Cytochrome C Oxidase is One of the Key Enzymes Providing the Ability to Synthesize Phenazines in Pseudomonas Chlororaphis Subsp. Aurantiaca

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Abstract

Phenazines are heteroaromatic compounds consist of a central pyrazine ring fused with two benzenes. Various functional groups linked to the dibenzopyrasine core cause differences in chemical, physical and biological properties of phenazines. The interest to these substances does not reduce in several decades. New biological activities and practical applications discovered in recent years force the researchers to study all aspects of phenazines synthesis, degradation and mechanisms of their action.

In this study, we demonstrated the involvement of *coxA* gene product (cytochrome *c* oxidase, su I) in phenazines biosynthesis in *P. chlororaphis* subsp. *aurantiaca.* Overlap PCR was used to knockout *coxA*gene and derived mutants were analyzed for their ability to grow on rich and minimal culture media, as well as for the phenazines production level. We showed that the product of *coxA* gene is necessary for the phenazines production in rich growth media. At the same time CoxA protein seems has no effect on phenazines production in M9 minimal salts medium. CoxA protein is one of the core proteins of large transmembrane protein complex cytochrome *c* oxidase found in bacteria, archaea, and mitochondria of eukaryotes. We demonstrated that the knockout of even one subunit of this complex multiunit protein leads to a significant decrease (to trace concentrations) or complete suppression of phenazine antibiotics production on rich PCA-medium in *P. chlororaphis* subsp. *aurantiaca*.

1. Introduction

Phenazine antibiotics (or phenazines) are a broad class of planar heterocyclic aromatic molecules with multiple biological activities. All phenazines and their derivatives can act as the electron shuttles and modulate the redox state of the cells. In natural environment these compounds are mainly synthesized by bacteria of *Pseudomonas, Streptomyces* species and a few other genera (Pierson 3rd and Pierson 2010). Some unique forms of phenazines are produced by marine bacteria and archaea. Nowadays there are many complex structures of phenazines which were chemically synthesized. Artificially synthesized phenazines, as well as natural products are actively studied now as the sources of pharmacological substances. These compounds demonstrate significant positive effects in the field of photodynamics, antifungal, antibacterial and antitumor therapy. Due to their ability to change the color depending on pH medium phenazines are used as efficient fluorescent probes to study the changes of biochemical processes inside cells and organisms (Yan et al. 2021).

The ability of phenazine-producing bacteria to suppress many plant-pathogenic microorganisms is also well known (Chin-A-Woeng et al. 2003; Yu et al. 2018). In eukaryotes phenazines can modify host cellular responses to environmental and internal clues. In plants, for example, these substances may influence growth and elicit induced systemic resistance (Bakker et al. 2007). But we still have lack of information concerning effects of phenazines on animal cells. Bacterial producers themselves are used phenazines for nutrient acquisition, signaling or inhibition of competitors, gene expression and redox balancing. In pathogenic species *Pseudomonas aeruginosa* one of phenazines, pyocyanin, is an important factor of virulence. It was demonstrated that pyocyanin synthesis aids survival of *P. aeruginosa* in antibiotic-exposed biofilms and confers a physiological condition of enhanced antibiotic tolerance (Schiessl et al. 2019).

Chemical synthesis of phenazines is quite expensive and complex, but it still could be useful to obtain derivatives with the desired activity or to enhance the special biological activity. Chemically synthesized phenazine derivatives are obtained in several-stage catalyzed reactions at high temperatures. Sometimes an inert atmosphere and expensive catalysts are also needed. The cheaper and more profitable way is to modify naturally synthesized phenazines purified from bacteria. The wider the diversity of natural phenazines, the more successful will be the attempts to create new perspective precursors for medicines of wide range of action. So the creation of natural producers of phenazines is still relevant.

The majority of bacterial producers need rich cultural media for phenazines production (Shanmugaiah et al. 2010). These compounds are commonly released during the stationary phase of bacterial culture growth. Their biosynthesis is tightly controlled on different cellular levels ranging from population densities to regulation via nutrients and feedback control. All producer`s genomes contain a conserved set of core biosynthesis genes *phzA/BCDEFG*, which provides synthesize one of two main phenazines that act as precursors for all other derivatives: phenazine-1-carboxylic acid or phenazine-1,6-dicarboxylic acid (Dar et al. 2020). There are several genes beyond the operon, which products are involved in modification of these precursors to form wide range of «simple» phenazines (Laursen and Nielsen 2004). Despite the intensive studies less is known about genes participating in formation of phenazines structures which includes vast isoprenoid side-chains and other complex substituents.

