

# The mystery of the fourth clone: comparative genomic analysis of four non-typeable *Streptococcus pneumoniae* strains with different susceptibilities to optochin

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**Abstract** Optochin-resistant pneumococci can be rarely caught in clinical microbiology laboratories because of the routine identification of all such strains as viridans group non-pneumococci. We were lucky to find four non-typeable *Streptococcus pneumoniae* clones demonstrating the different susceptibilities to optochin: one of them (Spn\_13856) was resistant to optochin, while the other three (Spn\_1719, Spn\_27, and Spn\_2298) were susceptible. Whole genome nucleotide sequences of these strains were compared to reveal the differences between the optochin-resistant and optochin-susceptible strains. Two adjacent genes coding maltose O-acetyltransferase and uridine phosphorylase which were presented in the genomes of all optochin-susceptible strains and missed in the optochin-resistant strain were revealed. Non-synonymous substitutions in 14 protein-coding genes were discovered, including the Ala49Ser mutation in the C-subunit of the F0 part of the ATP synthase rotor usually associated with pneumococcal optochin resistance. Modeling of a process of optochin interaction with the F0 part of the ATP synthase rotor

indicates that the complex of optochin with “domain C” composed by wild-type C-subunits is more stable than the same complex composed of Ala49Ser mutant C-subunits.

## Introduction

Optochin—an agent of the quinine family—was first exploited against *Streptococcus pneumoniae* more than one hundred years ago, when it was established that this agent possessed strong anti-pneumococcal activity (Bowers, 1955). However, the clinical use of optochin was restricted very shortly after, as it was found to be heavily toxic in humans. Nevertheless, starting from the mid-1950s, optochin susceptibility testing has found its stable niche in clinical laboratory diagnostics: for many decades, it allowed to confidently discriminate between optochin-susceptible pneumococci and optochin-resistant morphologically similar close relatives of the mitis group.

Despite there being no evident reason for the formation of wide-ranging resistance to optochin, due to its clinical irrelevance, reports concerning optochin-resistant *S. pneumoniae* have been sporadically appearing for the last several decades [1–8]. Possibly, optochin-resistant pneumococci are not so rare; however, when optochin susceptibility testing remains the only clinical test carried out for the identification of *S. pneumoniae* in many laboratories, we are ready to believe that such strains can be erroneously identified as mitis group non-pneumococci and discarded.

The mechanism of resistance to optochin has not been thoroughly studied. In the excellent work of Fenoll and co-workers [9], it was established that optochin resistance arises due to the point mutations

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that produce different amino acid changes at a number of different positions (48, 49, or 50) of the F1/F0 ATP synthase C-subunit. In later works, mutations in other positions of the subunit C of ATP synthase conferring resistance to optochin for *S. pneumoniae* isolates were discovered; also, it was established that AA substitution in the A-subunit of H<sup>+</sup>-ATP synthase can contribute to the formation of optochin resistance [10–12].

Now, we have a unique possibility to look at the entire genome of bacteria and try to follow what has occurred at the basal genetic level of *S. pneumoniae* when it becomes resistant. Actually, we have caught four *S. pneumoniae* strains which appeared to be exact genetic clones demonstrating the different susceptibilities to optochin: one of them (Spn\_13856) was resistant to optochin, while the other three (Spn\_1719, Spn\_27, and Spn\_2298) were susceptible. Thus, the purpose of this work was to obtain the whole genomes of the four pneumococcal strains and compare them, to reveal the possible differences in a set of protein-coding genes of optochin-resistant and optochin-susceptible variants.

## Methods

### Strains

Three clinical isolates of *S. pneumoniae* were recovered from the nasopharynx of pediatric patients hospitalized with different diagnoses in the Moscow Scientific Centre of Children Health. One isolate (Spn\_27) have been obtained from the laboratory collection of the Smolensk Institute of Antimicrobial Chemotherapy. All isolates were routinely characterized by the standard viridans group streptococci identification tests under acquisition.

After being transferred to our laboratory, isolates were streaked out on plates of Columbia agar (Oxoid Ltd., UK) supplied with 5 % sheep blood, to form isolated single colonies; pure cultures were subcultured from single colonies after overnight incubation at 37 °C in air with 5 % CO<sub>2</sub>. All strains were retested: the optochin susceptibility and bile solubility tests were carried out using standard diagnostic optochin or sodium deoxycholate disks (Research Centrum for Pharmacotherapy, St. Petersburg, Russia), respectively, in accordance with the manufacturer's instructions. The latex agglutination assay was accomplished by using the Slidex<sup>®</sup> pneumo-kit (bioMérieux, France). For all tests, the *S. pneumoniae* ATCC 49619 laboratory strain was used as a reference strain. All tests were repeated at least twice.

Strains were kept at –70 °C in brain heart infusion broth (BD, USA) containing 30 % fetal bovine serum (Gibco, USA) and 20 % glycerol.

### DNA extraction

For all genetic manipulations, total streptococcal DNA was extracted using the modified protocol of Miller et al. [13]. Briefly, an 18-h culture from whole blood agar plates was harvested and lysed in Promega Nuclei Lysis Solution buffer (Promega, USA). After that, the cellular proteins were removed by adding saturated NaCl solution, and the genomic DNA was concentrated and desalted by isopropanol precipitation. The final DNA pellet was re-suspended in 50–100 µL of TE buffer and kept at 4 °C. For whole genome sequencing, DNA was additionally purified by using minicolumns for DNA purification (Technoclon, Russia), in accordance with the manufacturer's instructions.

### MLSA and MLST analysis

Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) were performed as described by Enright and Spratt [14] and by Bishop et al. [15], respectively, with minor modifications for the MLSA scheme described earlier by Ikryannikova et al. [16]. The results were analyzed using the MLST (<http://www.mlst.net>) and MLSA (<http://viridans.emlsa.net/>) databases. The Vector NTI 9.0 and MEGA 6.0 software packages were used for the manipulations with gene fragments and phylogenetic evolutionary analysis.

