Carbapenemases fall into β-lactamase molecular classes A, B, and D. Class A carbapenemases include rare enzymes, such as IMI, NMC-A, and SME, but also the KPC variants, which are currently a major public health concern in the United States (17, 18) and are spreading elsewhere (19–22). These are primarily found in members of the Enterobacteriaceae, with KPC reported most often in *K. pneumoniae*. In contrast, although reported in Enterobacteriaceae, class B metallo-carbapenemases are more typically associated with *Pseudomonas* spp., *Acinetobacter* spp. and other non-fermenting genera. The class B enzymes include many intrinsic, chromosomally encoded carbapenemases (e.g., L1 enzyme of *Stenotrophomonas maltophilia*) as well as acquired metallo-carbapenemases. The latter include many variants of the IMP and VIM families (http://www.lahey.org/studies/webt.html), plus SPM-1 (which is a particular problem in Brazil) (23, 24), and rarer variants, such as GIM-1 (25) and SIM-1 (26). The class D (OXA) carbapenemases are mainly restricted to *Acinetobacter* spp., although OXA-48 enzyme mediates carbapenem resistance in diverse members of the Enterobacteriaceae in Turkey (27), while other carbapenem-hydrolysing variants (OXA-54 and -55) have been described in environmental bacteria of the genus *Shewanella* (28, 29).

To date, four phylogenetic sub-groups of OXA carbapenemases have been described in *Acinetobacter* spp. The major species of clinical concern is *A. baumannii*, and the OXA-23-like, OXA-40-like and OXA-58-like sub-groups have each been acquired by this species (30). It appears that genes encoding OXA-23-like carbapenemases have emerged in *A. baumannii* after their “escape” from the chromosome of *A. radioresistens* (31); the origins of the OXA-40-like and OXA-58-like subgroups are unknown. Over 50 variants of OXA-51-like enzymes have been described (30); these are encoded by intrinsic chromosomal genes

of *A. baumannii* and their detection may be used for identification to species level (32). Unlike the OXA-23-like, OXA-40-like, and OXA-58-like sub-groups, OXA-51-like enzymes do not usually confer clinically significant levels of carbapenem resistance, but they may do so if their expression is upregulated e.g., by an adjacent, upstream copy of an insertion sequence, such as IS*Aba1* (33).

Despite their public health importance, there are no good phenotypic tests for detecting all carbapenemases reliably in diagnostic bacteriology laboratories. Variations of the cloverleaf test (also known as the Hodge test; Fig. 1) are often used. This is a microbiological assay to detect carbapenem degradation by a test isolate (*see* legend of Fig. 1 for further details), but is time-consuming to set up, may lack specificity (often giving false-positive results e.g., for isolates of *Enterobacter* spp. where carbapenem resistance results from derepressed AmpC expression plus porin loss), and may not detect reliably the production of some class D enzymes. Also, reference laboratories may seek more information than can be obtained with such a test. They may, for example seek details of enzyme types to aid local detailed epidemiological investigations. This lies beyond phenotypic methods and requires molecular tests.

In this chapter, three multiplex PCR assays are described which are suitable for detecting and assigning to their phylogenetic subgroups genes that encode CTX-M ESBLs (34), OXA-type non-metallo-carbapenemases (35), and metallo-carbapenemases (36). These are ideal tools for monitoring the prevalence and dissemination of the resistance genes, and, when used with strain typing techniques,

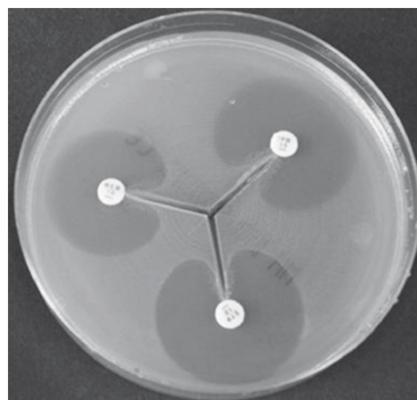

Fig. 1. A clover leaf (or Hodge) test can provide phenotypic evidence of carbapenemase production. The agar plate is inoculated with a carbapenem-susceptible *E. coli* strain, and the "test" isolate is inoculated into cuts made in the agar surface. Carbapenem disks are then applied and the plate is incubated. If the "test" isolate produces a carbapenemase (i.e., can hydrolyse carbapenems), local decreases in carbapenem concentration will allow the *E. coli* indicator strain to grow towards the carbapenem disks.

can aid rapid recognition of problematic bacterial strains, and track the emergence of new strains. If such techniques are implemented directly in diagnostic laboratories, their potential to influence patient management can be maximised.

2. Materials

1. Standard microbiological media/equipment for growing the bacteria of interest.
2. Block-based thermal cycler (e.g., Thermo P×2) and standard PCR reagents for amplifying the required target.
3. Separate stocks of dATP, dGTP, dCTP, and dTTP each at 10 mM (e.g., Invitrogen). The four stock solutions should be mixed in equal volumes, and aliquots should be stored at −20°C. The mixed dNTPs can be refrozen and used several times.
4. *Taq* polymerase, $MgCl_2$, and polymerase buffer (supplied together e.g., Invitrogen).
5. Target-specific primer pairs (Table 1). Individual primers should be dissolved in PCR quality water to a stock concentration of 1 μg/μL. Multiplex primer mixes are then prepared by mixing 5 μL of each required primer and adjusting the final volume to 100 μL with PCR quality water.
6. Molecular biology grade agarose (e.g., Bio-Rad, Sigma).
7. 0.5× Tris–borate–EDTA buffer pH 8.0 (e.g., Sigma).
8. DNA sub-cell and power supply for electrophoresis (e.g., Bio-Rad, Hoefer).
9. Ethidium bromide (or alternative DNA-staining dye) (Sigma).
10. UV transilluminator (37).

3. Methods

3.1. Amplification of DNA

1. Grow bacterial isolates overnight on appropriate agar plates.
2. There is no need to extract genomic DNA to use as PCR template. Crude DNA can be prepared by suspending two bacterial colonies in 100 μL PCR quality water, vortexing briefly, and "pulsing" on a microcentrifuge for 10–15 s to remove cell debris. Aliquots of 2 μL can be used as PCR template. Alternatively, if preferred, genomic DNA can be extracted rapidly using commercially available systems, e.g., Promega Wizard Genomic DNA Purification Kit.

Table 1
Primers suitable for detecting genes encoding CTX-M ESBLs, metallo-carbapenemases (MBLs) and OXA-type carbapenemases

Multiplex	Gene sub-group	Primer pairs (5′–3′)	Amplicon	Reference
$bla_{CTX\text{-}M}$	Group 1	5′-AAA AAT CAC TGC GCC AGT T C 5′-AGC TTA TTC ATC GCC ACG TT	415-bp	(34)
	Group 2	5′-CGA CGC TAC CCC TGC TAT T 5′-CCA GCG TCA GAT TTT TCA GG	552-bp	
	Group 8	5′-TCG CGT TAA GCG GAT GAT GC 5′-AAC CCA CGA TGT GGG TAG C	666-bp	
	Group 9	5′-CAA AGA GAG TGC AAC GGA TG 5′-ATT GGA AAG CGT TCA TCA CC	205-bp	
	Group 25	5′-GCA CGA TGA CAT TCG GG 5′-AAC CCA CGA TGT GGG TAG C	327-bp	
bla_{MBL}	bla_{IMP}	5′-GGAATAGAGTGGCTTAATTCTC 5′-CCAAACCACTACGTTATCT	188-bp	(36)
	bla_{VIM}	5′-GAT GGT GTT TGG TCG CAT A 5′-CGA ATG CGC AGC ACC AG	390-bp	
	bla_{GIM}	5′-TCG ACA CAC CTT GGT CTG AA 5′-AAC TTC CAA CTT TGC CAT GC	477-bp	
	bla_{SPM}	5′-AAA ATC TGG GTA CGC AAA CG 5′-ACA TTA TCC GCT GGA ACA GG	271-bp	
	bla_{SIM}	5′-TAC AAG GGA TTC GGC ATC G 5′-TAA TGG CCT GTT CCC ATG TG	570-bp	
bla_{OXA}	$bla_{OXA\text{-}51\text{-}like}$	5′-TAA TGC TTT GAT CGG CCT TG 5′-TGG ATT GCA CTT CAT CTT GG	353-bp	(35)
	$bla_{OXA\text{-}23\text{-}like}$	5′-GAT CGG ATT GGA GAA CCA GA 5′-ATT TCT GAC CGC ATT TCC AT	501-bp	
	$bla_{OXA\text{-}40\text{-}like}$	5′-GGT TAG TTG GCC CCC TTA AA 5′-AGT TGA GCG AAA AGG GGA TT	246-bp	
	$bla_{OXA\text{-}58\text{-}like}$	5′-AAG TAT TGG GGC TTG TGC TG 5′-CCC CTC TGC GCT CTA CAT AC	599-bp	

3. DNA from control strains should be included in every run of each assay. These control strains may be identified among local collections through empirical testing, or can be obtained from researchers in the field (including the author).