Phenazines are highly redox-active substances which could generate active oxygen species and promote susceptible organisms` death. The mode of phenazines action includes intercalation into DNA (and so that hindering DNA biosynthesis, replication or processing), apoptosis induction, and topoisomerase activity inhibition (Yan et al. 2021). There are a few reports that in natural environments there could be symbiotic relationships among organisms which help them to withstand phenazines` challenge (Dahlstrom and Newman 2021). Different *Mycobacterium* species possess several types of phenazine-degrading enzymes, some of which even help them to use phenazine-1-carboxylic acid as a sole carbon source (Costa et al. 2015). But the high concentrations of phenazines could be potentially toxic for producers as well. So the producers themselves must elaborate some kinds of protection against the high concentrations of their own phenazines. It is well known that the phenazines production in bacteria is associated with the great changes in producers` metabolism. The main point of these changes is the activation of different components of

antioxidant and excretion systems in the producers' cells (Veremeenko and Maksimova 2010; Zhao et al. 2021). Studying the genomes and metabolomes of already existing phenazine-producing strains could help us to understand the mechanisms of superproductivity formation.

Despite the existence of different modern techniques of producers` creation, chemical mutagenesis coupled with the appropriate selection strategy still remains one of the most effective. For many years the great disadvantage of this approach was that the researchers didn't know what genes had been exactly changed. Nowadays when NGS-technologies have come into practice it is possible to sequence the mutant strain genome and recognize the sites which have been changed. It opens the vast opportunities of discovering the new genes directly or indirectly enrolled in phenazines biosynthesis. The discovery of new genes at such approach demands not only the identification of mutation itself but also the reproduction of this mutation in wild type strain and precise analysis of produced phenotype.

In current research we studied in details one of genes which had been discovered as mutated in our former investigations in unique mutant strain of *Pseudomonas chlororaphis* subsp. *aurantiaca* B-162/17, capable of producing phenazines on minimal media (Liaudanskaya et al 2022) This strain contains several mutations but the deletion of four amino acid residues in the central part of cytochrome *c* oxidase subunit I was one of the most interesting issue. To date there was no direct evidence that cytochrome *c* oxidase may be somehow involved into phenazines production.

2. Materials And Methods

2.1. Bacterial strains and cultivation.

The wild type strain *P. chlororaphis* subsp. *aurantiaca* B-162, as well as mutant strain *P. chlororaphis* subsp. *aurantiaca* B-162/17 were obtained from the collection of the Department of Genetics, Belarusian State University. *Escherichia coli* strain BW 19851 was obtained from the collection of the Department of Microbiology, Belarusian State University.

Depending on the experimental goals the bacterial cultures were grown on LB Broth (Thermo Fisher Scientific, USA), Luria Broth agar (LB-powder, 1.5% agar, Thermo Fisher Scientific, USA), M9 minimal salts medium (4X) (KH_2PO_4 12 g/L, NaCl 2 g/L, Na_2HPO_4 24 g/L, NH_4Cl 4 g/L), and liquid PCA-medium (2% peptone from casein (AppliChem, Germany), 0.5% NaCl, 0.1% KNO3, 1% glucose, pH 7.2) (Levitch and Stadtman 1964), at 28°C (for *Pseudomonas* strains) or 37°C (for *Escherichia* strain). For phenazines extraction *P. chlororaphis* subsp. *aurantiaca* strains were cultivated without aeration, for 108 h.

2.2. Phenazines extraction

Phenazines extraction was conducted according to the method described in (Shapira et al. 2021). The presence of phenazine antibiotics was determined by absorbance at 369 nm (Taniguchi and Lindsey 2018).

2.3. DNA manipulation.

Genomic bacterial DNA was extracted using GeneJET Genomic DNA Purification Kit K0881 (Thermo Scientific, USA). In order to create deletion of *coxA* gene two pairs of primers F1 (Fragment 1.FOR)–R1 (Fragment 1.REV) and F2 (Fragment 2.FOR)–R2 (Fragment 2. REV) were designed (Table 1). Upstream Fragment 1 (602 bp) and downstream Fragment 2 (654 bp) were first amplified by polymerase chain reactions (PCR). A DNA thermal cycler CFX96™ system (Bio-Rad Co.,USA) was used for amplification. Fragment 1 and Fragment 2 were extracted from 1% agarose gel and purified using ArtDNA MiniSpin Gel kit (ArtBiotech, Belarus). A 1236 bp fragment fusion (Fragment 3) was amplified by overlap PCR. The fusion fragment (Fragment 3) was cloned into pKNG101 on BamHI restriction sites, creating the recombinant plasmid pKNG101/coxA$^-$.

Table 1
Primers used in this study

Primer name	Sequence*	Description
F1 (Fragment 1.FOR)	gc**ggatcc**cctgtatcgcggcca	contains BamHI restriction site (in bold)
R1 (Fragment 1.REV)	**tgttcatttcacttccggcg**tcgctcacagcgtt	contains a complementary region to the *coxC*-gene, polypeptide I (in bold)
F2 (Fragment 2.FOR)	cgccggaagtgaaatgaaca	
R2 (Fragment 2. REV)	gc**ggatcc**tccttatcgggcacgctt	contains BamHI restriction site (in bold)

*All primers were designed according to the sequence of *P. chlororaphis* subsp. *aurantiaca* B-162 (accession number CP050510.1).