### Whole genome sequencing and assembly

Whole genome nucleotide sequences of three stains (Spn\_1719, Spn\_27, and Spn\_2298) were obtained by using a Roche 454 Life Sciences Genome Sequencer FLX+ Genetic Analyzer (Roche 454 Life Science, USA), in accordance with the manufacturer's instructions. Strain Spn\_13856 was sequenced by using an Ion Torrent Personal Genome Machine (PGM) Genetic Analyzer (Life Technologies, USA). Genomes were assembled by GS De Novo Assembler v.2.8 (Roche, USA). Assembly data (contigs) were annotated using the RAST (Rapid Annotation using Subsystem Technology, USA, <http://rast.nmpdr.org/>) and NCBI (American National Center for Biotechnology Information) PGAP (Prokaryotic Genome Annotation Pipeline, USA, [http://www.ncbi.nlm.nih.gov/genome/annotation\\_prok/](http://www.ncbi.nlm.nih.gov/genome/annotation_prok/)) annotation servers, and published in the GenBank database of the NCBI under accession numbers AYRK00000000.2 (Spn\_13856), AYRL00000000.1 (Spn\_1719), AYRM00000000.1 (Spn\_27), and JHAN00000000.1 (Spn\_2298). Raw sequencing reads of the four genomes were published in the Sequence Read Archive (SRA) database of the NCBI under accession numbers SRP043266 (Spn\_13856), SRP043265 (Spn\_1719), SRP043267 (Spn\_27), and SRP043268 (Spn\_2298).

## Genome analysis

All newly sequenced genomes were checked once again for the correctness of the phylogenetic status of strains, by repeated verification of the MLSA and MLST genes. Looking for the MLSA or MLST genes in whole genome nucleotide sequences of the strains under study, as well as the fragments of capsule operon, was realized using the BLAST v.2.2.23+ software (USA), with default parameters.

Alignment of whole genome nucleotide sequences and estimation of the “core genome” size of our four strains, to evaluate a similarity rate among them, was done in the MUMmer v.3.0 package using a nucmer algorithm with default parameters. Briefly, the algorithm of comparison was as follows. At the first stage, the pseudo “pan-genome” of the four strains under study was created by choosing a seed (“reference”) genome sequence following stepwise comparison of each of the other non-seed genome sequences to the “pan-genome”, and adding fragments not present in the “pan-genome”. The expanded “pan-genome” was used for the comparison against the next genomes. After the creation, the pseudo “pan-genome” was subsequently compared against each of the genomes under study, to reveal the homologous and unique nucleotide sequence fragments. The results were obtained as a table containing a list of homologous or unique fragments attributed to each of the genomes.

Comparisons of annotated genomes and clustering of protein-coding sequences by their homology were done using the open web resource USEARCH v.7.0 realizing the UCLUST algorithm (<http://drive5.com/usearch/>). The identity threshold was chosen to be 0.5 or 0.7. The results were obtained as a table containing a list of the clusters of homologous protein-coding sequences attributed to each of the genomes. Also, we used the facilities of the SEED web resource (USA, [http://theseed.org/wiki/Home\\_of\\_the\\_SEED](http://theseed.org/wiki/Home_of_the_SEED)) as a convenient tool for a sequence-based or function-based comparison of two genomes.

Looking for the non-synonymous substitutions in the homological protein-coding sequences was realized by the comparison of protein sequences translated from the nucleotide sequences of our genomes during the annotation procedure. It was done using default options in BLASTP, which is a part of the standalone BLAST v.2.2.23+ package.

Visualization of the compared genomes was made by using the BRIG (BLAST Ring Image Generator) v.0.95 software (<http://brig.sourceforge.net/>).

## In silico modeling of the complex of the F0 part of the ATP synthase rotor and optochin

A homology model of the F0 part of the ATP synthase rotor (“domain C”) was constructed using ORCHESTRAR/SYBYL-X 1.2 (ORCHESTRAR™, Tripos International,

<http://www.tripos.com>). The RCSB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) contains several homologous crystal structures where the rotor consists of between 9 and 15 subunits C [PDB IDs: 4BE (C9), 2BL2 (C10), 2CYD (C10), 1YCE (C11), 3ZK1 (C11), 3ZO6 (C12), 2X2V (C13), 4CB4 (C13), 3V3C (C14), 2W5J (C14), 4MJN (C14), 2WIE (C15), 2XQS (C15), etc.]. Since the more favorable homolog (C12) has only one crystal structure (3ZO6; *Bacillus pseudofirmus*) with low identity (26 %) in alignment with the modeling structure (UniProtKB/Swiss-Prot P0A307.1), we also used 3ZK1 (*Fusobacterium nucleatum*, 39 % identity), 1YCE (*Ilyobacter tartaricus*, 39 %), 3V3C (*Pisum sativum*, 26 %), 2W5J (*Spinacia oleracea*, 24 %), and 2WIE (*Arthrospira platensis*, 24 %). The highest acceptable inter-Ca distance between equivalent residues within a sequence conserved region was established as being equal to 1.5 Å, with an RMS (root mean square) difference during minimization in the alignment procedure higher than 0.00001 Å considered as being significant. Three structures of single C-subunit were constructed by combining different homologs. All structures were very similar. A model of the mutant structure was constructed by virtual mutation of Ala49 to Ser. The full rotor model was performed by fitting the modeled structure to the structure of the C12 rotor from 3ZO6. The refinement of each structure was achieved by SYBYL's structure preparation tool with staged minimization. After that, a long final minimization of the whole F0 rotor part without any restriction was done.

The interaction between optochin and the F0 part of the ATP synthase rotor was simulated by the program Surflex-Dock (Surflex-Dock™, Tripos International, <http://www.tripos.com>). The binding site was defined by Protomol (Tripos International, <http://www.tripos.com>) as a set of amino acid residues located in 7 Å from Ala49 (or Ser49 for the mutant structure). During the docking process, a “protein movement” for heavy atoms was allowed; a maximum of ten conformers were considered and the lowest free energy of binding was selected as the final complex.

