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# The effect of levofloxacin concentration on the development and maintenance of antibiotic-resistant clones of *Escherichia coli* in chemostat culture

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**A levofloxacin-sensitive strain of *Escherichia coli* (broth MIC: 0.0625 mg $l^{-1}$) was grown in carbon-limited chemostat culture for 316 h ($D$=0.294 $h^{-1}$). Hyperresistant strains isolated after 58 and 91 generations of culture retained a 16- to 47-fold increase in tolerance to levofloxacin during antibiotic-free serial batch and continuous culture (20 generations, glucose-limited, $D$=0.2 $h^{-1}$). Isolates differed from the original strain in their maximum growth rates in the presence and absence of subinhibitory levels of levofloxacin, protein-banding profiles, and resistance to a range of antibiotics. Competition between resistant isolates and the original sensitive strain was studied in glucose-limited chemostat cultures ($D$=0.2 $h^{-1}$). At levofloxacin concentrations less than 0.03 mg $l^{-1}$, the sensitive strain outcompeted resistant isolates and displaced them from the culture, whereas the reverse was true at higher concentrations. These results have clinical and environmental implications for those administering levofloxacin.**
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**Keywords:** *Escherichia coli*; levofloxacin resistance; chemostat; minimum selection concentration; competition; selection

## Introduction

Levofloxacin is a second-generation fluoroquinolone composed of the optically active L-isomer of ofloxacin. The antibiotic has a twofold greater potency than its progenitor [34] and is active against both Gram-positive and, to a lesser extent, Gram-negative pathogens [26]. The clinical spectrum of use encompasses *Streptococcus pneumoniae* including penicillin-resistant *S. pneumoniae* (PRSP) and pathogens associated with urinary tract infections [1,18]. It is highly potent against clinical strains of *Pseudomonas aeruginosa* [31]. Although complications associated with administration have been reported [21], others have argued that levofloxacin is a relatively safe drug and may have additional analgesic properties, which would make it useful for the treatment of patients with painful infections [11].

Fluoroquinolones directly inhibit DNA synthesis in bacteria. They primarily target DNA gyrase (*gyrA*) in Gram-negative bacteria and topoisomerase IV in Gram-positives, trapping the enzyme/DNA complex during topoisomerisation, resulting in the release of lethal double-stranded DNA breaks [16]. Resistance in *Escherichia coli* largely results from mutations in the DNA *gyrA* gene and increased levels of resistance are believed to arise in a stepwise manner [16]. Secondary mutations in other loci result in a progressive elevated tolerance [6]. Horizontal transfer of resistance is not significant. To date, only one case of plasmid-borne resistance has been documented [25]. Low-level fluoroquinolone resistance is also mediated by a decreased intracellular accumulation of the antibiotic because of the overexpression of active MDR efflux systems, which can augment resistance brought about by mutations in DNA gyrase [6]. Increases in levels of resistance to fluoroquinolones over the past 5 years can be linked directly with patterns of administration [19]. Resistance to levofloxacin is also associated with cross-resistance to other fluoroquinolones [17]. The degree of selection pressure applied influences the type of resistant mutants selected. Zhou *et al* [36] reported that selection at low fluoroquinolone concentrations produced low-level resistant mutants of *Mycobacteria*. With increased selection pressure, a variety of *gyrA* variants with double mutations were selected. Epidemiological factors have been identified, which contribute to elevated levels of resistance in *Campylobacter jejuni*. The emergence of strains resistant to ciprofloxacin (among others) in humans coincided with, or followed the use of, fluoroquinolones in animal husbandry [9]. Increasing levels of fluoroquinolone-resistant *E. coli* in poultry may provide a natural reservoir for resistant organisms to colonise the human population [36].

The minimum inhibitory concentration (MIC) is operationally defined as the lowest concentration of an antibiotic that will kill or inhibit the growth of a microbial population, but this is only one aspect of the effects of antibiotic concentration on population dynamics. Low concentrations of antibiotic may affect competition between resistant and sensitive populations of bacteria. Subbacteriostatic concentrations of tetracycline can enrich for tetracycline-resistant clones in a chemostat population of sensitive and resistant *E. coli* [23]. The growth rates of sensitive clones were reduced when subbacteriostatic concentrations of tetracycline were added to a mixed population of resistant and sensitive *E. coli* [4]. A novel parameter, the minimum effect concentration (MEC), was introduced by Smith *et al* [33] to quantify such phenomena. They defined MEC as the minimum concentration of antibiotic capable of exerting a perceptible effect on the growth rate of sensitive bacteria. MEC was shown to be equivalent to the minimum selection concentration (MSC), which selected for resistant clones in a mixed population of *E. coli* [28].

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Whilst the use of levofloxacin for human therapy is still restricted, the advent of new-generation fluoroquinolones may result in its deregulation and a wider spectrum of application. Inevitably, the incidence of resistant bacteria will increase in both the human and animal populations. It is therefore important to determine the conditions under which resistant clones have a selective advantage and increase in frequency within a population.