The pKNG101/coxA$^-$ plasmid was transferred to *E. coli* BW 19851 by $CaCl_2$-transformation. Then biparental mating between *E. coli* BW 19851 and *P. chlororaphis* subsp. *aurantiaca* B-162 generated several mutant strains. To ensure accuracy, PCR analysis and sequencing were used to confirm the deletion.

2.4. PCR protocols.

PCR was carried out in a mixture of standard composition (Ausubel et al. 1993). The PCR-program was initial denaturation of 3 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C; and final extension for 3 min at 72°C for Fragment 1. Same PCR cycles were used for Fragment 2 amplification, except annealing temp. of 59°C. For overlap PCR primers F1 and R2 were used and Fragment 1 and Fragment 2 were used as a temple (instead of genomic DNA). The PCR-program for overlap PCR was initial denaturation of 3 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; and final extension for 3 min at 72°C. Binding sites for all primers are shown in Fig. 1.

All PCR-products were run on 1% agarose gel and visualized using UV transilluminator.

2.5. DNA sequencing

DNA sequencing was made on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

2.6. Bioinformatic techniques.

I-TASSER online platform was used for proteins structure prediction and structure-based function annotation (https://zhanggroup.org/I-TASSER/). The STRING (version 11.5) database and web resource was used to predict functional protein-protein network (https://string-db.org/). An open source software platform Cytoscape was used for visualizing molecular interaction networks and biological pathways.

3. Results And Discussion

In current research we tried to clarify the predicted role of cytochrome *c* oxidase in phenazine antibiotics synthesis in strains-producers *P. chlororaphis* subsp. *aurantiaca* B-162. These chemical compounds are considered to be the promising sources of next-generation antimicrobial and anticancer medicines. The development of such medicines is due to the increasing antibiotic resistance of pathogenic microorganisms and the emergence of multidrug resistant tumors. The introduction of new compounds for medical uses requires an accurate preliminary analysis of mechanisms of their action on cells of pro- and eukaryotic organisms, as well as the prediction of possible modes of resistance development. The natural resistance to phenazines in strains-producers might be an additional excellent model for prognosis of key steps of resistance development. The mechanism of phenazines` resistance in phenazine-synthesizing bacteria is not characterized well enough, as well as the mechanism of high productivity formation. We strongly believe that these two mechanisms are directly related.

In our former researches chemical mutagenesis followed by selection was used to derive the mutant strain *P. chlororaphis* subsp. *aurantiaca* B-162/17, capable of producing phenazines on minimal media (Shapira et al. 2021). We have also shown that this mutant strain demonstrated lower level of productivity on rich PCA-medium than the parental strain B-162/255, but it was still much higher than the wild type strain B-162 (Shapira et al. 2021). To identify mutations which could lead to such phenotype we made whole-genome sequencing and comparison of genomes of strains B-162/255 and B-162/17. The comparison of genome sequences of B-162/255 and B-162/17 strains helped us to discover the potentially novel candidate genes, which products might be involved in phenazine biosynthesis (Liaudanskaya et al 2022). Of the most interesting mutations there were two deletions directly influenced genes sequences (Liaudanskaya et al 2022). These deletions interfere with the sequence of cytochrome *c* oxidase subunit I gene (*coxA* gene) and arginine N-succinyltransferase, α-subunit gene (*astA* gene). In current research we focused on the *coxA* gene and its influence on phenazines production in *P. chlororaphis* subsp. *aurantiaca.*

It is known that *Pseudomonas species* possess one A-type (caa_3, *cox*-genes products) and multiple C-type (cbb_3) cytochrome *c* oxidases as well as two quinol oxidases for aerobic respiration (Osamura et al. 2017). These enzymes are the components of energy-converting chain that catalyzes the reduction of oxygen to water, thus participating in the buildup of the proton gradient across the cell membrane required for ATP synthesis (Kadenbach,2018). Cytochrome *c* oxidase is a large transmembrane protein, composed of several peptide chains possessing different activities. This complex can be found in prokaryotes and inside the mitochondria of eukaryotes.

In *Pseudomonas aeruginosa* A-type cytochrome *c* oxidases (*cox*-genes products) are expressed only under specific growth conditions (for example, nutrient starvation, and combination of starvation and high oxygen conditions) (Kadenbach 2018). A-type proteins are the members of the heme-copper oxidase superfamily and are closely related to the mitochondrial terminal oxidases (Pereira et al. 2001). Functionally active A-type enzyme in *Pseudomonas* species consists of three subunits. Subunit I is the main catalytic subunit of the enzyme, which forms the binuclear heme-Cu_B center. This center is responsible for O_2 binding and reduction. Subunit I (su I) also contains a second heme, which is necessary to facilitate the transfer of electrons to the binuclear center (Garcia-Horsman et al. 1994). The expression of *cox*-genes cluster is regulated by stationary phase sigma factor, RpoS (Kawakami et al. 2010). The same transcriptional activator is required for optimal phenazines $synthesis (Girardet al.\ 2006).\ Itmeanst\hat{t}\ hehighestconcentrationofC \otimes\ -\ prote \in swillc$ or $relatewiththe\pi ckofphenaz \in es$ concentration.

