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Assessment of a microplate method for determining the post-antibiotic effect in *Staphylococcus aureus* and *Escherichia coli*

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***Objectives*: The post-antibiotic effect (PAE) is an important parameter of antibiotic action that is widely used as a predictor of pharmacodynamic activity. Traditionally, PAE has been determined by a labour-intensive method involving determination of viable cell numbers. New methods using spectrophotometric procedures could offer significant advantages for PAE determinations, particularly in terms of speed. A number of such methods have been described in the literature, but extensive comparison with the classical procedure for determining PAEs has not been carried out. We have now compared PAE values obtained using a rapid microplate method with those achieved by the classical viable count procedure.**

***Methods*: We determined PAE values for a variety of antibiotics against *Staphylococcus aureus* and *Escherichia coli* following exposure to 5 × MIC drug concentrations for 60 min in Mueller–Hinton Broth (MHB). The duration of the PAE was obtained by following the recovery of bacterial growth in antibiotic-free MHB measured either as colony forming units on Mueller–Hinton agar, or as culture absorbance (600 nm) in a microplate reader.**

***Results*: For bacteriolytic agents there was poor correlation between the two methods for both *S. aureus* ($R^2 = 0.096$) and *E. coli* ($R^2 = 0.5456$). However, when PAEs for bacteriostatic agents and non-lytic bactericidal agents were compared, correlation between the two methods was high for both *S. aureus* ($R^2 = 0.7529$) and *E. coli* ($R^2 = 0.7687$).**

***Conclusions*: The spectrophotometric microplate method for determining PAEs may be a suitable alternative to the classical method for those antibiotics that do not induce bacterial cell lysis.**

Keywords: antibiotic action, *S. aureus*, *E. coli*

Introduction

Continued suppression of bacterial growth following limited exposure to an antimicrobial agent was first noted nearly 60 years ago.[1] The term post-antibiotic effect (PAE) has now become the accepted description of this phenomenon which results from prior exposure of organisms to an antibiotic, rather than persistence of sub-minimal inhibitory concentrations (sub-MICs) of drugs in the medium.[2] In the past 40 years, numerous studies have investigated the ability of antimicrobial agents to induce PAEs in bacteria.[2] Determination of the PAE is recommended in pre-clinical evaluation of all new antimicrobial agents[3] because it is a factor that influences optimal antimicrobial dosing intervals,[4] i.e. antibiotics without a PAE usually require more frequent administration than agents exhibiting PAEs. Recent examples of PAE determinations on new antimicrobial agents include studies with quinoline-indoles,[5] non-fluorinated quinolones,[6] TD-6424[7] and daptomycin.[8]

PAE periods are usually determined by a method, first established by McDonald and colleagues[2,9] which involves exposure of organisms to 5 × antibiotic MIC for 60 min, removal of drug by dilution, or re-suspension of organisms in fresh medium, followed by viable count determinations to monitor resumption of bacterial growth.[9] The PAE is then calculated using the standard formula PAE = $T-C$, where T is the time required for the treated cells to increase 10-fold (1 $\log_{10}$ cfu/mL) after washout of drug and C is the time required for a non-treated control to increase 10-fold (1 $\log_{10}$ cfu/mL) after washout with fresh medium.[9] PAE determination involving viable counting of organisms is labour-intensive and lengthy, and several new methods based

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on spectrophotometric techniques have been developed to measure more easily the resumption of bacterial growth after antibiotic exposure.[6,10–13] Spectrophotometric methods for PAE determination are particularly attractive because these measurements, when made in microplate format, could offer rapid, automated, high-throughput procedures for determination of PAEs.

However, spectrophotometric methods for determining PAEs have not been carried out with an extensive set of drugs and detailed comparison with the classical, viable count procedure, has not been conducted. Consequently, we devised a simple method for determining PAEs using a microplate reader and established its performance with a wide range of antibiotics active against *Staphylococcus aureus* and *Escherichia coli.*

Materials and methods

Bacterial strains

The *S. aureus* strain used throughout this work was 8325-4 (i.e. NCTC 8325 cured of phages 11, 12 and 13).[14] *E. coli* ATCC 25922 was obtained from the American Type Culture Collection (ATCC). *E. coli* 1411 (*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rspL*) and its derivative *E. coli* SM1411 (*acrAB*$^-$) which contains a Δ*acrAB: Tn903* Kan^r insert have been described in an earlier publication from this laboratory.[15]

