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# Effects of Clarithromycin at Sub-Minimum Inhibitory Concentrations on Early *erm*B Gene Expression, Metabolic Activity and Growth of an *erm*(B)-Expressing Macrolide-Resistant Strain of *Streptococcus pneumoniae*

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## ABSTRACT

**Aim:** To investigate the effects of exposure of a macrolide-resistant [*erm*(B)-expressing] strain of *Streptococcus pneumoniae* (strain 2507) to clarithromycin (0.5 and 5 mg/L) added at the outset and 6 hours after initiation of culture on early gene expression, energy metabolism, and growth. **Methods:** Bacterial growth was determined by turbidometric and colony counting procedures, energy metabolism by measurement of ATP, while analysis of gene expression was performed using reverse transcription-PCR and sequencing. **Results:** Addition of clarithromycin, at either concentration, at the outset of culture, caused transient suppression of growth of 10 - 12 hours duration, while delayed addition of antibiotic (during the logarithmic phase) resulted in an abrupt halt in growth followed by recovery. These inhibitory effects of clarithromycin on bacterial growth were associated with up-regulation of expression of *erm*(B), decreased ATP and protein synthesis, and were unaffected by inclusion of either catalase (500 and 1000 kunits/L), or competence-stimulating peptide (CSP-1, 0.5 mg/L). The inhibitory effects could, however, be overcome by pre-exposure of the bacteria to the antibiotic. Moreover, clarithromycin appeared to potentiate the antimicrobial actions of ceftriaxone, at sub-MIC concentrations, for strain 2507. **Conclusions:** Unlike several other common bacterial pathogens, the full expression of *erm*(B)-mediated macrolide resistance by the pneumococcus has a slow onset, which is associated with transient susceptibility to macrolides and inhibition of growth.

**Keywords:** Clarithromycin; Macrolide-Resistance; Pneumococcus

## 1. Introduction

Macrolide antibiotics are used in the management of patients with community-acquired pneumonia in a number of settings, for example as monotherapy in young, otherwise healthy patients in the outpatient setting, in whom macrolide resistance among pneumococci is unlikely to exist. They are also used in combination therapy, together with beta-lactam agents, in more severely ill, hospitalised patients being admitted for intravenous therapy. However, macrolide resistance is increasing worldwide and clearly limits the clinical utility of this class of antimicrobial agent, particularly when used alone. Nevertheless, it is noteworthy that therapeutic efficacy of these agents in the setting of macrolide resistance has been documented in a number of reports in humans and animal models of experimental infection [1-6]. Several mechanisms have been proposed to explain the susceptibility of ostensibly resistant pathogens to macrolides; these include possible non-ribosomal antimicrobial activities of macrolides [7-9] and/or beneficial immunomodulatory/anti-inflammatory effects of these agents [10-13]. In addition, we have recently proposed that delayed onset of inducible resistance mechanisms may result in transient

susceptibility to macrolides [14-16]. The current study has been designed to probe this latter possibility, with particular emphasis on the early effects of clarithromycin on early *erm*(B) gene expression, metabolic activity and growth of an *erm*(B)-expressing strain of the pneumococcus, as well as the effects of sub-minimum inhibitory concentrations of this agent in combination with ceftriaxone on growth.

## 2. Materials and Methods

### 2.1. Bacteria

Although focused primarily on strain 2507, one clinical macrolide-susceptible strain of *Streptococcus pneumoniae* (strain 172) and seven additional macrolide-resistant strains (strains 1791, 1832, 2235, 2506, 3328, 3502 and 4916), were also included in this study. Isolates 172, 2506 and 2507 have been described previously [14]. Isolates were collected as part of an active laboratory-based surveillance system for invasive pneumococcal disease. Isolates were selected from adult cases (>15 years of age) and had been isolated from blood culture specimens.

### 2.2. Phenotypic Characterisation of Bacterial Strains

Bacterial identification was performed using standardised methodologies [14]. MICs were determined by Etest (AB Biodisk, Solna, Sweden). Pneumococci were serotyped by the Quellung method using specific antisera (Statens Serum Institut, Copenhagen, Denmark).