3.1. Analysis of CoxA proteins in *P. chlororaphis* subsp. *aurantiaca* B-162 and B-162/17 strains.

According to the data of NGS-sequencing, the mutation in *coxA* of B-162/17 strain caused the deletion of 12 nucleotides without a frameshift, which led to the loss of 4 amino acids in the middle part of the protein (Fig. 2) (Liaudanskaya et al 2022).

The amino acids (228-231 positions) which were deleted in B-162/17 strain are marked in red

The deleted amino acids are the part of one of the transmembrane regions of cytochrome *c* oxidase su I which is made up of hydrophobic α-helices (Fig. 3).

Deleted amino acids (228-231 positions) are marked with the blue square. Flanking amino acids V and M are underlined with red. Small pink squares on 3-D models mark the V (position 227 in normal protein sequence) and M (position 232 in normal protein sequence) amino acids flanking the deletion site.

Correct α-helices formation is important for overall proteins structure, dynamics and regulation of their functions. Even subtle changes in the amino acid sequence of these structural elements can alter protein function and its interaction with the membrane and with the other parts of polypeptide cytochrome *c* oxidase complexes. In cytochrome *c* oxidase su I these elements of secondary structure are necessary for the formation of 12 transmembrane segments which form one of the key parts of the minimal functional unit (oxygen reducing part) (Branden et al. 2006). We clarified that according to the data of Protein Blast sequence analysis, deleted amino acid residues TM and L are highly conserved among the most well studied in this regard prokaryotes *Paracoccus denitrificans* and *Rhodobacter sphaeroides*. The same residues are highly conserved among yeast, which enzyme is similar to the human enzyme (Bratton et al. 2003), and *Homo sapiens* cytochrome *c* oxidase su I (48 % of identity). It means that these residues have an important biological function and might be engaged into assembly or functionality of the minimal functional unit. In *R. sphaeroides* these residues are located in helix V, which are supposed not to be involved into metal ligands binding and redox-active centers formation (Calhoun et al. 1994). But this subunit is still might be engaged into H-channel formation, which is the unique proton pump pathway in the mammalian mitochondrial oxidase and prokaryotic enzyme as well (Wikström et al. 2018). Plenty of mutations in cytochrome *c* oxidase su I reported in human patients suffering from a range of disorders had no direct effect on the catalytic activity of the enzyme but supposed to have influence on the whole enzyme complex assembly and interaction among subunits (Bratton et al. 2003). As it is obvious from 3-D models (Fig. 3), the distance between flanking amino acid residues V and M became shorter in mutant strain. We speculated that these changes might alter su I folding or its interaction with su II-III or membrane. Such changes in CoxA structure of B-162/17 strain potentially might be the reason for its lower phenazines yield on rich media comparing to parent strain B-162/255.

3.2. The role of cytochrome c oxidase subunit I in phenazines synthesis.

To further clarify the possible role of cytochrome *c* oxidase su I in phenazines synthesis in *P. chlororaphis* subsp. *aurantiaca* we knockouted the *coxA* gene in wild type strain B-162. The overlap PCR was used for this purpose. The schematic description of the experiment and the primers design are shown in Fig 4 (a–c).

Building the deletion of full-length *coxA* gene required two primer pairs. The first primer pair (F1 (Fragment 1.FOR) and R1 (Fragment 1.REV)) was target the genomic region upstream of the desired deletion point, and the second primer pair (F2 (Fragment 2.FOR) and R2 (Fragment 2. REV)) was target the genomic region downstream of the desired deletion point. The pair of primers F1 (Fragment 1.FOR) and R2 (Fragment 2. REV)) was used to create full-length construction for *coxA* knockout and for further identification of deletion mutants. The first primer pair was used to receive Fragment 1 (602 b.p.) and the second primer pair was used to receive Fragment 2 (654 b.p.).

Primers F1 (Fragment 1.FOR) and R2 (Fragment 2. REV) were used to receive Fragment 3. Fragment 1 and 2 were used as the templates in this PCR reaction. Fragment 3 included last 475 bp of 3`-end of cytochrome *c* oxidase su II gene, whole gene of cytochrome oxidase biogenesis protein Cox11-CtaG and several nucleotides of intergenic region between Cox11-CtaG gene and cytochrome *c* oxidase su III gene, which flanked *coxA* gene, but there was no *coxA* gene in its structure. Fragment 3 was cloned into pKNG101 plasmid into the BamHI sites, resulting pKNG101/coxA$^-$ recombinant plasmid. This recombinant plasmid was transferred into *E. coli* BW 19851 and clones were selected for ampicillin resistance.

