The role of bacterial hypermutation in biofilm formation and antibiotic resistance in urinary tract infections caused by pathogens of the *Enterobacteriaceae* family

PhD Thesis

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1. Introduction

Eradication of infectious diseases is constantly challenged by micro-organisms that develop new survival strategies. Previous studies suggest that mutational events play a predominant role in bacterial adaptation and confer a selective advantage [1, 2]. Early experiments detecting mutators used mutagenized laboratory strains of bacteria, which coupled with different selection strategies. LeClerc et al reported high mutation frequency among *Escherichia coli* and *Salmonella* pathogens, challenging the theory that mutators were rare among bacterial populations [3]. These findings demonstrated that natural populations could respond to environmental selection in two ways, i.e. by enhanced mutation frequencies and by recombination.

Proteins involved in the DNA mismatch repair pathway (methyl-directed mismatch repair (MMR)) help to replace nucleotides introduced erroneously into the replicated DNA and also hinder recombination between non-identical DNA sequences. Deficiencies in any of the DNA mismatch repair pathway mechanisms can lead to a hypermutator phenotype.

All the mutations present in a given population at a chosen time can be measured by determining the mutation frequency. Usually the mutation frequency of a bacterial population is of the order of 10^6 to 10^7, but numerous studies have described organisms which exhibit permanent mutation frequencies 10-1000-fold or even higher than the median value of their bacterial population, including clinical pathogens and animal sources [4] [5] [6]. These clones are termed as hypermutators. Baquero et al defined 4 categories of *E. coli* strains according to their findings on mutation frequencies (f), and named them as hypomutable ($f \leq 8\text{x}10^{-9}$), normomutable ($8\text{x}10^{-9} < f < 4\text{x}10^{-8}$), weak mutator ($4\text{x}10^{-8} \leq f < 4\text{x}10^{-7}$), and strong mutator ($f \geq$ to $4\text{x}10^{-7}$) [7]. Denamur et al proposed that a strain should be considered a mutator strain when the frequencies of mutations that conferred resistance to rifampicin were 10-fold higher than the median value of

mutagenesis observed for all the studied strains (these were termed '10-fold mutators'). Strains that displayed a >50-fold increase in mutagenesis were considered strong mutators (50-fold mutators) [4]. *E. coli* is far the most frequent pathogen in UTI, followed by other species of the *Enterobacteriaceae* family. The former is investigated in detail, but data about the mutation frequency of the latter group was missing in the literature.

Hypermutators make up about 0.1% of the natural *E. coli* population [3], but this incidence is sometimes higher in clinical strains [4, 7]. Denamur et al studied a population of 603 *E. coli* strains, some commensal (i.e. fecal samples collected from healthy unrelated human subjects) and some pathogenic (from patients with bacteraemia, from patients with UTIs, enteroinvasive or enterohaemorrhagic pathogens, or from newborn meningitis) [4]. They found no significant difference in the frequency of mutators between the two groups. However, mutator strains occurred significantly more frequently among UTI strains. These results were confirmed by Baquero, who found a higher frequency of weak mutators in urinary tract isolates (25 %) and in blood isolates (38 %).

The increasing resistance of the most common uropathogens against the majority of antibiotics used in UTI is a global problem. The mechanism of *Enterobacteriaceae* acquiring resistance has been widely studied and many of the modalities have been described. If mutators have a role in the emergence of antibiotic resistance under natural conditions, such strains are expected to occur in natural populations, including clinical specimens [8]. Although the relationship between hypermutability and the acquisition of antibiotic resistance has been extensively investigated, the evidence of the association among different species remains conflicting except for cystic fibrosis [7, 9]. From the available data, it is still not clear whether high mutation frequencies are particularly important for the global evolution of pathogen populations, including antibiotic resistance acquisition [3, 10-12].

