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Subinhibitory concentrations of penicillin increase the mutation rate to optochin resistance in *Streptococcus pneumoniae*

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***Objectives*: The aim of this work was to study the effect of subinhibitory concentrations of penicillin, chloramphenicol and erythromycin on the mutation rate of *Streptococcus pneumoniae*.**

***Methods*: The mutation rate to rifampicin and optochin resistance was estimated using fluctuation analysis in three capsulated *S. pneumoniae* strains, cultured both with and without different subinhibitory antibiotic concentrations. The *atpAC* and *rpoB* mutations that conferred optochin and rifampicin resistance, respectively, were identified by DNA sequencing.**

***Results*: The exposure to subinhibitory concentrations of penicillin increased the mutation rate (expressed as mutation per cell division) to optochin resistance between 2.1- and 3.1-fold for all three strains studied. In contrast, the rifampicin resistance assay showed no significant variations. To analyse the putative cause of the different responses between the optochin and rifampicin tests, mutations that conferred resistance in both cases were analysed. The difference may be explained by the genetic nature of the *atpAC* mutations, mostly transversions, which are not efficiently repaired by the HexAB mismatch repair system.**

***Conclusions*: We demonstrated that subinhibitory concentrations of penicillin significantly increased the mutation rate of *S. pneumoniae*, suggesting that exposure to this antibiotic could help this pathogen to acquire mutations that confer resistance to other antibiotics. The optochin test was useful to detect this phenomenon and it should be considered for further mutability analysis in *S. pneumoniae*.**

Keywords: mismatch repair system, mutability, rifampicin

Introduction

Streptococcus pneumoniae is not only a normal inhabitant of the upper respiratory tract of humans, but it is also the most common cause of invasive bacterial infections in children after the neonatal period, with high rates of morbidity and mortality. Since the 1980s, a worrying increase in pneumococci resistant to β-lactams and macrolides has been reported. Despite this alarming therapeutical situation, these antibiotics are still the first-line empirical therapy for community-acquired pneumonia.

After administration, antibiotics reach concentrations higher than those required for bacterial inhibition in the various host tissues. Nevertheless, the antibiotic concentration frequently decreases in some body compartments, both during treatment and after removal, thereby exposing the pathogens to subinhibitory antibiotic levels over long periods of time. It has been noted that exposure to subinhibitory concentrations of antibiotics produces multiple effects on bacterial cells, such as a decrease in biofilm formation, secretion of virulence factors, flagellin expression, toxin secretion, as well as an increase in bacterial adhesion, gene transfer, colicin synthesis and, in particular, mutation frequency.[1] The hypermutator phenotype described in several bacterial pathogens is due to permanent mutations in genes that encode the DNA repair systems, such as *hexA* and *hexB* genes in *S. pneumoniae*.[2,3] Under stress conditions, hypermutator strains have the advantage of rapid adaptation. Antibiotic-induced mutability is a transient physiological state, which allows bacteria to generate mutations and to survive a specific stress situation.

In this work, our main objective was to investigate the effects of the subinhibitory concentrations of penicillin, erythromycin and chloramphenicol on the mutation frequency of *S. pneumoniae*, using the optochin and rifampicin resistance tests to evaluate this phenomenon.

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Materials and methods

Bacterial strains

The following reference strains were used in this study: *S. pneumoniae* D39 NCTC 7466 (capsulated virulent strain, serotype 2), *S. pneumoniae* R6 ATCC BAA-255 (uncapsulated derivate from D39) and *S. pneumoniae* ATCC 49619 (capsulated strain, serotype 19F). We also used *S. pneumoniae* CBA7, a clinical serotype 14 strain.

Fluctuation analysis

The fluctuation analysis was performed as described previously.[4] The antibiotic concentrations used corresponded to 75% of the MIC determined for each compound and strain analysed [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The mutation rate to rifampicin and optochin resistance was determined for at least 20 replicas by spreading the entire 0.4 mL of each culture on brain heart infusion (BHI) agar plates containing 2 mg/L rifampicin and 6 mg/L optochin, respectively. The mutation rate determinations and the statistical analysis from the fluctuation assays were carried out as described by Gould *et al.*,[4] using the FT program created by the Sniegowski laboratory (available at http://www.bio.upenn.edu/faculty/sniegowski/#software).

