Mutator phenotype confers advantage in *Escherichia coli* chronic urinary tract infection pathogenesis

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Abstract

It has been suggested that mutator phenotype could be associated with an increase in virulence, but to date experimental evidences are lacking. Epidemiological studies have revealed that urinary tract infection isolates encompass the highest proportion of mutator strains within the *Escherichia coli* species. Using the uropathogenic strain CFT073 and its *mutS/C0* mutator mutant, we show that the mutator strain is selected in vitro in urine and in the late stages of infection in a mouse model having urinary tract infection. Thus, we report that, under specific conditions, i.e., urinary tract infection, the mutator phenotype may confer an advantage in pathogenesis.

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1. Introduction

Constitutive mutator strains, i.e., strains exhibiting constantly elevated mutation frequencies, represent a few percentage of natural isolates in several bacterial species such as *Escherichia coli* and *Salmonella* [1,2], *Pseudomonas aeruginosa* [3], *Helicobacter pylori* [4], *Haemophilus influenzae* [5,6], *Neisseria meningitidis* [7], *Streptococcus pneumoniae* [8] and *Staphylococcus aureus* [9]. This high frequency of mutators suggests the existence of condition selecting for higher mutation rate in nature [10]. In vitro and in silico studies showed that, even if they produce predominantly deleterious mutations, mutators can be selected in adapting populations due to their favourable (adaptive) alleles that they generate (second-order selection) [10]. An important issue is to understand what kind of environmental conditions requiring new adaptation helps the mutator to reach a high frequency in nature. It is clear that antibiotic pressure may be one of such conditions that favor mutator phenotype. *P. aeruginosa* [3], *Haemophilus influenzae* [5,6] and *S. aureus* [9] mutator strains isolated in cystic fibrosis patients receiving numerous antibiotic cures are more resistant than non-mutator strains. However, this association is not retrieved in *H. pylori* [4] and *S. pneumoniae* [8] strains, indicating that other conditions are involved. It has been suggested that infection could be one of them [11] as increase genetic variability can help to cope with the immune system of the host by increasing the antigen variation or adapting to stressful environments, such as pus, blood or urine. Indeed, *N. meningitidis* mutator strains displayed high phase...
variation rates [7] and a study of mutation rates in a large collection of commensal and pathogenic E. coli strains showed that uropathogenic strains had the highest frequency of mutators [12].

To test the hypothesis that E. coli mutator strains can be involved in urinary tract infection (UTI) pathogenesis, we constructed a mismatch repair mutant of the uropathogenic strain CFT073 and compared its fitness with the wild-type strain in urine and in a UTI mouse model.

2. Materials and methods

2.1. Bacterial strains

The fully sequenced pyelonephritis strain CFT073 [13] was used to construct a mutS strain. mutS belongs to the mismatch repair system and is the most frequently inactivated gene leading to the mutator phenotype in natural isolates, mainly by large deletions [1, personal data]. The method of Datsenko and Wanner [14] was used to replace a 728 bp mutS region (between nucleotides 1456 and 2184), coding for the C-terminal conserved domain involved in nucleotide/magnesium binding, by a chloramphenicol acetyl transferase gene of 1.1 kilobases. The chloramphenicol resistance cassette insertion within the mutant strain (CFT073 mutS::CmR) was checked by PCR using deletion external primers. No significant differences of the plating efficacy of the CFT073 mutator strain were observed with or without chloramphenicol. The resulting mutator phenotype of the mutant strain CFT073 mutS::CmR was revealed by a 100-fold increase in the frequency of mutations conferring rifampicin resistance (data not shown).

2.2. In vitro growth experiments

Bacterial cells were grown at 37 °C with agitation in 23 ml-glass tubes containing 10 ml of Luria-Bertani (LB) broth or urine. The urine was collected from several healthy donors without any history of UTI or medical treatment for 3 months, pooled, filtered (0.22 μm filter) and stored at −20 °C before use.

In competition experiments, co-isogenic mutator and wild-type strains were grown from single colonies in LB broth to stationary phase. Then they were mixed in the appropriate ratios and densities, inoculated into LB broth or urine, and grown as above. After an overnight culture, a serial transfer regime was performed in which populations were diluted (1:1000) once a day, 6 days a week, into 10 ml of fresh media. The 1:1000 dilution and regrowth to stationary phase permits 10 (log2 1000) cell generations per day. Cells were counted by plating appropriate dilutions on LB medium with and without chloramphenicol (10 mg l−1) once a week.