The idea to investigate the association between hypermutation and biofilm forming capacity as well as antibiotic resistance in urinary

tract infections (UTI) was based on several observations. The elevation of antibiotic resistance is a global problem, which affect most of the antibiotics used in UTI. *E. coli* hypermutators more frequently found in UTI than in other origin [4]. Both biofilm formation and acquisition of antibiotic resistance in urinary tract infections have an extensive literature. There is one clinical entity, in which significant relation between hypermutation and antibiotic resistance or between hypermutation and biofilm exists, namely cystic fibrosis (CF) lung infection. In CF the alveoli and bronchioli are blocked by a thick secretion, which is colonized and infected by bacteria. This mucus leads to the formation of bacterial microenvironments known as biofilms in the long run, which protect the pathogen against the penetration of antibiotics making infection even more difficult to eradicate.
Chronic infection, bacterial adaptation, presence of persisters and transient mutators, protection against antibiotics and antibiotic pressure are the phenomena which are both present in CF lung infection and in UTI, making these two entities similar in a certain respect. This gave me the idea to investigate hypermutation, biofilm formation and antibiotic resistance in the context of urinary tract infections.

2. Hypoteses and objectives

2.1 Hypoteses

(1) Clones of the most common uropathogens belong to the *Enterobacteriaceae* family with hypermutable phenotype are more likely to be resistant to antibiotics.
(2) The higher the mutation frequency of the studied population the greater the ability to initiate biofilm formation.

2.2 Objectives

(1) To show the frequency of mutation for the first time in a highly diverse collection of *Enterobacteriaceae* strains collected from urine of UTI patients and from blood stream isolates.
(2) To investigate the capacity of strains to initiate biofilm formation.
(3) To explore the relationship between mutation frequency and biofilm formation.
(4) To determine the antibiotic resistance of the isolates against the most common antibiotics used in UTI.
(5) To show a possible link between hypermutable strains and acquisition of antibiotic resistance.

3. Material and Methods

3.1 Bacterial strains

A total of 4000 clinical strains were screened for *Enterobacteriaceae* species admitted to the Urologic and Nephrologic Department of Pontchaillou University Hospital, in Rennes, France from January to December 2007. Samples with polymicrobial results were excluded.

3.2 Pulse-field gel electrophoresis (PFGE)

Preparation of the cellular DNA for PFGE followed the protocol of Allardet-Servent et al with minor modifications [13]. PFGE patterns were compared by calculating the Dice correlation coefficient with the Gel Compar II software (Applied Maths, St-Martens-Latem, Belgium) and were clustered into a dendrogram using the unweighted pair group matching method (tolerance, 2.0%). Two isolates were considered genetically related if their Dice coefficient was 85% or higher. Solely the unique patterns were considered for the statistical analysis.

3.3 Determination of mutation frequencies

Bacteria from the strain collection were spread onto agar plate (soy tryptase, TS) 18 hours (h), at 37°C to verify the purity of the strain. One isolated colony is incubated in 10ml Luria Bertani (LB) broth for 18h, at 37°C, continuously stirred at 150 rpm. Serial dilutions of the preculture made in sterile distilled water (10ml) 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} and 10^{-8} respectively. Then 100µL of dilutions 10^{0} and 10^{-1} are spread onto agar LB + rifampicin, and 10^{-6}, 10^{-7} and 10^{-8} onto agar LB without rifampicin. The mutation frequency corresponds to the number of bacteria resistant to rifampicin / the total number of bacteria.

The number of colonies was counted and mutation frequencies were estimated. We used two methods for the evaluation of mutation frequencies. We considered the categorization described by Baquero, where a strain determined as normomutable when the mutation frequency (f) was equal or close to the modal point of the distribution of mutation frequencies. The results for the different species were categorized as previously described for *E. coli* strains. If the value is less than or equal to ($N \leq$) $8x10^{-9}$, it means that the bacterium is hypomutable, if the value is $8x10^{-9} < N < 4x10^{-8}$, it means that the bacterium is normomutable, when it is $4x10^{-8} \leq N < 4x10^{-7}$, it is a week mutator, and finally, if it is $N \geq$ to $4x10^{-7}$, it means that the strain is a strong mutator [7]. According to Denamur et al, strains displaying a > 50-fold increase of the median value of mutagenesis were considered strong mutators and a 10- to 50-fold increase as weak mutators [4].

