

Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*

Ana Alonso, Ester Campanario and José L. Martínez

Author for correspondence: José L. Martínez. Tel: +34 91 5854539. Fax: +34 91 5854506.
e-mail: jlmtnez@cnb.uam.es

Departamento de
Biotecnología Microbiana,
Centro Nacional de
Biotecnología (CSIC),
Campus UAM, Cantoblanco,
28049 Madrid, Spain

***Pseudomonas aeruginosa* is one of the most important opportunistic pathogens involved in nosocomial infections, cystic fibrosis patients included. Hospital isolates frequently present multidrug-resistance (MDR) phenotypes as the consequence of constant antibiotic selective pressure. The kinetics of emergence of *P. aeruginosa* MDR mutants under antibiotic selective pressure indicated that long-term incubation in the presence of the bacteriostatic antibiotic tetracycline increases the mutation rate per cell per day of *P. aeruginosa* PAO1 by several orders of magnitude. The tetracycline-resistant mutants obtained were stable, showed decreased susceptibility to antibiotics belonging to different structural families, and contained an outer-membrane protein not present in the wild-type *P. aeruginosa* strain PAO1. These data are consistent with the hypothesis that incubation in the presence of tetracycline favours the emergence of MDR mutants in *P. aeruginosa*. The results are relevant for understanding the rapid emergence of antibiotic-resistant mutants among bacterial populations during infections. Their relationship to other models of increased mutagenesis under stress is discussed with respect to the adaptive mutation phenomenon.**

Keywords: antibiotic resistance, mutation rate, adaptive mutation, MDR, *Pseudomonas aeruginosa*

INTRODUCTION

Acquisition and spread of antibiotic resistance among pathogenic bacteria constitute a major threat in modern medicine (Bonhoeffer *et al.*, 1997). The effect of antibiotic usage on natural bacterial populations is the most important 'experiment' on evolutionary biology that can be faced in human (not geological) lifetimes (Shapiro, 1993; Baquero *et al.*, 1997; Baquero & Blazquez, 1997). Emergence of antibiotic-resistant mutants in bacterial populations under antibiotic selective pressure is thus a relevant field of study in evolutionary biology, medical practice and public health.

Antibiotic resistance can be achieved by either acquisition of foreign antibiotic-resistance genes, or mutations in indigenous genes encoding the target or the transport systems of the drugs. The actual concentration of antibiotics inside bacterial cells depends on the

permeability of bacterial envelopes and the presence of efflux pump systems capable of extruding the antibiotic (Nikaido, 1994). The latter systems are ubiquitous among bacteria (Saier *et al.*, 1998) and resemble multidrug-resistance (MDR) determinants involved in mammalian tumour cell resistance to anticancer chemotherapeutic agents (Gottesman *et al.*, 1995).

Both in bacterial and mammalian MDR, the genes for the synthesis of those systems are present in all cells/strains of the individual/species (Saier *et al.*, 1998), but their expression is usually down-regulated, at least under laboratory conditions. However, mutants showing high constitutive expression can emerge and, upon selection, render a population of MDR cells. Due to their ubiquity and their chromosomal localization, MDR mutants are suitable as a model for analysing the factors that regulate emergence of antibiotic-resistant mutants in bacteria. Since bacterial MDR mutants can be selected with bacteriostatic antibiotics, we could study the effect of physiological conditions such as long-term incubation with the selective agent on the emergence of antibiotic-resistant mutants.

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Abbreviation: MDR, multidrug resistance.

We have used *Pseudomonas aeruginosa* as a model organism. This opportunistic pathogen is involved in several infectious diseases (Quinn, 1998), and has a natural low susceptibility to antibiotics used in current medical practice (Quinn, 1998). Its low susceptibility is the consequence of an intrinsic low permeability to antibiotics and of MDR activity (Nikaido, 1994). Three different chromosomal MDR operons [namely *mexA-mexB-oprM* (Poole *et al.*, 1993), *mexC-mexD-oprJ* (Poole *et al.*, 1996), *mexE-mexF-oprN* (Köhler *et al.*, 1997a)] have so far been characterized in this species. Mutants with constitutive expression of these determinants are easily selectable and display a multiresistant phenotype. In the present work, we have analysed the effect of long-term incubation with tetracycline on the emergence of MDR mutants. Our results indicate that mutation rate leading to MDR is favoured by antibiotic challenge in *P. aeruginosa*.