Antibiotics and growth media

Ciprofloxacin, linezolid, mupirocin, meropenem, tiamulin and mecillinam were gifts, respectively from Bayer AG (Leverkusen, Germany), Pharmacia and Upjohn Inc. (Kalamazoo, MI, USA), GlaxoSmithKline Pharmaceuticals (Harlow, Essex, UK), AstraZeneca Pharmaceuticals (Macclesfield, UK), Biochemie (Vienna, Austria) and Leo Pharmaceuticals (Ballerup, Copenhagen). Other antibiotics were purchased from Sigma–Aldrich (Poole, Dorset, UK). Mueller–Hinton broth (MHB) and agar (MHA) were purchased from Fisher (Loughborough, UK).

Determination of susceptibility of S. aureus and E. coli strains to antimicrobial agents

MICs were determined by broth microdilution in MHB using an inoculum of 10^6 colony forming units per mL for *S. aureus* 8325-4 and 10^4 cells per mL for *E. coli* strains in a final volume of 70 μL. Microtitre plates (384 wells) containing triplicate two-fold dilution series were incubated for 16 h at 37°C in a Spectramax 384 plus microtitre plate reader (Molecular Devices, Abingdon, Oxfordshire, UK), running SOFTmax PRO 3.1.1 software. Optical density readings (600 nm) were taken at 10 min intervals. Plates were shaken for 30 s before each reading. The MIC was taken as the lowest concentration of antibiotic that prevented growth in the triplicate wells. Susceptibility determinations were carried out on three separate occasions and the mean of these values recorded as the MIC.

PAE determination by viable counting

Bacteria were grown to the early logarithmic phase (2×10^8 cells/mL) in MHB. Cultures were then divided into two aliquots, one of which received test antibiotic at 5 × MIC, the other serving as a drug-free control. The cultures were incubated for a further 60 min and the bacteria harvested by centrifugation (5000 **g**, 5 min) in a Sigma 3K18 laboratory centrifuge (Philip Harris Scientific, Ashby Park, Leicestershire, UK) that had been pre-warmed to 37°C. Bacteria were resuspended in fresh pre-warmed sterile MHB and washed three times by centrifugation as above. Finally, the washed cell pellets were resuspended in fresh pre-warmed sterile MHB using volumes equivalent to the original culture volumes. These new cultures were then incubated at 37°C.

Samples were removed for viable counting before washing, immediately after washing and at hourly intervals thereafter. Samples were serially diluted in ice-cold PBS and plated onto MHA. Colonies were counted after incubation at 37°C for 16 h. The viable count for each sample was determined from the average number of colonies on at least three plates containing between 30 and 300 colonies. The duration (D) of the PAE was calculated according to McDonald *et al.*[9] using $D = T - C$ where T is the time (h) required for the treated cell density to increase by 10-fold (by 1 $\log_{10}$ cfu/mL) and C is the time required for the non-treated control cell density to increase 10-fold (by 1 $\log_{10}$ cfu/mL).

PAE determination by optical density

Cultures were grown in MHB and exposed to antimicrobial agents at 5 × MIC for 60 min as described above. Samples (1 mL) were then removed from cultures containing antibiotics and drug-free controls and bacteria harvested by centrifugation at 16 000 **g** for 2 min in a microfuge. Supernatants were removed using a suction pump and the cell pellets resuspended in fresh pre-warmed MHB (1 mL) before the cells were again harvested by centrifugation. The

Table 1. Susceptibility of *E. coli* and *S. aureus* strains to antibiotics

	MIC mg/L			
	E. coli 1411	*E. coli* SM1411	*E. coli* 25922	*S. aureus* 8325-4
AMP	2	2	4	0.125
AMX	0.5	0.5	4	0.06
CHL	4	0.25	2	2
CRO	0.0156	0.0156	0.125	0.5
CTX	0.125	0.0625	0.0625	2
DCS	32	16	64	32
ERY	32	2	64	0.25
FUS	256	2	128	0.125
FOF	8	1	2	1
GEN	ND	ND	0.5	0.25
IPM	0.06	0.06	0.06	0.0078
LNZ	ND	ND	ND	4
MEC	0.0078	0.0078	0.06	ND
MEM	0.0078	0.0078	0.0156	0.03
MIN	2	0.0625	1	0.5
MUP	32	1	32	0.03
NIT	4	1	8	ND
NOV	64	1	16	ND
PUR	64	4	32	ND
RIF	4	2.5	4	0.016
TET	4	1	1	0.06
TIA	ND	ND	ND	0.25