### 2.3. Genotypic Characterisation of Bacterial Strains

Bacterial strains were boiled at 95°C for 10 min in order to extract chromosomal DNA, and screened for the presence of *erm*(B) and *mef*(A/E) by a duplex PCR [19]. The *erm*(B) gene was amplified using forward primer *erm*BF (5'-CTTAGAAGCAAACTTAAGAG-3') and reverse primer *erm*BR (5'-ATCGATACAAATTCCCCGTAG-3'). For each 50 μL reaction, 3 μL of chromosomal DNA was added to a mix containing 2.5 U of *Taq* DNA polymerase, 1x reaction buffer, 1.5 mM MgCl$_2$, 200 μM each of dATP, dCTP, dGTP and dTTP and 800 nM each of forward and reverse primer. Cycling parameters were: 94°C for 2 min; 94°C for 1 min, 52°C for 1 min and 72°C for 3 min for 30 cycles; and 72°C for 5 min. Amplified products were purified with QIAquick PCR purification kit (Qiagen, Surrey, UK) and the *erm*(B) gene sequenced using an Applied Biosystems 3130 Genetic Analyzer. Multilocus Sequence Typing (MLST) was performed as previously described [17]. Sequence types were assigned by submission of the allele sequences to the MLST website (http://spneumoniae.mlst.net).

## 2.4. Antimicrobial Agents and Other Chemicals

Clarithromycin was kindly provided by Abbott Laboratories, North Chicago, IL, USA. Clarithromycin and ceftriaxone were made to a stock solution of 5 g/L in sterile distilled water. The pneumococcal competence pheromone, competence-stimulating peptide-1 (CSP-1), was kindly provided by Dr. D. A. Morrison, Laboratory of Molecular Biology, University of Illinois, Chicago, IL, USA, while all other chemicals and reagents, including bovine liver catalase and ceftriaxone, were purchased from the Sigma Chemical Co, St. Louis, Missouri, USA.

## 2.5. Bacterial Growth Studies

Strains were grown in tryptone soy broth (TSB; Biolab Diagnostics, Johannesburg, South Africa) for 18 hours at 37°C in an atmosphere of 5% CO$_2$, after which the bacteria were pelleted by centrifugation, resuspended in 0.15 M phosphate-buffered saline (PBS, pH 7.4), and adjusted turbidometrically to a concentration of $1 \times 10^7$ colony-forming units (cfu)/mL. For all growth studies, TSB was inoculated with a standard inoculum of $2 \times 10^5$ cfu/mL of each strain and growth monitored for up to 24 hours using a microplate turbidometric method (*Powerwave$_x$* [Bio-Tek Instruments Inc., Winooski, Vermont, USA]), at a wavelength of 540 nm, and colony-counting procedures. The effects of clarithromycin at fixed, final concentrations of 0.5 and 5 mg/L on bacterial growth were investigated using two strategies. Firstly, continuous exposure of each of the eight resistant pneumococcal strains to clarithromycin, added from the outset, and secondly, addition of the antibiotic 6 hours after the initiation of culture. Because of the differences in rates of growth of the different strains, these results are expressed as the time taken for the clarithromycin-free control systems and the corresponding clarithromycin-treated (5 mg/L only, added at the outset) systems, to enter the exponential phase of growth.

Strain 2507 was subjected to pulsed addition of clarithromycin at 6 hourly intervals from time 0 to 24 hours. In addition, this strain was also investigated for the effects of pre-exposure to clarithromycin for 18 hours followed by dilution (1:20,000 approximately) or washing on growth during re-exposure to the antimicrobial agent. CSP-1 (0.1 - 0.5 mg/L) and catalase (500 - 1000 kunits/L) were used to investigate interference with quorum sensing and increased sensitivity to the auto-toxic actions of hydrogen peroxide as potential mechanisms of clarithromycin-mediated transient inhibition of growth of strain 2507.