3.4 Biofilm formation

The initiation of biofilm formation was assayed using polystyrene microtiter plates, as described previously, with some modifications [14]. Specifically, one fresh colony of each strain was inoculated into 10 mL tryptic soy (TS) broth and cultured for 2 h. Samples (150 µL) of exponential-growth-phase bacteria were removed and incubated overnight (18 h) at 37 °C in 96- well microtiter polystyrene plates

(Falcon MicrotestTM 96; Becton Dickinson, Meylan, France). After removal of the medium, crystal violet (0.4% solution; 150 µL) was added to the emptied wells to stain the biofilm if present. The biofilm was quantified at least in triplicate for each sample. Streptococcus gordonii (strain Challis/ATCC 35105/CH1/DL1/V288) and bovine Salmonella Heidelberg B182 [6] were used as positive controls. Escherichia coli HB 101 and sterile TS culture broth were used as negative controls. The mean OD570 nm value (ODm) was calculated for three wells. A cut-off value (ODc) was established as three standard deviations above the mean OD570 nm of the three negative controls in each plate. Biofilm production was calculated as the ODm/ODc ratio as recommended by Stepanovic et al [14]. The studied strains were classified into four categories: no biofilm producer (ratio $\leq$ 1), + biofilm producer (1 < ratio $\leq$ 2), ++ biofilm producer (2 < ratio $\leq$ 4) and +++ biofilm producer (ratio > 4). The raw value of the ratio was used for the correlation calculations.

3.5 Susceptibility testing

Susceptibilities of commonly used antibiotics for treatment of urinary tract infections or surgical prophylaxis were determined at admission, using agar diffusion and E tests methods. AmpC overproducers were detected by their characteristic antibiotype, Production of extended spectrum beta-lactamases (ESBL) was detected using double-disk synergy assay and E-tests (ESBL E-test, AB Biodisk).

3.6 Statistical analysis

Continuous data were expressed as mean values ± standard deviation or as median and percentile 25 and 75, and discrete data as percentages. Comparisons between two groups were performed by two-sided Fisher's exact test for count data or the Mantel–Haenszel chi-square test for stratified data or Student's t and chi-square tests, P value of <0.05 was considered as statistically significant. For the

purpose of analysis, “non-susceptible” count included both the intermediate and resistant categories.

4. Results

4.1 Pulse-field gel electrophoresis (PFGE)

Three-hundred sixty-nine clinical strains of *Enterobacteriaceae* were selected from urine samples of 218 patients for further analysis after purity examination and the exclusion of polymicrobial samples. The evaluation of PFGE determined 222 unique clones (isolated from 195 patients).

4.2 Mutation frequencies

The mutation frequency distribution was calculated for the whole collection. According to Baquero’s criteria, 10.3% hypomutable ($f \leq 8x10\text{-}9$), 64.8% normomutable ($8x\ 10\text{-}9 < f < 4x10\text{-}8$), 23% weak mutator ($4x10\text{-}8 < f < 4x10\text{-}7$), and 1.8% strong mutator ($f \geq 4x10\text{-}7$) strains was revealed. One-hundred weak mutators (57 UTI and 43 BSI strains) and 8 strong mutators were isolated [five UTI strains (one C. freundii and four *E. coli*) and 3 BSI strains (two *E. cloacae* and one *E. coli*)]. The distribution pattern of mutation frequencies for the *Enterobacteriaceae* collection without the *E. coli* strains showed close similarity to the distribution of the *E. coli* strains. Mutation frequency calculations using the criteria defined by Denamur et al helped to highlight 10-fold and 50-fold mutator *E. coli* strains: for the whole collection, 4.1% of the UTI and 1.9% of BSI isolates were 10-fold mutators, while 0.9% of the UTI and none of the BSI isolates were 50-fold mutators [4].

