ANALYSIS AND OPTIMIZATION OF THE SEQUENCING DATA PROCESSING FOR THE EFFECTIVE LOCALIZATION OF MUTATIONS RESPONSIBLE FOR THE ANTIBIOTIC RESISTANCE PROPERTY IN E.COLI ON THE EXAMPLE OF AMPICILLIN

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ABSTRACT

Antibiotic resistance has shown itself to be a complex, multifaceted and rapidly growing problem, despite the meager historical time since the beginning of industrial production and the active development of new antibiotics. This work is devoted to investigation of the antibiotic resistance mechanisms of a particular E. coli strain resistant to the antibiotic ampicillin.

The analysis was based on the alignment of the Illumina sequencing reads from shotgun sequencing of an ampicillin-resistant E. coli strain to the reference E.coli strain K-12 substrain MG1655 genome. Four identified missence single-nucleotide polymorphisms (SNPs) were found in the genes ftsI, acrB, mntP and envZ of the ampicillin-resistant strain and are supposedly involved in the mechanism of antibiotic resistance.

The mutated genes were examined to determine the mechanism of antibiotic resistance in each case. We conclude that in the treatment of a patient with this strain of E. coli Aminoglycosides, Ansamycins, Fluoroquinolones will be more reasonable and effective as a drug of choice with the obligatory condition of the sensitivity test.

Also, we present a tool automating a significant part of the performed work, which can ease the analysis of the antibiotics resistance mechanisms in other bacteria.

***K*eywords** Antibiotic resistance · Escherichia coli · Ampicillin · Variant calling

Contents

1 INTRODUCTION

A little less than a hundred years have passed since the official discovery of antibiotics, but numerous studies note the enormous impact of the phenomenon of antibiotic resistance not only on the health and duration of treatment of patients with antibiotic-resistant infections, but also on their quality of life.[1][2][3]. Moreover, this phenomenon makes an obvious and critical impact on the pharmaceutical industry, the economy and many other areas of society, which is emphasized by the World Health Organization [4]. It is widely agreed that in spite of continuous gathering of new knowledge, enabling the design of novel, even more effective therapeutics to combat bacterial infection, and the development of new research tools, antibiotic resistance remains a worldwide health care problem. [20].

In this regard, it is of extreme importance to understand the mechanisms of antibiotic resistance, timely monitor trends in its occurrence, and be conscious in choosing the type of antibiotics, prescribing, taking and selling them.

The main mechanisms of antibiotic resistance in bacteria include the following adaptations:

1. The target protein change yielding the lost of the antibiotic's ability to bind;
2. Inactivation or modification of the antibiotic itself (e.g. β-lactamase encoding);
3. Metabolic shunts;
4. Reducing the amount of the drug in the cell;
    - Kicking the drug out of the cell (efflux pumps)
    - Decreasing the permeability, so that the drug cannot enter the cell (altering pores to block hydrophilic drugs, altering membrane to block hydrophobic drugs).

Due to its relevance, the topic is very popular [5][6][7][8][20].

The purpose of this study was to find and understand the correlations between mutations in certain genes of an ampicillin-resistant strain of E. coli and the participation of products of these genes in the ampicillin resistance, to suggest ways to resolve E. coli ampicillin-resistance in clinical practice.

Another result of our work is a tool automating a significant part of the implemented research, which can be used to ease the analysis of the antibiotics resistance property in other bacteria.

2 METHODS

All the work was done in the bash [9] shell.

2.1 RAW DATA COLLECTION

The reference sequence of the parental (unevolved, not resistant to antibiotics) E.coli strain K-12 substrain MG1655 and the annotation were downloaded from NCBI FTP [10]

The Illumina sequencing reads from shotgun sequencing of an E. coli strain that is resistant to the antibiotic ampicillin were downloaded from `https://figshare.com/` [11]:

2.2 INSPECTING RAW SEQUENCING DATA WITH FASTQC

The extracted unmapped reads were processed with the FastQC [12] software (v0.11.9) for quality control (with the default parameters).

2.3 FILTERING THE READS

The FastQC report showed poor quality statistics for the base sequence. We used the Trimmomatic [13] software (v.39) to improve the overall quality of the sequencing read and obtain more representative subsequent analysis steps. We used the **trimmomatic** command with the following parameters:

```
trimmomatic␣PE␣-phred33␣2:30:10␣LEADING:20␣TRAILING:20␣SLIDINGWINDOW:10:20␣MINLEN:20
```

Raw sequence data had drops in the average PHRED quality score below 30. The average read quality was increased by trimming to a PHRED score of above 30. The data quality comparison before and after using the Trimmomatic software is illustrated in the Figures 1a, 2a, 3a and 4a.