4. Add 23 μL of a master mix containing appropriate pre-mixed primers (Table 1), dNTPs, $MgCl_2$, *Taq* polymerase, and polymerase buffer (see Note 1).
5. Transfer tubes/microtitre plates to a thermal cycler and amplify the target region.
6. Optimum conditions for the assays described are: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 25 s, 52°C for 40 s, and 72°C for 50 s; and a final elongation at 72°C for 6 min (see Note 2).
7. After amplification, confirm the specificity of the PCR by electrophoresing 4–10 μL of the PCR product on a 2% agarose gel. Stain with ethidium bromide (final concentration, 1 μg/mL) and visualise/photograph under UV light (see Notes 3 and 4).
8. The sizes of any bands amplified from "test" isolates should be compared visually with those amplified from control isolates. Co-migration with a "control" band is the criterion used to assign a genotype (Figs. 2–4) (see Notes 5–7).

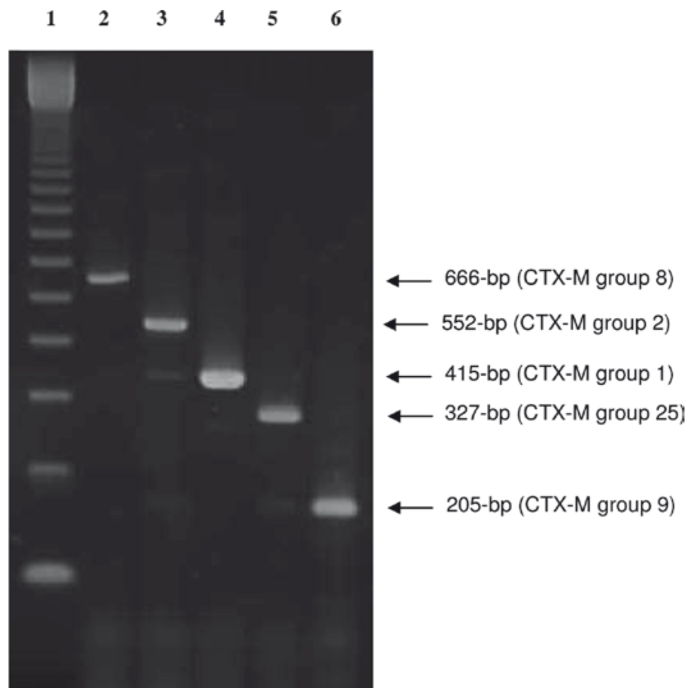

Fig. 2. Multiplex PCR assay for five sub-groups of bla_{CTX-M} alleles (34): amplification from control strains. Lane 1: group 1; lane 2: group 2; lane 3: group 8; lane 4: group 9; lane 5: group 26. The molecular size marker (lane 6) is a 123 bp ladder (Invitrogen, Paisley, UK).

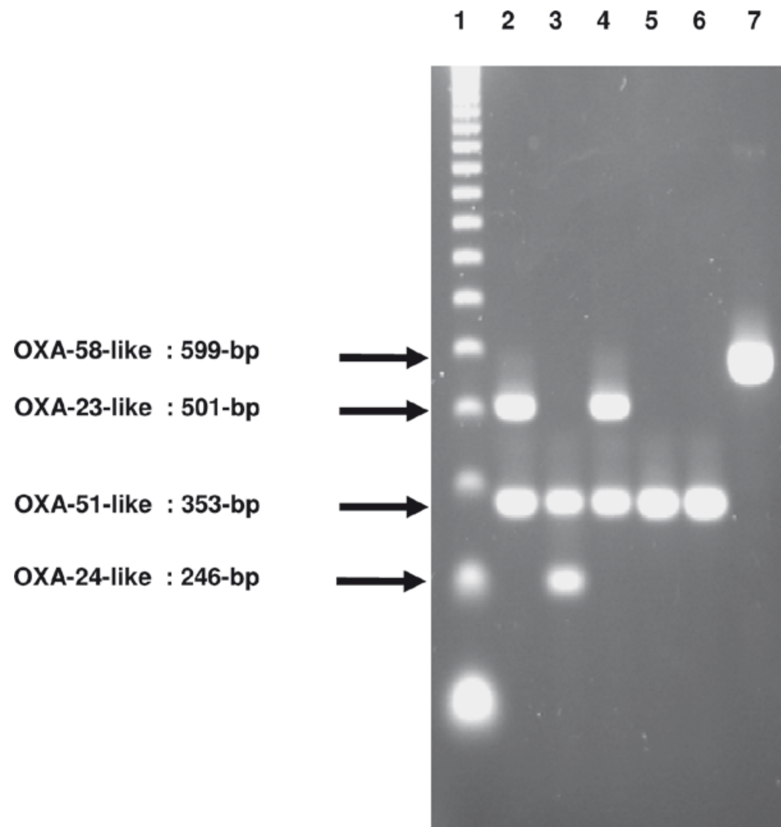

Fig. 3. Multiplex PCR assay for four sub-groups of OXA carbapenemases (35). Example isolates contain alleles encoding: OXA-23-like and OXA-51-like enzymes (lanes 2 and 4); OXA-24-like and OXA-51-like enzymes (lane 3); an OXA-51-like enzyme only (lanes 5 and 6); and an OXA-58-like enzyme only (lane 7). The molecular size marker (lane 1) is a 123-bp ladder (Invitrogen, Paisley, UK). Reproduced from reference (35) with permission from Elsevier.

4. Notes

1. A master mix suitable for 12×25 μL PCR reactions would include: 225 μL PCR quality water; 30 μL *Taq* buffer (supplied with *Taq* polymerase); 30 μL dNTP mix; 12 μL 50 mM $MgCl_2$; 12 μL appropriate multiplex primer mix; 7.5 U *Taq* polymerase.
2. The assay and the stated amplification conditions have proven to be robust. The assays have been transferred into several collaborating laboratories in different countries and using different thermal cyclers.

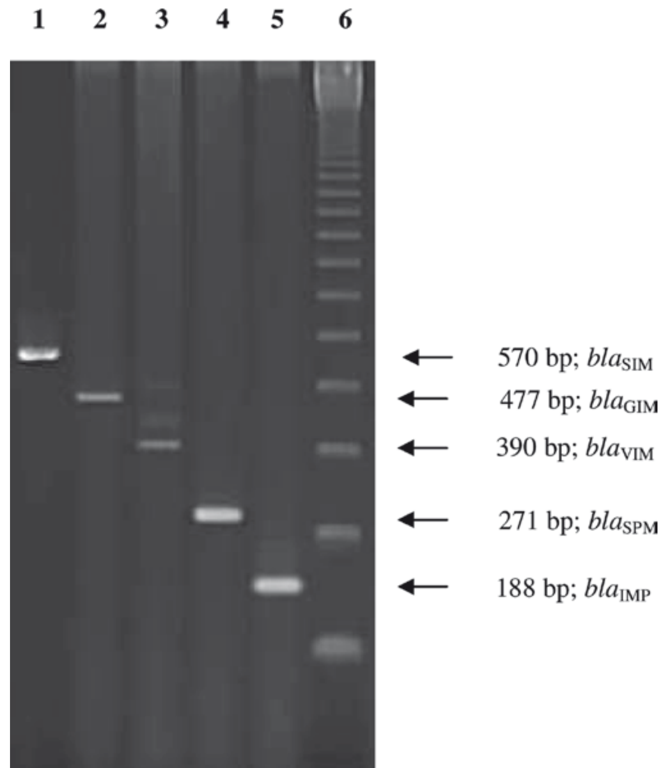

Fig. 4. Multiplex PCR assay for genes encoding acquired metallo-carbapenemases (36): amplification from control strains. The molecular size marker (lane 6) is a 123-bp ladder (Invitrogen, Paisley, UK).

3. Ethidium bromide is mutagenic and should not be handled during pregnancy. It should only be handled while wearing gloves and should be disposed of in accordance with local safety regulations.
4. Transilluminators are sources of strong UV radiation; minimum personal protective equipment is a full face shield if the transilluminator is to be used in, for example, a photographic darkroom. Alternatively, many commercial gel camera systems have the transilluminator fully enclosed, and the UV cannot be switched on until the "door" is closed, thereby protecting the user.
5. If desired, the assays described in this chapter could be transferred onto alternate amplification/detection platforms. This would require careful optimization and validation, and has not been done in the author's laboratory.
6. Detection and phylogenetic grouping of $bla_{\text{CTX-M}}$ alleles have been performed by other authors using real-time PCR (38, 39), and pyrosequencing (39, 40). Precise $bla_{\text{CTX-M}}$ alleles can be determined by reverse line hybridization (41) or by denaturing

high performance liquid chromatography (dHPLC) (42). DNA arrays can also be used to screen for these genes (43). Detection of metallo-carbapenemase genes has also been accomplished by real-time PCR (44).