Susceptibility of *E. coli* and *S. aureus* strains to antibiotics. AMP, ampicillin; AMX, amoxicillin; CHL, chloramphenicol; CRO, ceftriaxone; CTX, cefotaxime; DCS, D-cycloserine; ERY, erythromycin; FUS, fusidic acid; FOF, fosfomycin; GEN, gentamicin; IPM, imipenem; LNZ, linezolid; MEC, mecillinam; MEM, meropenem; MUP, mupirocin; MIN, minocycline; NIT, nitrofurantoin; NOV, novobiocin; PUR, puromycin; RIF, rifampicin; TET, tetracycline; TIA, tiamulin; ND, not determined.

washing and centrifugation steps were repeated a further two times. After washing, the bacteria were resuspended in fresh pre-warmed MHB (1 mL) and 70 μL of each culture was added to the wells of 384-well microtitre plates. Plates were then incubated as described for MIC determination with automated reading of culture turbidity every 10 min. PAE duration was calculated according to Odenholt-Tornqvist,[16] i.e. the time taken for antibiotic-treated cultures to reach 50% of the OD_{max} of the control culture, minus the time taken for the control culture to reach the same point.

Correlation between viable counting and optical density techniques as methods for PAE determination was calculated as R^2 values by the Pearson correlation calculation using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software in accordance with the manufacturer's instructions.

Results and discussion

As an alternative to PAE determination based on viable count procedures, several authors have explored the possibility that spectrophotometric methods might be used to monitor more rapidly the re-growth of organisms following exposure to antibiotics. Thus spectrophotometric methods have been used to determine benzylpenicillin-induced PAEs in streptococci,[10] gentamicin and ciprofloxacin PAEs in *E. coli*, *Pseudomonas aeruginosa* and *S. aureus*,[11] teicoplanin PAEs in staphylococci and enterococci,[12] quinolone PAEs in *Streptococcus pneumoniae* and *P. aeruginosa*[6] and ampicillin, tobramycin and ciprofloxacin PAEs in clinical isolates of *E. coli*.[13] However, direct comparison with the classical viable count procedure was only made in three of these studies.[10,11] In view of the very limited data comparing the old and new procedures, we measured PAEs using both methods with a wide range of antibiotics active against *E. coli* or *S. aureus*.

For *E. coli*, we used strain ATCC 25922, which has been widely used for PAE determinations based on viable count methods,[2] together with strains 1411 and SM1411 (*acrAB*$^-$). For *S. aureus*, we used strain 8325-4 which has been previously used in our laboratory to determine PAEs for candidate anti-staphylococcal agents.[5,17]

The MIC values for a variety of antibiotics against these strains are shown in Table 1. Some of these antibiotics have no clinical application for the treatment of infections caused by *E. coli* or *S. aureus*, but were nevertheless included in our studies to explore the validity of spectrophotometric microplate procedures for measuring PAEs. We have already used *E. coli* strains 1411 and SM1411 (*acrAB*$^-$) for other studies on the mechanism of the PAE.[18] In addition, the use of a mutant which is insertionally inactivated for *acrAB* more readily permits PAE studies in *E. coli* with antibiotics such as erythromycin and

