STUDY OF SOME MUTANT *ESCHERICHIA COLI* WITH ANTIBIOTIC RESISTANCE

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Keywords: *Enterobacteriaceae*, chromosomal antibiotic resistance, mutant

Abstract: Some of our experiments dealt with the production in vitro of mutant strains of *Escherichia coli* with increased resistance to various antibiotics. The mutant obtained was compared with the wild multi-antibiotic resistant strains, isolated from various pathological samples, using yet another prototrof strain of Escherichia coli, referred here as **C55**, sensitive to all the antibiotics tested, and which did not displayed the existence of any plasmide.

INTRODUCTION

The introduction of any new antibiotic in the day-to-day medical treatment becomes associated, sooner or later, with occurrences of pathogen microorganisms displaying resistance to that specific antibiotic. The bacterial resistance to the antibiotic precedes its introduction, but the antibiotic use leads to a selection of the resistant strains. The bacterial resistance to antibiotics like nitrofurans, novobiocine, rifampicins or quinolons, it seems not be mediated by plasmides but rather explained through a chromosomal mutation (Courvalin, 1990). Lately, frequent occurrences of *Escherichia coli* strains with resistance to multiple antibiotics, including quinolons, led us to examine, within the means available to us, the mechanism of this resistance in the case when it is not mediated by plasmides.

MATERIAL AND METHOD

Microorganism

Escherichia coli C55 strain, displaying antibiotic sensitivity and not displaying any plasmid, was isolated from urinary tract of a 52 years aged patient.

Culture conditions

In order to obtain mutant strains to quinolons action, the bacteria have been cultivated on two types of Muller-Hinton medium or on a minimal medium, containing sub-inhibiting concentrations of ciprofloxacin (0,02µg/ml) or concentrations four times higher than the MIC (minimal inhibitory concentration) value (0,32µg/ml). After 48 hours of cultivation at 38°C the frequency of resistant colonies was evaluated. In the both types of cultivation (lower or higher concentrations of quinolone), the frequency of occurrence of mutant colonies was similar. From the resistant colonies at ciprofloxacin, appeared on a medium with superinhibitor concentrations of antibiotics, we have selected three (M1, M2, M3) which had been then retested with regards to their sensibility towards other antibiotics (see table 1).

The data referring to outer membrane proteins role in the cellular permeability towards antibiotics (porin role) are contradictory enough. If numerous authors consider that OmpF is the main porin (not specific) in the case of *E. coli* (McEwen and Silverman, 1982; Komatsu et al., 1991; Piddock et al., 1992), lately there are data that even if they do not exclude the role of this protein , they show that other proteins from the outer membrane may have the same role – of a porine- as: OmpD2, OmpE or OmpH1 (Nicas and Kancock, 1980; Nikaido et al., 1991; Hamzehpour et al, 1991a, 1991b). It seems that these porines are specific to certain kinds of antibiotics: for example OmpD2 would be specific to the introduction of the imipenem and OmpH1 to gentamycin and polymyxin.

In the experiments we have made, we have tried to isolate the proteins from the outer membrane from different cultures of *E. coli*, sensitive or resistent at different antibiotics (especeally at quinolons). In order to isolate this protein, we have used an adapted Kropinski et al. method, taking into account their indication that the cultures should be boiled for five minutes in proof buffer which contains SDS, this treatment assuring their better desaggragation.

Because we have started with little quantities of bacterian cultures (50ml), the quantity of prelevated proteins was very poor (under 500mg/ml) thus, after coloring the polyacrilamid gels with Coomasie Brilliant Blue R250, only one or two proteical lines came to view and these very little colored, from analyse of which we couldn' t reach any conclusion. The gels were recolorated with silver which allowed visualisation of a greater number of proteic lines, even if the gel fond got a grey hue.

RESULTS AND DISSCUSIONS

The obtained mutant strains show variation in regards to quinolons resistance, and resistance to unrelated antibiotics (see table 1).