It is worth mentioning that by altering the parameters of Trimmomatic even better overall quality can be obtained. For example, with the following parameters the average read quality can be increased up to a PHRED score of above 32 and higher.

```
trimmomatic␣PE␣-phred33␣2:30:10␣LEADING:30␣TRAILING:30␣SLIDINGWINDOW:10:30␣MINLEN:30
```

However, this decreases the depth of the covering as a trade-off, and we preferred the former parameters.

2.4 ALIGNING SEQUENCES TO REFERENCE

The BWA-MEM [14] software (0.7.17-r1188) was used to index the reference file and then to align the trimmed, paired sequences to the reference genome (we used the default parameters). The resulting .sam file was compressed with the samtools software [15] (v.1.16.1 (using htslib 1.16)) with the parameters -S (for Statistics) and -b (for converting a .sam file to a .bam file).

We also used the **samtools flagstat** command (with default parameters) to get some basic statistics: we got 891649 (99.87%) mapped reads and 1127 (0.13%) unmapped reads.

The resulting .bam file was then sorted and indexed with the **samtools sort** and **samtools index** commands (with the default parameters)

2.5 VARIANT CALLING

We used the **samtools mpileup** command (with the parameters -f, –fasta-ref for faidx indexed reference sequence file) to make a mpileup intermediate file.

Then we used the VarScan [16] software (v.2.4.4) to call actual variants. The command **varscan mpileup2snp** (with the parameters –min-var-freq 0.2 –variants –output-vcf 1) was used to identify SNPs from the pileup file and the command **varscan pileup2indel** (with the parameters –min-var-freq 0.2 –variants –output-vcf 1) was used to identify indels from the pileup file.

2.6 AUTOMATIC SNP ANNOTATION

We used SnpEff Genomic variant annotations and functional effect prediction toolbox [17] (v.5.1d (build 2022-04-19 15:49), by Pablo Cingolani) for automatic SNP annotation. The file containing both annotation and sequence of reference genome, required to build a custom database, was downloaded from NCBI FTP [10] Then, we created database with the following steps:

1. Creating text file snpeff.config with one string: "k12.genome ecoli_K12"
2. Creating folder for the database and putting there unzipped .gbk file.
3. Creating database:

   ```
   snpEff␣build
   ```

   with parameters -genbank (use GenBank format).
4. Annotation:

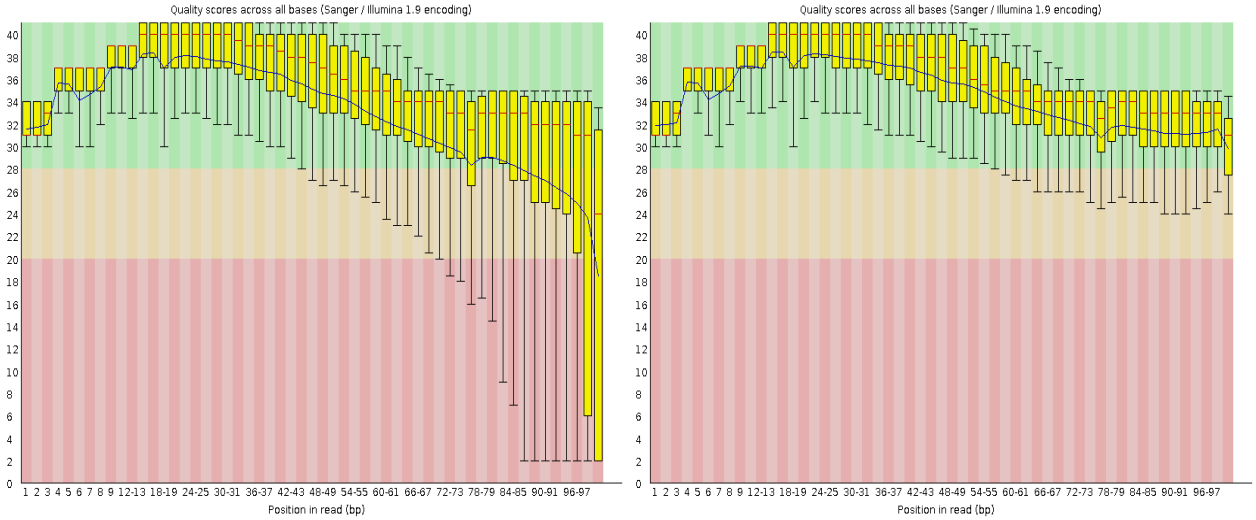
   ```
   snpEff␣build␣ann
   ```

   redirecting command output to a new .vcf file with additional field "ANN" (for "annotation"), describing all the effects for each SNP.