## Results

### Characteristics of strains

Four strains of viridans group streptococci were picked out for further investigation based on their divergent results of routine pneumococcal identification tests. In accordance with the primary and following identifications, three strains (Spn\_1719, Spn\_27, and Spn\_2298) were susceptible to optochin, but have given no agglutination with latex particles when using the Slidex® pneumo-kit, while one strain, Spn\_13856, demonstrated negative reactions on both the optochin and latex agglutination tests, so it was at first erroneously identified as belonging to the mitis group non-pneumococcus. Also, we

observed no clear lysis zones around the disks of sodium deoxycholate for all strains in a bile solubility test.

The results of the identification tests are summarized in Table 1.

In accordance with the results of the MLSA analysis, all strains under study appeared to be *S. pneumoniae*. Moreover, all of them reached the same position on a phylogenetic tree—in other words, all four strains were found to be MLSA clones. The MLST approach confirmed the clonal identity of these strains and allowed to define a sequence type for them, ST 2996, which was firstly assigned to strain 89 selected in 2006 in Arkhangelsk (Russia) and belonged to the large clonal family of unencapsulated or non-typeable (NT) pneumococcal strains (<http://www.mlst.net>).

### Comparison of genomes

The details of the whole genome sequencing are presented in Table 1. Regardless of the sequencing method, the estimated average size of genomes was 2.1–2.2 Mbp, with more than 17-fold coverage for all genomes, and the number of contigs fluctuating near 100.

No fragments of a capsule operon were determined in any genome under study.

#### *Comparison of whole nucleotide sequences of the four genomes under study, and looking for differences in a set of protein-coding genes of optochin-resistant and optochin-susceptible strains*

A comparison of whole genome nucleotide sequences was done for revealing homologous or unique fragments in the genomes under study. A pseudo “pan-genome” was created which was compared against each of the four genomes (see the “Methods” section).

Further, we estimated a similarity rate of our four clones: roughly, the size of the homologous fragments of all genomes (“core genome” of four strains) was near 2.08 Mbp; this is 95–98 % of genomes sizes. Therefore, the length of the non-homologous part varied from 2 to 5 % for each genome.

To visualize the compared genomes, we used the BRIG tool. The BRIG software demands a reference nucleotide sequence; to get this, we chose the Spn\_27 genome and aligned other genomes to it. The results of the comparison are plotted as a number of rings; each of them (excluding an inner ring, which is a reference sequence) represents a query sequence, which is colored to indicate the presence of hits to the reference sequence (Fig. 1). As can be seen, all four genomes under study are very similar. There are the obvious relationships between Spn\_27 and Spn\_2298 (purple and green rings), and Spn\_13856 and Spn\_1719 (red and blue rings) strains, respectively.

To reveal the differences in a set of protein-coding genes of optochin-resistant Spn\_13856 and optochin-susceptible strains, we used the UCLUST algorithm for protein clustering and comparison embedded in the USEARCH v.7.0 software (<http://drive5.com/usearch/>). For comparison, protein-coding sequences translated from the nucleotide sequences of the genomes under study during the annotation procedure were accepted. Also, we used the facilities of the SEED Viewer web resource ([http://theseed.org/wiki/Home\\_of\\_the\\_SEED](http://theseed.org/wiki/Home_of_the_SEED)) to verify the results. SEED Viewer gives the possibilities of a function-based or sequence-based comparison of genomes. The function-based option allows to compare the functioning parts of two organisms, giving a list of genes associated with a subsystem in the respective organism. The sequence-based algorithm allows to align the comparison organism to the reference genome, displaying the list of genes of the reference strain in chromosomal order with indication of the percentage nucleotide sequence identity. In the current state, the sequence-based module allows to compare all four genomes at once. Finally, all fragments discovered which differ from the optochin-resistant Spn\_13856 strain from the three optochin-susceptible strains were inspected manually.

An output of all approaches was the same: two adjacent genes coding maltose O-acetyltransferase (EC 2.3.1.79) and uridine phosphorylase (EC 2.4.2.3) were found to be present in the genomes of the optochin-susceptible Spn\_1719, Spn\_27, and Spn\_2298 strains

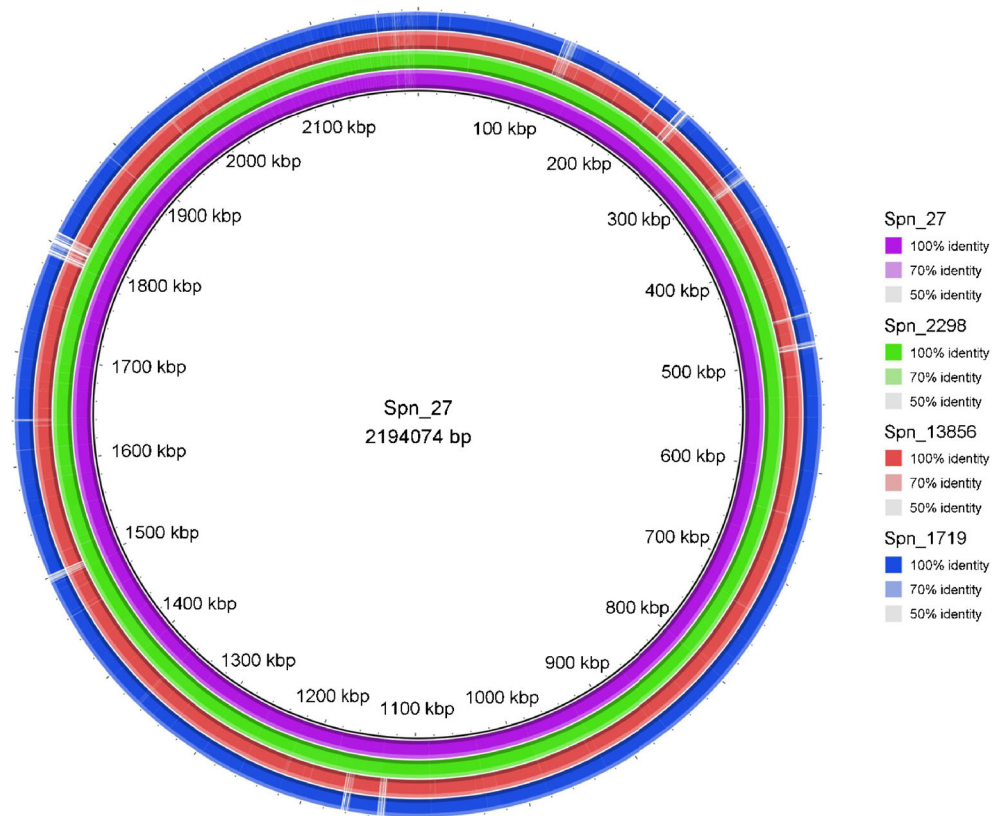
**Table 1** Characteristics of the four pneumococcal strains under study and details of whole genome sequencing