Table 1: Some *E. coli* mutants antibiotic resistance

	MIC (µg/ml)							
E.coli strain	CIP	OFX	NOR	NA	AP	CXM	IPM	GM
C55	0,015	0,3	0,3	2	4	0,125	0,2	2
M1	0,12	12	1,2	64	32	24	0,2	2
M2	1,5	32	16	4	32	12	0,2	2
M3	0,06	0,6	0,6	32	8	0,5	0,2	2

CIP=ciprofloxacin; OFX=ofloxacin; NOR=norfloxacin; NA= nalidixic acid; AP=ampicillin; CXM=cefuroxim; IPM=imipenem; GM=gentamycin

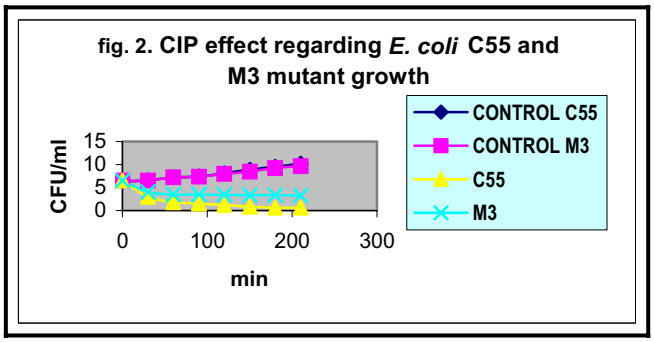
Mutant strain M1 has an increased resistance against all the quinolons tested as well as against beta-lactams, but remains susceptible to imipenem and gentamicyn. The mutant M2 has a 50 to 100 fold increased resistance to ciprofloxacin, ofloxacin and norfloxacin, but surprisingly remains susceptible to the nalidixic acid. M2 is also beta-lactams resistant. Mutant M3 has low quinolone resistance, MIC (Minimal Inhibitory Concentration) for these antibiotics being only 2 or 3 times higher than that of the original strain. It also has a decreased sensitivity to beta-lactams.

The M1 and M2 mutants have been analyzed after that to establish their resistance to multiple antibiotics. In case of M2 strain there was no re-occurrence to the sensitive phenotype after successive sub-cultivations on a medium without antibiotic. M1 strain maintained its sensitive phenotype in proportion higher than 60% of the analyzed colonies, cultivated on a medium without antibiotics. At the same time some bacteria did get back to the sensitive phenotype.

Mutant M3 cultivated on a medium without antibiotics maintained its apparent sensitive phenotype, but at higher quinolone concentrations then the MIC value, displayed an increased tolerance over time, a reduced killing rate becoming evident.

For M3 type mutants, which were manifesting a phenomenon of persistence, as well as for the original strain E. coli C55, we used an inoculum of aproximately 10CFU/ml, which was cultivated for one hour at 370C such that the bacteria could start the logarithmic growth. At that moment (To) was added a concentration of ciprofloxacin 10 times higher than MIC (0,6 µg/ml for M3 and 0.15 µg/ml for *E. coli* C55).

After the addition of the antibiotic CFU number was determinate by placing serial diluted samples on Luria agar plates. Mutant M3 displayed a drastically decrease of cells number in the first 30 minutes. After that we have noticed a long time survive of the bacteria comparing with the origin strain displaying gradual but constant decrease of survival rate (fig. 2) although M3 strain ciprofloxacin MIC is low (0,06µg/ml).



A similar long time "survive" process was noticed in presence of other beta-lactams too (such as ampicyline). Considering Kitano and Tomasz (1979) and Shimming and co. (1984) regarding the obtain and characterization of some mutant *E. coli* strains demonstrating "persistence" (hip mutants) we watch the behavior of M3 strain in presence of high ampicylin concentrations (100 µg/ml) normally deteminating sensitive cells lysis.

M3 strains were placed on ampicylin suplimentated agar plates (100µg/ml), next the original C55 strain, sensitive to all antibiotics, at the level of some ditches practiced in the medium.

After 24 hours of incubation at 37°C spraying the plates with penicillinase solution moved off the antibiotic. The incubation was continued for another 20 hours. After this time we observed a high growth of M3 mutant and a low growth of original strain (only 2-5 colonies/ditch).

Morphological changes produced by some antibiotics over different enterobacterial strains.