7. PCR products may also be sequenced if further characterization of alleles is needed for specific projects.

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Chapter 15

Rapid Methods for Testing Inhibitors of Mycobacterial Growth

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Abstract

Considering the increased concerns with controlling infectious epidemics such as tuberculosis, a global concerted effort (WHO) is now dead-lined to tackle the emergence of extensive drug resistance through identifying a novel line of therapeutics which will on the one hand shorten the course of treatment and on the other is also expected to be effective against the emerging resistant strains. Major problems with the preclinical drug screening against the uniquely slow-growing pathogen *Mycobacterium tuberculosis* are either found expensive, time-consuming, or require a highly complex laboratory setup. A rapid and convenient, although relatively inexpensive, method requiring very little consumption of inhibitors within a simple microbiology setup for antimycobacterial screening is thus timely. The spot-culture growth inhibition assay aims to test the biological activity of a number of newly discovered natural products and thousands of novel chemicals synthesized on the basis of basic structural and molecular biology studies. Many different classes of novel chemical entities are now independently prepared around the world by distinguished chemists on the chemical behavior of the group of molecules. To serve the purpose of antimycobacterials screening, we aim to describe a method in this chapter performed in a six-well plate format. This method can also be extended accurately to a 96-well plate format according to the necessity of the project. In addition to evaluating a range of prospective drug candidates, this method would also contribute to elucidate substrates for many putative endogenous pathways through comparing the chemical inhibition with the corresponding genetic modification.

Key words: Drug susceptibility test, Mycobacteria, Growth inhibition

1. Introduction

The presence of a rapid economical and significant method to test the antimycobacterial activity of certain compounds is important as new strains of mycobacteria have emerged. In addition, a number of novel anti-TB compounds that have been synthesized or purified from natural sources urgently need testing for their activity against *Mycobacterium tuberculosis.* The chemical biology

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approach, where a chemical inhibitor for an enzyme or a biochemical pathway targets an endogenous substrate of *M. tuberculosis*, would be vital to identify the role of a novel enzyme or the pathway involved in bacterial physiology and the degree of their essentiality.

Disk diffusion drug-testing method also known as zone of inhibition (ZOI) assay was first introduced as a rapid drug susceptibility test for identifying Gram-negative anaerobic bacilli (1); later on, it was developed for antibacterial susceptibility test method against a number of mycobacterial species (2). In this method, bacilli are swabbed onto an agar plate. A paper disk absorbed with different concentrations of drug is kept over the agar and then plates are incubated at 37°C to grow the bacilli. The zone of inhibition around each of the disks is measured as the diameter in millimetres (3). Results are qualitative in terms of resistance/susceptibility and susceptibility is defined as a zone size of >10 mm. Although it is comparable to broth dilution method, it does not promise success for all antibiotics and unknown compounds. Also, the spreading of hazardous pathogenic strains is quite risky.

The standard existent methods for testing drug susceptibility in slow-growing *M. tuberculosis,* using Lowenstein–Jensen (L–J) medium, are the proportion method, the absolute concentration method, and the resistant ratio method (4–6). Among these, the proportion method is the most preferable choice and is used as a reference method (5). In the proportion method, critical concentrations of the tested drugs or inhibitors are added to Middlebrook 7H10 agar or L–J agar media. After solidification, the plates were incubated with a diluted sample of *M. tuberculosis* and incubated until single colonies are obtained. The whole procedure is laborious, time-consuming, and requires at least 4 weeks of incubation. Minimal Inhibitory Concentration (MIC) is considered as the concentration where the number of colonies is <1% from a control plate where no drug or inhibitor was added (5).

In order to shorten the time the proportion method required in the susceptibility testing of *M. tuberculosis*, new techniques have appeared. One of the methods used more frequently is the BACTEC-460 radiometric susceptibility method (Becton Dickinson), which detects the production of radiolabeled CO_2 from mycobacteria (7). In this method, a specific liquid medium is used (BACTEC 12B vial) that contains radiolabeled ^{14}C. Growing mycobacteria produce $^{14}CO_2$ that is manifested by a Growth Index (GI) increase in the system. The MIC is determined as the lowest drug concentration that inhibited more than 99% of the mycobacterial population. This method has reduced the incubation time to 4–12 days (8). Disadvantages of the method are the biohazard radioactive waste management and potential user risk, as well as the cost, as it requires specific

equipment and consumables, which is a limiting factor in developing countries. In 1998, the same company (Becton Dickinson) introduced the automated nonradiometric BACTEC-MGIT (mycobacterial growth indicator tube) 960 Mycobacteria Culture System, available only in developed countries (9, 10). BACTEC-MGIT 960 contains a modified Middlebrook 7H9 broth, which supports the growth and detection of mycobacteria as well as a fluorescent compound embedded on the bottom of the tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The initial concentration of dissolved oxygen quenches the emission from the compound, and little fluorescence can be detected. Later, actively growing microorganisms consume the oxygen that allows the compound to fluoresce (BACTEC Manual). The BACTEC-MGIT 960 instrument continually monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the control (drug-free) tube is used by the instrument to determine susceptibility results. This method takes around 15 days and does not produce radioactive waste as the previous BACTEC 460TB, but again the high expense of this method makes it only available in developed countries (11–15).

During recent years, a number of colorimetric susceptibility methods have been introduced (16–19). Colorimetric methods are based on the reduction of a colored indicator added to the culture medium after *M. tuberculosis* has been exposed in vitro to different drugs. These methods use a Middlebrook 7H9 liquid medium, which is applied in a microtitre plate format. The most common redox indicators, such as tetrazolium salt MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), resazurin or the alamar blue, used to perform the colorimetric assays measure cell proliferation by detecting the level of oxidation during respiration (16–19). These assays have a high-throughput format, and by using liquid medium are quicker than the standard solid-based assays. The overall time for these assays is between 7 and 14 days (20–22). On the drawback, as these methods use a liquid medium, they are more prone to contamination of the sample during the assay manipulation. In addition, due to the complexity of the techniques, the reliability of the results sometimes affects the researcher who performs the experiment as results between two laboratories may vary.

Other methods at the developmental stage include the Etest (23–25) (AB BIODISK, Solna, Sweden), flow cytometry (8, 26), and phage-based techniques (4, 27, 28). Most of these new methods are complex to interpret, highly expensive, require the presence of specialized equipment and highly skilled employees. In addition, most of the above methods have been developed for the determination of mycobacterial susceptibility against the current anti-TB drugs but not the elucidation of the antitubercular phenotypes of

synthesized novel inhibitor(s) or purified natural product(s) in medicinal chemistry or molecular microbiology research. Furthermore, very importantly, on many occasions, use of those screening techniques are restricted due to limited availability of synthetic test compounds or endangered natural wealth.

In this chapter, we demonstrate a solid agar-based rapid and convenient high-throughput method for the determination of inhibitory concentration of crude natural extracts, purified fractions, pure semisynthetic or synthetic inhibitor molecules against slow-growing mycobacteria alone or in combination. The in vitro spot culture growth inhibition assay described here uses a six-well plate format; however, it can be replicated to a different multiwell plate equally accurate for a higher output. To our knowledge, this assay can be done with an amount of compound as low as 1 mg as compared to other available in vitro antimycobacterials screening methods. Thus, it is a very economic and high-throughput method that can be performed in a general microbiology laboratory within a basic setup. The assay is principally based on the nature of the bacilli growth, where growth is measured from generated spots grown from around 1,000 bacilli inoculated to the centre of the wells containing a range of different concentrations of inhibitors. Also, the compound mixed to the agar media has optimum access to the bacilli and will reflect on their biological property through growth inhibition or killing. Results are obtained within 14 days for both the slow-growing species *Mycobacterium bovis* BCG (29–32) and *M. tuberculosis* H37Rv (30, 32). We describe the *M. bovis* BCG growth inhibition results in this chapter.

2. Materials

2.1. *M. bovis* BCG Cell Culture

1. Middlebrook 7H9 liquid medium with 0.2% Glycerol and 0.05% Tween-80.
2. Albumin–Dextrose–Catalase (ADC; Difco), store at 4°C.

2.2. In Vitro Spot Culture Assay

1. *M. bovis* BCG cell culture at the mid-exponential phase (OD 600 nm ~1).
2. Middlebrook 7H10 agar medium with 0.5% Glycerol.
3. Oleic acid–Albumin–Dextrose–Catalase (OADC; Difco), store at 4°C.
4. Sterile DMSO, double-distilled water or any other solvent.
5. Natural extracts or compounds to be tested in dry powder form.
6. Six-well plates.
7. Isoniazid as a control.