To investigate possible interactive antimicrobial effects of clarithromycin and ceftriaxone, strain 2507 was treated with a combination of the macrolide (5 mg/L) and *β*-lactam (0.05, 0.1, 0.25 and 0.5 mg/L); clarithromycin

was added from the ouset, followed by ceftriaxone 45 min later, and the bacteria monitored for growth using turbidity measurement (540 nm) and colony-counting procedures after an incubation period of 16 hours at 37°C, 5% $CO_2$.

The effects of sub-MIC concentrations of clarithromycin (0.01 and 0.025 mg/L) on the growth of the susceptible strain, 172, were also investigated.

## 2.6. Measurement of Bacterial ATP

The effects of clarithromycin (0.5 and 5 mg/L) on the energy metabolism of strain 2507 were measured by a chemiluminescence procedure using numerically standardised bacterial suspensions in indicator-free tissue culture medium RPMI 1640 (Highveld Biological (Pty) Ltd., Johannesburg, South Africa). Bacteria cultured for 18 hours in TSB were pelleted by centrifugation, washed once, and resuspended to a concentration of $5 \times 10^7$ cfu/mL in RPMI 1640 without or with clarithromycin and incubated for 1 hour at 37°C, 5% $CO_2$ followed by addition of bovine serum albumin (5 g/L). After further incubation periods of 1 and 5 hours (total incubation periods of 2 and 6 hours respectively), viability was measured using colony counting procedures, while bacterial ATP concentrations were determined using the Bac-Titer-Glo™ Microbial Cell Viability Assay (Promega Corporation, Madison, WI, USA). Briefly, 0.2 mL of a 1:50 dilution of the bacteria were mixed with an equal volume of BacTiter-Glo™ solution (a composite mix consisting of a prokaryotic cell ATP extraction agent and luciferase/luciferin for the detection of ATP) and incubated for 5 min at room temperature followed by measurement of chemiluminescence using a $20/20^n$ chemiluminometer (Turner Biosystems Inc., Sunnyvale, CA, USA). The results are expressed as relative light units (rlu).

## 2.7. Bacterial Protein Synthesis

The effects of clarithromycin (0.5 and 5 mg/L) on protein synthesis by strain 2507 were measured using a radiometric procedure. Bacteria grown for 18 hours in TSB were resuspended to a concentration of $5 \times 10^7$ cfu/mL in RPMI 1640 supplemented with 0.5 mCi/L of a radiolabelled amino acid mixture (L-amino acid mixture $^{14}$C[U], 37 mBq; Du Pont-NEN Products, Boston, MA, USA) and incubated at 37°C, 5% $CO_2$. After 2, 4, 6 and 8 hours of incubation, the bacteria were pelleted by centrifugation and washed, followed by the addition of warm 5% trichloroacetic acid to release bacterial proteins and radioactivity in the lysates was measured by liquid scintillation spectrometry.

## 2.8. Quantification of *erm*(B) Gene Expression

The expression of the *erm*(B) gene was measured for

strain 2507. Three 50 mL aliquots of fresh Brain Heart Infusion broth (Oxoid, UK) taken from the same batch were each inoculated with 500 µL of a freshly thawed glycerol stock of the bacterium (total viable count = $\log_{10}$ 8.392 cfu/mL). These were incubated in a water bath at 37°C and the optical density (OD) monitored until it reached midlog.

At this point, 10 mL of each of the three cultures was removed and centrifuged at $5087 \times$ g at room temperature. The supernatant was decanted and the pellet immediately frozen in liquid nitrogen and stored at –80°C prior to RNA extraction.