Chemostats by their nature promote the evolution of microbial populations towards a climax of complete adaptation to the culture environment. They have been used extensively for the generation of strains with desired characteristics through a process of mutation and periodic selection [35]. The nature of the selection pressure applied can "direct evolution" and enrich for a population with the desired phenotype [12]. The use of agents such as antibiotics, which disadvantage nonresistant strains during chemostat culture, may be used to maximise the proportion of antibiotic-resistant clones [2].

In the present study, levofloxacin selection pressure was applied to a chemostat culture of *E. coli* ATCC 35218. An array of levofloxacin-resistant isolates possessing varying degrees of tolerance to the drug was generated because of mutation and enrichment. Continuous culture was further employed to determine the MSC for these levofloxacin-resistant strains in the presence of subinhibitory concentrations of levofloxacin.

## Materials and methods

### *Microorganisms, media, and antibiotic*

*E. Coli* ATCC 35218 was obtained from the American Type Culture Collection. All other strains were derivatives of *E. coli* 35218, generated by means of selective chemostat culture. Strains were stored at $-20^{\circ}$C in 20% (vol/vol) glycerol. Stocks were retrieved by thawing at room temperature for 10 min and colonies were isolated after 16 h of growth (37°C) on Isosensitest agar (Oxoid, Basingstoke, UK).

M9 minimal medium [24] was supplemented with 4.0 g $l^{-1}$ glucose and 0.5 g $l^{-1}$ casamino acids (Oxoid) for batch culture studies. The glucose concentration was reduced to 400 mg $l^{-1}$ for experiments using chemostat culture. The medium was further supplemented with various concentrations of levofloxacin where indicated. M9 agar was prepared by the addition of 1.1% (wt/vol) agar–agar (Oxoid).

Levofloxacin (as Tavanic®) was obtained from Hoechst Marion Roussel Ireland (Dublin, Ireland) as 100 ml of sterile 5.0 mg $ml^{-1}$ solution for intravenous infusion. Tavanic® also contained the following excipients: $Na^{+}$ ions at 154 mM $l^{-1}$ and HCl (pH 4.8). Levofloxacin was diluted to appropriate working concentrations with sterile deionised water.

### *Maximum specific growth rate ($\mu_{max}$)*

The $\mu_{max}$ determinations were carried out in M9 medium using the method described by Schwartz *et al* [30]. Portions (10 ml in 30-ml specimen bottles) of broth were inoculated with a single colony, and incubated with agitation (120 rpm) for 15 h at 37°C. These served as inocula (10 ml) for 90-ml broths in 500-ml baffled flasks. Levofloxacin was added to inocula and test broths where indicated. Optical densities (540 nm) were monitored over 6 h of growth (120 rpm, 37°C) using matching sterile cuvettes (1 ml, 1.0 cm path width) in a spectrometer (Milton-Roy Spectronic 20D). Lag phases were not observed and $\mu_{max}$ was determined from plots of the logarithm of optical density (OD) against time.

### *Antibiotic susceptibility testing*

MICs of levofloxacin were determined in M9 medium using the broth macrodilution susceptibility method [27] according to the published recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) with some modifications. A starting stock concentration of 10 mg $l^{-1}$ antibiotic (rather than 100 mg $l^{-1}$) for doubling dilution was used in experiments with *E. coli* 35218. This was further diluted with sterile deionised water, yielding assay concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1562, and 0.078 mg $l^{-1}$ levofloxacin. The standard assay procedure was unaltered for derivatives of *E. coli* 35218. MICs were also determined using the agar plate dilution method according to the published recommendations of the National Committee for Clinical Laboratory Standards [27]. M9 agar was employed for this purpose.

### *Enrichment chemostat culture*

The design of the enrichment chemostat apparatus has been described previously [12]. The culture was inoculated with *E. coli* 35218 grown overnight in 90 ml of M9 medium (37°C, 210 rpm). The vessel was brought to its working volume (510±10 ml) by the addition of antibiotic-free medium and allowed to reach steady-state ($OD_{540}$ 0.86, $D$=0.294 $h^{-1}$, 37°C). Glucose limitation was confirmed using the method of Goldberg and Er-El [13]. The culture was grown for a further 7.5 generations (25.5 h) after which the medium feed was supplemented with 0.25 mg $l^{-1}$ levofloxacin. This represented a fourfold increase in levofloxacin concentration relative to the MIC of *E. coli* 35218 (broth MIC, 0.0625 mg $l^{-1}$). Antibiotic concentrations were progressively increased when the OD of the culture exceeded 0.4–0.6 OD units. Changes to 0.5, 4.0, 12.0, 22.5, and 40 mg $l^{-1}$ levofloxacin were made during the course of the culture. The selection chemostat was operated continuously for 93 generations (316 h) when glucose limitation was again assured. Samples (1 ml) were withdrawn at intervals, diluted in phosphate-buffered saline, and plated on M9 medium with and without 1.6 mg $l^{-1}$ levofloxacin. The percentage of the population showing resistance to the drug was determined after incubation of plates for 24 h at 37°C. Further samples (1.5 ml) were removed from the chemostat at approximately seven-generation intervals and stored in glycerol (20%) at $-20^{\circ}$C for further MIC determinations.

