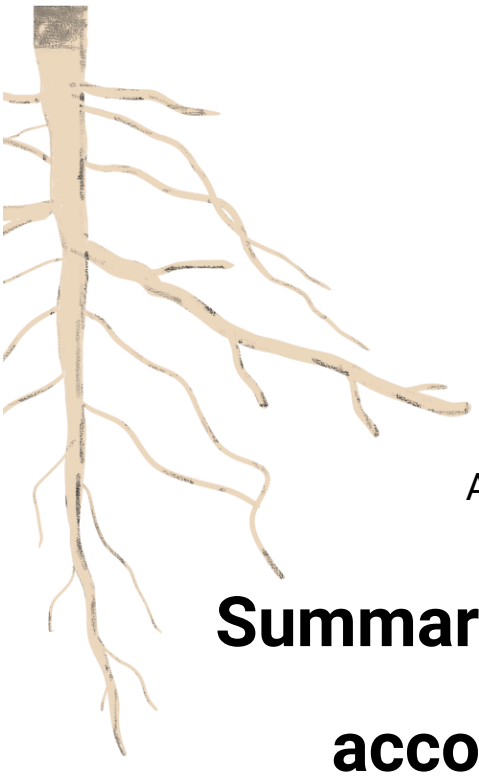
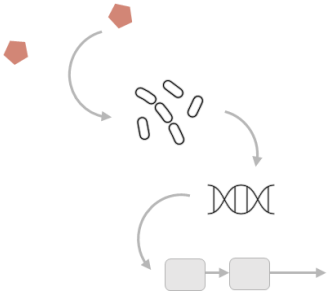


Uniwersytet Gdański

Attachment no. 3

Summary of professional accomplishments

dr Dorota Krzyżanowska



Laboratory of Biologically Active Compounds
Intercollegiate Faculty of Biotechnology
University of Gdańsk and Medical University of Gdańsk

Gdańsk 2023

1. **Name.**

Dorota Magdalena Krzyżanowska (Dorota M. Krzyżanowska)
ORCID: 0000-0003-0177-377X; LinkedIN: 59370533

2. **Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, and title of the Ph.D. dissertation.**

2015	Ph.D. in Biological Sciences in the field of Biochemistry Intercollegiate Faculty of Biotechnology UG and MUG Title of the Ph.D. dissertation: „The *in vitro* and *in planta* antagonism of *Pseudomonas* sp. strain P482 against bacterial phytopathogens of the genera *Pectobacterium* and *Dickeya*"
2009	Master's degree in Biotechnology Intercollegiate Faculty of Biotechnology UG and MUG
2007	Bachelor's degree in Biotechnology Intercollegiate Faculty of Biotechnology UG and MUG

3. **Information on employment in research institutes or faculties/departments or school of arts.**

01.02.2022-present	Lab manager Employment in a research project NCN SONATA BIS Laboratory of Biologically Active Compounds Intercollegiate Faculty of Biotechnology UG and MUG
20.02.2018-20.12.2021	Adiunkt (Postdoc) Employment in a research project NCN OPUS13 Laboratory of Plant Microbiology Intercollegiate Faculty of Biotechnology UG and MUG
07.04.2015-19.02.2018	Assistant (main contractor in a research project) Employment in a research project NCN OPUS7 Laboratory of Plant Microbiology (then: Laboratory of Biological Plant Protection) Intercollegiate Faculty of Biotechnology UG and MUG

01.10.2009- 07.04.2015	Ph.D. candidate Doctoral studies in Chemistry and Biochemistry at the Faculty of Chemistry of the University of Gdańsk

During my professional career, I gave birth to two children and took maternity and parental leave; 7 months in 2012/13 and 12 months in 2020/21.

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act

My main scientific achievement for the purposes of the habilitation procedure is a series of **four thematically related experimental publications.** In all of them, I am the first author. Other of my scientific achievements since I obtained my Ph.D. degree are presented in sections 4.4 and 5 of this document.

4.1. The title of the series of articles that meet the condition specified in Art. 219 section 1 point 2 point (b) of the Act

The application of next-generation sequencing and *in silico* data mining to gain insight into the properties of selected rhizobacteria with the potential for application in biological plant protection

4.2. List of publications constituting the main achievement, detailing the candidate's contribution

IF – Impact Factor from the year the work was published

MEiN/MNiSW – scoring of journals according to the Polish Ministry of Education and Science in accordance with the list valid at the time of publication of the works. Different journal scoring systems were used during different periods.