3. Methods

3.1. Preparation of Mycobacterial Broth Culture (33) for the Spot Culture Growth Inhibition Assay

1. *M. bovis* BCG are cultured at 37°C in 100 mL roller bottle flasks in an incubator with rotation at 2 rpm on Middlebrook 7H9 liquid medium supplemented with 10% (vol/vol) Albumin–Dextrose–Catalase (ADC; Difco), 0.2% glycerol and 0.05% Tween-80.
2. Mycobacterial cultures are grown until the mid-exponential phase when the OD_{600} is around 1.0 and used for the spot inhibition assay. (see Note 2)
3. For the quality control of the culture each time the bacilli were stained using modified Ziehl–Neelsen acid fast staining (TB color kit, BDH).

3.2. In Vitro Spot Culture Assay

1. Natural extract or compound to be tested is dissolved in sterile 100% DMSO, sterile double-distilled water, or any other solvent to make 50 mg/mL stock (see Note 3). DMSO at the final concentration of 0.1% does not cause any effect on the growth of *M. tuberculosis* H37Rv or *M. bovis* BCG.
2. From the original stock, four different working stock concentrations are prepared using serial dilutions (e.g. 20 mg/mL, 10 mg/mL, 1 mg/mL, and 0.1 mg/mL).
3. Pipette 5 μL of the stock concentrations (50 mg/mL, 20 mg/mL, 10 mg/mL, 1 mg/mL, and 0.1 mg/mL in this case) in the respective wells of a six-well plate. Pipette 5 μL of sterile 100% DMSO/solvent into the control well. All the original stock solutions will finally achieve 1:1,000 dilutions into the culture medium.
4. Boiled Middlebrook 7H10 agar medium is cooled down to 55°C in a prewarmed water bath.
5. Add 10% Oleic acid Albumin–Dextrose–Catalase (OADC; Difco) to the 7H10 agar medium and pour 5 mL in each well of the six-well plates.
6. Swirl the plate gently to accommodate the compound in the medium uniformly and leave to solidify for 5 min with the lid half-open.
7. Meanwhile, prepare serial dilutions of the mycobacterial culture in order to achieve the working concentration of 10^5 cells per mL.
8. Spot 10 μL of the diluted culture (1,000 bacilli) at the center of each well in the six-well plates, swirl the plate gently, and leave with the lid open for 5 min to absorb the culture within the medium.
9. Tape the side of the plates with parafilm and wrap with aluminum foil.

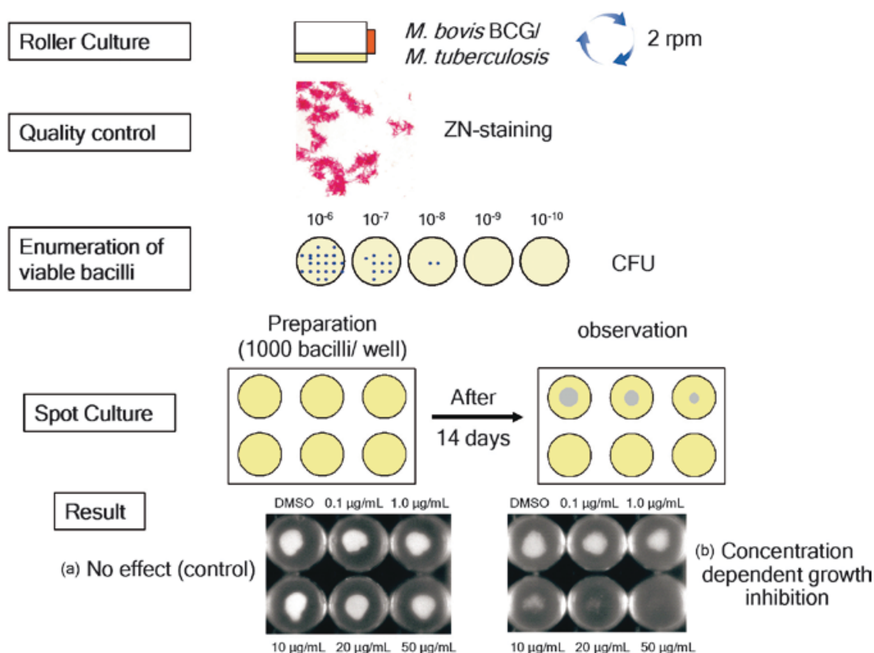

Fig. 1. Spot culture growth inhibition assay: a schematic diagram demonstrates the steps involved in the method (see Subheading 3 for details). Results showing (**a**) no growth inhibition and (**b**) growth inhibition with the increased concentration of an inhibitor. Picture was taken using a digital photo unit (Doc-It®LS, UVP).

10. Incubate the plate inverted at 37°C and observe them on 7th and 14th day following the experiment and take pictures where appropriate.
11. Observe the spots in the plate and determine the MIC on the 14th day as the lowest concentration at which there is no growth (Fig. 1) (see Notes 1 and 3).

4. Notes

1. There are different ways to illustrate the results of a spot culture assay (Fig. 1). First, by direct eye observation of the plate and the spots. Sometimes taking a sufficiently good-quality picture of the spots from Microbiology Safety Level – three Containments is more difficult because the plates are incubated within a sealed plastic bag. In that case, it is possible to analyze experimental results in different ways using specific software (Doc-It®LS, UVP). The second approach is to determine the area that a spot covers (2 πr/πd) and plot a graph comparing the control spot with the other spots. This is a useful method in cases where you observe a

concentration-dependent inhibition (Fig. 1) as well as a 100% killing. And finally you can also measure the density of the spots. Usually, the density of the spots needs to be calibrated against the control spot in order to generate the inhibition graph.

2. Spot Culture Growth inhibition assay can be performed using a culture grown at varied conditions such as restricted nutrient supply, oxygen supply to different stages of the growth cycle such as actively dividing bacilli or stationary phase bacilli.
3. A combination of inhibitor molecules can also be tested simultaneously to determine if there is any interaction in the form of synergism or antagonism of more than one inhibitor reflected on the growth or morphology of the generated spot growth. This can be obtaining by setting a normal set of spot inhibition assay including only one inhibitor in order to determine its MIC. Then another assay can be performed with the combination of the same inhibitor in the presence of a different drug or inhibitor. In that case, the Fractional Inhibitory Concentration (FIC) is calculated by comparing the MIC of the both inhibitors and the MIC of the inhibitor alone (ex. $MIC_{inhibitor+drug}/MIC_{inhibitor}$) (34). FICs values that are lower than 0.5 refers to a synergistic effect (35, 36).

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Chapter 16

Use of Biofilm Model Systems to Study Antimicrobial Susceptibility

Jonathan Pratten and Derren Ready

Abstract

There are many laboratory biofilm models available which can be used to assess the susceptibility of these distinctive resistant phenotypes. The complexities of these models vary considerably and indeed, the antimicrobial susceptibility of biofilms grown in these different models are also not standardised. It is clear that such methods are necessary for the testing of antibiotics and antimicrobial agents since these persistent communities are far more resistant than their planktonic counterparts. Therefore, it is now apparent that standardised tests such as MIC are no longer appropriate on their own to fully characterise susceptibility. There has also been a growing realisation that bacteria are growing as biofilms in almost every health-care setting and are, thus, a major contributing factor to the difficulty of treating infections. There is a pressing need for the models outlined in this chapter to test both current and novel anti-biofilm compounds and materials.

Key words: In vitro models, Laboratory testing, Sessile growth, Susceptibility

1. Introduction

The extensive use of antimicrobials both in the community and hospitals has accelerated the emergence of antibiotic-resistant organisms (1–3). This has caused increasing concern in the medical and scientific community as to the level of antibiotic use in the general population and the degree of inappropriate antibiotic prescription. The biofilm mode of growth can also affect antibiotic susceptibility. Bacteria living in biofilms in general are considered less susceptible to antibiotics than free-living bacteria (4). When *Pseudomonas aeruginosa* cells that were susceptible to tobramycin were grown as a biofilm, there was an approximately 1,000-fold reduction in susceptibility to this antibiotic (5). The attachment of *Klebsiella pneumoniae* to the surface of a glass slide

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resulted in a 150-fold decrease in susceptibility to hypochlorous acid (6). When treating biofilm-associated *Porphyromonas gingivalis* with metronidazole the minimum inhibitory concentration (MIC) values were 160 times higher than those obtained for planktonic cells (7). Another study has reported that in order to eliminate bacteria grown in biofilms, MICs greater than 500 times the MIC found in planktonic culture may be required (8). It is, therefore, clear from the literature that there is a need for laboratory methods testing the efficacy of antibiotics, antimicrobial agents, and chemical disinfectants against bacteria growing as biofilms.