### *Isolation and screening for hyperresistant strains*

Hyperresistance was characterised as a 15-fold increase in plate MIC and a 200-fold increase in broth MIC compared with values obtained for *E. coli* 35218. To determine the stability of the hyperresistance characteristic, strains were inoculated into M9 broth (20 ml in 30-ml specimen bottles) and grown for 24 h with shaking (37°C, 120 rpm). Aliquots (1 ml) were transferred to fresh medium and cultured for a further 24 h. The MIC was then determined using the agar plate dilution method. Strains that showed an MIC >25 mg $l^{-1}$ were retained for further study. The stability of the MIC for *E. coli* 35218 was determined in a similar manner.

The MIC stability of original and hyperresistant strains was also determined after chemostat culture without antibiotic. Strains were inoculated into 10 ml of antibiotic-free M9 medium (30-ml

specimen bottles) and cultured overnight on an orbital shaker (37°C, 120 rpm). The contents of the specimen bottles were transferred to preequilibrated 100-ml chemostat vessels containing 90 ml of antibiotic-free M9 medium (400 mg $l^{-1}$ glucose, 37°C). The cultures were allowed to grow as batch cultures until an $OD_{540}$ of 0.4 was obtained. Medium feed was then initiated and the cultures were allowed to reach steady state (20 h, $D$=0.2 $h^{-1}$). Chemostat cultures were grown for 96 h (19.2 generations). Carbon limitation was assured as described previously. Samples (2.5 ml) were withdrawn from the chemostats after 1, 20, 50, and 96 h of steady-state culture. Broth and plate MICs were then determined as described previously.

### *Resistance/sensitivity to antibiotics*

Antibiogram analyses of original and chemostat-derived strains were carried out on M9 agar (37°C, 24 h) using Mastring M26 discs (Mast Diagnostics, Liverpool, UK). The sensitivity/resistance profile was determined for ampicillin (25 $\mu$g), chloramphenicol (50 $\mu$g), colistin sulphate (100 $\mu$g), kanamycin (30 $\mu$g), nalidixic acid (30 $\mu$g), nitrofurantoin (50 $\mu$g), streptomycin (25 $\mu$g), and tetracycline (100 $\mu$g).

### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell extracts*

SDS-PAGE protein profiles for *E. coli* 35218 and its derivatives were determined using 10% polyacrylamide gels in a Mini-Protean III® electrophoresis module (Bio-Rad, Hemel Hempstead, UK). Cultures were grown overnight in Isosensitest broth without antibiotic (20 ml in 30-ml specimen bottles, 37°C). OD (540 nm) was standardised at 0.7 OD units using sterile phosphate-buffered saline. Sample preparation, electrophoresis, and band visualisation were carried out as described previously [22].

### *Competition studies using chemostat culture*

A diagram of the apparatus used for chemostat competition studies is shown in Figure 1. It had a 100±2 ml working volume and consisted of a glass vessel (200×45 mm) to which air (100 ml $min^{-1}$) and medium were supplied through a medium break. Vessels were immersed in a temperature-controlled water bath at 37±0.5°C. Aeration also served as a means of agitation. Medium flow was controlled using a peristaltic pump (Watson-Marlow 505U, Falmouth-Cornwall, UK). Chemostat working volumes were maintained by means of positive pressure displacement through a central overflow tube. Samples were withdrawn using a 5-ml syringe attached to a T-sampling device. Sample lines were cleared after sampling using filter-sterilised air (0.2 $\mu$m pore size; Millipore, Cork, Ireland). Chemostats were operated at a dilution rate of 0.2 $h^{-1}$.

Individual chemostat cultures of strains designated for competition studies were established from overnight cultures grown in antibiotic-free M9 medium. Chemostats were allowed to reach steady state ($D$=0.2 $h^{-1}$, 20 h) and glucose limitation was assured. Mixed cultures were established in a new chemostat vessel using equal volumes (50 ml) of steady-state cultures. The medium feed without or containing subinhibitory concentrations of antibiotic (0.02, 0.03, or 0.04 mg $l^{-1}$) was established immediately after culture mixing. Chemostats were operated at a dilution rate of 0.2 $h^{-1}$ for 4.5 h. Samples (1 ml) were withdrawn at 30-min intervals, diluted in phosphate-buffered saline, and plated on M9 medium with and without 1.6 mg $l^{-1}$ levofloxacin. The relative