RESEARCH ARTICLE 1

Krzyżanowska D.M., Maciąg T., Ossowicki A., Rajewska M., Kaczyński Z., Czerwicka M., Rąbalski Ł., Czaplewska P. Jafra S.;

Ochrobactrum quorumnocens sp. nov., a quorum quenching bacterium from the potato rhizosphere.

***PLoS ONE* 2019**, 14(1): e0210874. DOI: 10.1371/journal.pone.0210874

IF 2,740; MEiN 100

Contribution of the applicant:

- *I participated in creating the research concept*
- *I carried out phylogenetic analysis based on the ANIb and GGDC parameters and the core genome*
- *I obtained bacterial growth curves in various culture conditions*
- *I carried out phenotyping of the tested strains using the BIOLOG GENIII*
- *I performed tests for the inactivation of signaling molecules of the acyl-L-homoserine lactones (AHL) type*
- *I conducted an analysis of the phenotype-genotype relationship, including a comparison of gene clusters encoding urease in various Ochrobactrum strains and an analysis of the presence of genes encoding the flagellum*
- *I conducted a comparative analysis of the core genome and pan-genome for various Ochrobactrum strains*
- *I prepared all the figures and tables*
- *I wrote the original draft of the manuscript*
- *I had a leading role in preparing responses to reviewers and participated in introducing corrections to the finally accepted version of the manuscript*

RESEARCH ARTICLE 2

Krzyżanowska D.M., Supernat A., Maciąg T., Matuszewska M., Jafra S.;

Selection of reference genes for measuring the expression of *aiiO* in *Ochrobactrum quorumnocens* A44 using RT-qPCR.

***Scientific Reports,* 2019**, 9, 13129

IF 3,998; MEiN 140

Contribution of the applicant:

- *I participated in the creation of the research concept, including the selection of the appropriate set of samples for experiments*
- *I participated in the design of the starters.*
- *I conducted some qPCR experiments and related analyses (including Cq range of reference genes, analysis of the expression stability of reference genes using the BestKeeper and Normfinder methods).*
- *I prepared all the drawings and tables*
- *I wrote the original version of the manuscript*
- *I had a leading role in preparing responses to reviewers and participated in introducing corrections to the finally accepted version of the work*

RESEARCH ARTICLE 3

Krzyżanowska, D.M., Iwanicki, A., Czajkowski, R., Jafra, S.;

High-quality complete genome resource of tomato rhizosphere strain *Pseudomonas donghuensis* P482, a representative of a species with biocontrol activity against plant pathogens.

***Molecular Plant-Microbe Interactions,* 2021**, 34:12, 1450-1454

IF 3,422; MEiN 140

Contribution of the applicant:

- *I analyzed the genome for the presence of clusters encoding secondary metabolites (antiSMASH)*
- *I conducted a comparative analysis between two versions of the P482 genome obtained several years apart, using different methods*
- *I piloted a sequencing service*
- *I submitted genomic data to Genbank*
- *I prepared all the drawings and tables*
- *I wrote the original version of the manuscript*

RESEARCH ARTICLE 4

Krzyżanowska D.M., Jabłońska M., Kaczyński Z., Czerwicka-Pach M., Macur K.,Jafra S.

Host-adaptive traits in the plant-colonizing *Pseudomonas donghuensis* P482 revealed by transcriptomic responses to exudates of tomato and maize.

***Scientific Reports*, 2023**, 13(1), 9445

IF 4,6; MEiN 140

Contribution of the applicant:

- *I participated in the development of research methodology and coordinated experimental work*
- *I participated in plant breeding in gnotobiotic conditions*
- *I prepared plant exudates for analysis*
- *I prepared standards of chemical compounds for chromatographic analysis (GC-MS)*
- *I cultured bacteria in the presence of exudates and isolated RNA*
- *I coordinated the order for the RNAseq service*
- *I analyzed RNAseq data after differential expression, including analysis of the enrichment of metabolic pathways and gene networking.*
- *I analyzed gene expression using the RT-qPCR method*

- *I submitted data to the NCBI GEO database*
- *I conducted an experiment to determine the efficiency of colonization of plant roots by the P482 strain (pot experiment)*
- *I prepared all the drawings and tables*
- *I wrote the original version of the manuscript*
- *I prepared responses to the reviewers and introduced most of the corrections to the finally accepted version of the work*