3 RESULTS

3.1 RAW READS QUALITY IMPROVEMENT

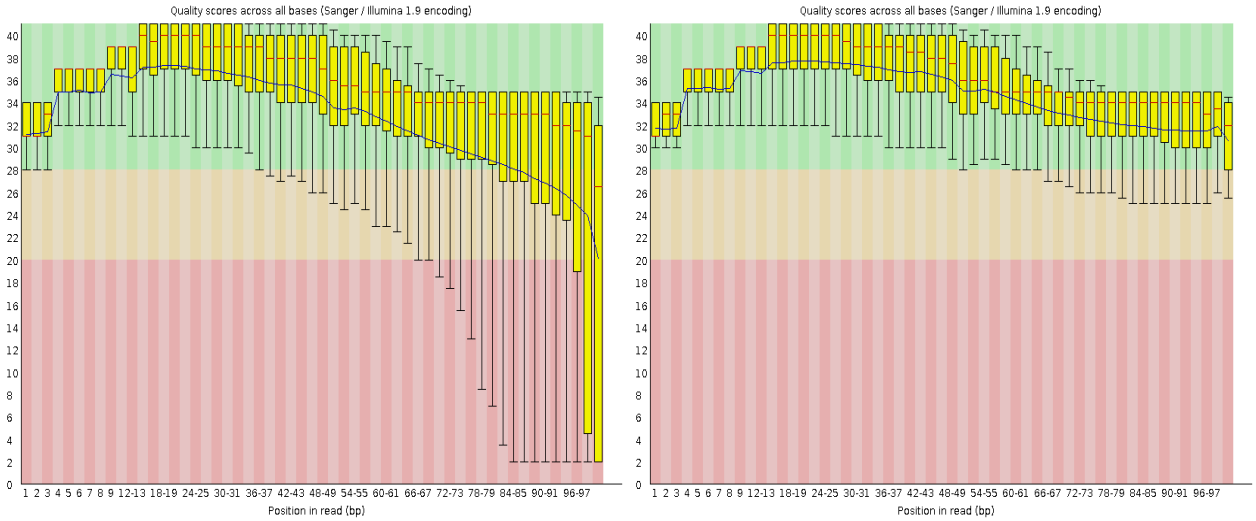
FastQC visualization before and after running Trimmomatic on raw Illumina sequencing reads from shotgun sequencing of an E. coli strain that is resistant to the antibiotic ampicillin.



(a) forward reads before trimming

(b) forward reads after trimming

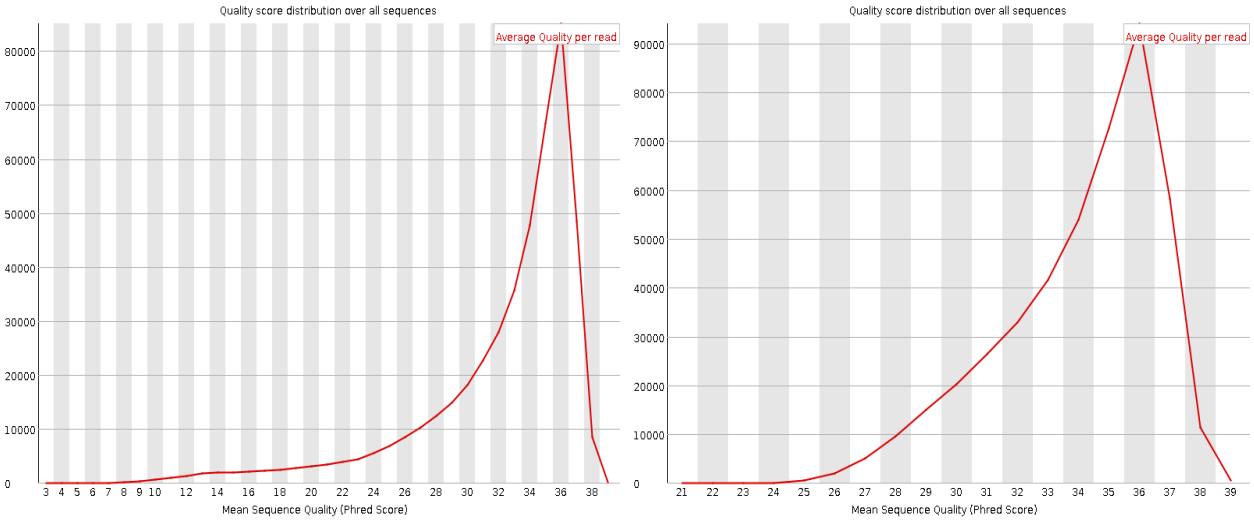
Figure 1: FastQC report: per base sequence quality of Illumina sequencing forward reads before and after filtering with Trimmomatic