Strain	Identification tests*			Whole genome sequencing			
	Susceptibility to optochin	Latex agglutination assay	Bile solubility test	Sequencing platform	Sequencing depth	Number of large contigs	Genome length, bp
Spn_13856	non-susc.	neg.	neg.	Ion Torrent PGM™	43×	120	2111028
Spn_1719	susc.	neg.	neg.	GS FLX+	29×	84	2146816
Spn_27	susc.	neg.	neg.	GS FLX+	16×	108	2187514
Spn_2298	susc.	neg.	neg.	GS FLX+	17×	117	2181986

\*Typical pneumococci are susceptible to optochin, agglutinate in the Slidex® pneumo-kit test, and demonstrate a zone of lysis around sodium deoxycholate (bile) disks



**Fig. 1** Comparative BRIG image of the four genomes under study. In this figure, the provisional reference strain (Spn\_27, purple ring) is positioned against the other three genomes



and missed in the optochin-resistant Spn\_13856 strain. One gene coding the mobile element protein was found additionally in the sequence-based module of the SEED Viewer, differing between the optochin-susceptible and optochin-resistant strains.

#### *Looking for the non-synonymous SNPs in the protein-coding sequences of optochin-resistant and optochin-susceptible strains*

Looking for the non-synonymous substitutions in the homologous protein-coding sequences was realized by the comparison of protein sequences translated from the nucleotide sequences of our genomes during the annotation procedure. The aim was to find significant single nucleotide polymorphisms (SNPs), leading to the amino acid substitutions, in the optochin-resistant Spn\_13856 strain compared to the three optochin-susceptible strains. The results are presented in Table 2.

As one can see, point mutations resulting in the changes of amino acids were found in 14 protein-coding sequences. Among others, the replacement of alanine by serine at position 49 of C-subunit of the membrane part of the ATP synthase rotor, which was assumed in previous works to lead to the appearance of optochin resistance, was revealed. To reveal the relevance of these amino acid changes, we additionally inspected a number of

homologous proteins of related streptococcal strains/species stored in the NCBI database. For the majority of proteins (protein nos. 2–4, 6–8, 10–14), the mutations found are unique, i.e., these mutations are only present in the strain Spn\_13856. The Thr691Asn mutation in the isoleucyl-tRNA synthetase (protein no. 1) is unique for the Spn\_13856 strain; however, sporadic replacement of Thr by Ile at position 691 occurred in the *S. pneumoniae* (1.6 %) and *S. mitis* (4.8 %) strains. A similar situation is observed in the case of protein no. 9: Lys124Arg was found for the Spn\_13856 strain only, but the Lys124Glu mutation was revealed in 1.5 % of *S. pneumoniae* strains and nearly half of the *S. pseudopneumoniae* or *S. mitis* strains. One exception is the hypothetical protein no. 5: about half (52 %) of all *S. pneumoniae* isolates inspected carried the Leu30Trp substitution.

#### **Models of the complex of optochin and the F<sub>0</sub> part of the ATP synthase rotor with or without Ala49Ser mutation in C-subunit**

In this work, we have tried to model a process of binding of optochin to the membrane F<sub>0</sub> part of ATP synthase.

Six prototypes from the RCSB Protein Data Bank which are homologous crystal structures of different sizes (from 9 to 15 subunits C) were used for the creation of the model of the F<sub>0</sub> part of the ATP synthase rotor

**Table 2** A list of proteins carrying the amino acid substitutions in the optochin-resistant Spn\_13856 strain compared to the three optochin-susceptible strains

No	NCBI/RAST IDs <sup>a</sup>	Category <sup>b</sup>	Subcategory	Subsystem	Role	AA substitution	Dissimilarity index <sup>c</sup>
1	gi 564902471/ fig 6666666.48119.peg.175	Protein metabolism	Protein biosynthesis	tRNA aminoacylation, Ile	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	Thr691Asn	0
2	gi 564902472/ fig 6666666.48119.peg.176	Cell division and cell cycle	Cell division and cell cycle - no subcategory	Bacterial cytoskeleton	Cell division initiation protein DivIVA	Ala274Ser	1
3	gi 564902108/ fig 6666666.48119.peg.609	Cofactors, vitamins, prosthetic groups, pigments; membrane transport	NAD and NADP; membrane transport - no subcategory	NAD and NADP cofactor biosynthesis global; ECF class transporters	Substrate-specific component NiaX of predicted niacin ECF transporter	Ala144Val	0
4	gi 564902027/ fig 6666666.48119.peg.679	-	-	-	ATP synthase C chain (EC 3.6.3.14)	Ala49Ser	1
5	gi 564901884/ fig 6666666.48119.peg.838	-	-	-	hypothetical protein	Leu30Trp	-2
6	gi 564901700/ fig 6666666.48119.peg.1078	Motility and chemotaxis; carbohydrates	Motility and chemotaxis - no subcategory; di- and oligosaccharides; polysaccharides	Bacterial chemotaxis; maltose and maltodextrin utilization; alpha-amylase locus in <i>Streptococcus</i>	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE	Arg104Ser	-1
7	gi 15459437/ <sup>d</sup> fig 6666666.48119.peg.1395	-	-	-	MATE efflux family protein DinF	His229Tyr	2
8	gi 564901027/ fig 6666666.48119.peg.1717	DNA metabolism	DNA metabolism - no subcategory	DNA structural proteins, bacterial	Chromosome partition protein SMC	Ser792Phe	-2
9	gi 564900932/ fig 6666666.48119.peg.1801	Carbohydrates	Monosaccharides	Deoxyribose and deoxynucleoside catabolism	Hypothetical protein, phosphotomutase (EC 5.4.2.7)	Lys124Arg	2
10	gi 564900937/ fig 6666666.48119.peg.1806	-	-	-	Glutamate transport ATP-binding protein	Leu163Ile	2
11	gi 564900940/ fig 6666666.48119.peg.1810	-	-	-	ATP-dependent Clp protease, ATP-binding subunit ClpE	Cys6Tyr	-2
12	gi 564900772/ fig 6666666.48119.peg.1867	RNA metabolism	RNA processing and modification	ATP-dependent RNA helicases, bacterial	ATP-dependent RNA helicase YqfR	Thr211Met	-1
13	gi 564900409/ fig 6666666.48119.peg.1932	Phosphorus metabolism	Phosphorus metabolism - no subcategory	Phosphate metabolism	Phosphate starvation-inducible protein PhoH, predicted ATPase	Leu200Phe	0
14	gi 564899359/ fig 6666666.48119.peg.2187	-	-	-	Hypothetical protein	Asp123Glu	2