As well as the need for biofilm-based susceptibility testing there are further complexities to consider when devising apparatus for antimicrobial testing of biofilms, as it has been shown that the manner in which the biofilms are grown, or more precisely, the manner in which the bacteria become associated with a surface, will also influence the susceptibility of biofilms (9). Indeed, from the Buckingham-Meyer study (9) it was shown that using a CDC biofilm reactor system that creates a turbulent flow, biofilms were less susceptible than if grown in either a drip flow biofilm reactor system that created slow laminar flow, or a static biofilm system.

Biofilm models including laboratory, animal, and human in situ models, contrast noticeably in their microbiological complexity. The ethical implications for carrying out animal and human studies must be considered before their use. Infections are often localised and the biofilms at these sites are heterogeneous which can further lead to problems with representative sampling. These restrictions have led to the development of a range of laboratory-based models which simulate biofilm growth in vitro. Models are often chosen based on either their simplicity or those that reflect growth and environmental survival conditions of the bacterial species of interest. For example, models mimicking those biofilms within the human body, e.g. *P. aeruginosa* within the cystic fibrosis lung (10, 11) may be very different from those associated with environmental survival. Not only must a biofilm disinfection test method include all the biological, chemical, and analytical components of conventional suspension or dried surface tests, but the method also requires some engineered apparatus, such as a biofilm reactor, for growing a reproducible biofilm. Moreover, the laboratory biofilm should be grown so that it possesses the key attributes of the naturally occurring biofilm where the disinfectant will be applied. Table 1 gives an overview of the biofilm models available which have been used to determine the susceptibility of biofilms to antibiotics and antimicrobials.

The use of polystyrene micro-titre multi-well plates offers the advantage of producing a high number of replicates and therefore make high throughput testing possible (28). However, as the biofilms

Table 1
In vitro biofilm models that have been used to study antibiotic and antimicrobial action

Biofilm model	Examples of organisms tested	Culture	Substratum	Advantages	Reference
Annular reactor	Sulfate-reducing bacteria, *Pseudomonas aeruginosa*	Continuous culture	Various	Versatile surface shear, ASTM Standard Method	12, 13
CDC Biofilm Reactor	Gram-negative bacteria	Continuous culture	Plastic connectors	High shear, removable coupons, ASTM Standard Method	14, 15
Constant Depth Film Fermentor (CDFF)	*Listeria* spp., *Pseudomonas* spp., *Staphylococcus* spp., *P. aeruginosa*, multi-species biofilms	Continuous culture	Various	Longitudinal studies, intermittent pulsing of the antimicrobial or antibiotic	16–18
Flow-cell (Flow- chamber)	streptococci, *Pseudomonas alcaligenes*	Continuous or batch culture	Various	Direct visualisation	19, 20
MBEC Assay™	*P. aeruginosa*, *Staphylococcus aureus*, *E. coli*, enterococci	Batch culture	Plastic pegs	High throughput and simultaneous MIC	21–23
Membrane filters	*Enterococcus faecalis*, *P. aeruginosa*, *Escherichia coli*	Solid medium	Membrane filter on agar	Simple to grow, high biofilm mass	24–26
Microtiter plate assay	*S. aureus*, *Burkholderia cepacia*	Batch culture	Plastic	High throughput, use in standard spectrophotometers	27, 28
Modified Robbins device	*Porphyromonas gingivalis*, *P. aeruginosa*	Continuous or batch culture	Various	Flow device with removable coupons for antimicrobial testing	29–31
Sorbarod filter	*S. aureus*, *P. aeruginosa*	Continuous culture	Filter plug	Large biofilm mass, a number of replicates	32

are derived from organisms growing in batch culture there is a crucial limitation that only single-species biofilms can be grown. The MBEC Assay™ (formerly the Calgary Biofilm Device) (21) also uses the 96-well plate concept. The model was developed to investigate the antibiotic susceptibility of attached bacteria and has been used to test the several clinically relevant genera including, *Pseudomonas* spp., *Staphylococcus* spp., and *Mycobacterium* spp. This system has also been used in a number of studies including the evaluation of antimicrobials used for food and food contact surfaces (33). Biofilms grow on pegs which form part of the lid and suspend into the wells of the micro-titre plate. MIC values derived from the planktonic organisms in the plate wells have been validated against CLSI standards. The minimum biofilm eliminating concentration is determined from the biofilm growing on the 96-peg lid.

The flow-cell or flow-chamber is often the system of choice for short-term adhesion and colonisation studies. The advantage of this model is that it enables the non-destructive microscopic observation of biofilm development in real-time (20). The crucial elements of the flow-cell system are a liquid growth medium reservoir and the flow-cell itself. The system can be inoculated either by adding a culture to the medium reservoir (effectively a batch culture), by a chemostat, or by initially passing a culture through the flow-cell to allow cells to adhere before feeding with sterile medium. Thus multi-species biofilms can be generated if required. The flow-cell may be constructed in a number of ways, but typically consists of a transparent chamber of fixed depth through which the growth medium passes. When mounted on a microscope stage this system allows biofilm development inside the chamber to be observed in real-time and the dynamics of microbial killing can be determined by confocal laser microscopy (33). Although this model has been used extensively to study biofilms, few studies have used this method to evaluate antibiotic or antimicrobial activity. This is perhaps because it is a closed system and, in order to determine the susceptibility of the biofilm by any other means than visualisation with a viability stain, the device would need to be dismantled. A further disadvantage of this system for susceptibility studies is that that few replicates are available although multi-channel systems are now available to allow direct side-by-side comparisons.

The Modified Robbins Device (MRD) has been used extensively for in-line growth studies and antimicrobial testing (29, 34). The advantage of this model over the flow-cell is that coupons of various substrata can be removed during an experiment for susceptibility testing. An MRD consists of six or more individual ports in a linear array along a channel of rectangular cross-section, with dimensions of 10 mm (width), 145 mm (length), and 3.5 mm (depth), respectively. Each port accepts a press-fit

plug holding a sample coupon with a surface area of approximately 40 mm^2. Coupons, containing biofilms, can be removed from the device over time and used for susceptibility testing. Flow devices, such as the flow-cell and MRD are particularly useful for assessing the antimicrobial efficacy of antibiotic-releasing materials to kill biofilms or inhibit microbial adhesion.

In order to study biofilm development and perturbation it is useful to generate steady-state systems. One approach to produce biofilms in a steady-state is to develop a constant depth reactor where the surface growth is periodically removed to maintain a constant geometry (35). Such a device, the CDFF, was first described by Coombe et al. (36) to investigate the growth of dental plaque organisms and further developed by Peters and Wimpenny (37). The CDFF consists of a glass vessel with stainless steel end-plates; the top-plate has ports for the entry of the growth medium, gas and for sampling, while the bottom plate has a medium outlet port (Fig. 1). The vessel houses a stainless steel turn table containing 15 polytetrafluoroethylene (PTFE) sampling pans and this rotates under a PTFE scraper bar which smears the incoming medium over the 15 pans and maintains the biofilms, once formed, at a constant pre-determined maximum depth. Each sampling pan has five cylindrical holes containing PTFE plugs which are recessed to a set depth to create a space in

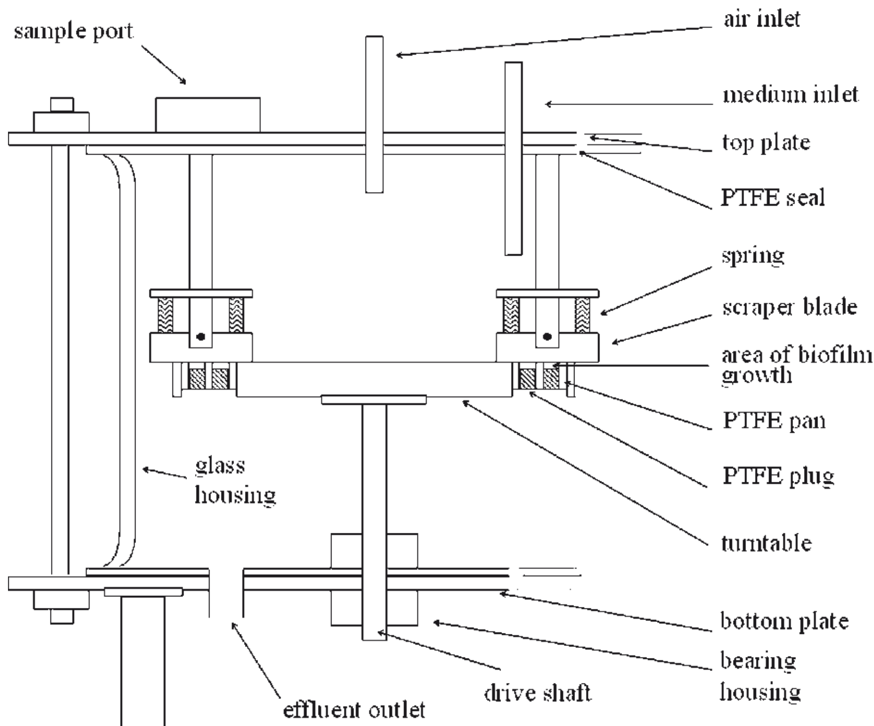

Fig. 1. Vertical schematic of the CDFF highlighting one of the removable PTFE pans.

which the biofilms form. The sampling pans can be removed aseptically during the course of an experimental run allowing longitudinal studies to be undertaken.

