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► **To cite this version:**

A. Martins, C. Iversen, L. Rodrigues, G. Spengler, J. Ramos, et al.. An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators. *International Journal of Antimicrobial Agents*, 2009, 34 (6), pp.602. 10.1016/j.ijantimicag.2009.06.029 . hal-00556355

HAL Id: hal-00556355

<https://hal.science/hal-00556355>

Submitted on 16 Jan 2011

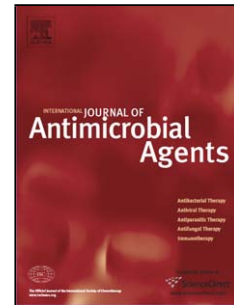
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PII: S0924-8579(09)00354-9
DOI: doi:10.1016/j.ijantimicag.2009.06.029
Reference: ANTAGE 3089

To appear in: *International Journal of Antimicrobial Agents*

Received date: 29-6-2009
Accepted date: 30-6-2009

Please cite this article as: Martins A, Iversen C, Rodrigues L, Spengler G, Ramos J, Kern WV, Couto I, Viveiros M, Fanning S, Pages JM, Amaral L, An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2009.06.029

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An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators

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ARTICLE INFO

Article history:

Received 29 June 2009

Accepted 30 June 2009

Keywords:

Overexpression

Efflux pumps

MDR phenotype

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ABSTRACT

In this study, we aimed to answer the question the following question: ‘How does a bacterium become so resistant to a given antibiotic even though the levels of antibiotic to which it has become resistant remained constant in the patient?’

Escherichia coli AG100 strain induced to high-level resistance due to overexpression of an AcrAB efflux pump was serially cultured in 10 mg/L tetracycline for 60 passages. Between each passage it became increasingly resistant to tetracycline, β -lactams and quinolones with concomitant restoration of wild-type AcrAB activity. Because the multidrug-resistant phenotype could not be reversed with transfer to drug-free medium or with efflux pump inhibitors, it may have resulted from activation of a ‘mutator gene’ system that reduced the ‘energy consumption’ associated with an overexpressed efflux pump system.

1. Introduction

Antibiotic therapy commonly results in the appearance of resistance to the agent in the infecting bacterium [1]. Although a variety of mechanisms account for distinct forms of resistance [2], the mechanism now recognised as playing a major role in the resistance of clinical isolates is overexpression of efflux pumps that extrude the antibiotic before it reaches its target [3]. Because the tripartite efflux pumps of Gram-negative bacteria, for reasons yet to be understood, have the capacity to recognise and extrude a wide variety of unrelated compounds, such as antibiotics from different classes, biocides and other noxious agents like bile salts [4], their overexpression results in a multidrug-resistant (MDR) phenotype that presents difficulties for the therapy of the MDR bacterial infection [5]. The mechanism by which these multidrug resistance efflux pumps are overexpressed has been studied in the laboratory; gradual and prolonged exposure of the bacteria to increasing concentrations of the antibiotic that are just below its minimum inhibitory concentration (MIC) promote the overexpression of individual efflux pumps, which is accompanied by increasing resistance to the antibiotic as well as to other non-related antibiotics [6–8]. Transfer of the now MDR phenotypic bacterium to drug-free medium restores initial susceptibility to the inducing antibiotic over time as well as eliminates its MDR phenotypic status [6,7]. Nevertheless, these studies do not entirely explain how MDR phenotypes develop in a clinical setting, since therapy does not involve progressive increases in dose levels. Furthermore, because the level of resistance of the MDR clinical isolate to a given antibiotic may be hundreds of times greater than that of its wild-

type reference strain, it is difficult to reconcile laboratory studies that induce high-level resistance with continuous exposure to increasing concentrations of an antibiotic with the high-level resistance present in MDR clinical isolates that have never been exposed beyond a level of drug compatible with therapeutic dosage. There must be more to the process by which multidrug resistance develops in a clinical setting. Therefore, in this study we attempted to simulate the relationship between exposure of a Gram-negative bacterium to an antibiotic, in this case *Escherichia coli* to tetracycline, under conditions expected to exist when a patient infected with this organism is treated for a prolonged period of time with a constant dose of the antibiotic.