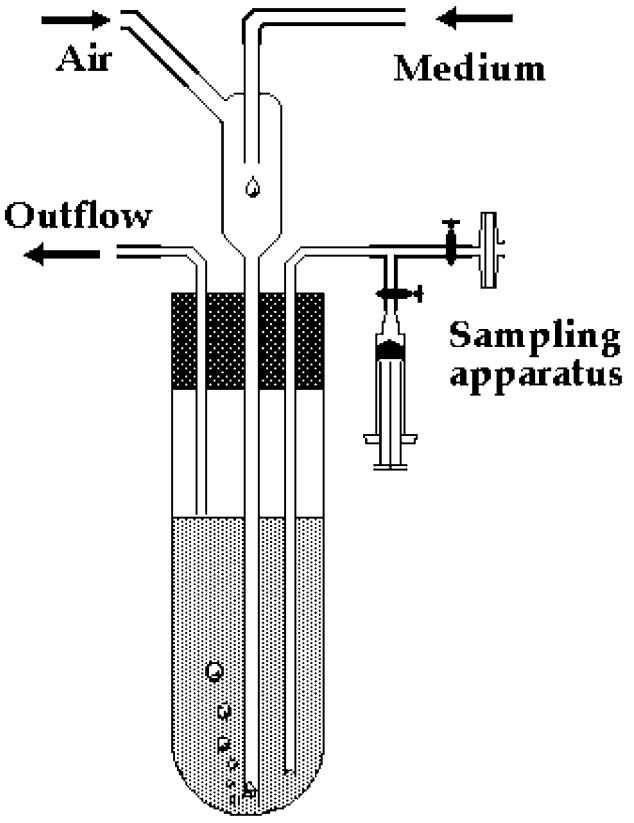


**Figure 1** Schematic diagram of 100-ml chemostat culture vessel used for strain competition studies.

proportions of competing strains were determined after incubation of the plates (37°C, 20 h). A selection coefficient ($S$, $h^{-1}$) was calculated using the equation of Dykhuizen and Hartl [8]:

$$\mathrm{Ln}[X1_{(t)}/X2_{(t)}] = \mathrm{Ln}[X1_{(0)}/X2_{(0)}] - St$$

where $X1_{(t)}$ and $X2_{(t)}$ represent the relative proportion of the two competing strains at time $t$. Selection coefficients were expressed on a selection-per-hour rather than on a per-generation basis since time is continuous and generations overlap in chemostat culture. The strain designated $X1$ is favoured, neutral, or disfavoured relative to the strain designated by $X2$ as $S>0$, $S=0$, or $S<0$. Significance in differences between the median of selection values was determined by Mann–Whitney nonparametric tests (InStat 1990–1998 statistics package).

## Results and discussion

### *Original strain characteristics and enrichment chemostat culture*

The original strain (*E. coli* 35218) had an MIC of 1.6 mg $l^{-1}$ levofloxacin on M9 agar and 0.0625 mg $l^{-1}$ in M9 broth. These parameters established breakpoint concentrations for the detection of hyperresistant clones during the course of selection studies. Medium composition, in particular the presence of relatively high concentrations of divalent cations, can result in the underestimation of MICs for quinolones such as oxalinic acid [32]. The MICs reported here, however, concur with those determined by the NCCLS for *E. coli* 35218, cultured on either Mueller–Hinton agar

or in Mueller–Hinton broth [27]. A degree of robustness apparently exists between the nature of the assay medium and MICs for levofloxacin.

*E. coli* 35218 was grown in a carbon-limited chemostat (400 mg $l^{-1}$ glucose, $D$=0.294 $h^{-1}$) for 316 h with increasing levels of levofloxacin in the medium feed. The evolution of hyperresistant strains was triphasic in response to levofloxacin levels. Medium supplementation with antibiotic levels less than 4 mg $l^{-1}$ failed to enrich for a population predominantly resistant to 1.6 mg $l^{-1}$ levofloxacin on plates (Figure 2). The period prior to 20 generations (68 h) was also characterised by a population MIC akin to that of the original strain (Figure 3). Subsequent increases in levofloxacin concentration at 22 (75 h, 4 mg $l^{-1}$) and 42 generations (143 h, 12 mg $l^{-1}$) resulted in the development of a population that was largely resistant (>90%) to the threshold level of drug. Clones with a plate MIC characteristic some 50- to 100-fold greater than *E. coli* 35218 evolved during this phase. Whilst the medium reservoir was supplemented with 4 mg $l^{-1}$ levofloxacin at 22 generations (75 h), the change in antibiotic concentration in the culture was governed by mixing and overflow so that levels approaching 4 mg $l^{-1}$ would not have been found in the culture until at least a further eight generations had elapsed. Thus, a significant proportion of the population between 22 and 30 generations (*ca.* 25%) was still sensitive to plate levels of 1.6 mg $l^{-1}$ levofloxacin (Figure 2). Medium supplementation with drug concentrations of 22.5 mg $l^{-1}$ (64 generations, 218 h) and 40 mg $l^{-1}$ antibiotic (83 generations, 282 h) maintained a population that exhibited the resistant phenotype. The population MIC (broth) was some 400- to 800-fold greater than that of the original strain during the latter stages of culture (Figure 3). It is known that spontaneous mutations leading to enhanced maintenance of antibiotic resistance by *E. coli* are more likely to occur when the chemostat population is predominantly of the resistant phenotype [15]. We have demonstrated that the selection for hyperresistance to levofloxacin occurred when the chemostat population was resistant to a level of antibiotic greater than the MIC (plate MIC, 1.6 mg $l^{-1}$) of the sensitive strain.