The CDFF is not principally designed to study early biofilm formation (although biofilms can be removed during early development) or for studies where a high shear-rate is required. Instead, the device is more useful for producing reproducible steady-state biofilms under controlled conditions. It is important to use a method where the biofilm is grown under fluid flow conditions similar to the environment where the disinfectant will be applied (9). However, some of the advantages of this model are that:

- It allows intermittent pulsing (or continuous application) of the antimicrobial agents and/or additional nutrients.
- It allows easy sampling of the biofilm (with large numbers of replicates) at various intervals during the course of an experiment.
- Biofilms of different thickness can be investigated.
- Pure or mixed cultures of bacteria can be used as an inoculum.
- A number of substrata can be investigated.
- It enables the determination of the effects of long-term exposure of biofilms to antibiotics as longitudinal studies can be carried out by removing replicate biofilms from the same CDFF experiment over time.

A protocol for the CDFF will be described in this chapter.

2. Materials

2.1. Materials Supplied When Purchasing the Fermentor AC Service Group, Poole, UK

1. Constant depth film fermentor with motor and power supply unit.
2. 15 PTFE pans, each with 5 plugs.
3. Recess tools and spanner.
4. Sampling tools.

2.2. Additional Materials Required and Suggested Suppliers

1. Incubator with ports at the top and side (for media in and effluent out) or a 37°C room (Various).
2. Peristaltic pump and pump tubing (Watson–Marlow, Falmouth, UK).
3. 3 mm and 5 mm bore silicone tubing, tubing connectors, and tubing clips (Various).
4. Couplers for connecting pump tubing to 3 mm tubing (Value Plastics Inc., Fort Collins, CO, USA). (Part: N220/210-6).
5. Nylon straps for securing tubing (Various).

6. Pyrex glass bottles, 1, 10, or 20 L depending on the length of experiment and flow rates used (Various).
7. Silicone rubber stoppers for medium and effluent bottles (Various).
8. Stainless steel tubes for the CDFF inlet ports and vessels.
9. Needle tubing 8G ×12″ SLS Scientific laboratory supplies Order no: SRY700 8H.
10. Coupling inserts and bodies to connect tubing (Sigma-Aldrich, Poole, UK). (Inserts Z12, 654-3 and bodies Z12, 651-9).
11. Air filters for the CDFF and vessels (Whatman, Maidstone, UK (Hepa-vent)).
12. Glass grow-back traps (Hampshire Glassware Ltd, Southampton, UK).
13. Retort stand and clamp (Various).
14. Portable butane burner (Various).
15. Silicone grease (Various).

3. Methods

3.1. Substratum

Pan assembly can be carried out as part of the fermentor assembly especially when using an inert substratum. Examples of such suitable substrata are shown in Fig. 2 (see Note 1).

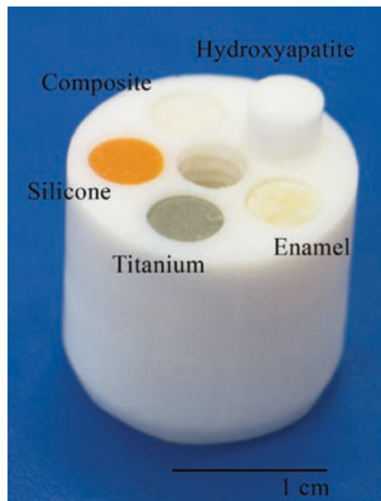

Fig. 2. Photograph depicting one of the CDFF pans. Four different 5-mm discs of materials have been recessed (Titanium, Silicone, Enamel, and Composite) while a disc of hydroxyapatite sits on one of the PTFE plugs.

1. The PTFE pans have five holes (each 5 mm in diameter) arranged around a central threaded hole into which the sampling tool is inserted. When assembling the pans it is important to have the central threaded hole uppermost.
2. Place the plugs into the holes in the pans with their flat surface uppermost.
3. If PTFE is being used as the substratum, push the plugs level with the top of the pan and use the recess tool to push the plug down by the required depth (two recess tools are supplied allowing depths of 100, 200, 300, and 400 μm).
4. For other substratum place a small amount of silicone grease between the PTFE and the chosen substratum to hold the material in place during autoclaving. Start with the plugs slightly raised above the pan, place a small amount of silicone grease on the PTFE plug, place the disc on top of the plug, push down, and wipe off excess grease. Push the plug up again slightly from underneath and then push down with the appropriate recessing tool.
5. Place the pans into the holes of the stainless steel turntable and ensure they are flush.

3.2. Inoculum

3.2.1. Single-Species

1. Add a 10 mL culture of the desired organism of an optical density (OD_{600}) of 0.6–1 L of medium.
2. This should then be used to inoculate into the CDFF for a minimum of 8 h depending upon the expected growth of the particular organism. For fast growing organisms the inoculum vessel can then be removed after this time; however, for slower-growing organisms the vessel can remain attached for up to 24 h.
3. The flow rate for inoculation should also be adjusted to coincide with the growth of the organism to ensure that the inoculum doesn't expire.

3.2.2. Mixed-Species

1. Confluent cultures of each organism should be grown on a suitable growth medium and transferred into 10 mL of a suspension medium containing 10% glycerol.
2. 1.0 mL aliquots of the resulting mixed suspensions are stored at –80°C (see Note 2).
3. Two cryovials of the desired bacterial suspension should be thawed and the contents transferred to 10 mL of sterile growth medium and vortex-mixed.
4. Using a 20-mL sterile pipette, the suspension is then carefully added to the rotating pans of the CDFF via the sample port.
5. Leave to rotate for a further hour and after this time connect the CDFF to a reservoir of sterile medium.

3.2.3. Microcosm Biofilms

Samples derived from the environmental system or body site can be used to provide an inoculum for multi-species biofilm consisting of microorganisms found in that environment, i.e. to produce a microcosm of that site or system (38). Environmental samples to be used as inocula for the CDFF would preferably be pooled to aid in reproducibility.

1. Add 10% glycerol to the environmental sample and aliquot into 1 mL amounts to be stored at −80°C for subsequent use.
2. For inoculation two aliquots should be added to 500 mL of sterile growth medium and pumped into the CDFF at an appropriate flow rate, for a minimum of 4 h (see Note 2).

3.3. CDFF Set-Up

3.3.1. Assembly

The CDFF pan assembly can be carried out as part of the fermentor assembly especially when using an inert substratum. However, certain materials may need to be kept wet and it may be more convenient to assemble the fermentor and add the pans at the last moment before autoclaving. If the material cannot be autoclaved it is possible to sterilise the CDFF by autoclaving and, in a sterile flow hood, remove the top-plate of the CDFF and place in pans/substratum which have been sterilised using an alternative method suitable for the material of choice (see Note 1).

1. When putting the CDFF together ensure that the glass housing and PTFE seal are liberally greased so that no leakage occurs.
2. The top-plate of the fermentor has three inlet ports – all need stainless steel tubes (Table 2) passing through the silicone bungs, all of these require silicon tubing and connectors.
3. The port in the middle of the top-plate is for the air-inlet, the silicone tubing attached here should have an air filter attached. The CDFF should be autoclaved prior to use.

Table 2
Additional materials required and suggested suppliers

Material	Supplier
Incubator with ports at the top and side (for media in and effluent out) or a 37°C room	Various
Peristaltic pump and pump tubing	Watson–Marlow, Falmouth, UK
3 mm and 5 mm bore silicone tubing, tubing connectors and tubing clips	Various

3.3.2. Atmospheric Requirements

The CDFF should be maintained at a constant temperature, e.g. 37°C by housing in an incubator. The atmosphere of the CDFF can also be altered depending on the requirement of the organisms or the environment the user is trying to model. For example an anaerobic atmosphere of 5% CO_2, 95% N_2 can be provided by connecting a gas cylinder to the fermentor air inflow port. The pressure used should be 1 bar and the flow rate 100 cm^3/min. Reinforced PVC tubing 5/16 mm internal diameter is needed to connect the gas pressure gauge and the flow metre. Silicon tubing with an 8 mm internal diameter should be used to connect the flow metre to the CDFF. The gas should be passed through air filters to maintain sterility. A “Y” branch tubing connector can be used to supply gas to both the fermentor and the inoculum flask if required.