It is very well known the effect of some antibiotics, especially beta-lactamic type, over the bacteria morphology. Thus, beta-lactams produce especially lengthening cell phenomena, as well as spheroplasts formation, the effect depending on the possibility of linking each beta-lactamic antibiotic to the linking proteins of the penicillin (PBP).

The bacterial filaments appearing under the antibiotic action are the result of the development, of the multiplication but not separation of the resulted cells, thus these forms contain many genotypes.

In example, the *E. coli* C55 strain, isolated from a patient with urinary infection, sensitive at the majority of the tested antibiotics, cultivated in the presence of cephachlore (reduced concentrations) presents very long filaments (fig. 3), resulted by inhibiting complete septation of the cells after having been divided, probably by linking PBP3 antibiotics (Chase et al., 1981)

In our experiments, we have noticed the fact that, if after 18 hours of incubation in the presence of this antibiotics (4µg/ml) the bacteria are passed on a non antibiotical medium, the cells are coming back to the initial form and dimensions. On the contrary, their maintanance in the presence of the antibiotic for a longer time leads to the cells lysis.

These morphological transformations of the bacterial cells could be due to the linking of the antibiotic (due to the high concentration and to the long time of incubation) both at PBP3 (which determins the elongation of the cells) and to PBP2 (which determins the ovoidal or spherical form of some cells).

Utilisation of augmentine for the tests determins the appearance of the filamentous forms as well as of some thickening or burgeoning aspects at the sensitive strains as it is *E. coli* C55 (fig. 4).

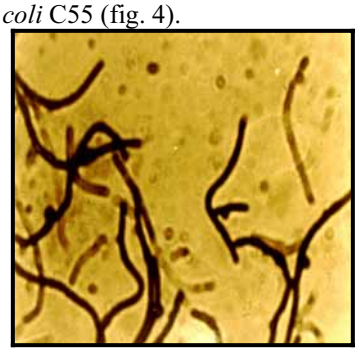


Fig. 3: Lengthening and burgeoning of *Escherichia coli* C55 cells due to cephachlorine action (60x)

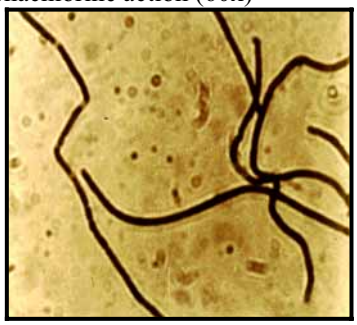


Fig. 4: Lengthening of *Escherichia coli* C55 cells due to augmentine action (60x)

Similar changes were noticed also for unrelated antibiotics as are the quinolons. The effect of quinolons differs in terms of intensity depending on the antibiotic and on the tested strain.

It is to underline the fact that some strains, even sensitive at quinolons, they resist for a long time (they are not killed not even after 24-48 hours of incubation) thus, returned to a medium without antibiotics, bacteria regain their form and normal characteristics. The aspect is of major importance for the medical practice because it explains the persistance of some pathogenic strains after having been treated with an antibiotic towards which the bacteria manifested sensitivity (by examining the antibiogramme). This is due to the fact that too small quantity of antibiotic was administered to the patient, and to the fact that not always MIC for the different isolated bacterial strains was determinated, establishing a rapid selection of some persistent mutant strains.

The effect pretty similar of the beta lactams and quinolons over different bacterial strains, tested before, is due to the inhibition of the cellular division, suggesting either the existance of a commom situs either the superposition (total or partial) of the accessing ways to the bacterial metabolism. It is possible that the filamenting produced by the quinolons to be determined by activating the SOS system or the others which interfere with the formation of the division septum, while the same effect produced by the beta-lactams is determined by joining the PBP3 with the antibiotic. There hasn't been established yet which is the relation among these mechanisms. (Wolfson et al., 1990).

However the less intensity of the morphological changes induced by quinolons could be explained by the fact that the acting mechanism of these antibiotics is more complex, supposing in the first place affecting the DNA gyrase and/or the cell permeability and probably, in a secondary plan, determining changes of the bacterial cell forms.