4.3 Hypermutation and biofilm formation

Higher biofilm-forming capacity was observed in UTI strains than in BSI strains: 140/222 (63.1%) and 90/213 (42.3%), respectively (Table 3-4.). The results were similar for the *E. coli* subpopulation in UTI strains (87/161; 54.0%) and BSI strains (71/149; 47.7%). The Mantel–Haenszel chi-squared test for stratified data (with *E. coli* in stratum 1, and other *Enterobacteriaceae* in stratum 2) gave a chi-square statistic = 18.4, a P-value < 0.001 and an Odds ratio = 2.26; 95% CI [1.55–3.28]. As expected among the urease producer *Proteus mirabilis strains*, 10/10 (100%) and 0/8 were biofilm producers in UTIs and BSIs, respectively (P < 0.001). Of the *Klebsiella pneumoniae* isolates, which are also urease positive, 14/15 (93.3%) of UTI and 3/13 (23.1%) of BSI isolates were biofilm producers (P < 0.001). No significant difference in biofilm production was demonstrated for *E coli* UTI strains (87/161; 54.0%) vs. BSI strains (71/149; 47.7%). Some biofilm-producing species (i.e. *P. mirabilis*) are particularly isolated from urine during pyelonephritis associated with bacteraemia, compared with digestive translocation or other origin. The great majority of *K. pneumoniae* UTI strains are biofilm producers; however, this is not the case for BSI strains (urinary, pulmonary or digestive origin). In our series, only 35.2% of the BSI strains had a proven urinary tract origin. Higher percentage of urine sample isolates presented in every level of biofilm production. The largest difference between urine and blood strains experienced among the strongest biofilm producers. There was no significant correlation (Pearson's R correlation coefficient and P-values < 0.05 were considered statistically significant) between mutation frequency and the capacity to initiate a biofilm (R = 0.030), irrespective of the source of the strain (urine: R = 0.022; blood: R = 0.057) or the group of species (*E. coli*: R = 0.038; other *Enterobacteriaceae*: R = 0.020).

The highly biofilm-producing strains were normomutators except one weak mutator (*E. coli*), and 6 out of 7 had a urinary origin (three *K. pneumoniae*, two *E. coli*, one *K. oxytoca* and one *S. marcescens*). Most of the *P. mirabilis* (7 out of 10) had a weak mutator phenotype,

and all of them were biofilm producers (one was +biofilm producer, five were ++biofilm producers, and two were +++biofilm producers).

4.4 Susceptibility testing

Resistance rate increased by 21% for amoxicillin, 9% for amoxicillin combined with clavulanic acid, 3.8% for third generation cephalosporins, 15.5% for cotrimoxazole, 8,7% for first-generation quinolones, and 13.6% for norfloxacin, and 9,1% for ciprofloxacin.

4.5 Hypermutation and antibiotic resistance

No significant difference in the distribution of mutator (strong and weak) and non-mutator (normo and hypo) phenotypes was observed between the antibiotic resistant and susceptible isolates for any of the tested antibiotics, neither if we examined the samples as a whole, nor as species by species. The number of strong mutators was too low to demonstrate a possible relationship between antibiotic resistance and acquisition of a strong hypermutator phenotype, although strong mutators were more frequently found in resistant strains, particularly: i) for quinolones, ii) a little for cefotaxime-ceftazidime, and iii) importantly for gentamicin.

4.6 'Highlights' of the clone dendogram

There were patients with multiple isolates up to 12, but they had three PFGE patterns at most. We examined patients with multiple isolates even though they had similar PFGE pattern.
One third of the patients had the same PFGE pattern with the same antibiogram through their whole series of isolates. One patient's strong mutator clone changed to hypomutable after one and a half month, with a 100% similarity of its PFGE pattern. Another clone changed to weak mutator from strong mutator in three months' time. The third sample got hypomutable from strong mutator in one month, than consequently showed hypomutatbility along with the same antibiogram for six months. One isolate became hypomutable from

strong mutator after seven months, with a 97% PFGE similarity. Our last example showed five isolates with the same PFGE pattern during four months, started with weak mutator phenotype which turned to hypomutable, than normomutable followed by a weak mutator phenotype, which altered to hypomutable in the end.