The resulting plasmid pKNG101/coxA$^-$ was introduced into the *P. chlororaphis* subsp. *aurantiaca* B-162 by biparental mating. Then *P. chlororaphis* subsp. *aurantiaca* B-162 strains with the plasmid pKNG101/coxA$^-$ were selected on LB agar plates with streptomycin and ampicillin. The final step of knockout procedure was to select against merodiploids and to identify the desired mutants. For this purpose colonies from the previous stage were streaked on no-salt LB agar that contains 15% (wt/vol) sucrose. We managed to select 6 strains on no-salt LB agar with 15% sucrose (presumably B-162/coxA$^-$ strains). Then each single colony isolated on sucrose agar was screened for the *coxA* deletion. PCR analysis was *carried out* to identify the mutant allele. PCR products, which corresponded to the size of the mutant allele and that had been generated from sucrose resistant and antibiotic sensitive colonies, were subsequently sent for Sanger sequencing to confirm the mutation.

It was shown that all mutant strains have a culture density and CFU comparable to that of the wild type strain, so the viability of the mutant strain was comparable to that of wild type strain. We speculate that the normal viability of mutant strains could be explained by the existence and activity of the other types of cytochrome *c* oxidases in *Pseudomonas* genome, which are usually active under the normal growth conditions (Osamura et al. 2017). These enzymes might completely or partially replace the function of the knockouted gene in such conditions. We next analyzed the level of phenazines production in 6 resulting B-162/coxA$^-$-strains. Analysis revealed that practically all of the B-162/coxA$^-$-strains lost the ability to produce phenazines in rich PCA-medium. Only one of B-162/coxA$^-$strains (strain № 6) generated up to 26.5 mg/L after 5 days of cultivation that is 38 times

lower than the wild-type strain B-162 (Fig. 5). This mutation didn`t let to acquire the ability to produce phenazines in M9 minimal salts medium as well.

The loss of the ability to produce phenazines in rich PCA-medium by the B-162/coxA$^-$–strains demonstrated the existence of the connection between phenazines biosynthesis and energy-converting chain. Previously it was shown that cbb3-type cytochrome oxidase subunit had supported *Pseudomonas aeruginosa* biofilm growth (Jo et al. 2017). Biofilm formation is controlled by the PhzR/I QS that is also critical for phz-operon activity (Pierson 3rd and Pierson 2010). So, the mature biofilm formation strongly correlates with enhanced phenazines production. Jo et al. (2017) demonstrated that *cbb*3-type cytochrome oxidases were required for phenazine reduction in hypoxic biofilm subzones, but till now there were no evidences that the disruption of cytochrome *c* oxidase genes could block phenazines production.

How exactly cytochrome *c* oxidase is involved in the production of phenazines is not yet fully understood. It is unlikely that this protein is directly involved in the reactions of shikimate pathway or the reactions of phenazine pathway. The involvement of this protein can occur at the level of antioxidant defense systems of the bacterial cell, which are also very sensitive to changes in the concentration of phenazines and reactive oxygen species formed in their presence. A possible violation of the redox balance upon deletion of *coxA* leads to the activation of direct or indirect mechanisms of blocking phenazines$synthesis.\ Itsho\underline{d}bementio \neq dt\hat{t}\,he\ directrelationship\ betweenphenaz \in es$ production and activity cytochrome *c* oxidase su I has not been shown till current research.

To identify the possible interconnection between cytochrome *c* oxidase su I and phenazines production a protein-protein network was built using a web resource STRING (version 11.5). The most probable functional partners of cytochrome *c* oxidase su I are shown at Fig. 6. The majority of functional partners are the components of cytochrome *c* oxidase complex or the other proteins of energy-converting chain. One of the most interesting issues is EY04_29170-protein which associates with cytochrome *c* oxidase and belongs to a large group of transporter proteins found in all organisms – major facilitator superfamily (MFS). It is known that the multidrug efflux pumps of this superfamily help to excrete a large variety of microbial metabolites from bacterial cell, including antibiotics and redox-active substances (hydroperoxide, potassium superoxide, many singlet oxygen-generating compounds) (Chen et al. 2017).

H. Huang et al. showed that the exposure of pathogenic fungus *Phellinus noxius* to low-level phenazine-1-carboxylic acid (PCA) cause up-regulated gene expression of seven tested genes, among which there were genes of MFS-transporters and cytochrome *c* oxidase, su I (*cox1,* homologue of *coxA*) (Huang et al. 2016). The authors suggested that those genes could be involved in PCA detoxification and improvement of the pathogen cell viability. The similar pattern of transporters gene expression was observed in *Pseudomonas aeruginosa*. The genes of efflux pomp MexGHI-OpmD, which was proved to be a main transporter of 5-methylphenazine-1-carboxylate, were induced in the presence of this phenazine species (Sakhtah et al. 2016). So, EY04_29170-protein might be involved into phenazines transport form *P. chlororaphis* subsp. *aurantiaca* cells, protecting them from toxic phenazines effects. Taking into account the fact that this protein is functionally connected with cytochrome *c* oxidase we speculate that the disruption of *coxA* gene might abolish this level of producer`s defense that leads to complete block of phenazines synthesis. If so, this level of defense could play a key role in providing the ability of bacterial cells to synthesize phenazines and, possibly, the other redox-active secondary metabolites.