All experiments were repeated at least 3 times.

2.3. UTI mouse model

We used the ascending, unobstructed UTI mouse model developed by Hagberg et al. [15]. Briefly, 8-week-old female CBA mice were infected into the bladder, with an inoculum of 0.05 ml-containing 10⁹ bacteria, through an urethral catheter immediately removed after the inoculation. Animals were sacrificed at different times after inoculation and spleen, kidneys and bladder were aseptically taken out. Half of each organ was homogenized, and bacterial cultures were performed. Cells were counted as above and expressed as CFU/g of tissue. The other half of the organs was used for histological examination. Paraffin-embedded sections were stained with hematoxylin and eosin and examined using light microscopy. The degree of inflammation was graded by the same pathologist (M.P.), blinded to the infecting organism, using the criteria of Hopkins et al. [16].

3. Results and discussion

3.1. In vitro growth experiments

Firstly, we looked at the growth rate of individual (wild-type and mutant) strains to see if important differences in fitness could exist between strains. We studied such growth rates in a rich synthetic medium, LB broth as a control, and in human urine. Growth rate in urine was shown to be an important factor for uropathogenic strains [17]. Bacterial cells from an overnight culture were inoculated at an initial quantity of 10⁴–10⁵ cells ml⁻¹, grown at 37 °C with agitation in LB broth or urine. In LB broth and in urine, the wild-type CFT073 strain stationary-phase density averaged 1.1 ± 0.4 × 10⁹ and 1.0 ± 0.2 × 10⁸ cells ml⁻¹, respectively. No difference was observed in the exponential phase growth rate and in the plateau density between the wild-type CFT073 strain and its mutS⁻ otherwise isogenic mutant (data not shown).

Secondly, we performed competition experiments to evidence possible more discrete gain in fitness of the strains, undetected in the previous experiments. Initial inoculum density was 10⁵–10⁶ cells ml⁻¹ for the starting mutS⁻/mutS⁺ ratio of 1, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁸ cells ml⁻¹ for the 10⁻⁶ ratio. As shown in Fig. 1, there is a clear difference in the behaviour of the strains between LB broth and urine. In LB broth, the initial mutS⁻/mutS⁺ ratio of 1 remains constant after 240 generations. In contrast, in urine, the initial ratios are modified over time, with an advantage for the mutator population frequency dependent. At mutS⁻/mutS⁺ ratios above 10⁻⁵, there is a progressive dominance of the mutator population. Alternatively, at a ratio below 10⁻⁷, the mutator population is displaced. These results
are in perfect agreement with the data of Chao and Cox [18] obtained from competition experiments between E. coli mutT+ and mutT− strains in minimal synthetic medium. The observation of a similar advantage for the mutator population when starting at an initial ratio of 1 in M9 minimal medium (data not shown) argues for a role of nutritional factors in the selection of mutators. Altogether, these data demonstrated that, in a non-optimal medium, the adaptive mutations conferring higher fitness produced by the mutator will reach fixation after a lag. This advantage can be eliminated by reducing the mutator population to a size where no mutants would be present. In this case, there is fixation of a higher fitness mutant in the wild-type population [18]. This advantage for the mutator population is not observed in a rich medium as LB broth where advantage conferred by adaptive mutations should be low. 

In conclusion, even in an already uropathogenic human isolate, mutator strains can be selected in human urine due to the adaptive mutations that they generate.

3.2. UTI mouse model experiments

Although it has been shown that E. coli CFT073 grown in human urine partially mimics growth in vivo [19], we used the ascending, unobstructed UTI mouse model developed by Hagberg et al. [15] to strengthen the in vitro results in more pathophysiologically conditions. As in vitro experiments, we first analyzed the behaviour of the wild-type and mutant strains separately. 61 and 52 mice have been inoculated with the wild-type and mutS− CFT073 strains, respectively, and sacrificed at days 2, 4, 6, 10, 15, 20, 25, 30 and 45 with at least 5 mice per group. No difference was observed between the two groups of mice in terms of percentage of infected mice, level of infection within the organs and degree of inflammation. There was a spontaneous clearance of infection over time with 55% of mice still infected on day 25. The mean level of infection at day 20 in the bladder and the kidneys is of 6.89 ± 2.13 log CFU/g and 5.39 ± 0.45 log CFU/g, respectively. The spleens were not infected. The inflammation scores indicated moderate inflammation responses (grades 1 and 2), but stable during the time at the opposite of the infection decrease.