The remaining culture was split into paired 18 ml aliquots and clarithromycin was added to a final concentration of 5 mg/L to one of each pair, and both samples reincubated at 37°C. After 15 minutes (when the cultures were still in the logarithmic growth phase), 10 mL of culture from each pair were extracted, centrifuged at room temperature and frozen in liquid nitrogen as above. RNA extraction began by adding 200 µL of fresh lysozyme TE buffer to the frozen pelleted cultures and continued using an RNeasy® Mini kit (Qiagen Ltd., Surrey, UK) according to the manufacturer's instructions, including the use of Qiagen RNase-Free DNase, while the RNA was on the column and a further DNase treatment with Ambion TURBO DNA-free™ (Applied Biosystems, UK) at the conclusion of the extraction. The quality of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, UK) which identified all samples as having an RNA Integrity Number between 9.8 and 10. cDNA was synthesised during an overnight incubation with Superscript III (Invitrogen, UK). cDNA concentrations were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, USA) and the cDNA was then used in a one-step real time (RT)-PCR reaction using SYBR® Green and a Roche Lightcycler® 480 (Roche Applied Science, UK). The primers used to determine *erm*(B) expression levels are forward 5'-GAA-AAGGTACTCAACCAAATA-3' and reverse 3'-AGT-AACGGTACTTAAATTG TTTAC-5' as previously described [18]. Standard curves were constructed, RT-PCR reaction efficiencies were calculated using Microsoft Office Excel 2003 (Microsoft®, UK) and comparison of the mean normalised gene expressions in the presence and absence of 5 mg/L clarithromycin at the same time point was calculated using QGene [19]. The following controls were used: 2 negative controls (nuclease free water and RNA used to synthesize the cDNA-no RT control), and a positive control (TIGR4 cDNA). The TIGR4 control was positive for pneumolysin and *gyr*-A as expected; *gyr*-A was used as a positive control and comparison gene from strain 2507 to determine the level of expression of *erm*(B).

## 2.9. Statistical Analysis

The results of each series of experiments are presented as

the mean values ± standard errors of the means (SEMs). Levels of statistical significance were calculated using the Student's *t*-test (paired *t* statistic). *P*-values < 0.05 were considered statistically significant.

## 3. Results

*Phenotypic and genotypic characterisation*:

Strain 172 (serotype 23F) was macrolide susceptible (clarithromycin MIC 0.064 mg/L [14]), while all of the other eight strains displayed the macrolide-lincosamide-streptogramin B (MLS$_B$) phenotype. The MICs for clarithromycin and erythromycin, as well as the serotypes of the macrolide-resistant strains are shown in **Table 1**.

All macrolide-resistant strains were *erm*(B)-positive and *mef* (A/E)-negative. Sequencing of the *erm*(B) gene of these strains demonstrated that there were no mutations in the leader sequence (control peptide) of the gene. For all strains, *erm*(B) was wild-type when compared with the *erm*(B) gene of the transposon TN1545 (Gen-Bank accession number ×52,632). MLST sequence types (ST) for the resistant strains are shown in **Table 1**.

*Effects of immediate*, *delayed or pulsed exposure to clarithromycin on the growth of pneumococcal strain* 2507:

The effects of exposure of strain 2507 to sub-MIC clarithromycin (0.5 and 5 mg/L) from the outset and at 6 hours after initiation of culture are shown in **Figures 1(a)** and **(b)** respectively. In the absence of clarithromycin, there was an exponential increase in bacterial growth over 9 - 12 hours followed by a levelling off (**Figures 1(a)** and **(b)**). Exposure of the bacteria to clarithromycin from the time of initiation of culture resulted in a lag phase of about 10 - 12 hours, followed by an exponential increase in growth which peaked at 18 hours, reaching values equivalent to those of the antibiotic-free control system

**Table 1. The phenotypic and genotypic characterization of the *erm*(B)-expressing macrolide-resistant strains of *S. pneumoniae***