(a) reverse reads before trimming

(b) reverse reads after trimming

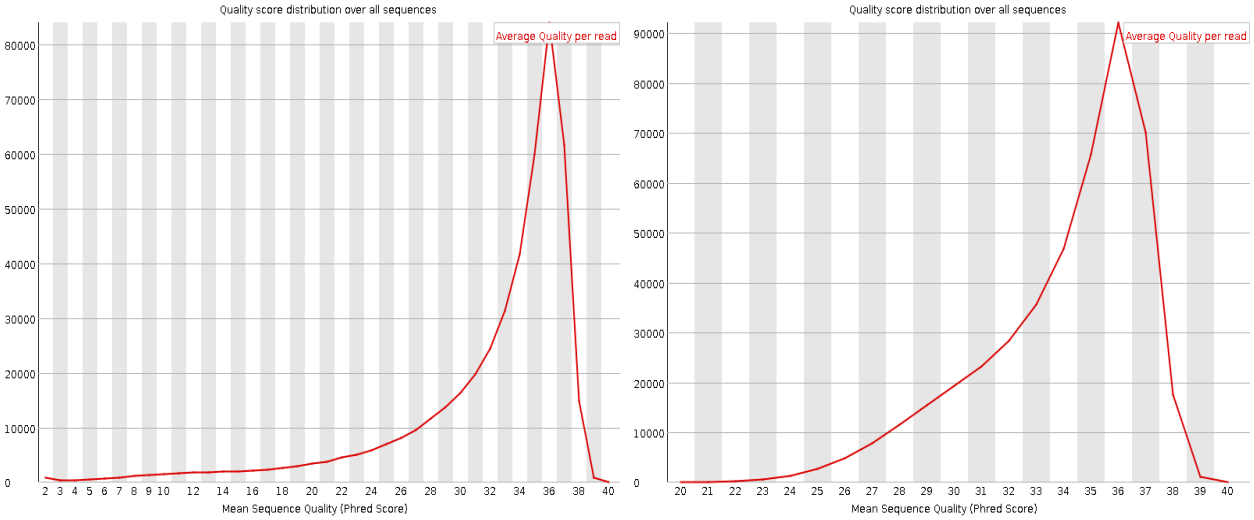
Figure 2: FastQC report: per base sequence quality of Illumina sequencing reverse reads before and after filtering with Trimmomatic



(a) reverse reads before trimming

(b) reverse reads after trimming

Figure 3: FastQC report: per sequence quality scores of Illumina sequencing forward reads before and after filtering with Trimmomatic



(a) reverse reads before trimming

(b) reverse reads after trimming

Figure 4: FastQC report: per sequence quality scores of Illumina sequencing reverse reads before and after filtering with Trimmomatic

3.2 CONTROL OF THE NUMBER OF READS DURING DATA PROCESSING

We started working with 455876 reads in each of forward and reverse raw reads and 445524 (97,73%) of them both survived after filtering with Trimmomatic. Then, there are 890190 (99.87%) mapped reads received after aligning to reference. These results are summarized in Table 3.2.

Table 3.2: Changing the number of reads during data processing

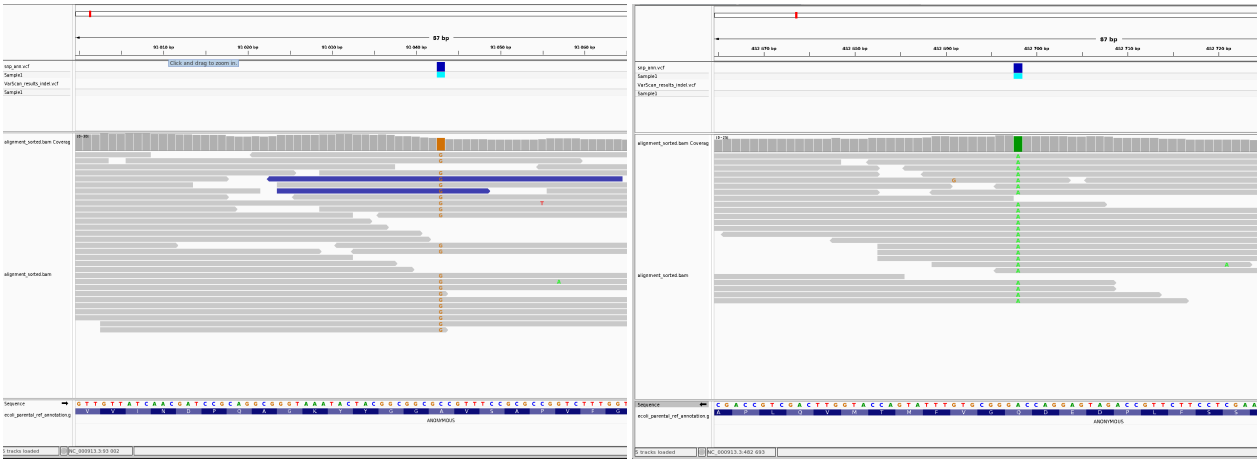
heightStage	File content	File type	Number of reads
2.1 RAW DATA COLLECTION	Illumina sequencing reads of an ampicillin -resistant E. coli strain: forward	.fasta	455876
2.1 RAW DATA COLLECTION	Illumina sequencing reads of an ampicillin-resistant E. coli strain: reverse	.fasta	455876
2.3 FILTERING THE READS	Reads both survived after Trimmomatic filtering	.fastq	445524
2.3 FILTERING THE READS		.fastq	445524
2.4 ALIGNING READS TO REFERENCE	Reads mapped to reference after alignment	.bam	890190