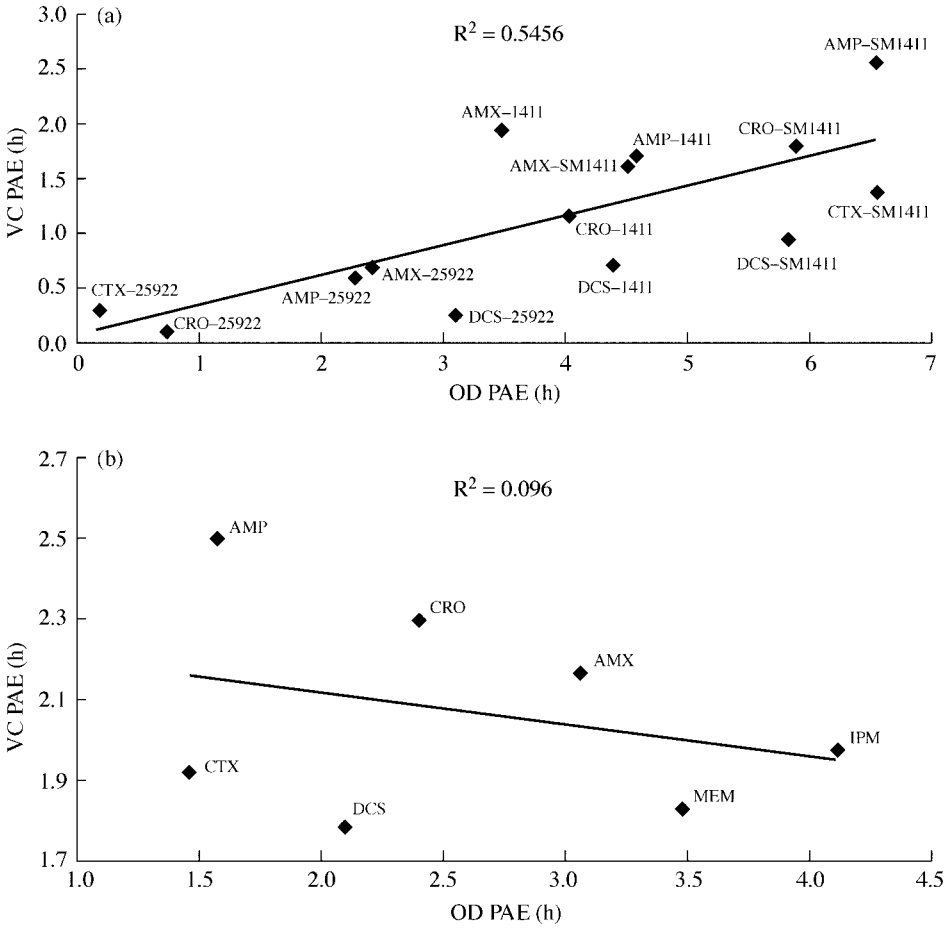

Figure 1. Relationship between PAE determined by two methods for bacteriolytic agents against *E. coli* (a) and *S. aureus* (b).PAE periods (h) were determined for a variety of antibiotics against the indicated strains using the viable count (VC) and rapid optical density (OD) procedures described in Materials and methods. Antibiotic abbreviations are defined in the footnote to Table 1.

novobiocin that normally have poor activity against *E. coli* due to AcrAB-mediated efflux. The benefit of using an *acrAB*$^-$ mutant for PAE determinations with certain antibiotics in *E. coli* is illustrated by the MIC data presented in Table 1.

PAEs were obtained by both methods using antibiotics that cause lysis by inhibiting peptidoglycan synthesis in *E. coli* and *S. aureus*. There was relatively poor correlation between the two methods for *E. coli* ($R^2 = 0.5456$) (Figure 1a) and extremely poor correlation for *S. aureus* ($R^2 = 0.096$) (Figure 1b). However, when PAEs for non-lytic agents were compared (Figure 2), correlation between the two methods was substantially improved [*E. coli*, $R^2 = 0.7687$; Figure 2(a); *S. aureus* $R^2 = 0.7529$, Figure 2(b)].

The inhibitors of peptidoglycan synthesis used here caused substantial cell lysis (data not shown). Accordingly, the time required for the survivors in these cultures to resume growth and reach a culture density detectable in the microplate reader inaccurately overestimated the PAE compared to the established viable count procedure. Indeed, this artefact could easily be mimicked in non-antibiotic-treated cultures simply by following the period required for diluted cultures to reach a threshold culture absorbance detectable by the plate reader (data not shown). The difficulty of measuring PAE for bacteriolytic agents by the spectrophotometric procedure described here might be overcome by using larger culture volumes during the antibiotic exposure phase, so that more-concentrated cell suspensions could be obtained after the washing stage that would be detectable in the plate reader. However, such methods might be compromised by interference from high levels of cellular debris present in the concentrated cell lysates. A further difficulty would relate to determining the growth of the non-antibiotic-treated control cells since these bacteria would need to be resuspended at very high cell densities to maintain strict parity with the dilution factors used for the cells exposed to lytic antibiotics.