<sup>a</sup>The identifiers are given for the Spn\_27 strain

<sup>b</sup>Categories, subcategories, and subsystems are given in accordance with the RAST partitioning

<sup>c</sup>The dissimilarity index is indicated in accordance with the BLOSUM62 substitution matrix [17]

<sup>d</sup>The identifier is given according to the *S. pneumoniae* R6 strain. Relative to the *S. pneumoniae* R6 strain, there is a stop codon inside the DinF protein (TGG100TAG) in the Spn\_27 strain as well as in all the other strains of this study. Therefore, there is no identifier for this protein in a standard PGAP annotation procedure. The presence of a stop codon inside this protein is typical for the large phylogenetic family of unencapsulated pneumococci

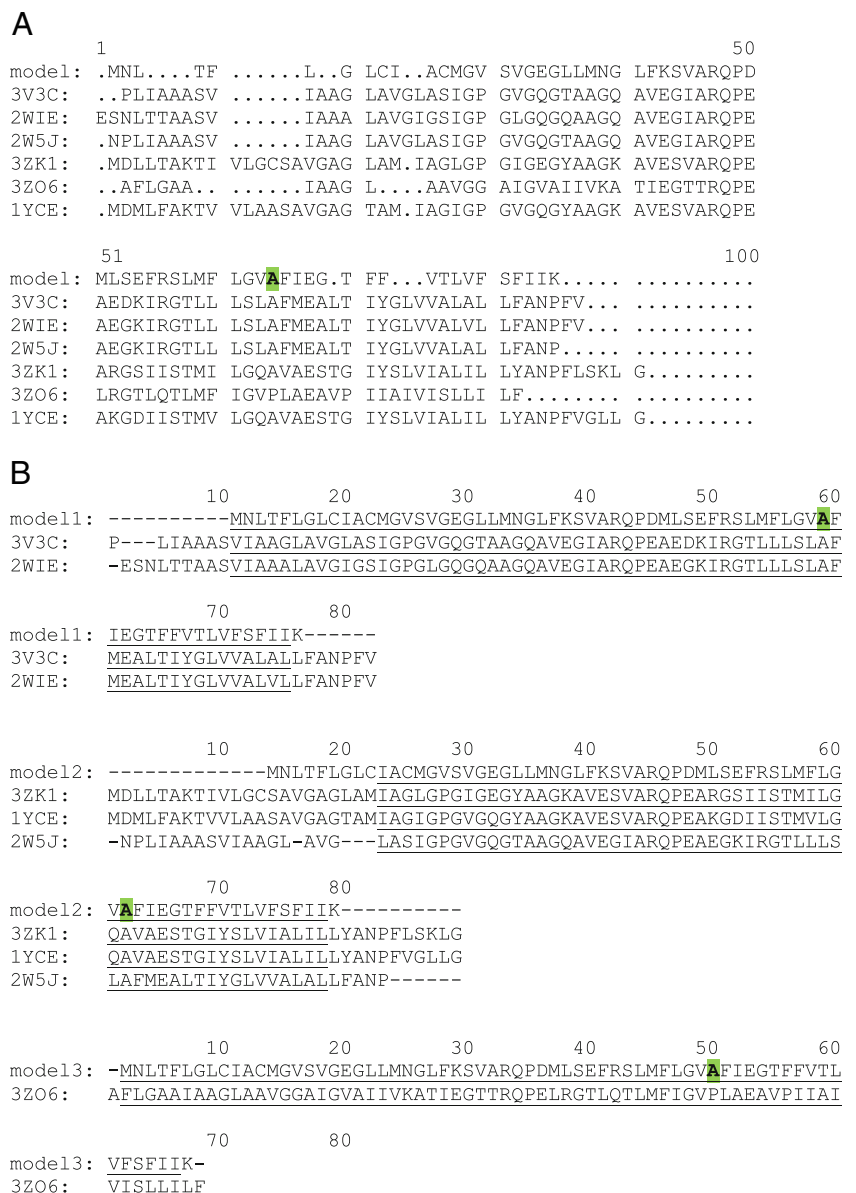
(“domain C”) (see the “Methods” section). Actually, it wasn’t impossible to align all 3D structures of the selected prototypes proteins with acceptable inter-Ca distance, but in this case, only two-thirds of the amino acid residues are included in the structurally conserved region (Fig. 2a). It was clear that an easier way to reconstruct a rotor “domain C” (modeled structure of 12 C-subunits) is to use the structure 3ZO6 only. The question was will the structure of the protein be adequate if we use only one protein prototype? So, three variants of a single rotor subunit were modeled (Fig. 2b). First (the prototypes were 3V3C and 2W5J) and second (3ZK1, 1YCE, and 2W5J) variants were based on the subunits of rotors of different sizes. The third variant was based on 3ZO6 only. All three

structures were found to be very similar (Fig. 3a), so we can further use the third model.