3.3 DATA VISUALIZATION

For data visualization and analysis we used Integrative Genomics Viewer (IGV) [18] [19] software (v.2.14.1 09/02/2022 12:22 AM). Reads were mapped to the reference and scanned manually and automatically (using SnpEff Genomic variant annotations and functional effect prediction toolbox) to identify positions that likely contained mutations. The results of automatic data processing confirmed the results of manual analysis. We found four missence mutations in which were present in the genes ftsI, acrB, mntP, envZ, one mutation in non-protein-coding sequence in rybA region and one synonimous mutation in the gene rsgA. We also found three indels: deletion and insertion in pseudogenes and insertion in non-coding anonymous region. These results are summarized in Table 3.3.

Table 3.3: The SNPs analysis results

heightGene ID	Position	Reference (codon, amino acid)	Mutation (codon, amino acid)	Type of mutation	Gene
944799	93043	GCC (A)	GGC (G)	missence	ftsI
945108	482698	CAG (Q)	CTG (L)	missence	acrB
2847681	852762	TTT	TCT	non-protein-coding	rybA
946341	1905761	GGT (G)	GAT (D)	missence	mntP
947272	3535147	GTA (V)	GGA (G)	missence	envZ
948674	4390754	GCC (A)	GCA (A)	synonymous	rsgA

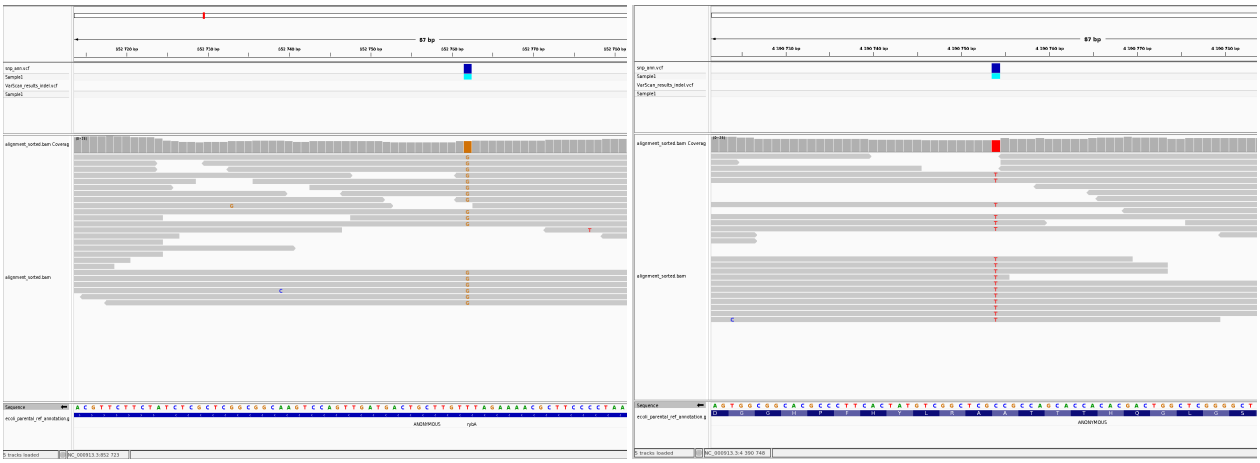


(a) missence mutation: gene ftsI (b) missence mutation: gene acrB

Figure 5: Missence mutations visualization: IGV

(a) missence mutation: gene mntP (b) missence mutation: gene envZ

Figure 6: Missence mutations visualization: IGV



(a) mutation in non-protein-coding sequence in rybA region (b) synonimous mutation in the gene rsgA

Figure 7: Mutation in non-protein-coding sequence in rybA region and synonimous mutation in the gene rsgA. visualization: IGV