| *Strain* | *MIC* (mg/L) | *Serotype* | *MLST sequence type (ST)* |
|---------|-------------|-----------|--------------------------|
| 1791 | >256 | 14 | ST4171 |
| 1832 | >256 | 6B | ST4172 |
| 2235 | >256 | 14 | ST1492 |
| 2506 | >256 | 23F | ST81 |
| 2507 | >256 | 23F | ST81 |
| 3328 | >256 | 14 | ST230 |
| 3502 | >256 | 6A | ST4193 |
| 4916 | >256 | 6A | ST2289 |
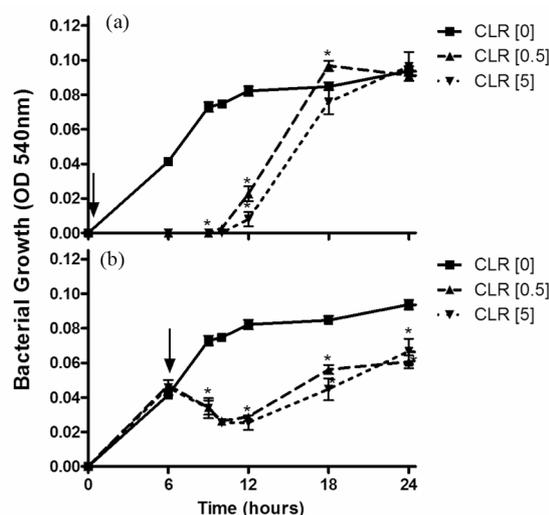


*P* < 0.05 for comparison with the corresponding clarithromycin-free control system.

**Figure 1. Effects of clarithromycin (CLR) at concentrations of 0.5 and 5 mg/L on the growth of the macrolide-resistant strain 2507 of *S. pneumoniae*. Clarithromycin was added at the outset (a) or 6 hours after initiation of growth (b) as indicated by the arrows (↓). The results are presented as the mean values ± SEM of 4 different experiments, with 3 replicates for each system in each experiment.**

(**Figure 1(a)**). Delayed exposure of strain 2507 of the pneumococcus to clarithromycin caused an abrupt suppression of bacterial growth, which persisted for about 6 hours and was followed by recovery of growth (**Figure 1(b)**). In both experimental systems, the inhibitory effects of 0.5 and 5 mg/L clarithromycin were comparable. The effects of pulsed exposure to clarithromycin, at either 0.5 or 5 mg/L, at 6 hourly intervals from time 0 or from 6 hours after initiation of culture, did not differ from those of a single exposure effective from the outset or 6 hours later (results not shown).

Exposure of the macrolide-susceptible strain 172 of the pneumococcus to clarithromycin at either 0.5 or 5 mg/L resulted in complete inhibition of bacterial growth, while exposure to sub-MIC concentrations of 0.01 and 0.025 mg/L resulted in a slowing of growth (data not shown). Exposure of all test strains containing wild-type *erm*(B) genes to clarithromycin at the outset of culture resulted in transient suppression of bacterial growth of up to 10 - 12 hours duration. The effects of clarithromycin (5 mg/L only) on the growth of the *erm*(B)-expressing strains of the pneumococcus are shown in **Table 2**.

*Effects of pre-exposure of strain 2507 of the pneumococcus to clarithromycin on bacterial growth*:

Prior exposure of the bacteria to clarithromycin, for 18 hours followed by washing of the bacteria, attenuated the transient inhibition of growth on re-exposure to the antimicrobial agent. The values for bacterial growth (turbidity) measured 6 hours after initiation of culture for the

**Table 2. The effects of clarithromycin (CLR, 5 mg/L) on the growth of *erm*(B)-expressing, macrolide-resistant strains of the pneumococcus.**

| *Strain*: | *Time to onset of the exponential phase of growth* (hours) | |
|---|---|---|
| | **CLR [0]** | **CLR [5]** |
| 1791 | 1.54 ± 0.247 | 10.25 ± 0.116* |
| 1832 | 4.92 ± 0.651 | 11.54 ± 0.470* |
| 2235 | 3.08 ± 1.214 | 5.14 ± 1.345* |
| 2506 | 2.20 ± 0.839 | 8.23 ± 0.713* |
| 2507 | 1.75 ± 0.075 | 12.17 ± 0.379* |
| 3328 | 2.59 ± 0.748 | 5.15 ± 0.370* |
| 3502 | 4.67 ± 1.752 | 6.24 ± 1.526* |
| 4916 | 1.20 ± 0.330 | 4.85 ± 1.271* |