PAE data are often conflicting due to the use of various methods for determination of PAE and different conditions of exposure to the test antibiotic. In view of the importance of PAE as a pharmacodynamic predictor, it would be advantageous to develop a standard, rapid, method for PAE determination. Spectrophotometric methods have recently been used to determine PAEs. However, there has been no systematic evaluation of these methods and little comparison with the classical, viable-count-based, procedure of McDonald *et al.*[9] In this paper, we have shown that PAE values, comparable to those obtained by the classical procedure, can be obtained with *S. aureus* and *E. coli* for non-lytic antibiotics using a rapid spectrophotometric method which involves a plate reader. Nevertheless, although the rapid method described here could have a useful application in the determination of PAEs for bacteriostatic and non-lytic bactericidal agents against these organisms, it appears that it will still be necessary to use the classical, viable-count-based, procedure for studies with lytic agents such as the β-lactam antibiotics. Further work will be needed to assess the usefulness of the microplate method for determining PAEs of non-lytic agents

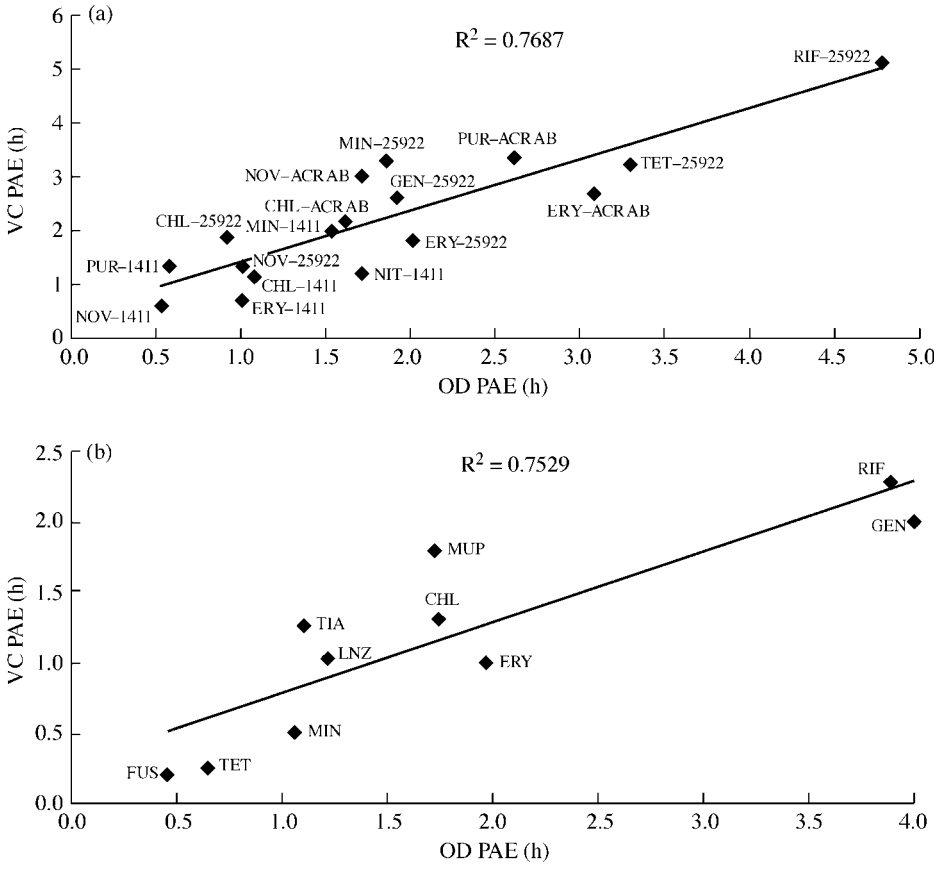

Figure 2. Relationship between PAE determined by two methods for non-bacteriolytic agents against *E. coli* (a) and *S. aureus* (b). PAE periods (h) were determined for a variety of antibiotics against the indicated strains using the viable count (VC) and rapid optical density (OD) procedures described in Materials and methods. Antibiotic abbreviations are defined in the footnote to Table 1.

against other Gram-positive and Gram-negative organisms, including clinical isolates.