A steady-state OD was not achieved at any stage during culture. OD values typically fluctuated in a saw tooth pattern before 40 generations (136 h) of culture. These changes ranged between 0.6 OD units ($38\times10^6$ CFU $ml^{-1}$) prior to increase in antibiotic concentration to 0.15 OD units ($12\times10^4$ CFU $ml^{-1}$) some three generations thereafter. Fluctuations in OD have been attributed to the dynamics of competition between resistant and sensitive cells in chemostat cultures challenged with antibiotic [3]. They are more commonly associated with the generation and periodic selection of

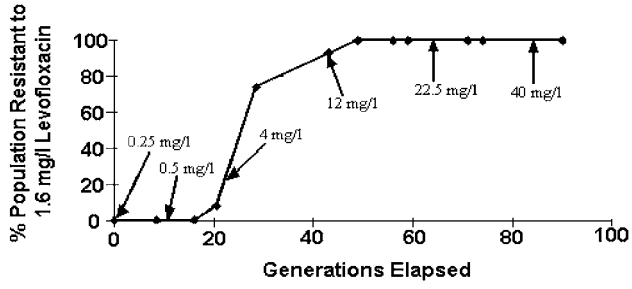


**Figure 2** Resistance to 1.6 mg $l^{-1}$ levofloxacin (plate MIC) in a selection chemostat culture inoculated with *E. coli* 35218. Arrows denote increases in antibiotic concentrations in the medium feed.

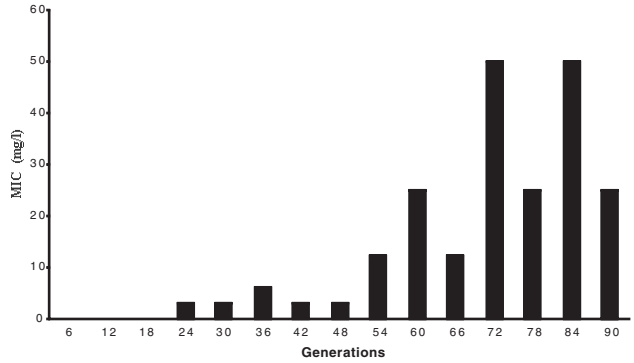


**Figure 3** Broth MIC (mg $l^{-1}$ levofloxacin) characteristics of selection chemostat populations.

mutants in long-term continuous cultures [8,12]. The magnitude of OD perturbations (indicative of mutation and selection) was less pronounced (<0.3 OD units) after 57 chemostat generations (173 h).

The original strain was also grown in chemostat culture without levofloxacin for 20 generations ($D$=0.2 $h^{-1}$, 100 h). Whilst results of this experiment will be discussed later in the context of MIC stability, fluctuations in OD, which were previously attributed to periodic selection events, were remarkably absent from the system. The chemostat maintained a steady-state OD (0.38 OD units) for the duration of the culture. Samples taken from the chemostat after 1, 20, 50, and 96 h had MIC characteristics identical to that of *E. coli* 35218 (broth MIC, 0.0625 mg $l^{-1}$ levofloxacin). If levofloxacin-resistant mutants were generated during the course of chemostat culture without antibiotic, they were competitively disadvantaged and lost from the system.

### *Isolation and characteristics of hyperresistant strains*

Eighteen strains were obtained from the selective chemostat culture. Two strains, from samples taken prior to 15 generations (51 h; Figure 2) possessed a plate MIC characteristic similar to that of the original strain (1.6 mg $l^{-1}$ levofloxacin). Six strains were isolated from samples taken between 20 (68 h) and 56 generations (190 h), which delineated a period of evolving resistance to levofloxacin (phase 2; Figure 2). With the exception of one isolate (MIC 12.5 mg $l^{-1}$), these had plate MICs of 6.25 mg $l^{-1}$. A further 10 strains were isolated between 58 (197 h) and 93 generations (316 h; Figure 2). Six had a plate MIC of 25 mg $l^{-1}$, a 16-fold increase over the original strain. A further four isolates exhibited plate MICs between 50 and 100 mg $l^{-1}$. These MICs represented a 31- to 63-fold enhanced level of resistance to the drug. Strains showing a plate MIC greater than 25 mg $l^{-1}$ were deemed to be hyperresistant.

Isolates L9 (51 h), L14 (96 h), L18 (197 h), and L27 (311 h) were chosen at random as being broadly representative of each grouping. The MIC and growth rate characteristics of these and the original strain are shown in Table 1. Isolates L18 and L27 exhibited plate MICs some 16- and 47-fold greater than *E. coli* 35218. Strain L27, which was isolated after 91 generations, had a broth MIC (25 mg $l^{-1}$) less than the selection pressure applied at the time of isolation (40 mg $l^{-1}$). We observed a similar phenomenon in chemostat cultures of *Bacillus subtilis* where progressively higher feed levels of chloramphenicol were required to maintain selection pressure for plasmid maintenance [12]. This was attributed to enzymatic degradation of antibiotic as a result of expression of the