In early research it was shown that some phenazines were tend to accumulate in mitochondria of rat liver cells (French et al. 1973). This accumulation depends on electron flow through an energy-converting chain and coupled with phenazines` reduction and consequent storage of reducing equivalents in the mitochondria (French et al. 1973). A-type proteins are closely related to the mitochondrial terminal oxidases (Pereira et al. 2001). It can be assumed that a similar process also takes place in bacterial cells, despite the absence of mitochondria. The key role at this process seems to be played by the electron transport chain and its components, primarily A-type cytochrome *c* oxidase. Phenazines are redox-active compounds and cytochrome *c* oxidases are involved in maintaining the redox balance in the cell. Like inside rat liver cells, A-type cytochrome *c* oxidase of bacterial cells can participate in phenazines reduction and detoxification. It might be the second point of cytochrome *c* oxidase engagement into producers` self-resistance development to phenazines. This level of protection might function in tight bound with MFS-transporters. Based on our data, we believe that the intracellular reduction of phenazines in bacterial cells can lead to their conversion into redox-inactive forms and thus ensures their detoxification and safety for principal cell molecules and metabolites. Then the inactivated phenazines might be excreted through different types of MFS-proteins. The proposed protection mechanism would be especially effective under the shortage of stronger oxidizing agents (for example, oxygen), which can be observed into the microbial biofilms. This mechanism is also might be engaged in eukaryotic cells defense and should be taken into account while developing phenazines-containing medicines. According to the results of our research, cytochrome *c* oxidase might play the leading role at this process. The absence of this protein reduces the effectiveness of given protective mechanism that leads to a complete blockage of phenazine production in bacteria, particularly in *P. chlororaphis* subsp. *aurantiaca*.

Declarations

Ethical statements

There are no ethical issues relevant to this work.

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Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Katsiaryna G. Verameyenka contributed to the study conception and design. Material preparation, data collection and analysis were performed by Katsiaryna G. Verameyenka and Volga A. Naumouskaya. The first draft of the manuscript was written by Katsiaryna G. Verameyenka and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

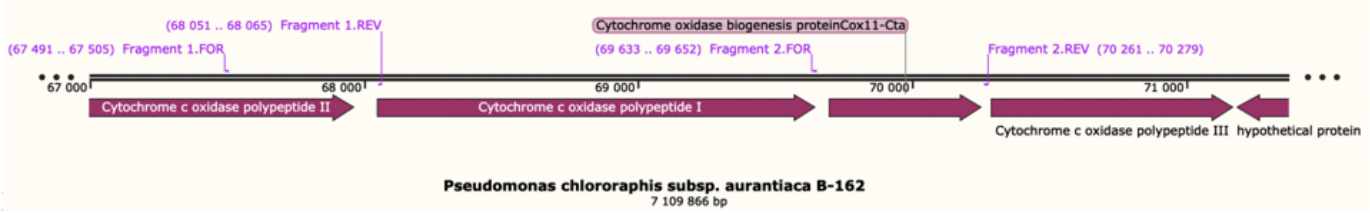


Figure 1

Scheme of primers` binding sites location on chromosome of *P. chlororaphis* subsp. *aurantiaca* B-162