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References

1. Eagle, H. & Musselman, A. D. (1949). The recovery of bacteria from the toxic effects of penicillin. *Journal of Clinical Investigation* **28**, 832–6.

2. Craig, W. A. & Gudmundsson, S. (1996). Postantibiotic effect. In *Antibiotics in Laboratory Medicine* (Lorian, V., Ed.), pp. 296–330. Williams & Wilkins, Baltimore, MD, USA.

3. Beam, T. R., Gilbert, D. N. & Kunin, C. M. (1992). General guidelines for the clinical evaluation of anti-infective drug products. *Clinical Infectious Diseases* **15**, S5–32.

4. Vogelman, B., Gudmundsson, S., Leggett, J. *et al.* (1988). Correlation of antimicrobial pharmacokinetic parameters with therapeutic efficacy in an animal model. *Journal of Infectious Diseases* **158**, 831–47.

5. Oliva, B., Miller, K., Caggiano, N. *et al.* (2003). Biological properties of novel antistaphylococcal quinoline-indole agents. *Antimicrobial Agents and Chemotherapy* **47**, 458–66.

6. Odenholt, I., Lowdin, E. & Cars, O. (2003). Postantibiotic, postantibiotic sub-MIC and subinhibitory effects of PGE-9509924, ciprofloxacin and levofloxacin. *Antimicrobial Agents and Chemotherapy* **47**, 3352–6.

7. Pace, J. L., Krause, K., Johnston, D. *et al.* (2003). *In vitro* activity of TD-6424 against *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **47**, 3602–4.

8. Pankuch, G. A., Jacobs, M. R. & Appelbaum, P. C. (2003). Postantibiotic effects of daptomycin against 14 Staphylococcal and Pneumococcal clinical isolates. *Antimicrobial Agents and Chemotherapy* **47**, 3012–4.

9. McDonald, P. J., Craig, W. A. & Kunin, C. M. (1977). Persistent effect of antibiotics on *Staphylococcus aureus* after exposure for limited periods of time. *Journal of Infectious Diseases* **135**, 217–23.

10. Lowdin, E., Odenholt-Tornqvist, I., Bengtsson, S. *et al.* (1993). A new method to determine postantibiotic effect and effects of subinhibitory antibiotic concentrations. *Antimicrobial Agents and Chemotherapy* **37**, 2200–5.

11. Dominguez, M. C., de la Rosa, M. & Borobio, M. V. (2001). Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar. *Journal of Antimicrobial Chemotherapy* **47**, 391–8.

12. Odenholt, I., Lowdin, E. & Cars, O. (2003). *In vitro* studies of the pharmacodynamics of teicoplanin against *Staphylococcus epidermidis* and *Enterococcus faecium*. *Clinical Microbiology and Infection* **9**, 930–7.

13. Rescott, D. L., Nix, D. E., Holden, P. *et al.* (1988). Comparison of two methods for determining *in vitro* postantibiotic effects of three antibiotics on *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **32**, 450–3.

14. Novick, R. (1967). Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. *Virology* **33**, 155–66.

15. O'Neill, A. J., Bostock, J. M., Morais Moita, A. *et al.* (2002). Antimicrobial activity and mechanisms of resistance to cephalosporin P1, an antibiotic related to fusidic acid. *Journal of Antimicrobial Chemotherapy*, **50**, 839–48.

16. Odenholt-Tornqvist, I. (1993). Studies on the postantibiotic effect and the postantibiotic sub-MIC effect of meropenem. *Journal of Antimicrobial Chemotherapy* **31**, 881–92.

17. Oliva, B., O'Neill, A. J., Miller, K. *et al.* (2004). Antistaphylococcal activity and mode of action of clofazimine. *Journal of Antimicrobial Chemotherapy* **53**, 435–40.

18. Stubbings, W. J., Bostock, J. M., Ingham, E. *et al.* (2002). Deletion of the multiple drug efflux pump (AcrAB) of *Escherichia coli* prolongs the postantibiotic effect. In *Program and Abstracts of the Forty-second Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA*, 2002. Abstract C1-429, p.65. American Society for Microbiology, Washington, DC, USA.