Scientometric data for the articles included in the series:

Total IF: 14,8 (2-year); 17 (5-year)

Total MEiN: 520

4.3. Scientific aims of the works mentioned in point 4.2 and the obtained results

4.3.1. Introduction and reasons for undertaking research

The composition of microflora has a significant impact on the well-being of plants. The plant root zone, also called the rhizosphere, is a niche with a particularly high diversity of microorganisms and a dense network of interactions between them. Due to this fact the rhizosphere is sometimes referred to as the 'gut inside out' (Ramírez-Puebla et al., 2013). Bacterial strains showing a positive influence on the condition and health of plants are collectively called the PGPR (Plant Growth-Promoting Rhizobacteria)(Lugtenberg and Kamilova, 2009). The concept of using beneficial microorganisms, in particular PGPR, to improve crop yield and protect plants against diseases fascinated me since the early years of my studies. The idea itself is not new, but it is currently enjoying a growing interest both from scientists and companies. Public awareness has increased that excessive use of chemicals for plant protection fertilization has a negative impact on the environment and human health. At the same time, there is a need to increase agricultural production, especially in developing parts of the world. This challenge gets even bigger due to rapidly progressing climate change (FAO data). Microorganisms have the potential to be applied as an eco-friendly means to support agriculture. This includes the application of preparations containing single microbial strains or mixtures of strains, as well as the use of plant varieties and agricultural practices that lead to the formation of a beneficial endemic population of microorganisms on the target plant (so-called rhizosphere engineering) (Ahkami et al., 2017). The "Holy Grail" is a scenario in which a farmer will be able to easily check what plant-PGPR system (plant variety + microbial inoculant), and in combination with what agricultural practices (including fertilization) will ensure high crop yield in conditions of a given agricultural field (soil properties, climate), and taking into account the currently observed pathogen pressure. **Importantly, the effective use of microorganisms to improve the well-being of plants requires thorough knowledge on the**

biology of microorganisms and their fate in the target ecosystem. Due to the high biodiversity of microorganisms associated with plants and the complexity of the studied plant-microorganism-environment systems, the transition from observing/investigating a systems to the mastery that enables the design of an efficient systems requires numerous studies. In this regard, **my contribution to the development of the represented scientific discipline includes expanding knowledge about the biology of selected microorganisms associated with plants, mechanisms underlying the antagonism between PGPR and the pathogens, as well as the possibility of using microorganisms for biological protection of plants against diseases.**

My main research achievement for the purposes of the habilitation procedure is a **series of four thematically related scientific articles. These are all experimental works describing the results of research I conducted after my Ph.D. defense (June 2015). In each of the presented works, I am the first author and I had a leading role in their creation. None of the publications in the series have been previously used in the procedure for awarding a doctoral or habilitated doctor's degree**. In total, I am the co-author of 16 articles in scientific journals, one chapter in a book, 3 granted patents, and two manuscripts at the stage of review. Eleven of the 16 articles were published after my doctoral defense. Four of the eleven works were included in the series (my main achievement), while the remaining ones, along with other achievements, are briefly presented in point 4.4 (Other scientific achievements) and point 5 (Information on significant scientific activity carried out at more than one university).

The result of research described in the series of articles is the elucidation of many aspects of the biology of two Gram-negative bacterial isolates: ***Ochrobactrum quorumnocens*** **A44 and *Pseudomonas donghuensis* P482. These two strains, although phylogenetically distant, have important common features.** Both microorganisms were **obtained from the rhizosphere of crop plants from the *Solanaceae* family**: the *O. quorumnocens* A44 strain from the rhizosphere of potato (*Solanum tuberosum* L.) (Jafra et al., 2006), and the *P. donghuensis* P482 strain from the rhizosphere of tomato (*Solanum lycopersicum* L.)(Krzyzanowska et al., 2012b)(***publication from Ph.D.***). Moreover, **both strains have the ability to reduce disease symptoms caused on plants by the pectinolytic bacteria *Pectobacterium* spp. and *Dickeya* spp., currently included in the SRP group** (Soft Rot Pectobacteriaceae). SRP pathogens cause soft rot of many species of crop and ornamental plants, including potato, leading to economically significant losses (Mansfield et al., 2012). Meanwhile, methods of controlling these pathogens are limited predominantly to preventive measures (Czajkowski et al., 2011b). **The lack of targeted agents to combat SRP pathogens became the basis for me and my colleagues to conduct research on microorganisms showing antagonism towards SRPs.**