*$P < 0.05$ for comparison with the corresponding clarithromycin-free system. The results are expressed as mean time ± SEM taken to enter the logarithmic phase of bacterial growth, in the antibiotic-free, and antibiotic (5 mg/L, added at outset)-treated systems, (data from 3 - 4 experiments with 3 replicates for each system in each experiment). In the presence of clarithromycin, all strains showed a significant increase in time to onset of the exponential growth phase. The combined mean ± SEM maximal ODs for all the strains for the clarithromycin-free and clarithromycin-treated systems were 0.258 ± 0.030 and 0.231 ± 0.024 respectively (no significant differences).

untreated control system, and for clarithromycin-treated systems without and with prior 18 hour exposure to the antibiotic (5 mg/L) were 0.02 ± 0.001, 0.003 ± 0.002, and 0.03 ± 0.002 respectively (data from 4 different experiments; $P < 0.05$ for comparison of the control and the pre-exposed systems with the exposed only system).

*Effects of CSP-1 and catalase on clarithromycin-mediated inhibition of the growth of strain 2507 of the pneumococcus*:

Addition of CSP-1 to the culture medium, either from the outset or at 3 and 6 hours after initiation of culture failed to attenuate the inhibitory effects of clarithromycin on bacterial growth (results not shown). Although inclusion of catalase in the growth medium augmented the growth of the pneumococcus, the anti-oxidative enzyme did not affect the suppressive effects of clarithromycin on bacterial growth (results not shown).

*Interactive effects of clarithromycin and ceftriaxone on the growth of pneumococcal strain 2507*:

The effects of clarithromycin (0.5 mg/L) added from the outset, followed 45 min later by the addition of ceftriaxone (0.05, 0.1, 0.25 and 0.5 mg/L, with an MIC of 0.5 mg/L) are shown in **Figure 2**. Exposure of the pneumococcus to clarithromycin alone did not affect growth, while exposure to ceftriaxone (≥0.1 mg/L) alone resulted in a decrease in the growth of strain 2507; however, the combination of exposure to clarithromycin followed by ceftriaxone potentiated the inhibitory effect of ceftriaxone (≥0.1 mg/L) significantly.

*Effects of clarithromycin on microbial ATP levels and protein synthesis*:

Results are shown in **Figure 3**. Exposure of strain 2507 ($5 \times 10^7$ cfu/mL in RPMI 1640) to clarithromycin at 0.5
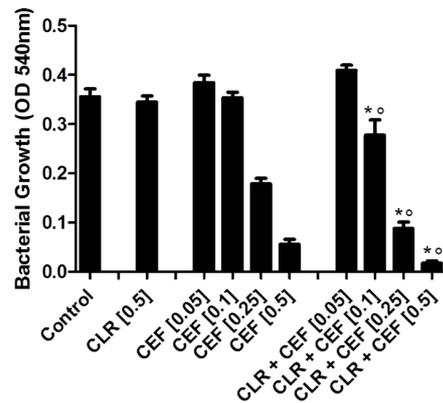


*$P < 0.05$ for comparison with the corresponding clarithromycin (CLR) treated system; °$P < 0.05$ for comparison with the corresponding ceftriaxone (CEF) treated system.

**Figure 2. Effects of clarithromycin (CLR) at a concentration of 0.5 mg/L added from the outset, followed 45 min later by the addition of ceftriaxone (CEF, 0.05, 0.1, 0.25 and 0.5 mg/L) on the growth of the macrolide resistant strain 2507 of *S. pneumoniae* after 16 hours. The results are presented as the mean ± SEM of 5 different experiments with 3 replicates in each system in each experiment.**

and 5 mg/L for 2 and 6 hours resulted in a substantial decrease in microbial ATP levels (**Figure 3(a)**) which was associated with decreased viability (**Figure 3(b)**), and were comparable for both concentrations of antibiotic. The effects on microbial ATP levels were paralleled by significant reductions in protein synthesis (**Figure 3(c)**).