**Table 1** MIC and maximum growth rate ($\mu_{max}$) characteristics of *E. coli* 35218 and chemostat-derived strains

| Strain | Chemostat selection culture | | MIC | | $\mu_{max}$ ($h^{-1}$) |
|---|---|---|---|---|---|
| | Time of isolation [generations (h)] | Levofloxacin feed concentration ($mg\ l^{-1}$) | Broth ($mg\ l^{-1}$ levofloxacin) | Plate ($mg\ l^{-1}$ levofloxacin) | |
| 35218 (original) | 0 (0) | 0.0 | 0.0625 | 1.6 | 0.66±0.02 |
| L9 | 15 (51) | 0.5 | 0.39 | 1.6 | 0.31±0.01 |
| L14 | 28 (96) | 4.0 | 3.3 | 6.25 | 0.25±0.01 |
| L18 | 58 (197) | 12.5 | 12.5 | 25 | 0.41±0.006 |
| L27 | 91 (311) | 40.0 | 25 | 75 | 0.33±0.02 |

MIC determinations were identical for ≥3 determinations. $\mu_{max}$ values were determined in the absence of antibiotic and are an average of ≥5 determinations±SEM.

plasmid-encoded chloramphenicol acetyltransferase resulting in lower levels in the culture than in the feed. Resistance to levofloxacin, however, cannot be attributed to enzymatic breakdown of the selective agent [7]. *E. coli* is less sensitive to the inhibitory effects of ofloxacin (the progenitor of levofloxacin) at lower maximum specific growth rates [10]. Growth under nutrient-restrictive conditions in the chemostat may have resulted in a similar phenomenon.

Strain characteristics, which are selected in chemostat cultures, may rely on the system for their maintenance [29]. Isolates L18 and L27 retained their broth and plate MIC characteristics after 48 h (six generations) of serial batch culture in the absence of antibiotic, as did the original strain (*E. coli* 35218). Others have demonstrated that hyperresistant ciprofloxacin mutants of *Staphylococcus aureus* generated during the course of batch culture enrichments also maintained their MIC characteristics for 100 generations of serial batch culture in antibiotic-free medium [20]. *E. coli* 35218, L18, and L27 were also grown in chemostat culture ($D=0.2\ h^{-1}$) for 96 h without antibiotic. The MICs (broth and plate) of samples withdrawn from the chemostats after 20, 50, or 96 h were identical to those of samples obtained after 1 h of culture. All strains maintained their initial MIC attributes over 32 days of storage as glycerol stocks at −20°C.

In antibiotic-free cultures, hyperresistant strains had maximum specific growth rates significantly lower than that of *E. coli* 35218 (Table 1). With the exception of L18, isolates grew at half the maximal growth rate of the original strain. It is generally accepted that there is a metabolic burden associated with antibiotic resistance [14]. Table 2 shows the maximum specific growth rates of *E. coli* 35218, L18, and L27 when grown in the absence and presence of levofloxacin. The growth rate of the original strain was retarded by subinhibitory levels of levofloxacin. Drug concentrations as small as 0.02 $mg\ l^{-1}$ (1/3 MIC) elicited a growth rate one-half of that in media without antibiotic. Others have reported that subinhibitory concentrations of tetracycline some 1/10 to 1/40 the MIC of the sensitive strain caused a growth rate reduction in *E. coli* [4,23,28]. The hyperresistant strains, however, had identical growth rates in the absence and presence of drug. It would appear that the

**Table 2** Effect of subinhibitory concentrations of levofloxacin on maximum specific growth rates ($\mu_{max}$) of *E. coli* 35218 and chemostat-derived strains

| Strain | Levofloxacin ($mg\ l^{-1}$) | $\mu_{max}$ ($h^{-1}$) |
|---|---|---|
| 35218 | 0 | 0.66±0.03 |
| | 0.0125 | 0.36±0.04 |
| | 0.02 | 0.33±0.04 |
| | 0.0625 (MIC) | 0.00±0.00 |
| L18 | 0 | 0.41±0.04 |
| | 0.0125 | 0.41±0.05 |
| | 0.02 | 0.41±0.04 |
| | 0.04 | 0.41±0.04 |
| | 12.5 (MIC) | 0.00±0.00 |
| L27 | 0 | 0.33±0.04 |
| | 0.0125 | 0.33±0.02 |
| | 0.02 | 0.33±0.04 |
| | 0.04 | 0.33±0.04 |
| | 25.0 (MIC) | 0.00±0.00 |

MIC denotes broth MIC of isolate (see Table 1). $\mu_{max}$ values are an average ≥4 determinations±SEM.