METHODS

Bacterial strains and growth conditions. Strain PAO1 (Ostroff *et al.*, 1989) is a wild-type clinical isolate currently used as a model for virulence studies in *P. aeruginosa*. Cultures were grown in Luria broth (LB) (Atlas, 1993) at 37 °C.

Frequency of emergence of mutants as a function of time. The emergence of tetracycline-resistant mutants was tested in LB-agar plates containing tetracycline at 20 µg ml⁻¹, which is approximately three times higher than the MIC for *P. aeruginosa* PAO1 (6 µg ml⁻¹). Known amounts of exponential-phase *P. aeruginosa* cells were spread on the selection plates. The plates were incubated at 37 °C, and the emergence of new tetracycline-resistant colonies was recorded daily through 5 d.

To estimate the viability of bacteria during incubation under selective conditions, agar plugs (0.6 cm diameter) were removed each day from regions lacking visible colonies. The plugs were crushed in 1 ml sterile PBS, and serial dilutions of the cell suspension were plated on LB-agar to determine the number of viable cells present (Mittler & Lenski, 1990). The values obtained were normalized to the size of the Petri dish (8.5 cm diameter) to determine the total number of viable bacteria on the plate each day.

Mutation rate per cell per day was inferred by applying the function developed and explained in detail by Mittler & Lenski (1990), $u(t) = c(t')/d(t)$, where $u(t)$ is the time-dependent mutation rate, $d(t)$ is the number of *P. aeruginosa* cells at risk of mutation at time t , and $c(t')$ is the rate of appearance of tetracycline-resistant mutants at a later time t' .

Stability of the mutants and reconstruction experiments. To test whether the colonies that emerged were stable tetracycline-resistant mutants, representative clones were picked with a tooth-pick each day and seeded in LB medium without antibiotic. The fully grown cultures were diluted (1 in 1000) in fresh non-selective medium and incubated again. After these two rounds of growth, bacteria had doubled approximately 20 times without antibiotic selective pressure. The resulting bacterial populations were plated on LB-agar with tetracycline (20 µg ml⁻¹) and inspected for bacterial growth after incubation overnight at 37 °C.

To analyse whether the colonies that emerged several days after plating were slow-growing variants or post-plating mutants, reconstruction experiments were performed as

described by Shapiro (1984). Wild-type *P. aeruginosa* PAO1, as well as clones obtained at day 4 after plating, were grown to mid-exponential phase in LB medium at 37 °C. At this stage, 6×10^7 cells from strain PAO1 were independently mixed with approximately 400 cells from each clone, and the mixtures were plated on LB-agar containing tetracycline (20 µg ml⁻¹). As a control, another plate was seeded with 6×10^7 *P. aeruginosa* PAO1 cells. The emergence of colonies was recorded after overnight incubation and 24 h later.

Determination of MICs. The MICs of different antibiotics were determined by the dilution method on Mueller-Hinton agar plates (Atlas, 1993) as recommended by the National Committee for Clinical Laboratory Standards (1997). The replicator prong delivered approximately 10^4 c.f.u. per spot. Sixteen different isolates (15 mutants and the control wild-type) were tested on each plate.

Outer-membrane protein analysis. Outer membranes were prepared by differential solubilization (Fukuoka *et al.*, 1991) of cells grown overnight. Cultures were centrifuged and the pellet was washed and resuspended in 25 mM Tris/HCl (pH 7.2) buffer. Cells were sonicated and the suspension was centrifuged at 2000 g to remove unbroken cells. Membranes were recovered by centrifugation of the supernatant at 110000 g at 4 °C for 50 min, and resuspended in water. Triton X-100 was added to a final concentration of 1.5%, and the suspension was cooled on ice for 30 min. Outer membranes were recovered by centrifugation at 40000 g at 10 °C for 1 h and resuspended in distilled water. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce) using bovine serum albumin as a standard. Samples (10 µg) of outer-membrane proteins were examined by 10% SDS-PAGE (Bio-Rad Protean minigel system and broad range protein molecular mass markers).