MNAVSDEHGHAVVGDHDHTHGPAKGLMRWVLTTNHKDIGTLYLWFAFSMFLLGGSFAMVIR

AELFQPGLQIVQPEFFNQMTTMHGLVMVFGAVMPAFVGLANWMIPLMVGAPDMALPRMNNFSFWLL

PAAFLLLVSTLFMPGGGPNFGWTFYAPLSTTYAPESVTFFIFAIHLMGISSIMGAINVIATILNLRAPGMTL

MKMPLFVWTWLITAFLLIAVMPVLAGCVTMMLMDIHFGTSFFSAAGGGDPVLFQHVFWFFGHPEVYI

MILPAFGAVSAIIPAFSRKPLFGYTSMVYATASIAFLSFIVWAHHMFVVGIPLVGELFFMYATMLIAVPT

GVKVFNWVSTMWQGSLTFETPMLFAVAFVILFTIGGFSGLMLAIAPADFQYQDTYFVVAHFHYVLVPG

AIFGIFASAYYWLPKWTGHMYDETLGKLHFWLSFIGMNMAFFPMHFVGLAGMPRRIPDYNLQFADFN

MVSSIGAFTFGATQIFFLFIVIKCIRGGQPAPAKPWDGAEGLEWSVPSPAPYHTFTTPPEVK

Figure 2

Sequence of CoxA protein in *P. chlororaphis*subsp. *aurantiaca*

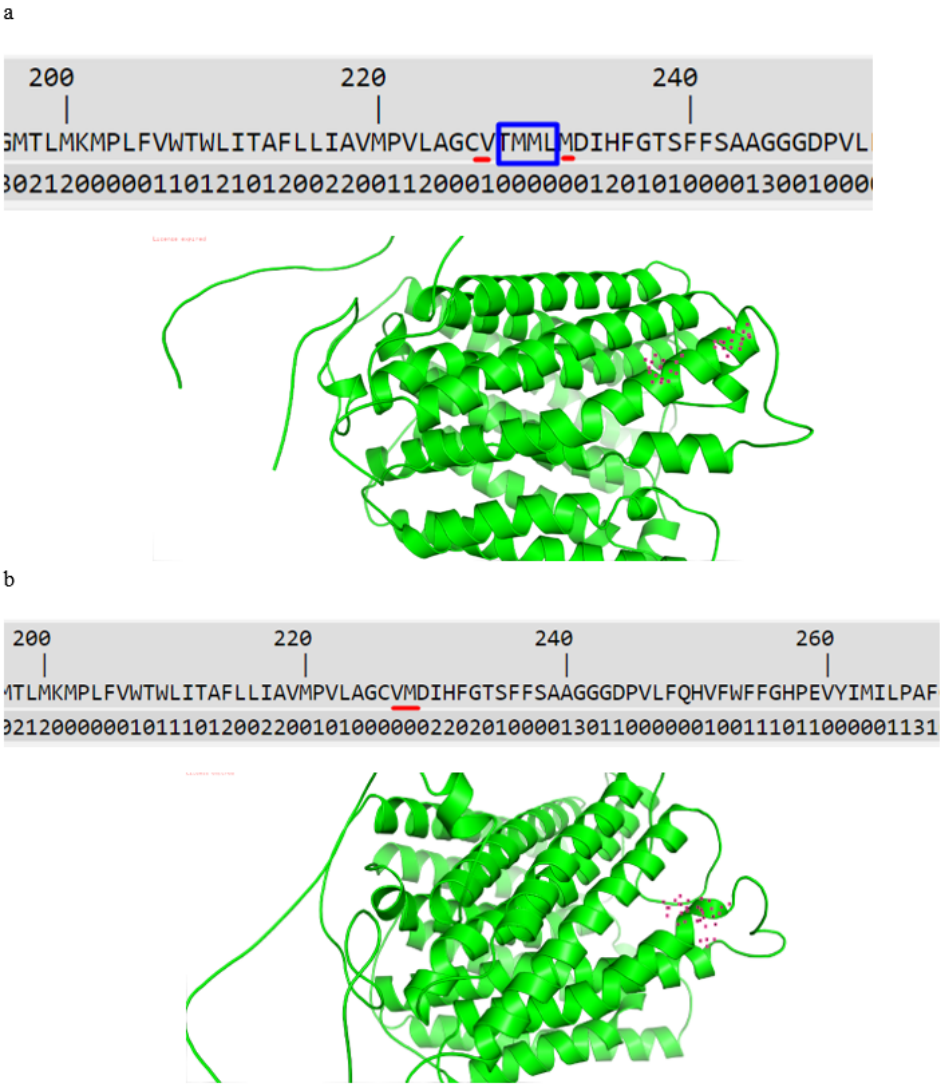
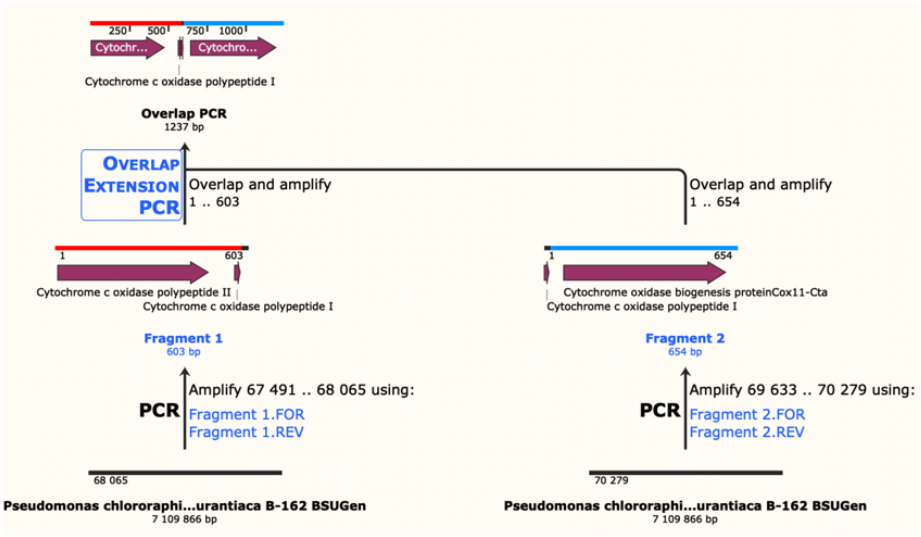