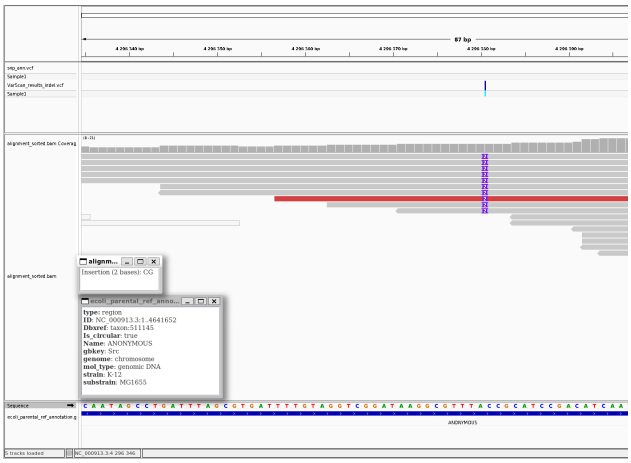
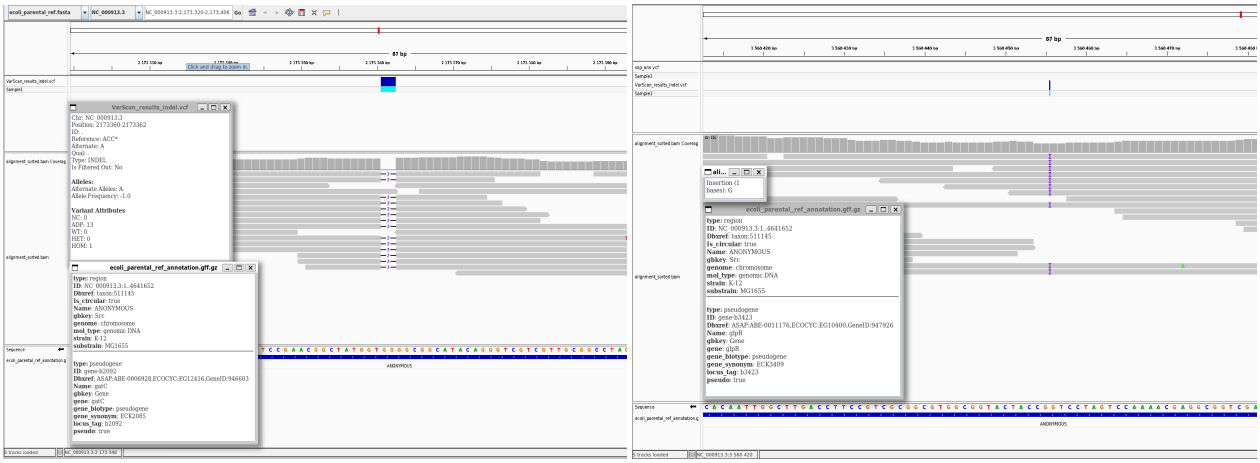


Figure 8: Insertion in non-coding anonymous region



(a) deletion in pseudogene

(b) insertion in pseudogene

Figure 8: Indels visualization in pseudogenes: IGV

4 DISCUSSION

4.1 DATA

As it can be seen from section 2 METHODS: 2.2 INSPECTING RAW SEQUENCING DATA WITH FASTQC, we received fairly good quality raw data. The FastQC report showed unusual indicator only for per base sequence quality for both raw forward and raw reverse reads, and for of per tile sequence quality for raw forward reads. The latter cannot be corrected, since it is very similar to be a quality problem directly on the cartridge during sequencing. However, the other indicators are good and in addition and the fact that this problem did not drag on to the end of the tile and went away, we can accept this data for the experiment. As for per base sequence quality, we managed to improve this indicator to excellent values due to Trimmomatic software filtering.
Indeed, further verification of the change in the number of reads during the experiment (section 3 of RESULTS: 3.2 CONTROL OF THE NUMBER OF READS DURING DATA PROCESSING) showed that the number of raw reads coincided with Basic Statistics from FastQC Report: 455876 for each of the raw forward and reverse reads, 445524 for each of forward and reverse reads after processing by Trimmomatic software. After alignment of reads to the reference genome, we also got a very high mapping percentage: 890190 (99.87%). Thus, we can count on fairly accurate and reliable further data processing steps.

4.2 VISUALIZATION. MANUAL AND AUTOMATIC DATA ANALYSIS

How it can be concluded from the section RESULTS: 3.3 DATA VISUALIZATION, due to the small number of mutations found, we were able to examine them manually and then confirm these results using the SnpEff software.
We have focused on four missense mutations in genes of ftsI (peptidoglycan DD-transpeptidase FtsI), acrB (multidrug efflux pump RND permease AcrB), mntP (Mn(2(+)) exporter) and envZ (sensor histidine kinase EnvZ) as those that could provide enough data and evidence to suggest the effect of their products on ampicillin resistance within the framework of the materials and methods of this work.