RESULTS

To analyse the effect of prolonged incubation under antibiotic selective pressure on the emergence of MDR mutants, *P. aeruginosa* cells from the strain PAO1 were spread on LB-agar containing tetracycline in five Petri dishes (10^7 cells per plate) and the emergence of mutants was followed over 5 d. The number of mutants increased with time (Fig. 1). To control the actual selective potency of the antibiotic through the experiment, LB-tetracycline-agar Petri dishes were pre-incubated at 37 °C, and seeded daily with 10^7 *P. aeruginosa* PAO1 cells. The number of resistant colonies that emerged did not change by pre-incubation of the plates at 37 °C (not shown), indicating that the increase in the number of mutants is not a consequence of antibiotic degradation.

The kinetics of emergence can be explained by post-plating mutation events (McKenzie *et al.*, 1998). Tetracycline-resistant *P. aeruginosa* colonies can grow within 1 d of incubation (see the reconstruction experiments below and Table 1), so only mutants that emerge on the first day after plating (none under the experimental conditions of Fig. 1) would be present in the bacterial population before plating. The other mutants must have originated during incubation on the selection plates. The increase in frequency over time indicates that increased mutation frequencies under stress, i.e. adaptive mutation (Cairns & Foster, 1991; Rosenberg, 1997; Shapiro, 1997), might be involved.

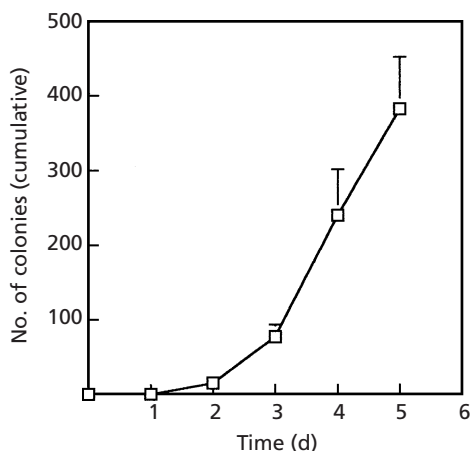


Fig. 1. Kinetics of emergence of MDR mutants from *P. aeruginosa* PAO1. The tetracycline-sensitive *P. aeruginosa* strain PAO1 (10^7 cells) was spread on tetracycline LB-agar in Petri dishes and incubated at 37 °C; the number of colonies was recorded daily. The squares represent the cumulative number of colonies and the bars represent the SEM of the results obtained from five different plates. Bars are not shown where the SEM was below 3.

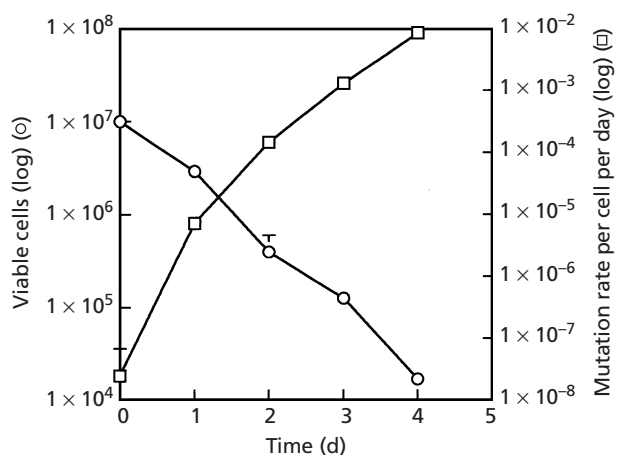


Fig. 2. Viability and mutation rate per cell per day of *P. aeruginosa* on tetracycline selection plates. Agar plugs were removed daily from the plates and the number of viable cells was determined as described in Methods. Mutation rates were calculated as described by Mittler & Lenski (1990). The circles represent the viability, and the squares represent the mutation rates of *P. aeruginosa* PAO1. The bars show the SEM of the results obtained from five different plates. Bars are not shown where the SEM was below 0.1 logarithmic units.