Given the no difference observed when the strains were independently tested, we then inoculated 62 mice with a mix of mutS− and wild-type CFT073 strains at a ratio of 1. Mice were sacrificed at days 4, 10, 15, 20, 25, 45 and 55. In mice showing an infection, there was a significant initial displacement of the mutator during the first month with 3/17 bladders (χ² test, p = 0.002) and 4/20 kidneys (χ² test, p = 0.0001) infected by a population with a mutS−/mutS+ ratio > 1. However, later on, within the infected mice, the fraction in which the mutator was predominant increased, reaching 50% (Fig. 2). Remarkably, from day 10, the dominance of one type of strain

![Fig. 1. Changes in the ratio of CFT073 mutS− (mutator)/CFT073 mutS+ (wild-type) over time at varying starting ratios in LB broth (dotted line) and filtered human urine (unbroken line). Experiments were repeated at least three times. As similar results were obtained, only one typical experiment result per condition is shown for clarity. For the experiments starting with a mutS−/mutS+ ratio of 1 in urine, 10−1 and 10−2, there are at least 500 times more mutator than non-mutator strains at both generations 180 and 240. For the experiments starting with a mutS−/mutS+ ratio of 10−4, the mutator strain was below our detection threshold (approximately mutS−/mutS+ ratio of 4×10−7) at 180 generations.](image)

![Fig. 2. Results of the competition experiments between the CFT073 mutS− (mutator) and the CFT073 mutS+ (wild-type) strains in the ascending, unobstructed UTI mouse model [15]. The number of inoculated mice is indicated above the graph. For clarity, results from bladders and kidneys have been pooled as no significant difference was observed between the organs (data not shown). Two organs were considered for each mouse, the bladder and the “kidney”. When only one kidney was infected in a mouse, the organ “kidney” was considered as infected. The percentage of organs is expressed as a function of the days after inoculation at a starting ratio of 1. Three categories of organs are considered: uninfected organs (hachured), infected organs with a predominance of the mutator strain (mutS−/mutS+ ratio > 1) (black), infected organs with a predominance of the wild-type strain (mutS−/mutS+ ratio < 1) (stippled).](image)
(i.e., the mutator or the wild-type strain) was clear with at least 10 times the ratio (data not shown). It seems that under these pathophysiological conditions, the mutator can be selected in the long term.

When all mice were considered (infected and non-infected), an interesting pattern emerged. The proportion of mice where the non-mutator was dominant decreases in time (from 100% to 0% for the bladders and 60% to 15% for the kidneys) whereas the proportion of mice where the mutator was dominant remained constant around 20% in both organs (Fig. 2). This indicates that, when the mutator is fixed early, it persists chronically. It can be hypothesized that the passage to chronic state in UTI requires numerous mutations that the mutator will be able to produce more easily. It has been shown that a subpopulation of E. coli strains is able to persist for months in murine model of UTI within bladder epithelial cells [20]. These strains have to cope with host inflammatory response and to colonize the exposed transitional epithelium [21].

Finally, to test the hypothesis that adaptive mutations leading to chronic infection were fixed in mutator strains, we took strains from the bladder and the kidney of different mice sacrificed after 45 days of infection to inoculate new mice. Twenty-one mice were infected with a mutator strain that was dominant in the bladder (10 mice) and the kidney (11 mice) whereas 22 mice were infected with a wild-type strain that was dominant in the bladder (11 mice) and the kidney (11 mice). As a control, 16 mice were infected with either the naïve mutator or wild-type strain. All the mice were sacrificed at day 45. The bacterial load in both groups of mice (first and second passages) was similar (data not shown). However, a significant enhancement of the virulence in the second passage as compared with the first passage was observed only for the mutator strain (37.5% of infected mice infected, 80.9% versus 63.6% of infected mice in the second passage for the mutator and wild-type strains, respectively) ($\chi^2$ test, $p < 0.01$) (Fig. 3). This confirms a better adaptation to chronicity for the mutator strain.

3.3. Concluding remarks

To date, there was no clear experimental evidence for the role of mutator in virulence [22,23]. We report here for the first time that, under the specific conditions suggested by an epidemiological approach [12], i.e., UTI, the mutator phenotype may confer an advantage in pathogenesis. Our results suggest that a high level of mutagenesis is associated to the development of chronicity. Increased mutagenesis could play an important role in the development of persistent intra cellular reservoirs observed in UTI [20,21].

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