Construction of the hexB mutant

PCR-ligation mutagenesis was used for the construction of the *hexB* insertion–deletion mutant. The *hexB* 5′-flanking region was amplified with primers FhexB1 (5′-TAAGCGGTTGCCAAAGTTGAAGAGC-3′) and RhexB1 (5′-GCGAATTCAGGAAAAGCTGATTGTCCAAGCACC-3′) and the 3′-flanking region was amplified with primers FhexB2 (5′-GCGAATTCTGTTCAAAAACCTTGATTTTATGCG-3′) and RhexB2 (5′-AACAAAGAAAAATCGAATGGGTCAC-3′). Both DNA fragments were digested with *Eco*RI and ligated to the kanamycin resistance gene, *aphA3*, excised with the same restriction enzyme from the plasmid pGEM-ΩKm. This plasmid contains the *aphA3* gene, which was amplified with primers FKmMeg (5′-CCGGGCCCAAAATTTGTTTGATTTTAGCTTCTGGTGTATAATTAAATACTGTAGAAAAGAGGAAGG-3′) and RKmMeg (5′-GGACAGTTGCGGATGTACTTC-3′), cloned in pGEM-T easy®. The ligation mixture was used to transform strain D39, and the selection of mutants was made in BHI 5% sheep-blood agar plates supplemented with kanamycin at 250 mg/L. The presence of the desired mutation was confirmed by PCR.

Transformation assays

The R6 and D39 strains were genetically transformed using a procedure described previously.[5] Cells were transformed with PCR-amplified *atpAC* or *rpoB* genes, and transformants were selected on Mueller–Hinton agar plates supplemented with 5% defibrinated sheep blood, containing 6 mg/L optochin (Sigma, St Louis, MO, USA) or 2 mg/L rifampicin (Sigma), respectively.

Nucleotide sequence accession numbers

The nucleotide sequence data were deposited in the GenBank database under accession numbers: EU256624-30 and EU256632-36.

Results

Antibiotic effects on the mutation rate of S. pneumoniae

Based on previous reports describing antibiotics inducing a transient mutability increase in bacteria,[1] we investigated whether this phenomenon could also occur in *S. pneumoniae*, by testing antibiotics that are frequently used for the treatment of pneumococcal infections, such as penicillin and erythromycin, and also chloramphenicol, which is a non-related antibiotic. Rifampicin resistance testing (or rifampicin test) is the most common assay used to assess the mutation frequency in bacteria, which has also been tested on *S. pneumoniae*.[6]

Here, we evaluated the antibiotic effects on the mutation rate of *S. pneumoniae* by using an additional test, an optochin resistance assay, where optochin resistance is conferred by point mutations in the *atpAC* genes that encode subunits of $F_0.F_1$-ATPase.[7] Optochin is a quinine derivate with antimicrobial activity against *S. pneumoniae*, and the optochin disc test is used in clinical laboratories for its identification. Three capsulated strains (D39, ATCC 49619 and CBA7) were exposed to subinhibitory concentrations of antibiotics, corresponding to 75% of each MIC value [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], and the mutation rate to optochin resistance was estimated. The results obtained (Figure 1 and Table S1) showed that erythromycin and chloramphenicol did not alter this mutation rate. However, when we compared the exposed and unexposed cells for penicillin, this antibiotic increased the average rate of the optochin-resistant mutants 2.6-fold for the D39 strain (3.4×10^{-8} versus 1.3×10^{-8} mutations per cell division), 3.1-fold for the ATCC 49619 strain (2.2×10^{-7} versus 7.2×10^{-8} mutations per cell division) and 2.1-fold for the CBA7 strain (2.9×10^{-8} versus 1.4×10^{-8} mutations per cell division). In parallel, the mutation rate of *S. pneumoniae* was also estimated by the rifampicin test, but the differences found between cells either with or without penicillin treatment were not significant (D39, 3.1×10^{-8} versus 3.2×10^{-8} mutations per cell division; ATCC 49619, 1.6×10^{-7} versus 1.9×10^{-8} mutations per cell division; and CBA7 strain, 1.0×10^{-8} versus 1.2×10^{-8} mutations per cell division), indicating a different sensitivity between both tests in determining the mutation rate of *S. pneumoniae*.

Analysis of the penicillin-induced mutation types that conferred optochin and rifampicin resistance

The differing results obtained between the optochin and rifampicin tests raised the question about the putative cause of this difference. Thus, we investigated the penicillin-generated mutation types in the *atpAC* (encodes subunits of the $F_0.F_1$ ATPase) and *rpoB* (encodes the β subunit of the RNA polymerase) genes that conferred optochin and rifampicin resistance, respectively. To characterize the penicillin-induced *atpAC* mutations, 20 Opt^R colonies were randomly picked from plates containing a total of 40–50 Opt^R colonies, which were plated from a pool of penicillin-induced Opt^R mutants generated *in vitro* from the D39 strain. The *atpAC* genes of these Opt^R mutants were amplified and transformed into the optochin-susceptible R6 strain. Then, those PCR products able to confer optochin resistance were sequenced, and we found the G14S, A49T, A49S, F45V and F50L *atpC* mutations, which have been