2. Materials and methods

2.1. Media

Mueller–Hinton (MH) in powder form was used for the preparation of broth and agar. Antibiotic-containing disks (Kirby-Bauer disk diffusion method) were from Difco (Detroit, MI). Antibiotic powders and Phe-Arg- β -naphthylamide (PA β N) were from Sigma-Aldrich Química SA (Madrid, Spain).

2.2. Bacteria

Wild-type *E. coli* K-12 AG100 strain [*argE3 thi-1 rpsL xyl mtl* Δ (*gal-uvrB*) *supE44*] [9] induced to high-level tetracycline resistance (AG100_{TET8} strain) [6,7], when transferred to drug-free medium or to medium containing the antibiotic and PA β N

assumes the tetracycline susceptibility of its parent [6,7]. The AG100_{TET8} strain was exposed to 10 mg/L tetracycline for 60 serial passages in 500 mL of MH broth to produce strain AG100_{TET10}.

2.3. Methods

MICs of antibiotics and PA β N against the strains employed in this study were determined by the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [7].

Antibiotic susceptibility to tetracycline during exposure of *E. coli* to tetracycline was determined by broth dilution [7] and Etest (AB BIODISK, Solna, Sweden) in accordance with the manufacturer's instructions and CLSI guidelines [7].

Assessment of the activity of global regulator genes and genes that code for the AcrB transporter of the tetracycline-exposed strains relative to the unexposed control AG100 parent was conducted by real-time reverse-transcription polymerase chain reaction (rt-RT-PCR) as described previously in detail [6,7].

Phenotype MicroArrayTM plates (Biolog, Hayward, CA) were inoculated with cell suspensions of the wild-type AG100 and its tetracycline-exposed progeny (AG100_{TET8} and AG100_{TET10} strains), according to the manufacturer's instructions. All of the plates were incubated for 48 h at 37 °C and data were recorded using an OmniLog instrument (Biolog). The areas under the metabolic

curves were analysed and an increase of $\geq 20\,000$ tetrazolium dye reduction units in comparison with the wild-type was used to designate a significant increase in resistance.

The 'fitness' of AG100_{TET10} was determined by inoculating 500 mL of MH broth with equal numbers of AG100 and AG100_{TET10} followed by incubation at 37 °C for 16 h. A 0.01 mL aliquot was serially diluted in saline to 10^{-3} dilution and 0.1 mL aliquots of each dilution were plated on MH agar lacking or containing 10 mg/L tetracycline. The plates were incubated at 37 °C for 16 h, colony-forming units were counted for each dilution and the number of bacteria of each strain was extrapolated to the final product of the mixed culture.

3. Results

As shown in Table 1, serial passage of AG100_{TET8} in medium containing 10 mg/L tetracycline resulted in an increase in resistance to the antibiotic [MIC increase from 10 mg/L to >200 mg/L (AG100_{TET10})]. The Gram-negative bacterial efflux pump modulator PA β N (40 mg/L) restored susceptibility to tetracycline up to the 8th passage in 10 mg/L tetracycline. However, after passage number 10, PA β N has no effect on the MIC of tetracycline (data not shown).

Although lack of any effect of PA β N suggests that resistance to tetracycline is due to a non-efflux pump mechanism, we nevertheless used rt-RT-PCR to

assess the activity of the global regulator genes and genes that code for the transporters of eight efflux pumps in AG100_{TET8} and AG100_{TET10} relative to those of the AG100 strain unexposed to tetracycline, as previously described [6,7]. Whereas the activity of the regulators *marA* and *ompX*, the stress genes *rob* and *soxS* and the transporter genes *acrB* and *acrF* of the parental AG100_{TET8} strain were significantly elevated, those of AG100_{TET10} were practically identical to those of the wild-type AG100 strain (Fig. 1), suggesting that resistance in AG100_{TET10} was not mediated by the efflux pumps of that strain.

Evaluation of the AG100_{TET8} and AG100_{TET10} strains for resistance to other antibiotics (e.g. ampicillin, ciprofloxacin, norfloxacin, nalidixic acid, streptomycin, etc.) demonstrated that both strains were resistant to these antibiotics and therefore maintained a MDR phenotype (data not shown). Evaluation of phenotypic resistance by microarray of AG100_{TET8} and AG100_{TET10} relative to that of the wild-type AG100 is summarised in Table 2. Briefly, increased resistance to antibiotics that target the cell envelope, gyrase and the ribosome is shown for AG100_{TET8}; resistance to these antibiotics is even greater for AG100_{TET10}.