3.3.3. Inoculation

1. To inoculate the CDFF a vessel containing the inoculum needs to be attached via one of the connectors on the top-plate.
2. The inoculation vessel will require tubing and connectors as well as an air filter.
3. Put the inoculum vessel into the incubator on a magnetic stirrer and pump in the inoculum using a peristaltic pump.
4. When the inoculum starts to drip onto the CDFF turntable switch on the motor at approximately 3 rpm.
5. An effluent bottle is required for the waste which is attached via an outlet port on the base of the CDFF. The vessel can sit on the laboratory floor to allow flow via gravity. If this is not possible an additional peristaltic pump may be required.
6. Once the CDFF is inoculated the inoculum vessel can be removed and the system fed from sterile media via one of the unused ports. This vessel will require the appropriate tubing, connectors, and air filters to allow the medium to be pumped into the CDFF.

The rate at which the medium enters the CDFF can be changed and should be a relevant rate (see Note 3) to the environmental situation.

3.3.4. Sampling

1. To remove sample pans from the CDFF, stop the turntable from rotating by reducing the power such that the desired pan comes to rest under the sample port.
2. Remove individual pans aseptically via the sample port using the screw-threaded tool by screwing this into the thread of the pan.
3. Remove the substratum from the sample pan by aseptically pushing each plug from below and removing the disc with forceps.

Biofilms can then be processed either intact or vortexed to remove the biofilm from the substratum.

3.4. Antibiotic Delivery

Pulse the agent into the CDFF via one of the free inlet ports at appropriate times to mimic in vivo use. For example, for the administration of tetracycline:

1. Pump in 100 mL of sterile medium containing 2 μg/mL tetracycline (Sigma, Poole, U.K.) for 2 h via a second peristaltic pump.
2. After this period, increase the concentration of tetracycline to 12 μg/mL for 1 h and subsequently decrease to 2 μg/mL for 2 h (39). These levels of antibiotic were selected as they mimic the peak concentration of tetracycline found in gingival fluid after a single systemic dose.

Additionally, biofilms can be removed from the CDFF and placed into an agent. In this case it is advisable to place the whole PTFE pan into the solution (5 mL in a universal to cover pan) in order to reduce disruption of the biofilms. Using this method it is possible to generate minimum inhibitory concentration-like data for the biofilms.

4. Notes

1. Substrata to be used in the fermentor cannot always be autoclaved at 121°C for 15 min with the rest of the apparatus. In this instance, it is advisable to sterilise the discs in some other way, e.g. dry heat, radiation, 70% ethanol, ethylene oxide, or low-temperature gas plasma which are currently used for a variety of materials used in medicine. The Fermentor can be autoclaved and the materials added afterwards in a sterile cabinet.
2. If the composition of the final biofilm needs to be regulated, it may be advisable to have different starting amounts of organisms in the inoculum. Preliminary studies would need to be performed to determine the conditions required for these populations. These studies may need to include standard growth curves, the growth of single-species biofilms, growth of the organisms in different atmospheres and using different nutrient sources. When considering the criteria for a community study, the organisms selected need to be from a pool of species common to the oral environment and important components of that community. Further, the group of species should form a stable community where, under standard conditions, all members are retained over the course of

the experiment. To further produce biofilms which are more realistic of the in vivo scenario the establishment of laboratory microcosms is useful in association with a suitable substratum and nutrient source.

3. An important consideration is the medium to be used and the flow rate at which it enters the fermentor. As well as being the nutrient source for the organisms, the chemical and physical properties, of the medium will exert a profound influence on the adhesive interactions that occur. Due to the obvious difficulty in using large volumes of human-derived media, a synthetic substitute will often be needed to reproduce features of materials which are considered important. These should be dripped into the fermentor at rates which would normally be associated with the body site or the environment the user is trying to mimic.

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Chapter 17

Rapid Analysis of Resistant Mutant Genotypes Using Pyrosequencing®

Catherine Arnold

Abstract

The rapid detection of nucleotide mutations conferring drug resistance is especially important for organisms with long generation times. *Mycobacterium tuberculosis*, an organism thought to infect around one-third of the global population, is probably the most important of these slow-growers. Multidrug-resistant (MDR) strains of *M. tuberculosis*, indicated by resistance to rifampicin, are emerging and their rapid detection is crucial for treatment and control of this pathogen. Single nucleotide polymorphisms (SNPs) in the rifampicin resistance determining region (RRDR) of *rpo*B, an 81-bp region of the RNA polymerase B gene, confer resistance to rifampicin in the vast majority of cases. Combining PCR of this region and rapid short read sequencing using Pyrosequencing® enables rapid high-throughput MDR strains to be detected from sputum and early cultures.

Key words: Pyrosequencing®, *Mycobacterium tuberculosis*, Rifampicin, Drug resistance, RRDR, *rpo*B

1. Introduction

The appearance of large numbers of strains of *Mycobacterium tuberculosis* resistant to more than one of the first-line agents used to treat the disease is of significant concern. Mortality associated with a multidrug-resistant (MDR) strain of tuberculosis (MDR-TB) infection is reported to be high and is effectively no different from the mortality of tuberculosis in the preantibiotic age. In the UK, the first-line treatment of tuberculosis is a 2-month course of isoniazid, rifampicin (RIF) and pyrazinamide, often with the addition of ethambutol, followed by isoniazid and rifampicin for a further 4–6 months. The marker used for MDR-TB is RIF resistance. Drug resistance in *M. tuberculosis* occurs following spontaneous mutation in target genes. In a study of 26 structural

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cgatcacacc gcagacgttg atcaacatcc ggccggtggt cgccgcgatc aaggagttct tcggcaccag
ccagctgagc caattc atgg **A**ccagaacaa cccgctgtcg gggttgaccc **A**caagcgccg actgt**C**ggcg
ctggggcccg gcggtctgtc acgtgagcgt gcc

Fig. 1. The RRDR of wild-type (susceptible) *rpo*B in *M. tuberculosis.* Common polymorphic nucleotides corresponding to amino acid positions 516, 526, and 531, respectively, are shown in bold capital letters. The Pyrosequencing® primer is shown in italics.

genes of 842 strains of *M. tuberculosis*, more than 95% of nucleotide substitutions caused amino acid replacements or other mutations in genes linked to antibiotic resistance (1). The majority of the mutations seen were single nucleotide polymorphisms (SNPs), defined as single base pair positions in genomic DNA at which different sequence alternatives or alleles exist. Although originally the term was used only in human genome studies where an alternative allele exists in normal individuals in some population(s) with a frequency of 1% or greater, the term SNP is now usually used more loosely and single base variants in all genomes are classed as SNPs. SNPs identified in a short region of the *rpo*B gene confer resistance to RIF in *M. tuberculosis*. RIF inhibits transcription by binding to the RNA polymerase (*rpoB*) beta subunit (2). Resistance to RIF is encoded by mutations in the RRDR of the *rpoB* gene, an 81-bp region of DNA encoding amino acids 507–533. The most common mutations (70% of resistant strains (3)) are at codons 526 and 531 and are associated with high levels of resistance (>100 µg/ml). Lower-frequency mutations, particularly codon 516, are associated with a lower level of resistance. Figure 1 shows the region of interest in *rpo*B. SNPs in other genes (including *inh*A, *kat*G, and *pnc*A) also confer resistance to the other first-line drugs used to combat tuberculosis, including isoniazid and pyrazinamide. The methods used to detect SNPs are varied but the choice of a rapid sequence-based method such as Pyrosequencing®, a proven SNP-detection technology rather than a hybridization or DNA migration approach is best suited for a rapid high-throughput system, based on the quantitative detection of pyrophosphate released following nucleotide incorporation into a growing DNA chain (4). We developed a molecular screen capable of use on primary clinical samples to detect MDR-TB, based on mutations associated with RIF drug resistance (5).

2. Materials

2.1. PCR Setup

1. Boiled lysed *M. tuberculosis* culture or processed decontaminated (NaOH treated) sputum sample (see Note 1).
2. Sigma PCR Mastermix (Sigma-Aldrich). Store at –20°C.

3. Forward PCR primer, 5′–3′, CGATCACACCGCAGACG-TTGAT. Store at −20°C in 100 μM aliquots.
4. Reverse PCR primer, 5′–3′. This primer is biotinylated at the 5′ end. GGCACGCTCACGTGACAGACC. Store at −20°C in 100 μM aliquots.
5. Pyrosequencing® primer, 5′–3′, ACCAGCCAGCTGAGCCAA-TTC. Store at −20°C in 100 μM aliquots.

2.2. PCR Product Preparation

1. Streptavidin-coated Sepharose beads (Amersham Pharmacia Biotech). Store at +4°C.
2. Annealing buffer, binding buffer, denaturation solution, and wash buffer (Biotage AB, Uppsala). Seventy percent Analar ethanol, freshly made with sterile distilled water.
3. PyroGold SQA kit and reagent cartridge (Biotage, Uppsala). Once resuspended in sterile distilled water, the enzyme and substrate mixes from the kit can be stored at +4°C for several days or can be frozen once for longer storage. The nucleotides supplied can be stored at +4°C for extended periods (weeks).