4.3 ftsI

The ftsI-encoded multimodular class B penicillin-binding protein 3 (PBP3) is a key element of the cell septation machinery of Escherichia coli. [24] It enables penicillin binding processes, peptidase activity, enables peptidoglycan glycosyltransferase activity, enables serine-type D-Ala-D-Ala carboxypeptidase activity, is involved in cell wall organization [10]. All this functions are very important for ampicillin-resistance mechanisms. Ampicillin belongs to the group of β-lactam antibiotics, which act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity.[21]. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by DD-transpeptidases, also known as penicillin binding proteins (PBPs). PBPs vary in their affinity for penicillin and other β-lactam antibiotics. The number of PBPs varies between bacterial species [22]. Escherichia coli has six murein synthases which enlarge the sacculus by transglycosylation and transpeptidation of lipid II precursor. A set of twelve periplasmic murein hydrolases (autolysins) release murein fragments during cell

growth and division. [23]. Many authors confirm that PBP3 is an essential cell-division protein for E. coli. Kong KF et al. note that supporting evidence for its role in cell division came from the use of PBP3-specific β-lactam antibiotics, furazlocillin and piperacillin. PBP3 is recruited to the septal ring where it functions as a transpeptidase to crosslink the peptidoglycan at the division septum.
Thus, we have reason to assume that this mutation is involved in the mechanism of resistance due to decreasing in the affinity of PBP for antibiotics, in other words, is is target site alteration mechanism.
NB: we mentioned piperacillin, also belonging to the class of beta-lactam antibiotics. Aedo SJ came to similar conclusions about piperacillin and ampicillin-resistance in E. coli in their work. [25]

4.4 acrB

The multidrug efflux pump RND permease AcrB has been studied most intensively as the prototype of RND pumps. It has an extremely wide specificity, including practically all types of antibacterial agents (except aminoglycosides), detergents, microbicides, dyes, free fatty acids, and even simple solvents. [26] AcrB is one of the key components of multidrug resistance (MDR) system. The MDR system effectively expels antibiotics out of bacteria causing serious issues during bacterial infection. [27] Seeger MA et al. also notes that in E. coli, the tripartite efflux system AcrA/AcrB/TolC is the pump in charge of the efflux of multiple antibiotics. [7]

Li XZ et al. noted that there has been a steady increase in the resistance of E. coli isolates to the agents mentioned above. In a survey covering 30 years of isolates in Sweden, the prevalence of isolates showing "non-wild-type" MICs (minimum inhibitory concentration) of ciprofloxacin increased from 0% to 40% in 2009. Drugs that have become essentially useless in recent years include ampicillin (70% showing non-wild-type MIC values), tetracyclines, and trimethoprim (up to 60%). It is even more alarming that these statistics are from Sweden, a country with one of the lowest frequencies of drug-resistant bacteria. The prevalence of E. coli isolates resistant to extended-spectrum cephalosporins was 4.4% in Sweden in 2012 but was much higher in some other European countries, e.g., 31% in Slovenia. Efflux mechanisms likely contribute to such a rapid emergence of resistance in the presence of antimicrobial selection pressure [26]. These facts are very interesting for us in the framework of our work and confirm the involvement of a mutation in the acrB gene in the development of the mechanism of ampicillin resistance in Escherichia coli. Thus, it is very likely that the mutant acrB gene is involved in the mechanism of ampicillin resistance in Escherichia coli as an efflux pump.

4.5 mntP

The mntP Mn(2(+)) exporter enables manganese ion transmembrane transporter activity, involved in ion transport. [10] Foti C et al. notes the following: due to the widespread use of aminopenicillins as antibiotics, knowledge of their complexation behavior and speciation is of fundamental importance for understanding their pharmacokinetics and pharmacodynamics. For the correct assessment of the bioavailability of a drug or a trace element in the presence of that drug, it is essential to know the possible interactions occurring in body fluids. Accordingly, the knowledge of speciation and sequestering ability of two drugs employed as antibiotics, such as ampicillin and amoxicillin with Mn2+, are necessary to evaluate the action mechanism of these ligands and the bioavailability of the metal cation in the presence of the drugs under physiological conditions. In this work the sequestering ability of ampicillin and amoxicillin towards Mn2+ was evaluated under different conditions, including physiological ones. The assessment of the sequestering capacity is of fundamental importance as it takes into account the competitive processes of the metal cation and the ligand and facilitates comparisons among several ligands towards the same metal cation or the same ligand towards several cations under the same conditions of pH, temperature and ionic strength.[28]
Vera Valovicova et al. describe the successful preparation of the laboratory-obtained manganese dioxide/montmorillonite (MnO2/MMT) composite for removal of two types of antibiotics – amoxicillin and ampicillin – from aqueous solution and demonstrated the removal of two types of broad-spectrum β-lactam antibiotics from aqueous solution to a sufficient degree.[29]
The association of a mutation in this gene with antibiotic resistance is not as obvious as in the case of other genes. However, these works and our observation of a persistent missense mutation in this region in an ampicillin-resistant strain of Escherichia coli are the reason for further more detailed studies in order to confirm or refute our assumption about the involvement of this gene in the mechanism of antibiotic resistance, as a hypothesis, as an alter metabolic pathway to compensate.