Outer membrane proteins changes

In order to test the possibility to isolate proteins from the outer membrane we used many *E. coli* mutant strains. For *E. coli* HB101 strain, which shows sensitivity to all the tested antibiotics, we have noticed a number of proteins (fig. 5), among which two major lines with molecular weight of aproximately 38-40 KDa can be easily distinguished, possible corresponding to the OmpA proteins (38KDa) and OmpF (40 KDa) (in conformity to the indications of Komatsu et al., 1991). Together with these lines there can be noticed also other proteic lines, with slower migration, so with a heavier molecular weight of over 70 KDa, as well as other smaller ones of aproximately 20 KDa.

There had been tested some mutant strains : M1, M2 and M3, as well as *E. coli* C1. (fig. 5). The examination of the proteins separated by poliacrylamide gel-electrophoresis shows that M1 strain, which manifests grown resistance to all the tested quinolons as well as to beta-lactams,display a quantitative reduction of one of the major proteins, that of biggest dimension (40KDa), as well as the dissolution of the proteical line of 20 KDa.

A similar aspect shows *E. coli* C1 strain wich display in addition a quantitative reduction of higher weight proteical lines, of over 70 KDa (fig. 5).

We cannot specify the mechanism of the quantitative reduction of 40 KDa protein (maybe OmpF), or the dissolution or the appearance of some of the proteical lines for some mutants. Otherwise not even in the specialised literature there is no uniformity regarding the changes of proteical composition of outer membrane at the mutant strains of *E. coli* antibiotics multiresistant .

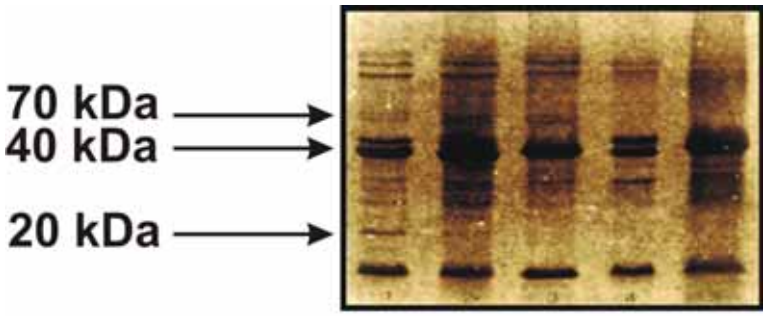


Fig. 5: Outer membrane proteins Poliacrylamide gel-electrophoresis: ***E. coli*** **HB101** (1), ***E. coli*** **C1** (2), ***E. coli*** **M1** (3), ***E. coli*** **M3** (4), ***E. coli*** **M2**(5)

CONCLUSIONS

The most effective quinolonic antibiotic over the *Echerichia coli* strains, isolated from different pathological products, is the ciprofloxacin which has a bactericide action in small concentrations.

There had been isolated many types of *E. coli* mutants manifesting resistance to quinolons, and other antibiotics. Some of them, M3, presented a "persistance" phenomenon.

Both isolation and the electrophoretic characterisation of the outer membrane proteins are useful methods to characterise some antibiotic resistant mutants, showing variations at the level of the proteical composition of the outer membrane, correlated with the grown resistance to the action of some antibiotics.

Studies on modified mutant strains, with grown resistance to quinolons, showed the fact that this resistance is due either to some DNA-gyrase genes mutations, either to some membranerian changes (changes in the proteical composition of the outer membrane), either to the two joint mechanisms.

Our results agree with Moyed and Bertrand (1983) results. They considered this bacteria type due to hip (high persistence) gene mutation (hipA or hipQ).

The mechanism for this phenomenon is not well understood. Nevertheless, bearing in mind that both beta-lactams and quinolons have an inhibiting effect on cellular division, as indicated by the formation in the earlier stages of filamentous cells, then it can be assumed that common action situs exists, or multiple bacteria killing modes are superimposed.

Moreover, it is possible that the persistence phenotype to be similar to the one of tolerance described by Kitano and Tomasz (1979), although Moyed and Bertrand (1983) view them as distinct phenomena. The persistence phenotype may be due to mutations affecting the first stages of the cell wall synthesis, when the ampicillin action is very strong, with further pleiothropic effects over other genes responsible to sensitivity to other antibiotics.

Also the mutation that induces that phenotype may be located at the level of 1between 15 genes identified at *E. coli* being responsible for cell septum and wall synthesis.

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