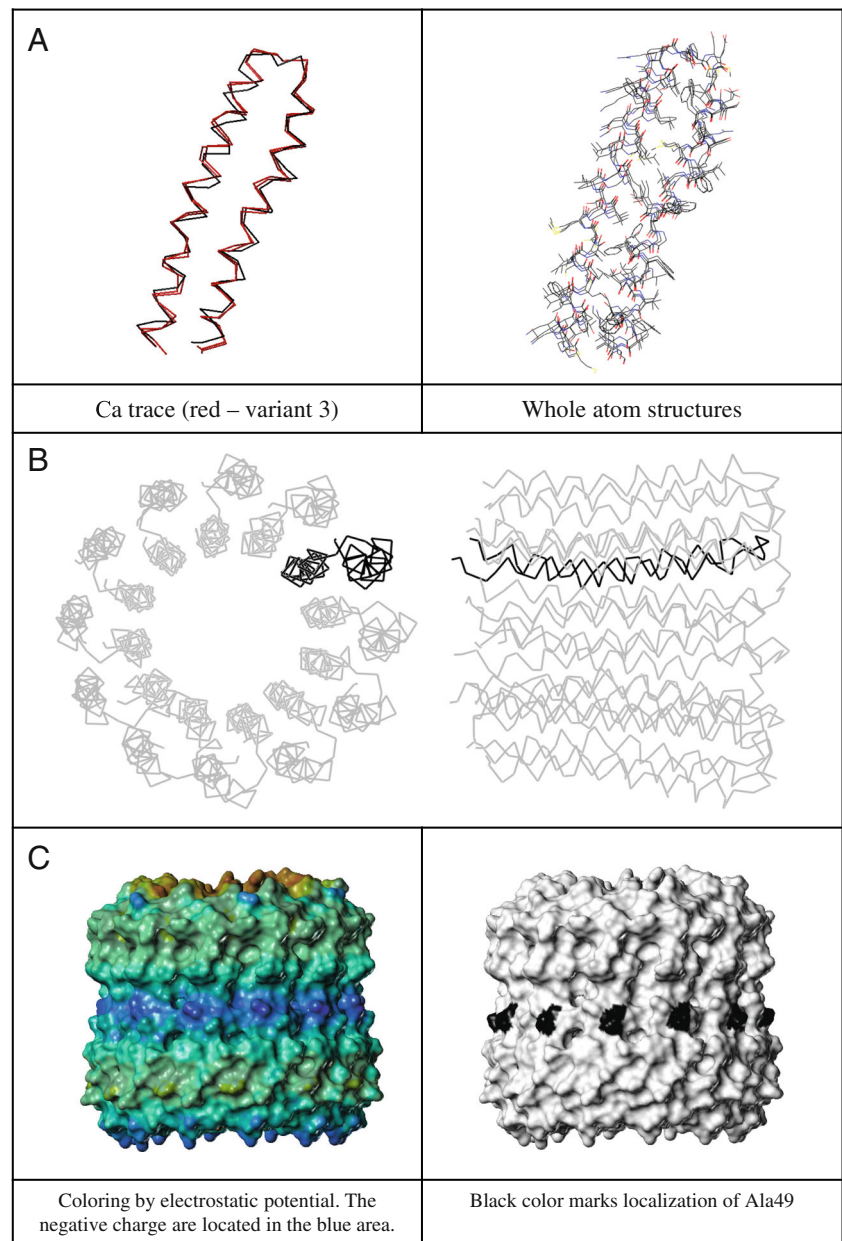
The restored structure of the F<sub>0</sub> part of the ATP synthase rotor is shown on the Fig. 3b. The charge distribution on the surface of “domain C” corresponds to the expected localization of the maximum negative charge in the estimated area of H<sup>+</sup> transfer (Fig. 3c).

The modeled complexes of optochin with “domain C” obtained by an automated docking procedure have significant differences in ligand location and orientation in the binding site (Fig. 4). So, the values of scoring functions for both complexes also vary (Table 3). All values indicate that the complex with the wt “domain C” is more stable.

**Fig. 2 a** The alignment of the prototypes (homological AA sequences of known crystal structures) set used for modeling a single subunit C of the membrane part of the ATP synthase rotor. **b** The variants of prototypes sets used for modeling a single C-subunit. In all alignments, the position of Ala49 is marked by the color and bold type



**Fig. 3** **a** Aligned variants of the model of the C-subunit of the membrane part of the ATP synthase rotor. **b** The restored rotor structure (“domain C”) of the F0 part of ATP synthase of *S. pneumoniae* (orthographic projection). The domain is composed of 12 C-subunits; black color highlights one of the single C-subunits. **c** A molecular surface of “domain C” of the F0 part of *S. pneumoniae* ATP synthase