3. Methods

Pyrosequencing® is a rapid high-throughput short sequencing method based on a chemical light-producing reaction triggered by the enzymatic incorporation of a complementary nucleotide in a growing chain. Essentially, the method allows sequencing of a single strand of DNA by synthesizing the complementary strand by cyclic incorporation of A, C, G, or T, triggering a cascade of enzymatic reactions, which in turn generate a light signal. A single-stranded DNA template, generated very rapidly and easily using a vacuum prep tool, is hybridized to a Pyrosequencing® primer and automatically incubated with the enzymes and substrates. When DNA polymerase incorporates the correct complementary dNTP into the template, pyrophosphate (PPi) is released. ATP sulfurylase quantitatively converts this pyrophosphate to ATP, which initiates luciferase-mediated conversion of luciferin to oxyluciferin. This generates light signal proportional to the number of nucleotides incorporated (e.g., twice the amount of ATP would be produced by the incorporation of two nucleotides together in a chain at once, so a light signal twice the amount would be produced). During the minute-long cycle of each individual nucleotide addition, unincorporated nucleotides and ATP are degraded by apyrase, and the reaction can resume with another nucleotide in the next cycle. The light produced in

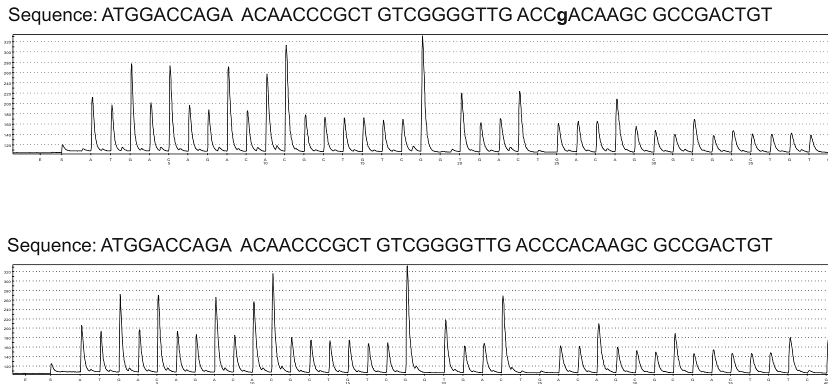

Fig. 2. Examples of *rpo*B Pyrograms®. Sequence variation from wild-type shown as lower case.

the luciferase-catalyzed reaction is automatically detected by a charge coupled device camera and generates a Pyrogram® (see Fig. 2).

3.1. PCR Setup

1. In a 50-μl final volume set up the following: 10 pmol of each primer, 25 μl of PCR Sigma Mastermix, 1 μl of sample template, make up to 50 μl with sterile distilled water (see Note 2).
2. Cycle the reaction as follows:

94°C – 12 min	1 cycle
94°C – 30 s 40 cycles 56°C – 1 min	
72°C – 2 min	

Store the products at +4°C until use.

3.2. PCR Product Preparation

The single-stranded DNA templates for Pyrosequencing® can be easily made using a solid phase template preparation system with streptavidin-coated magnetic beads. Streptavidin-coated sepharose beads are bound (by mixing) to biotinylated PCR products, generated by one of the PCR primers being synthesized with biotin at the 5′ end. This is the format for all Pyrosequencing® assays, making design very simple as either the forward or the reverse primer can be biotinylated. Using a 96-filter vacuum prep tool, the large sepharose beads, now coated with double-stranded PCR product, can be taken up by the tool but are too large to pass through the filter pins. Solutions can then be passed through the filters to make the bound double-stranded PCR product single-stranded using NaOH to denature and remove the non-biotinylated strand in preparation for Pyrosequencing®. The solutions used are contained in five troughs and the handheld pin tool is passed from trough to trough after a few seconds have elapsed to allow sufficient solution to pass through each filter.

1. Mix 20 μl PCR product with 3 μl streptavidin-coated sepharose beads, 40 μl binding buffer, and 17 μl H_2O in a PCR plate. Cover the plate with sealing tape or strip caps.
2. Incubate the mixture for 5–10 min at room temperature while shaking at 1,400 rpm (see Note 3).
3. Prepare a Pyrosequencing® plate containing 45 μl of 0.3 μM sequencing primer in annealing buffer per well. The "well layout" should correspond to the layout of the PCR plate.
4. With the beads in suspension, aspirate the solution using the 96-pin vacuum prep tool. The beads will stick to the filters of the pin tool. Keep the tool and PCR plate together and tilt them to check that all solution has been aspirated (see Note 4).
5. Transfer the tool to the first trough and aspirate 70% ethanol for 10 s (see Note 5).
6. Transfer the tool to the second trough and aspirate denaturation solution for 10 s to denature the DNA.
7. Transfer the tool to the third trough and aspirate washing buffer for 10 s.
8. Pick up the tool and turn upright to allow liquid to drain from the probes, and then return to a horizontal position.
9. Place the pin tool above the Pyrosequencing® plate containing the annealing buffer and Pyrosequencing® primer. Release the vacuum (see Note 6).
10. After releasing the vacuum, wriggle the tool in the Pyrosequencing® plate to release the beads with single-stranded PCR product. The liquid takes on a very slightly milky appearance once the beads have been released from the pin.
11. Anneal the Pyrosequencing® primer by heating the plate at 80°C for 2 min and then allow it to cool down to room temperature (see Note 7).
12. Shake the Vacuum Prep Tool in the fourth trough, containing sterile distilled water, to release any leftover beads.
13. When all plates have been prepared, transfer the tool to the fifth trough and aspirate sterile distilled water for 20–30 s to wash the tool and always store dry (see Note 8).

3.3. Pyrosequencing®

1. Reconstitute the enzyme and substrate mixes in 620 μl sterile distilled water each (see Note 9).
2. Set up the run on the computer. For an SNP run, select new SNP run by right-clicking on the mouse and complete the sample sheet, selecting the correct entry for each run (see Note 10). For a sequencing run, do the same except select SQA run. For both types of run, select appropriate instrument

parameters and name the run. Selecting "view" will automatically calculate the volume of each reagent required.

3. Add enzyme mix, substrate mix, and the four deoxynucleotides to the reagent cartridge.
4. Load the plate and the cartridge into the instrument.
5. Start the run by clicking "run".
6. When the run is finished, analyze the results by clicking the "analyse" button.
7. Compare the sequence with the data shown in Fig. 1 and assign resistance profile. Data can be exported as tab-delimited data, which can then be stored in an EXCEL file.

N.B. There is software available to automate this procedure to expedite the identification process.

4. Notes

1. Both boiled lysate and processed sputum sample (liquefied, decontaminated, and concentrated prior to culture) have been used successfully. Sputum samples that are negative by microscopy are unlikely to produce sufficient PCR product for this assay.
2. Ten picomoles of each primer is probably the maximum amount that can be used. When the biotinylated product is bound to the sepharose beads for purification, excess unincorporated primer can also bind to the beads preferentially, effectively reducing the concentration of the purified product.
3. The exact time for shaking is not important but the beads do fall out of suspension relatively quickly so shaking does need to be vigorous. Shaking times of 30 min or more do not affect the quality of the sequence generated. Multiple plates can be prepared, shaken, and then stored at +4°C prior to Pyrosequencing® with no loss (in some cases, a slight gain) of signal generated.
4. Occasionally, filters can become blocked, as determined by the presence of a liquid in a well of the 96-well sample plate after sample preparation. These filters can be changed manually quite easily. The samples not picked up by the faulty filters can be transferred to another plate and retested with no detriment to signal.
5. The amount of time to place the pin tool in each solution may vary depending on the age of the pins. This time can be gauged by watching for liquid being aspirated into the waste reservoir.

As soon as liquid can be seen to have passed through the pin tool it indicates that sufficient solution has been used.

6. Always ensure that the vacuum on the prep tool is switched off before placing the pins into the Pyrosequencing® plate. The beads will not usually fall off the pins before they are put into the plate unless they have been dried out excessively (i.e., the vacuum left on without solution passing through the pins).
7. The amount of time needed to cool the samples will vary from assay to assay as some will benefit from a slower cooling. Usually, the time taken to fill in the sample sheet (around 10 min) is enough. The samples must be at room temperature before being analyzed as the enzymes used are not thermophilic.
8. Pins rarely need changing but if the tool is not stored dry, or rinsed with water after use, the pins can become blocked.
9. Do not use diethylpyrocarbonate (DEPC)-treated water to resuspend the enzyme and substrate as it can affect enzyme activity. Sterile distilled water is sufficient.
10. Pyrosequencing® can be used for both short read sequencing and SNP detection. Unlike traditional Sanger sequencing, the Pyrosequencing® primer can sit right next to the SNP in question which can then be detected in the first minute cycle of nucleotide addition.

Acknowledgment

Pyrosequencing® and Pyrogram® are registered trademarks of Biotage.

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