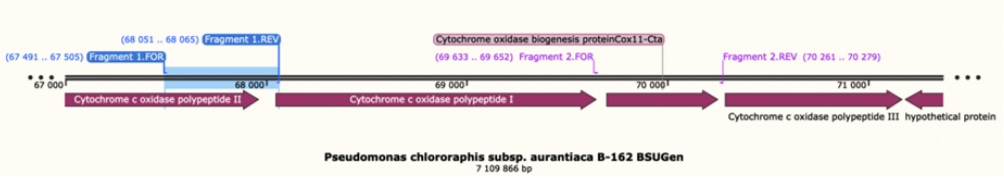
Figure 3

Predicted secondary and 3-D structure for wild type (a) and mutant (b) CoxA protein.

a



b



c

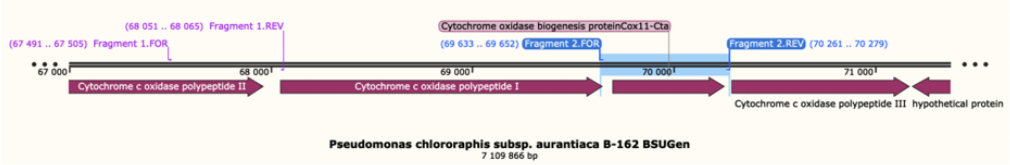


Figure 4

Creation the construction for *coxA* gene knockout

a – The whole scheme of experiment, b – location of primers for Fragment 1 (in blue). c – location of primers for Fragment 2 (in blue)

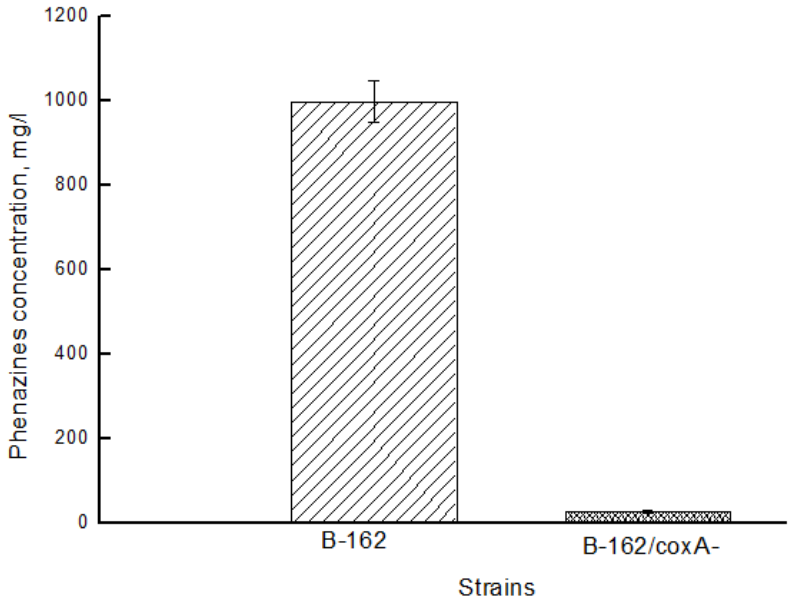


Figure 5

Phenazines production in the wild-type strain and in one of the B-162/coxA$^-$–strains (№ 6)

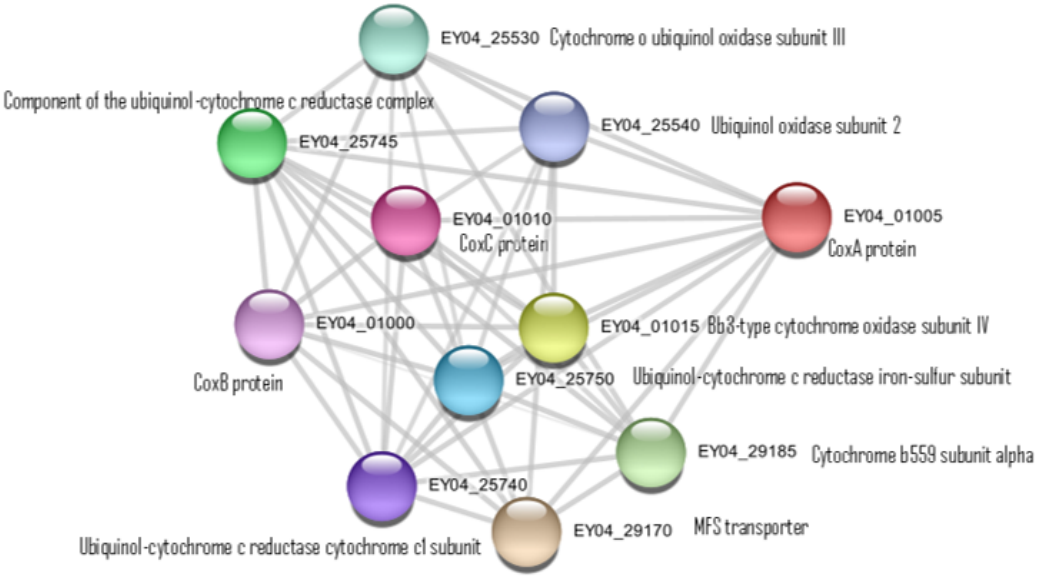


Figure 6

Protein-protein interaction network for CoxA protein

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