5. Discussion

5.1 Mutation frequencies

The distribution of mutation frequencies for the *E. coli* strains was in close agreement with the distribution reported by Baquero et al, who found a high rate (25%) of weak mutator and 0.7% of strong mutator strains in a collection of 696 *E. coli* isolated from urinary tract infections [7]. Polymorphisms in the rifampin resistance mutation frequency for the whole *Enterobacteriaceae* collection included the blood stream isolates showed similar pattern as well, even in case of excluded *E. coli* species. These results confirm that the mutation frequency classification developed for *E. coli* by Baquero and applied to *Stenotrophomonas* [15] can also be extended to other *Enterobacteriaceae* species.

5.2 Hypermutation and biofilm formation

By definition, hypermutable strains are expected to have higher capacities for adaptation, and some mutations might be linked to an increase in biofilm formation [16]. Numerous studies, especially with P. aeruginosa isolated from respiratory samples in patients with cystic fibrosis, showed a higher proportion of hypermutable strains in biofilms. Several authors have attempted to explain the large amount of hypermutators in a bacterial population known to readily form biofilms. Bacterial biofilm formation can be induced by DNA damaging agents (involved in mutations) triggering the SOS response, through a connection between stress-inducible biofilm formation and the RecA-LexA interplay [17]. Damaging agents have been described,

including silver nanoparticles used for their antibacterial properties [18], oxidative product created by other bacteria present in the biofilm (i.e. *S.gordonii* producing H2O2) [19], or some antibiotics such as fluoroquinolones [20].
To explain the large amount of hypermutators in biofilms, some authors have involved the formation of persister cells, in a quiescent state, rest in the biofilm or inside urothelial cells in the bladder [21] [22]. The appearance of persister cells in the biofilm promotes survival and may be related to the hypermutator phenotype. Persister cells are especially found in late cultures, and therefore in chronic infections. Considering all the above information, we could expect a relationship between the mutation frequency of a collection of clinical *Enterobacteriaceae* strains and their capacity to initiate a biofilm.
Contrary to what could be expected, there was no significant correlation between mutation frequency and the capacity to initiate a biofilm. However, association was realized in a small series. As expected with the urease producer *Proteus mirabilis*, 10/10 (100%) and 0/8 were biofilm producers in UTIs and BSIs, respectively ($P < 0.001$). Nevertheless, 7 out of 10 isolates of *P. mirabilis* from UTIs showed a weak mutator phenotype, all of which were biofilm producers in a different level. Thus, solid conclusions may be obtained for weak mutators, but not for MMR deficient strong mutators ($n = 8$). Moreover, among the few highly biofilm-producing strains, only one weak mutator type (*E. coli*) was detected, while seven were normomutators among other strains (three *K. pneumoniae*, two *E. coli*, one *K. oxytoca* and one *S. marcescens*). These strains were mostly isolated from urine samples (6 out of 7).

5.3 Hypermutation and antibiotic resistance

The relationship between the acquisition of antibiotic resistance and hypermutator phenotype is controversial. Mutation frequencies can vary among studies and often did not correlate well with antibiotic-resistant strains known to have developed mutational resistance [23].
In this study, no significant difference in distribution of mutation frequencies (hypo- and normomutable strains *versus* weak and strong

mutators) was observed between the resistant and susceptible isolates for any of the examined antibiotics, even if the strain was ESBL or AmpC overproducer, or had fluoroquinolone resistance.
However, when statistical analysis compared strong mutators *versus* all the other strains, a statistical difference was observed only with fluoroquinolones. This data correspond to the fact that point mutation in *gyrA* gene can result quinolone resistance. Strong mutators were also found in resistant strains a little more frequently for cefotaxime-ceftazidime. Mutation in AmpR transcription regulator may induce the AmpC β-lactamase [24, 25], which resulted in clones with similar features than ESBL clones, except the fact that they are resistant against β-lactamases. The association of strong mutators and gentamicin resistance is suggested in this study. The number of resistant species was only 12, out of which 30% were mutators. The very low resistance rate is welcomed considering the human population, but not enough to draw a solid conclusion. Another interesting data was that pathogens kept showing low resistance rate against fosfomycin, and we found only one mutator amongst them.