To analyse whether the tetracycline-resistant colonies were stable mutants, four different isolates were picked each day and tested for the maintenance of the tetracycline-resistant phenotype in the absence of selective pressure. In all cases the clones retained the tetracycline-resistant phenotype, indicating that they were stable mutants and not the result of a phenotypical induction of tetracycline resistance.

Although tetracycline is a bacteriostatic antibiotic, some residual growth might occur and the higher number of

mutants after several days could be due to more viable bacterial cells being present on the plate, and not to post-plating mutation events. To test this possibility, plugs of agar were obtained each day from the plates, and the number of viable cells on each plug was determined (Fig. 2). The viability of *P. aeruginosa* PAO1 decreased rapidly under tetracycline selective pressure; only 0.17% of bacterial cells remained viable 4 d after inoculation. Therefore, the increasing number of tetracycline-resistant colonies that emerged each day could not be due to overall growth of the population on the selection plates.

From the number of mutants and their viability, we estimated the mutation rate per viable cell per day following Mittler & Lenski (1990) criteria. The results (Fig. 2) indicated that the mutation rate to tetracycline resistance increased by 10^5 times after 4 d incubation under tetracycline challenge. These results could be explained by either increased mutagenesis under stress or the assumption that late-arising resistant colonies are a jackpot of slow-growing mutants that need several days to grow. These mutants should be present in the bacterial population prior to inoculation onto selection plates, and not the result of mutation events after plating. To test this, we performed reconstruction experiments as described by Shapiro (1984), in which the same number of wild-type cells as plated in the experiment shown in Fig. 1 was mixed with known numbers of late-arising mutants. If the tetracycline-resistant mutants were slow-growing, the bulk of them would need 4 d to produce visible colonies. However, if the mutants arose during incubation on the selection plate, they should be capable of forming colonies within the first day, even in the presence of excess wild-type competitor cells. Five tetracycline-resistant colonies that grew on day 4 were isolated. Approximately 400 cells of each were mixed with 6×10^7 cells of the wild-type strain and the mixtures were spread on selection plates containing tetracycline. A control plate was inoculated with 6×10^7 cells of the wild-type alone. The results (Table 1) indicate that all five mutants gave visible colonies the day after plating, whereas no colonies grew on the control plate. Since these experimental conditions were the same as in Fig. 1, the mutants that arose 4 d after plating were not slow-growing ones, but originated under tetracycline challenge.

Altogether these results indicate that the emergence of tetracycline-resistant mutants increases under antibiotic challenge in *P. aeruginosa*. We have used tetracycline for isolating antibiotic-resistant mutants, because it is bacteriostatic and allows prolonged incubation of bacteria in the presence of the selective agent. Because it is also an antibiotic used for selecting *P. aeruginosa* MDR mutants in the laboratory, the antibiotic susceptibility of mutants that emerged on different days after plating was analysed. Fifteen different mutants obtained from the experiment described in Fig. 1 (three from each plate) were picked each day and their antibiotic-resistance phenotypes were analysed. Since the mutation rate to tetracycline resistance is below 10^{-8} in *P. aeruginosa*

Table 1. Emergence of tetracycline-resistant colonies from clones selected 4 d after plating on tetracycline selection plates

| <i>P. aeruginosa</i> strain | c.f.u. in reconstruction mixture with clones: | | | | | |
|-----------------------------|---|-----------------|-----------------|-----------------|-----------------|-----------------|
| | A | B | C | D | E | – |
| PAO1 | 6×10^7 | 6×10^7 | 6×10^7 | 6×10^7 | 6×10^7 | 6×10^7 |
| Mutant | 512 | 237 | 305 | 342 | 297 | – |
| Time of incubation (h) | Tetracycline-resistant colonies | | | | | |
| 18 | 415 | 201 | 210 | 52 | 282 | 0 |
| 48 | 180 | 12 | 60 | 201 | 35 | 5 |
| Total | 595 | 213 | 270 | 253 | 317 | 5 |

Table 2. MIC ($\mu\text{g ml}^{-1}$) profiles of tetracycline-resistant *P. aeruginosa* mutants obtained at different times after plating on tetracycline selection plates

Eryth, erythromycin; Chlor, chloramphenicol; Norf, norfloxacin; Imip, imipenem; Ceft, ceftazidime; Carb, carbenicillin; Cefp, cefpirome.