## Discussion

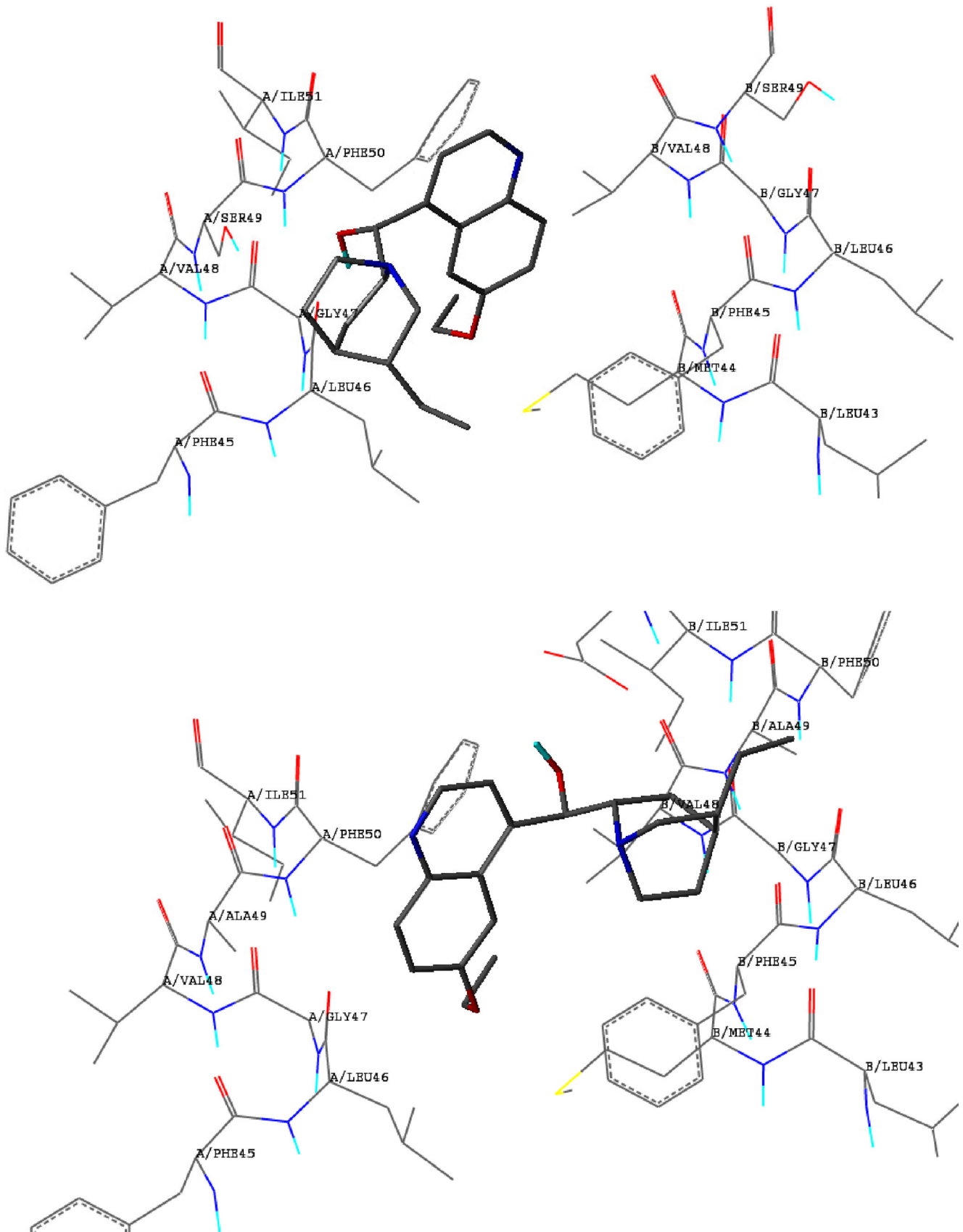
The cases when clonally relative subpopulations of *S. pneumoniae* with different optochin susceptibilities were discovered “in the same Petri dish” were described earlier for the different pneumococcal serotypes [3, 11]. In our case, four strains of *S. pneumoniae*, which are genetic MLST clones, were taken from different patients. Three of those strains were collected within the same clinic, while the fourth was picked up from another town.

Our strains appeared non-typeable or unencapsulated: none of them have given a positive reaction in a latex agglutination test; moreover, we succeeded in finding no fragments of the capsule operon under analysis of their whole genome

sequences. So, NT *S. pneumoniae* strains can also develop resistance to optochin under favorable conditions.

Only two genes coding maltose O-acetyltransferase and uridine phosphorylase enzymes, which were present in the genomes of all optochin-susceptible strains and missed in the optochin-resistant strain, were revealed as a result of the comparative genome analysis. Maltose O-acetyltransferase is an enzyme which catalyses the reaction of acetylation of maltose or other sugars at the C6 position, while uridine phosphorylase catalyzes the reversible phosphorylytic cleavage of uridine and deoxyuridine to uracil and ribose- or deoxyribose-1-phosphate. Note that this is a maltose binding protein (MBP) MalE of maltose/maltodextrin ABC transporter, which is presented in the list of 14





**Fig. 4** The best variants of complexes of optochin with “domain C” of ATP synthase (wt below and mutant Ala49Ser above)

**Table 3** The scoring functions values for complexes of optochin with “domain C” (modeled structure of 12 C-subunits) of the F0 part of the ATP synthase rotor

Scoring function	Ala49	Ala49Ser
Surflex-Dock (higher is better)	4.80	3.89
D_SCORE (lower is better)	-101.80	-88.80
PMF_SCORE (lower is better)	-42.75	-25.86
G_SCORE (lower is better)	-186.14	-172.05
CHEMSCORE (lower is better)	-23.54	-22.31

proteins carrying mutations in the optochin-resistant strain compared to the optochin-susceptible strains (Table 2). Both of the genes coding MBP and maltose O-acetyltransferase are presumably a part of the maltose/maltodextrin metabolism regulon [18]. The Arg104Ser mutation, where hydrophilic arginine is replaced by non-hydrophylic serine, takes place in one of the 17 amino acid residues which constitute the active site of MBP, as it was shown in detail for *Thermoactinomyces vulgaris* [19]. It is quite possible that this mutation changes the conformation of the active site of MBP, preventing interaction with maltose when missing part of the utilization mechanism of this substrate. However, in our case, this is just an assumption to be proven.

As follows from Table 2, AA mutations were found in 14 proteins distinguishing the optochin-resistant and optochin-sensitive strains. The most significant is the Ala49Ser mutation of subunit C of the ATP synthase F0 complex (see below). Some more proteins which are somehow referring to the ATPase activity appeared in this list. One of these proteins, PhoH, belonged to the Pho regulon, which regulates phosphate uptake and metabolism under low-phosphate conditions. PhoH is a cytoplasmic protein which could bind ATP and it was probably involved in the uptake of phosphate under conditions of phosphate starvation. It was assumed that there are several different potential functions for PhoH, but the most probable is ATPase function because of the conserved nucleoside triphosphate hydrolase domain [20]. The Phe200Leu mutation is found inside the P-loop containing the nucleoside triphosphate hydrolase domain. Another protein, ClpE ATPase, is an HSP100 family protein possessing two nucleotide-binding domains and an N-terminal C4-type zinc fingers motif, a small functional fragment that coordinates zinc ion to stabilize its structure through four cysteine residues. In *S. pneumoniae*, ClpE seems to be the major thermotolerance Clp ATPase and is also partly involved in virulence [21, 22]. The Cys6Tyr mutation differing the optochin-resistant Spn\_13856 strain from the optochin-susceptible strains is situated inside a zinc fingers domain. This domain keeps the four cysteine residues at positions 3, 6, 29, and 32. It could be assumed that the replacement of one cysteine by

tyrosine at position 6 destroys the zinc finger, leading to the destabilization of the protein structure and, also, possible protein–protein interactions. Actually, it was shown by Miethke et al. [22] that the alteration of two cysteine codons of the ClpE zinc finger at positions 29 and 32 to serines leads to a ten-fold loss of basal ATPase activity in vitro, showing that this structure element is crucial for the basic ClpE function.