*Erm*(B) *gene expression*:

In the case of strain 2507, expression analysis revealed that the baseline level of *erm*(B) expression was 1.97 ± 0.07, whereas after exposure to clarithromycin (5 mg/L) for 15 minutes it increased to 8.25 ± 2.09 ($P = 0.02$). These results represent the mean ± SEM of three biological replicates.

## 4. Discussion

In the current study, exposure of the macrolide-resistant strain, 2507, of the pneumococcus to clarithromycin (MIC > 256 mg/L), at concentrations of 0.5 and 5 mg/L, resulted in transient inhibition of bacterial growth which persisted for up to 10 - 12 hours, followed by a rebound in growth which reached levels comparable with those of the corresponding antibiotic-free control systems. Similar transient inhibitory effects of the antibiotic on growth were observed using 7 other resistant strain, differing phenotypically by serotype and genotypically by MLST sequence type. All contained wild-type *erm*(B) genes and displayed extended lag phases in the presence of sub-MIC concentrations of macrolides. Addition of the antibiotic during the logarithmic phase resulted in abrupt, transient suppression of bacterial growth followed by recovery, albeit at a slower rate than that of the control system,
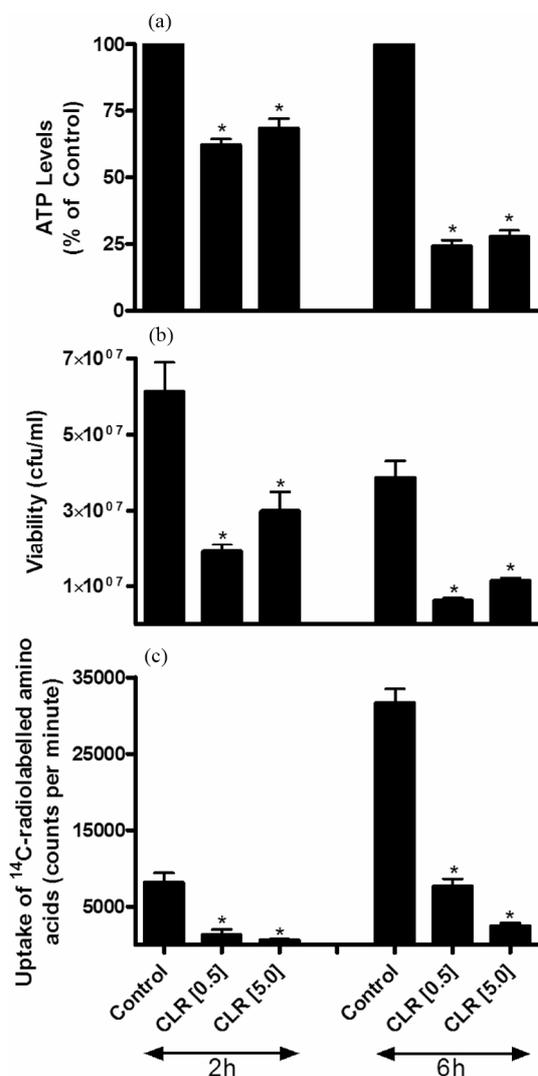
**Figure 3. Effects of exposure (for 2 and 6 hours) of a numerically standardised suspension of strain 2507 of *S. pneumoniae* in tissue culture medium RPMI 1640 to clarithromycin (CLR, 0.5 and 5 mg/L) on bacterial ATP levels (a), viability (b) and protein synthesis (c). The results of 2 (a and b) or 3 (c) different experiments are presented as the mean values ± SEM with 4 ((a) and (b)) or 3 (c) replicates for each system in each experiment.**

possibly due to antibiotic-induced stress and slow recovery of proliferative capacity. Although the results are not included, similar effects were observed with erythromycin in a more limited series of experiments. As displayed in the representative strain 2507, these inhibitory effects were unaffected by the inclusion of catalase in the culture medium, excluding increased sensitivity to hydrogen peroxide, which is produced in low millimolar concentrations by the catalase-negative pneumococcus [20], as a possible mechanism of macrolide-mediated inhibition of bacterial growth. The lack of involvement of hydrogen

peroxide was confirmed in an additional series of experiments (not included) in which we observed that exposure of a pyruvate oxidase gene knockout mutant of a macrolide-resistant variant of pneumococcal strain D39 also resulted in transient inhibition of bacterial growth.