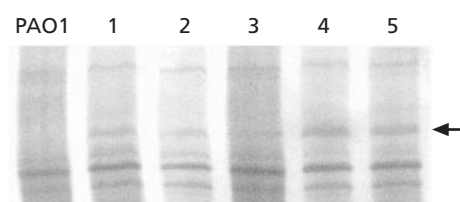
| <i>P. aeruginosa</i> strain | Eryth | Chlor | Norf | Imip | Ceft | Carb | Cefp |
|-----------------------------|---------|----------|------|------|------|---------|------|
| Wild-type | 256 | 64 | 0.5 | 0.5 | 2 | 32 | 2 |
| <i>nalB</i> type* | 256–512 | 256–1024 | 2–4 | 0.5 | 4–8 | 128–256 | 4–16 |
| <i>nfxB</i> type† | 2048 | 128–256 | 16 | 0.5 | 1–2 | 32–64 | 16 |
| <i>nfxC</i> type‡ | 256–512 | 64–128 | 2 | 4.0 | 1–4 | 32–64 | 8 |

* Values from 68 different tetracycline-resistant mutants: 14 from day 1, 15 from each day 2–4 and nine from day 5. No major differences were observed between the MIC values of pre-plating (from day 1) and post-plating mutants.

† Values from two different tetracycline-resistant mutants, one from day 1 and one from day 5.

‡ Values from five different mutants, all of them from day 5.

PAO1, no tetracycline-resistant mutants grew in our experimental conditions 1 d after plating. To obtain such mutants and to compare their phenotype with those of the other mutants obtained at different days after plating, 5×10^9 *P. aeruginosa* PAO1 cells were spread on tetracycline selection plates; mutants that grew after overnight incubation were used as representative of 24 h mutants. Antibiotics were included (Köhler *et al.*, 1997a, b) that could distinguish the three known MDR mutants of *P. aeruginosa*, namely *nalB*, *nfxB* and *nfxC*, which are associated with over-expression of *mexA–mexB–oprM* (Poole *et al.*, 1993), *mexC–mexD–oprJ* (Poole *et al.*, 1996) and *mexE–mexF–oprN* (Köhler *et al.*, 1997a), respectively. The MIC values confirmed that the mutants presented typical MDR phenotypes (Table 2). Sixty-eight out of 75 mutants presented a *nalB* phenotype, two mutants (one from day 1 and another from day 5) had a *nfxB* phenotype, and four mutants (all of them from day 5) had a *nfxC* phenotype. MDR is linked in *P. aeruginosa* to the expression of outer-membrane proteins with molecular masses in the 50–54 kDa range. When the presence of such proteins was tested by PAGE in representative MDR isolates, all of them showed a protein in the predicted molecular mass range not

**Fig. 3.** Outer-membrane proteins of tetracycline-resistant *P. aeruginosa* clones obtained on days 1–5 after plating. The presence of a new outer-membrane protein diagnostic for the expression of MDR systems is indicated with an arrow.

present in the wild-type antibiotic-susceptible strain (Fig. 3).

From these experiments we conclude that antibiotic selective stress can increase the rate of emergence of MDR mutants in *P. aeruginosa*.

DISCUSSION

Selection of mutants during long-term incubation in the presence of the selective agent is the basis of the adaptive mutation phenomenon (Cairns & Foster, 1991).

Although it was previously claimed that the presence of selective agent was necessary, more recent work has shown that selective substrates are not essential for the emergence of mutants during the stationary growth phase, providing strong evidence against directed mutation in those systems (Maenhaut-Michel & Shapiro, 1994; Mittler & Lenski, 1990; Sniegowski, 1995). Nonetheless, increased mutagenesis under stress can be advantageous for bacterial populations, and represents an important departure from assumptions in conventional evolutionary theory about the mutational process.