Single amino acid replacements were found in the curvature-sensitive membrane binding spatial regulator DivIVA [23] and upstream isoleucyl-tRNA synthetase. One more protein of the SMC (structural maintenance of chromosomes) family contains Ser792Phe mutation in the coiled-coil region separating the ATPase “head” domain from the central “hinge” domain [24].

Also, we revealed mutations in two ABC transporters. One of them, the maltose binding protein of maltose/maltodextrin ABC transporter, was discussed above. In another, the ATP-binding protein of the glutamate ABC transporter, the Leu163Ile mutation is placed in a highly conserved six-AA Walker B motif, which is thought to function to bind directly or indirectly a metal ion important in catalysis and provides a critical glutamate for positioning the hydrolytic water molecule [25]; the replacement of leucine by isoleucine takes place at the third position of this motif. The Walker B motif looks like a XXXXDE sequence, where X is a hydrophobic amino acid; so, the replacement of hydrophobic leucine by the hydrophobic and functionally similar isoleucine could unlikely influence considerably the properties of the catalytic center. In one more ECF (energy coupling factor)-type niacin transporter, amino acid mutation occurs inside the substrate-specific component (S component), a small integral membrane protein assigned to capture a specific substrate.

As seen, there are quite a number of modified proteins in the optochin-resistant strain compared to the optochin-susceptible strains that are expected to be functionally stable. These modifications may be related to the formation of resistance to optochin or may follow this process, or it may be the individual features of strain Spn\_13856. The evidence-based answer to this question is not possible in the frame of this work. Experiments using the artificial genetic constructions mutant for each of the query proteins are needed, but this is a task for future work.

As mentioned, the most significant finding is certainly the Ala49Ser mutation in the C-subunit of the F0 ATP synthase domain. It is one of the three point mutations in *atpC* producing amino acid changes at the positions 48, 49, or 50 which was found by Fenoll et al. and as proved to lead to optochin resistance [9]. Other mutations in C- or A- subunits of the ATP synthase F0 complex have also been described [10–12, 26, 27]. It is assumed that these AA substitutions can influence proton translocation through the ATP synthase membrane F0 complex. The putative model for the F0 complex of ATP synthase consists of a membrane-embedded cylinder made

up of 9–14 C-subunits, at that one A-subunit positioned within the membrane matrix on the outer surface of the cylinder. Each C-subunit consists of two transmembrane  $\alpha$ -helices joined by a short conserved cytoplasmic loop, like a hairpin. The cylinder structure is stabilized by hydrogen bond interactions between  $\alpha$ -helices and the adjacent C-subunits. The protein cylinder acts as a rotor embedded in the membrane. The proton pathway through the membrane is thought to be formed by an opening of the interface between adjacent C-subunits by the swiveling of helix 1, thus allowing hydrogen from the protonated glutamate 52 carboxyl side chain on helix 2 to transfer to the A-subunit on the outer surface of the cylinder [11, 28].

The molecular mechanism determining how optochin interacts with the F<sub>0</sub> complex of ATPase remains unclear. It is supposed that the proton-translocating subunits of ATPase may be disrupted in the presence of optochin, resulting in the proton pump failure and pneumococcal cell death. So, amino acid changes within the A- or C-subunits in optochin-resistant strains may prevent optochin from disrupting the proton transport pathway. To confirm this idea, we modeled an interaction of an optochin molecule with the “domain C” of the ATP synthase rotor composed of 12 C-subunits. The values of scoring functions calculated for the complexes of optochin with the “domain C” composed by wt or mutant Ala49Ser C-subunits indicate that the complex with the wt variant of “domain C” is more stable. Despite the fact that there are no strong correlations between scoring functions and dissociation constants, usually the best scoring corresponds to the best constant. Thus, we can assert that the results of in silico modeling are consistent with the ideas about the role of amino acid substitutions in the C-subunit of the ATP synthase F<sub>0</sub> complex.

As mentioned at the beginning of this paper, there is no evident reason for the formation of resistance to optochin, because this drug is not used in clinical practice, so the question of the origin of optochin resistance is quite interesting. Without claiming to be an exhaustive coverage of this issue, note that the current concepts tend to associate the origin of optochin resistance with the use of antimalarial chemotherapy in endemic areas: actually, it was shown that there is a cross-resistance to the different antimalarial agents of the quinine family related to the mutations in C- or A-subunits of the ATP synthase F<sub>0</sub> complex [26, 29]. Also, it was demonstrated [30] that the exposure to subinhibitory concentrations of penicillin significantly increased the rate of mutation conferring the resistance of *S. pneumoniae* to optochin. The authors explained this phenomenon by the genetic nature of the *atpAC* mutations, mostly transversions, which are not efficiently repaired by the HexAB mismatch repair system. In contrast to the antimalarial drugs, penicillin and its derivatives are the antibiotics commonly used for the treatment of different infections, including pneumococcal infections, so it could be a reason for

*S. pneumoniae* to acquire mutations conferring resistance to optochin.

In conclusion, we would like to emphasize that the current manuscript aimed to provide only a brief overview of changes occurring in the genome of the optochin-resistant *S. pneumoniae* strain compared to the optochin-susceptible strains. A detailed study of the role of each changed protein is a challenge for future works.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical statement** Submission of manuscript: All authors have contributed sufficiently to the scientific work presented in this manuscript and, therefore, share collective responsibility for the results. All authors agree with the final version of the manuscript under submission. This manuscript is not under consideration by any other journal. No parts of the data have been previously submitted for publication.

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