The 'fitness' status of AG100_{TET10} compared with that of the wild-type AG100 tetracycline-susceptible strain was evaluated and was shown to be significantly lower; within 1 day of mixed culture of the AG100 and AG100_{TET10} strains, <5% of the bacteria retrieved from this mixed culture were AG100_{TET10}. By the end of the

second serial passage of the contents of the former mixed culture, <0.005% of the cells were AG100_{TET10}.

4. Discussion

This study shows that serial culture of an efflux pump overexpressing *E. coli* strain in medium containing a constant amount of the antibiotic to which the strain had been induced to high-level resistance results in the restoration of the activity of genes that regulate and code for the efflux pump transporters relative to those of the antibiotic-susceptible AG100 wild-type strain. Accompanying this restoration is the continued increase of resistance to the antibiotic even though the bacterium had not been exposed to higher concentrations of the antibiotic to which it had been made resistant. This strain also displayed progressively increased resistance to compounds that target cell envelope constituents, gyrase and ribosomes, supporting the assumption that a large number of mutated targets resulted from continuous subculture in medium containing a constant concentration of an antibiotic. The inability of the AG100_{TET10} strain to revert in drug-free medium to initial susceptibility to tetracycline or to the antibiotics that contributed to its MDR status, in contrast to its AG100_{TET8} parent [6], supports the contention derived from phenotypic array studies that indeed mutations had taken place.

Bacteria obey the second law of thermodynamics, namely conservation of energy. Maintenance of an overexpressed efflux pump system must consume a

large, yet undefined, amount of energy given the dependence on metabolic energy as the main source of protons contributing to the proton-motive force [10]. One may suppose that following exposure of the bacteria to an environment that remains constantly noxious, the genetic system of the organism responds by activation of a mutator system [11] that results in the accumulation of mutations that render the organism MDR. Although as long as the organism remains in that environment its survival is assured, if the environment returns to that initially present the organism cannot compete with its counterpart that has its relevant targets fully functional.

Our study therefore demonstrates 'evolution of a bacterial strain' within a laboratory environment and may parallel what takes place within a patient who is infected with a bacterium and is treated for a prolonged period of time with a constant dose of a given antibiotic, ultimately resulting in increased resistance of the bacterium to that given antibiotic as well as to other non-related antibiotics. However, as of this writing, supposedly due to mutations, overexpressed genes that code for efflux pumps of MDR clinical strains have not been reported to return to wild-type levels of activity. Lastly, this study demonstrates for the first time that an overexpressed efflux pump provides the opportunity by which other mechanisms of resistance may ensue.

Funding: This work was partially supported by grants EU-FSE/FEDER-POCI/SAU-MMO/59370/2004 and EU-FSE/FEDER-PTDC/BIA-MIC/71280/2006

provided by the Fundação para a Ciência e a Tecnologia (FCT) of Portugal. GS, AM and LR were supported by grants SFRH/BPD/34578/2007, SFRH/BD/19445/2004 and SFRH/BD/24931/2005 (FCT, Portugal), respectively.

Competing interests: None declared.

Ethical approval: Not required.

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Fig. 1. Activities of the regulator, stress and transporter genes of *Escherichia coli* strains AG100_{TET8} and AG100_{TET10}. AG100_{TET8} and AG100_{TET10} strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.6, centrifuged, washed with saline and the OD₆₀₀ adjusted to 0.3. Then, 0.5 mL aliquots were used for the extraction of mRNA and the extracts were evaluated for the activities of *acrB*, *acrF*, *ompX*, *rob* and *soxS* genes by real-time reverse-transcription polymerase chain reaction (rt-RT-PCR) as previously described in detail [6]. The activities of *rob* and *soxS* genes of AG100_{TET10} were not determined since the activities of the regulators and transporter genes of this strain were similar to those of the wild-type strain.