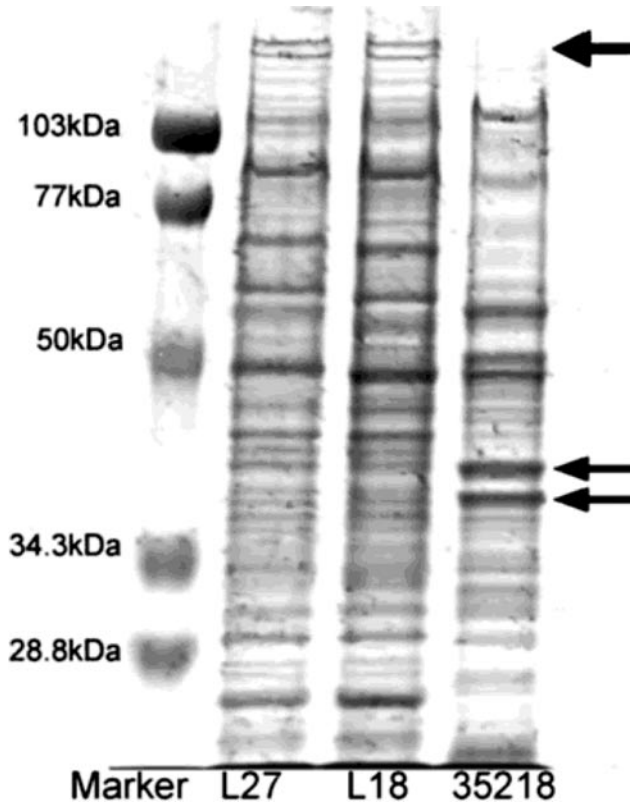


**Figure 4** SDS-PAGE protein profiles of *E. coli* 35218 and chemostat derivatives L18 and L27. Marker denotes molecular weight markers. The protein standards used were: phosphorylase, 103 kDa; bovine serum albumin, 77 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 34.3 kDa; soybean trypsin inhibitor, 28.8 kDa. Arrows indicate bands mentioned in the text.

**Table 3** Antibiogram profiles of *E. coli* 35218 and chemostat-derived strains

| Antibiotic | Ap | C | Co | K | Na | Ni | S | T |
|---|---|---|---|---|---|---|---|---|
| 35218 | R | R | S | S | S | S | R | S |
| L18 | R | S | S | R | S | R | R | R |
| L27 | R | S | S | R | R | R | S | R |

Ap, ampicillin (25 $\mu$g); C, chloramphenicol (50 $\mu$g); Co, colistin sulphate (100 $\mu$g); K, kanamycin (30 $\mu$g); Na, naladixic acid (30 $\mu$g); Ni, nitrofuratonin (50 $\mu$g); S, streptomycin (25 $\mu$g); T, tetracycline (100 $\mu$g). R denotes resistance. S denotes sensitivity.

hyperresistant strains pay an equal metabolic cost regardless of the presence or absence of antibiotic.

Whole cell protein extracts of the original and hyperresistant strains (L18 and L27) were compared using SDS-PAGE. Results are shown in Figure 4. A greater degree of divergence in banding patterns was found between *E. coli* 35218 and chemostat isolates, than between isolates themselves. This is exemplified by the presence of a banding pattern in 35218, which was absent from L18 and L27 (Figure 4, lower arrows), and bands that were absent from the original strain but present in the hyperresistant isolates (Figure 4, upper arrow). Whilst the SDS profiles provided a synopsis of differences between strains, further analyses will need to be carried out to characterise and identify differences in specific proteins.

Strain differences were further characterised by resistance/sensitivity profiles to antibiotics other than that used for selection. Strains L18 and L27 acquired an altered spectrum of resistance to antibiotics as a result of chemostat experience (Table 3). The original strain showed resistance to three (ampicillin, chloramphenicol, and streptomycin) of the eight antibiotics tested. Strains L18 and L27 had become sensitive to chloramphenicol but had acquired a common resistance to kanamycin, nitrofurantoin, and tetracycline. It is well established that resistance to a single fluoroquinolone can also confer a degree of cross-resistance to other antibiotics. Cross-resistance to antibiotics such as tetracycline is associated with reduced OmpF expression and the multiple antibiotic resistance (MAR) phenotype in *E. coli* [5].

Whilst investigations into the nature of mutations leading to hyperresistance were beyond the scope of this study, we propose the following model for the emergence of hyperresistant strains during the course of the selective chemostat culture. Medium feed supplementation with low concentrations of levofloxacin (0–42 generations, 0.25–4 mg $l^{-1}$) resulted in the appearance of a

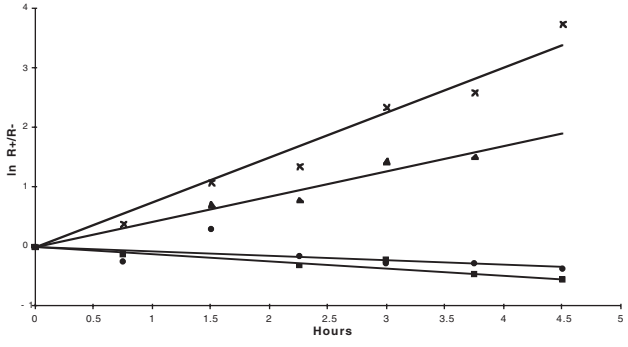


**Figure 5** Competition between *E. coli* 35218 and selection chemostat isolate L18. Competition was carried out in the absence (●) and presence (■) of 0.02, 0.03, and 0.04 mg $l^{-1}$ levofloxacin. Studies were conducted in 100-ml chemostats ($D$=0.2 $h^{-1}$) with glucose-limited M9 medium.