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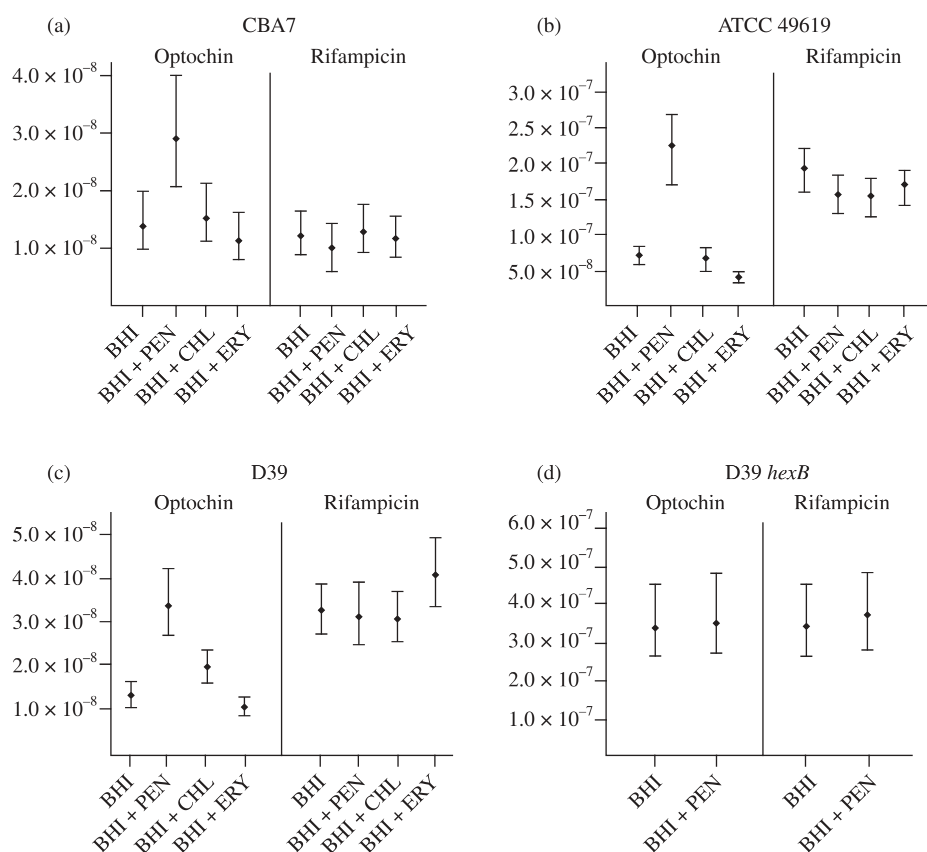

Figure 1. Effect of subinhibitory antibiotic concentrations on the mutation rate of three pneumococcal strains. The pneumococcal strains were grown in BHI alone or with the addition of antibiotics. The mutation rates corresponding to optochin and rifampicin resistance were calculated as described in the Materials and methods section. The corresponding values and the confidence limits are indicated in Table S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Each distribution corresponds to the analysis of at least 20 replicates for each strain and condition analysed. PEN, penicillin; CHL, chloramphenicol; ERY, erythromycin.

reported[7,8] (Table 1). A similar protocol was used to characterize the penicillin-induced *rpoB* mutations. We analysed only cluster I of the *rpoB* gene[9] from 20 Rif^R colonies, and we found three mainly conserved substitutions in the rifampicin-resistant strains, S482F, H486Y and H486N, and two new mutations, Q473R and R500H (Table 1). The amino acid positions were assigned according to the GenBank accession no. NC_008533, corresponding to the genome sequence of strain D39. Following the search for putative causes that could explain the differences found between the optochin and rifampicin tests, we then focused our attention on the kind of mutations, and we found 60% transversions and 40% transitions for the Opt^R mutations and 35% transversions and 65% transitions for the Rif^R mutations (Table 1 and Table S1). The incidence of transversions/transitions between both populations showed opposite trends, and we proposed that the genetic nature of the genes studied may explain

Table 1. *rpoB* and *atpAC* mutations identified in rifampicin- and optochin-resistant strains obtained from strain D39

Rifampicin-resistant mutations[a]				Optochin-resistant mutations[b]			
codon changes	amino acid changes	frequency[c]	mutation type[d]	codon changes	amino acid changes	frequency[c]	mutation type[d]
CAG→CGG	473 Q→R	2	TNS A→G	GGC→AGC	14 G→S	3	TNS G→A
TCT→TTT	482 S→F	8	TNS C→T	TTT→GTT	45 F→V	8	TNV T→G
CAC→TAC	486 H→Y	2	TNS C→T	GCC→TCC	49 A→S	4	TNV G→T
CAC→AAC	486 H→N	7	TNV C→A	GCC→ACC	49 A→T	2	TNS G→A
CGT→CAT	500 R→H	1	TNS G→A	TTT→CTT	50 F→L	3	TNS T→C

[a]Point mutations identified in the *rpoB* gene that confer rifampicin resistance.
[b]Point mutations identified in the *atpAC* genes that confer optochin resistance.
[c]Frequency is the number of mutations from a total of 20 mutations.
[d]TNS, transition; TNV, transversion.