Table 1

Effect of serial exposure of *Escherichia coli* AG100_{TET8} strain to 10 mg/L tetracycline on the minimum inhibitory concentration (MIC) of tetracycline ^a

	AG100 _{TET8}		AG100 _{TET10}				
	Passage	Passage	Passage	Passage	Passage	Passage	Passage
	1	10	20	30	40	50	60
MIC	10	64	64/96	96	96/128	128	256

^a AG100_{TET8} strain was serially cultured in Mueller–Hinton (MH) broth containing 10 mg/L tetracycline and at the end of each culture the MIC of tetracycline was determined by Etest. For purposes of clarity, because the AG100_{TET8} strain retains its ability to reverse its resistance to the antibiotic in drug-free medium or with 40 mg/L Phe-Arg-β-naphtylamide, it remains identified as AG100_{TET8} in Table 1. After the tenth serial culture the strain cannot revert its resistance to tetracycline and to the antibiotics that contributed to its multidrug-resistant status, therefore the strain is named AG100_{TET10}.

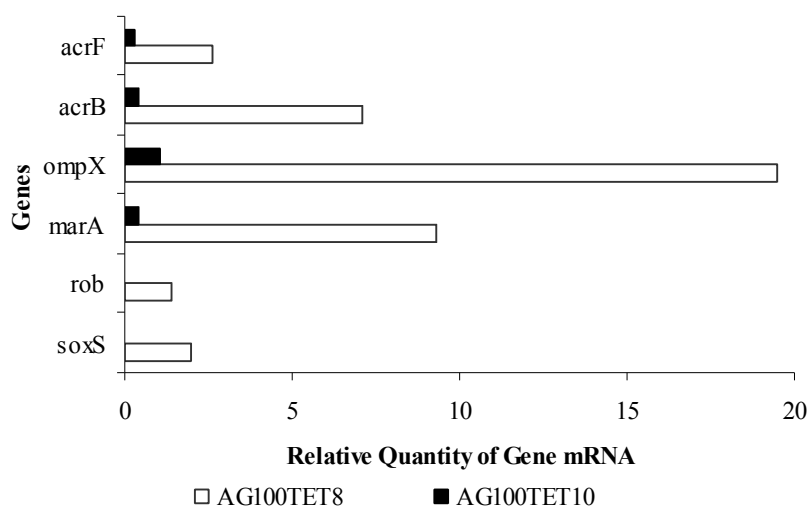
Table 2Phenotypic array evaluation of AG100_{TET8} and AG100_{TET10} strains

Antibiotic agent/target	Increase in TDRU ^a	
	AG100 _{TET8}	AG100 _{TET10}
Fluoroquinolones/DNA unwinding (gyrase and topoisomerase)		
Lomefloxacin	19450	41154
Enoxacin	40488	44797
Ofloxacin	18895	21316
Norfloxacin	20519	21240
Ciprofloxacin	21984	21352
Quinolones/DNA unwinding (gyrase and topoisomerase)		
Nalidixic acid	36125	41336
Oxolinic acid	19749	21048
Cinoxacin	38867	40375
Pipemidic acid	19522	21029
Aminoglycosides/protein synthesis (30S ribosomal subunit)		
Kanamycin	18700	20991
Sisomicin	19532	21523
Tobramycin	21158	22107
Tetracyclines/protein synthesis (30S ribosomal subunit)		
Chlortetracycline	18629	21997
Demeclocycline	41074	45607
Penimepicycline	18013	20270
Rolitetraacycline	38366	40913
Oxytetracycline	20910	20558
Aminoglycoside/protein synthesis		
Geneticin [®] (G418)	20325	21749
Tetracycline/protein synthesis		
Doxycycline	19558	20840
First-generation cephalosporins/cell wall		

Cefazolin	19308	22623
Cefalothin	19623	23278
Second-generation cephalosporin/cell wall		
Cefuroxime	20110	22397
Third-generation cephalosporins/cell wall		
Cefotaxime	58973	61403
Cefoperazone	60936	60582
β -Lactams/cell wall		
Amoxicillin	19914	23548
Cloxacillin	40098	44947
Nafcillin	19322	21372
Oxacillin	37677	41972
Carbenicillin	41948	45618
Aztreonam	20157	20760
Phenethicillin	40768	40166

TDRU, tetrazolium dye reduction units.

^a The increase in the area under the kinetic plot in comparison with the wild-type parent strain AG100 is given; an increase of $\geq 20\ 000$ TDRU is considered significant. The highlighted TDRU are highly significant and suggest high resistance to the corresponding agents.



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