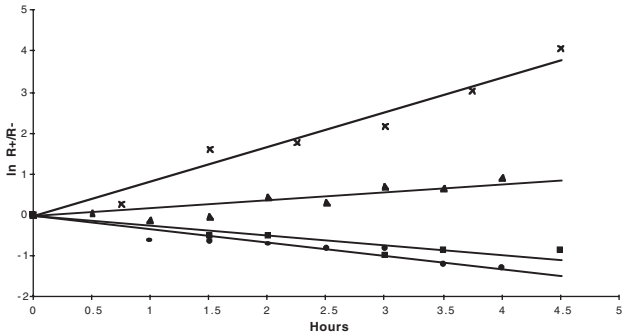


**Figure 6** Competition between *E. coli* 35218 and selection chemostat isolate L27. Competition was carried out in the absence (●) and presence (■) of 0.02, 0.03, and 0.04 mg $l^{-1}$ levofloxacin. Studies were conducted in 100-ml chemostats ($D$=0.2 $h^{-1}$) with glucose-limited M9 medium.

population exhibiting a low to medium resistance phenotype (broth MIC, 3.125–6.25 mg $l^{-1}$). These clones were generated through mutation and selection as evidenced by changes in culture OD and MIC characteristics during the early stages of selective chemostat culture (Figures 2 and 3). With increasing selection pressure and culture experience, the proportion of the population harbouring the hyperresistance characteristic (MIC>12.5 mg $l^{-1}$) increased by virtue of their enhanced growth rates in the presence of the drug (Table 2). The reduced fluctuations in culture OD after 57 generations and similarity between SDS-PAGE (Figure 4) and antibiogram profiles of isolates (Table 3) suggest that the type and nature of mutations leading to increased fluoroquinolone resistance became more stringent with elevated selection pressure. This is consistent with the observations of others [36].

## *Chemostat competition studies and determination of MSC*

Hyperresistant strains L18 and L27 were competed against *E. coli* 35218 in glucose-limited chemostats ($D$=0.2 $h^{-1}$). Competition studies were carried out in the absence and presence of subinhibitory concentrations of levofloxacin. Studies were carried out over a relatively short time (4.5 h). As has already been shown, no significant change in the MIC characteristics of the competing strains should take place over such a short time. Competition between *E. coli* 35218/L18 and 35218/L27 in the absence or presence of 0.02 mg $l^{-1}$ levofloxacin resulted in dominance by the

**Table 4** Selection coefficients ($S$) derived from chemostat competition studies between *E. coli* 35218 and chemostat isolates grown in the absence and presence of subinhibitory concentrations of levofloxacin

| Competing strains | Levofloxacin (mg $l^{-1}$) | $S$ ($h^{-1}$) |
|---|---|---|
| L18 *vs.* 35218 | 0.00 | −0.070 |
| | 0.02 | −0.118 |
| | 0.03 | 0.423 |
| | 0.04 | 0.754 |
| L27 *vs.* 35218 | 0.00 | −0.328 |
| | 0.02 | −0.239 |
| | 0.03 | 0.194 |
| | 0.04 | 0.841 |

Positive $S$ values denote competition in favour of the hyperresistant chemostat derivatives. Values are derived from the slopes of graphs in Figures 5 and 6 as described in Materials and methods.

parent strain (Figures 5 and 6). Though *S* values (Table 4) were significantly different (95% confidence limit) from zero (competitive neutrality), they were not significantly different from each other. The competitive advantage shown by *E. coli* 35218 in the absence of antibiotic is reflected in its growth rate differential to chemostat isolates (Table 1). Hyperresistant strains, however, outgrew the parent strain at drug concentrations equal to or greater than 0.03 mg $l^{-1}$ levofloxacin (Figures 5 and 6). Selection values (Table 4) were significantly different from zero and from each other at the 95% confidence level. The competitive advantage shown by hyperresistant strains can be attributed to the superior growth rates of these clones in media supplemented with subinhibitory concentrations of levofloxacin (Table 2). Since an inflection between negative and positive selection coefficients occurred between 0.02 and 0.03 mg $l^{-1}$ levofloxacin, the actual MSC lies somewhere between these two concentrations. The MIC/MEC ratio we observed for levofloxacin (2.1) is significantly higher (0.1) than those calculated from reports with tetracycline [23,28]. Whilst this infers that relatively greater subinhibitory concentrations of levofloxacin are required to select for hyperresistant clones, these concentrations are significantly less than those used clinically.

According to the reservoir hypothesis, bacteria may acquire antibiotic resistance as a result of exposure to the drug and should show a competitive advantage in its presence. The relative frequency of these resistant bacteria is therefore predicted to increase when the population is challenged with antibiotic [14]. Results presented here show that hyperresistant strains could arise from a sensitive population as a result of antibiotic challenge. In an environment where the antibiotic selection pressure is suspended, susceptible microorganisms are competitively advantaged and return to prominence at the expense of the resistant phenotype. If a reservoir of resistant strains survives, a threshold level of antibiotic (usually defined as MIC of the susceptible organism) will allow them to return to clinically important levels. Results from the present study have shown that antibiotic concentrations significantly less than the MIC of the sensitive strain provided sufficient selection pressure for resistant phenotypes to outgrow the sensitive strain. These results have clinical and environmental implications for those administering levofloxacin.

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