Adaptive mutation may be involved in the emergence of quinolone-resistant mutants in *Escherichia coli* (Riesenfeld *et al.*, 1997). However, the mutagenicity of quinolones (Clerch *et al.*, 1992) might bias the results. For this reason we used the non-mutagenic antibiotic tetracycline as the selective agent. The test organism, *P. aeruginosa*, frequently has a stressful lifestyle. It exists in environmental habitats, but is also an opportunistic pathogen (Quinn, 1998) with a major role in the final outcome of cystic fibrosis (CF) (Govan & Nelson, 1992). This human genetic disease is caused by mutations in the CF transmembrane conductance regulator (Kerem *et al.*, 1989; Sferra & Collins, 1993). Chronic lung infection, most frequently by *P. aeruginosa*, is the most important cause of morbidity and mortality in CF patients (Govan & Nelson, 1992). Treatment with antibiotics reduces the intensity of the disease, but bacteria gradually acquire resistance to the therapeutic repertoire, and a fatal outcome results. A higher mutation rate in *P. aeruginosa* under stress could account for the emergence of antibiotic-resistant mutants in the lungs of CF patients. This situation probably also applies to other bacteria involved in infectious diseases, particularly for chronic infections, where bacteria grow under prolonged stress.

That the selected mutants were resistant to multiple antibiotics belonging to different structural families makes our analysis more relevant from a clinical point of view. Multidrug efflux pump systems are ubiquitous among bacteria (Saier *et al.*, 1998), and their constitutive expression usually produces only small variations in the MIC values of antibiotics. In the last few years the idea that mutations in MDR systems, together with mutations in other targets of antibiotic action, are the basis of clinically relevant antibiotic-resistance phenotypes has grown. One example of this situation is quinolone resistance. Current knowledge supports the idea that resistance to these synthetic drugs is mainly due to mutations in the genes encoding bacterial topoisomerases (Wiedemann & Heisig, 1994). Nonetheless, mutations in other genes contribute to the final outcome of the quinolone-resistant phenotype (Martínez *et al.*, 1998). Constitutive expression of MDR determinants increases the MIC values of quinolones in *P. aeruginosa*, but MIC values are still too low to be clinically relevant (Köhler *et al.*, 1997b).

However, careful analysis of clinical quinolone-resistant mutants from different bacterial species demonstrates that they carry mutations in both topoisomerase and

MDR genes (Everett *et al.*, 1996; Deguchi *et al.*, 1997). Since the mutation rates in these are 10^{-8} – 10^{-9} , bacteria undergoing mutations in both genes at the same time would emerge with a rate of 10^{-16} – 10^{-18} . This rate of emergence would require a larger number of bacteria than is present in the human body during an infection to generate mutants; thus selection of bacteria carrying both mutations is unlikely. In spite of this, mutations in several genes seem to be the rule rather than the exception in clinical quinolone-resistant mutants (Everett *et al.*, 1996; Deguchi *et al.*, 1997; Kanematsu *et al.*, 1998; Wang *et al.*, 1998). The results that we describe here may account for the apparent paradox. If the mutation rate under stress increases to 10^{-2} – 10^{-3} , bacteria carrying mutations in several different genes could emerge in population sizes attainable during infection. The existence of hyper-mutable subpopulations in non-growing cells has been previously demonstrated. It has also been shown that those subpopulations present a high rate of coincident mutations in different genes of the same cell under stress conditions (Torkelson *et al.*, 1997). Higher mutation under stress could then favour the selection of clinically relevant quinolone-resistant mutants that otherwise would not be present in the population.

Most research on the effect of stress on the emergence of mutants has been performed with model bacteria under controlled laboratory conditions (e.g. Cairns & Foster, 1991; Maenhaut-Michel & Shapiro, 1994; Kasak *et al.*, 1997; McKenzie *et al.*, 1998). However, little work has been done to examine the phenomenon under situations that bacteria usually face *in vivo*. The model which we explore here could be important for understanding the population dynamics of pathogenic bacteria under antibiotic selective pressure.

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