Macrolides, at sub-MIC concentrations, have been reported to suppress quorum-sensing systems in *Pseudomonas aeruginosa* by inhibiting the production of acylated homoserine lactone autoinducers [8]. As the competence system is mediated by protein synthesis, inhibition of CSP production by macrolides would be predicted. We therefore investigated the potential of the pneumococcal autoinducer, CSP-1, to antagonise the inhibitory effects of clarithromycin on the growth of strain 2507. Addition of CSP-1 to the culture medium, either from the outset or during growth, did not, however, attenuate the inhibitory effects of clarithromycin on bacterial growth. Although these observations appear to exclude pneumococcal pheromones as potential selective targets of clarithromycin, we do concede that the use of a single CSP, as opposed to a range of pneumococcal auto-inducers, is a limitation of our experimental design.

The inhibitory effects of clarithromycin on the growth of strain 2507 could be largely overcome, however, by prior overnight exposure of the bacteria to the antibiotic. This is compatible with an inducible mechanism of *erm*(B)-mediated macrolide resistance and a relatively slow acquisition of the resistance phenotype. The existence of such a mechanism is supported by three lines of evidence. Firstly, exposure of strain 2507 to clarithromycin, in RPMI 1640 medium, for 6 hours resulted in a substantial decrease in bacterial energy metabolism, protein synthesis and viability. Secondly, sequencing of the *erm*(B) gene of strain 2507 revealed that the leader sequence of the *erm*(B) gene is wild-type and therefore likely to be inducibly expressed [21,22]. Thirdly, exposure to clarithromycin for 15 min resulted in a four-fold increase in transcription of the *erm*(B) gene. Importantly, clarithromycin inhibits bacterial protein synthesis by binding to the peptide exit tunnel of the large ribosomal subunit and blocking peptide chain elongation [9], while expression of the *erm* gene is predominantly regulated post-transcriptionally [23,24]. Therefore, although there is an in- crease in mRNA shortly after addition of the antibiotic, the production of the methylase enzyme will be delayed, as it is hindered by clarithromycin, hence the long lag phase before acquisition of the complete resistance phenotype. This contention is supported by our earlier observation that transformation of a pneumococcal strain from inducible to predominantly constitutive *erm*(B)-mediated resistance resulted in attenuation of the transient inhibitory effects of erythromycin on bacterial growth [16]. In addition, exposure of *S. pneumoniae* to macrolide antibiotics may also affect global bacterial

gene expression that could affect outcome of infection [25].

The clinical significance of clarithromycin-mediated transient inhibition of the growth of inducible *erm*(B)-expressing macrolide-resistant strains of the pneumococcus remains to be established. Together with other potentially beneficial activities such as high-level tissue penetration and accumulation, and anti-inflammatory properties, these inhibitory effects of macrolides on the growth of macrolideresistant pneumococci may tip the host vs pathogen balance in favour of the former, as has been described in an animal model of experimental infection [4]. Furthermore, it is noteworthy from the current study that combining clarithromycin with ceftriaxone potentiated the activity of the $\beta$-lactam for the macrolideresistant strain of the pneumococcus. The present treatment strategy for patients with severe pneumococcal pneumonia is a combination of a macrolide and a $\beta$-lactam [16].

In conclusion, exposure of *erm*(B)-expressing macrolide-resistant strains of the pneumococcus is associated with decreased metabolic activity and transient inhibition of growth apparently as a consequence of the slow acquisition of the full *erm*(B)-mediated resistance phenotype. We do concede, however, that this phenomenon of delayed acquisition of resistance may be limited to *erm*(B)-expressing pneumococci and of lesser relevance in other bacterial pathogens.

## 5. Acknowledgements

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