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the differences in mutation rates assessed by the optochin and rifampicin tests.

Effect of the mismatch repair system (MRS) on the penicillin-induced mutation rate

It has been shown previously that the MRS of *S. pneumoniae* has a lower repair efficiency for transversions.[10] Considering that the Opt^R mutations showed a predominance of transversions compared with the Rif^R mutations, we hypothesized that this fact could explain the increased mutability detected only by the optochin resistance assays. To analyse the putative effect of the MRS on the penicillin-induced mutator phenotype, we constructed the *hexB* mutant by insertion–deletion mutagenesis in the D39 strain. This mutant was exposed to subinhibitory concentrations of penicillin (75% MIC) and the mutation rate to optochin resistance was determined. The mutation rate was higher than the wild-type strain, increasing 11- and 26-fold by rifampicin and optochin resistance assays, respectively (Figure 1d). When the *hexB* mutant was exposed to penicillin, we detected no differences in the mutation rates to optochin and rifampicin resistance compared with the data obtained from the unexposed cells, suggesting that the lesser ability of the MRS to repair transversions could be how penicillin-induced mutator phenotypes are detected by the optochin resistance assays in wild-type strains (Figure 1a–c).

Discussion

In this work, we found that subinhibitory concentrations of penicillin increased the mutation rate to optochin resistance between 2.1- and 3.1-fold for the three strains studied. This rise was detected by the optochin resistance assay, but not by the classical rifampicin resistance assay. We estimated the mutation rate (as mutation per cell division) using fluctuation analysis because it is more accurate and reproducible than mutation frequencies (ratio of mutants/total cells in the population), which have a reduced level of reliability as demonstrated by Luria and Delbruck.[11]

The effect of subinhibitory concentrations of different antibiotics on the frequency of mutation in *S. pneumoniae* has been reported.[12] The authors found that ciprofloxacin and streptomycin increased the mutation frequency to rifampicin resistance between 2- and 5-fold for three isolates, but neither erythromycin nor ampicillin had any effects on any isolate. These findings were consistent with our results, because when the rifampicin test was used to evaluate erythromycin and penicillin, we did not find any modification in the mutation rate to rifampicin resistance.

To explain the different mutation rates obtained by optochin and rifampicin resistance assays, we explored the possibility that the MRS was less efficient due to the genetic nature of the Opt^R mutations. Comparing the mutations that confer optochin and rifampicin resistance, we observed a predominance of transversions over transitions among the Opt^R mutations, which could explain the increased mutation rate. We propose that the predominance of transversions among the Opt^R mutations could explain the increase in mutation rate to optochin resistance due to repair inefficiency of the MRS. In this sense, the *hexB* mutant showed no differences in its mutation rate when cells were exposed to subinhibitory concentrations of penicillin, suggesting that the MRS is involved in this phenomenon.

Here, we demonstrated that the optochin test was more useful for the analysis of the mutability state of pneumococcus, and we propose that this test should also be considered in future studies of adaptive mutability for *S. pneumoniae*. Coincidently with this proposition, mutation rate to optochin resistance was recently used to identify mutator phenotypes of clinical strains of *S. pneumoniae*.[4]

Penicillin is an antibiotic commonly used for the treatment of pneumococcal infections. Because antibiotic concentrations frequently diminish during the treatment or removal, we propose that the penicillin effect on the mutation rate of *S. pneumoniae* should have multiple consequences caused by mutations acquired during this transient mutator state. It is known that bacteria could transiently increase their mutation rate in response to stress conditions, thus generating a hypermutator subpopulation of cells containing multiple mutations.[13] This subpopulation would be able to acquire specific phenotypes to allow survival and proliferation by increasing its mutation rate or its transformability. In this sense, Prudhomme *et al*.[14] reported that *S. pneumoniae* stressed with aminoglycoside and fluoroquinolone antibiotics induces transformation, favouring genetic exchange that could increase antibiotic-resistant clones.

Because the penicillin effect on the mutation rate is transitory, maintaining a low mutation rate is an advantage because mutation accumulation produces detrimental effects in bacteria.[13] In this work, we have demonstrated that penicillin is able to induce a transient mutator state. We hypothesized that penicillin is a putative factor generating mutations that *S. pneumoniae* could exploit to enable survival in host tissues. Our results suggest that penicillin exposure could facilitate the appearance of mutations that confer resistance to other antibiotics.

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Transparency declarations

None to declare.

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Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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