5.4 'Highlights' of the clone dendogram

PFGE allowed to compare the isolates, which let us define the similarity of the bacteria isolated from different patients or from the same host. Multiple urinary tract infections affecting a patient within a year could be caused by reinfection or relapse. The latter case presumes the continuous presence of the pathogen. Chronicity is an issue in the mutagenesis and transient mutator status, which is thought to be involved in the adaptation of bacteria to the environment. Thus we examined all the species belonging to a patient and all the species with the same PFGE pattern. Comparison of mutation frequency changes and biofilm forming capacity, or possible alteration in the antimicrobial susceptibility was determined.
Our results showed examples for mutation frequency changes along with constant PFGE pattern. Variation of shifting included all the possibilities, involving the phenomenon of transient mutator status, which may involve reversion or recombination within the mutator

alleles or depletion of the MMR system proteins, allows the organism to temporarily benefit from the elevated mutation frequency for adaptation while reducing the risk of accumulating deleterious mutations. Antibiograms might alter together with changes of the PFGE pattern or mutation frequency as well, although we could not define any association.

5.5 Critical remarks, limitation of this study

A significant number of patients had recurrent infection, which presumes former antibiotic consumption. Previous antibiotic treatment possibly contributes to selection of mutators in in vitro experiments. Mutators can be favored under such conditions because they generate antibiotic resistance conferring mutations at a higher rate than what is generated by nonmutators. In addition, they also generate more mutations that compensate for the fitness reduction associated with antibiotic resistance [4]. We could not control the prescriptions made by other hospitals or the general practitioners and did not have information about the antibiotic consumption and about the course of healing, which rises several unanswerable questions like what kind of antibiotics did they receive and how long, were these infections due to resistant strains, whether the selected patients got the sufficiently prolonged therapy for that certain infection, was there a therapy failure, is there impairment between therapy failure and the development of resistance. Consequently, the effect of previous antibiotic treatment on our results is inestimable.

6. Conclusions

We were the first to confirm that the mutation frequency classification developed for *E. coli* by Baquero et al and applied to Stenotrophomonas [15] can also be extended to other *Enterobacteriaceae* species isolated from urinary tract infections.

We could not show linear dependence or significant correlation between mutation frequency and the capacity to initiate a biofilm. However, association was realized in a small series of *Proteus mirabilis*, where 7 out of 10 isolates were weak mutators, all of which were biofilm producers. This association may support our basic idea of this research, as cystic fibrosis and certain urinary tract infections are similar in a certain respect. Further investigation with larger *P. mirabilis* sample size might refine this relationship. Not finding a correlation between increased mutation frequency and initiation of biofilms in *Enterobacteriaceae* might be directly linked to the fact that strains were mainly isolated from acute infections, and does not mean that there is no relevant effect of mutation frequency in other stages or aspects of biofilm growth.

We found statistical difference between the antibiotic resistant and susceptible isolates for the tested antibiotics in case of quinolones, when the analysis compared strong mutators *versus* all the other strains, but we found no significant difference in the distribution of mutator (strong and weak) and non-mutator (normo- and hypo-) phenotypes. Although the number of strong mutators was too low to demonstrate a possible relationship between antibiotic resistance and acquisition of a strong mutator phenotype. The same conclusion has to be drawn for gentamicin, although the suggested association is an unexpected finding and would be a promising novelty, the sample size is sparse.

Alteration in mutation frequency and antibiotic resistance in isolates with identical PFGE patterns belonging to the same patients suggests the importance of chronicity and transient mutator status. Further investigation of these features might help to better understand the mechanisms of bacterial adaptation.

7. List of publications concerning the subject

I. **Kovacs B**, Le Gall-David S, Vincent P, Le Bars H, Buffet-Bataillon S, Bonnaure-Mallet M, Jolivet-Gougeon A.
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3. Tenke P, Ludwig E, Köves B, Bálint P, **Kovács B**, Hagymási N
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