4.6 envZ

The sensor histidine kinase EnvZ is an osmosensing histidine kinase located in the inner membrane, and one of the most extensively studied Escherichia coli histidine kinases. [30] It enables phosphoprotein phosphatase activity, phosphorelay

sensor kinase activity, protein histidine kinase activity. [10] Li M et al mention that in recent years, the active efflux mechanism is the main reason for the multiple drug resistance of bacteria. Since most of the effluent system transports substrates widely, and many active effluent systems can exist in the same bacteria, this system can lead to bacterial resistance to various antibacterial drugs with completely different structures, namely multiple resistance. In Marlen Adler's study, mutations in the ftsI gene alone did not increase antibiotic resistance, whereas ftsI and envZ gene mutations increased the MIC of antibiotics multiple times. In passive defense, bacteria make itself dormant, reduce the vitality of life and block the combination of antibiotics and target to reduce the killing effect of antibiotics. In active defense, they increase the activity of efflux pump to increase the efflux of antibiotics and reduce the accumulation of antibiotics in bacteria, thereby reducing the killing effect of antibiotics on bacteria. This study suggests that the resistance of E. coli to AMP is a combination of active defense systems and passive defense systems. Drug resistance can occur shortly before the bacterial MIC value reaches the drug resistance threshold. Genes frdD, ftsI, acrB, OmpD, marR, VgrG, and envZ are associated with ampicillin resistance.[5]

Heininger A point to another possible realization of the same mechanism: the Escherichia coli sensor kinase EnvZ modulates porin expression in response to various stimuli, including extracellular osmolarity, the presence of procaine and interaction with an accessory protein, MzrA. Two major outer membrane porins, OmpF and OmpC, act as passive diffusion-limited pores that allow compounds, including certain classes of antibiotics such as β-lactams and fluoroquinolones, to enter the bacterial cell. Even though the mechanisms by which EnvZ detects and processes the presence of various stimuli are a fundamental component of microbial physiology, they are not yet fully understood. [31]
Thus, we can conclude that envZ is part of two-component systems (TCS), which interferes with gene expressions when environmental stresses require a bacterial adaptation/defense. TCS systems (CpxAR, Rcs, BaeSR, PhoPQ, and EnvZ/OmpR) (RocS2-RocA2, ParR-ParS, AmgR-AmgS, CzcR-CzcS and CopR-CopS) can sense the external medium modifications such as pH, osmotic strength, oxidative stress, nutrient starvation, and toxic chemicals, etc. [32]. It can respond to the presence of ampicillin and regulate the activity of other genes such as PBP of ftsI, efflux pomp of gene acrB, porins of OmpF and OmpC (decreasing membrane permeability).

4.7 POTENTIAL SOLUTIONS TO THE PROBLEM OF ANTIBIOTIC RESISTANCE

Summarizing all of the above, we can conclude that the current situation with antibiotic resistance in bacteria looks alarming. Massive ill-considered prescribing and use of antibiotics, self-medication and poor control over the antibiotics dispense has led to the emergence of antibiotic-resistant strains of bacteria around the world. This situation must be dealt with on a global scale, this problem requires strict adherence to the advice of the international health organization, responsible and serious attitude of doctors, patients and legislators.
With respect to the specific case of E. coli resistance to ampicillin, the solution to this problem depends on the course of the disease, anamnesis, concomitant diseases. A doctor should not prescribe antibacterial drugs, especially broad-spectrum antibiotics for mild and erased disease. We believe that in this case it would be enough to follow a diet and take probiotics to maintain normal intestinal flora. However, in severe and moderate form of the course of the disease, we consider it necessary to combine therapy with the restoration and maintenance of the water-salt balance, enterosorbents and probiotics. In this case, antibiotics are indicated, however, to prescribe them, it is imperative to perform sensitivity tests to drugs, which in this case can be antibiotics with other mechanisms of action: Aminoglycosides (binding to the bacterial ribosomal subunit, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, leaving the bacterium unable to synthesize proteins vital to its growth.), Ansamycins (Block DNA transcription, either via inhibiting DNA-dependent RNA polymerase by binding to the β-subunit), Fluoroquinolones (Inhibits the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription).

4.8 SUPPLEMENTARY

The lab journal with the detailed pipeline, the tool for automating the process and all other supplementary data can be found in the github repository `https://github.com/IlyaOlkhovsky/BI_Project_1`.

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