O. quorumnocens A44 and *P. donghuensis* P482 can protect plant tissue against maceration resulting from the activity of SRPs, but the mechanism underlying this protection is different for both isolates. **Strain A44 inactivates signaling molecules of the N-acyl-homoserine lactones (AHL) type.** AHLs are low molecular weight compounds secreted into the environment and detected by many Gram-negative bacteria in a regulatory mechanism called quorum sensing (QS) (Fuqua and Winans, 1994). In QS, the expression of target genes

is modulated after the cell detects a threshold concentration of signaling molecules in its immediate environment. QS is known to regulate important metabolic processes, including the production of virulence factors and biofilm formation (Papenfort and Bassler, 2016). **The ability to inactivate AHL enables strains such as A44 to disrupt QS and thus QS-dependent phenotypes in other bacterial species, including pathogens.** It has been shown that by inactivating AHLs, strain A44 is able to attenuate the QS-dependent virulence of *Pectobacterium parmentieri* and *P. carotovorum,* and thus can protect plant tissue against soft rot caused by these pathogens (Jafra et al., 2006).

The *O. quorumnocens* A44, previously known as *Ochrobactrum* sp. A44, first became the object of my research during my master's thesis. As part of the tasks carried out at that time, I managed, among others, to clone the *aiiO* gene encoding AHL hydrolase from A44 and obtain heterologous expression of the AiiO protein encoded by this gene in *Escherichia coli*. The results I obtained were included in the publication in which we showed, for the first time, the mechanism of AHL inactivation by *O. quorumnocens* A44 (Czajkowski and Krzyżanowska et al., 2011a)(***publication from the results of M.Sc. thesis***; *equal contribution of the authors).

After obtaining my PhD, I returned to research on the A44 strain. My first goal was to determine the exact taxonomic position of A44 and obtain the genomic sequence of the strain to enable further research using molecular tools. **Based on the analyzes I performed with my colleagues, we proposed the establishment of a new species, *Ochrobactrum quorumnocens*, with strain A44 as the type strain. Moreover, we presented the first comparison for genomes derived from different species of *Ochrobactrum*** (**PUBLICATION 1**). **As part of the above-mentioned publication, I also managed to link the phenotypic differences among the analyzed strains in terms of urease production and motility with the genetic background of these differences**. What I was also interested in when it comes to A44 strain was whether the hydrolysis of AHLs by the AiiO protein produced by this strain is the main activity of AiiO or rather a manifestation of the substrate promiscuity of this enzyme. The role of AiiO in the environmental adaptation of A44 was also unknown. As part of broader research addressing these questions, **together with my colleagues I selected genes that are stably expressed in the A44 strain and can serve as reference genes in studies of gene expression in this strain using the reverse transcription quantitative polymerase chain reaction (RT-qPCR)** (**PUBLICATION 2**). Moreover, **as part of the same work, I showed that the expression of the *aiiO* gene in A44 is not induced in the presence of AHL molecules, thus demonstrating the lack of a clear connection between AiiO and the AHL molecules as the target substrate of this enzyme.**

It is worth mentioning that in 2020, Hordt and colleagues proposed the transfer of the whole *Ochrobactrum* genus to genus *Brucella* (Hördt et al., 2020). This proposal has generated controversy and strong opposition from many researchers (Moreno et al., 2023, Moreno et al., 2022). Therefore, we decided to comply with the classification that considers the two taxa being separate.

As mentioned above, the second plant rhizosphere isolate I investigated, the *P. donghuensis P482*, also shows antagonism towards the SRP pathogens. However, this is based on a different mechanism than in the case of strain A44. **The mechanism of antagonism**

of the P482 towards the SRP pathogens in the *in vitro* and *in planta* conditions was the subject of my doctoral thesis. As a result, I made a significant contribution to the identification of the gene cluster determining the antibacterial activity of this strain against SRPs (Krzyzanowska et al., 2016) (***work published after my Ph.D. defense, but containing many results the results from my Ph.D. thesis***). Moreover, as a result of tests performed by me and my colleagues on potato tuber tissue and the leaves of chicory heads, I **showed that the ability of P482 to protect plant tissue depends on the type of protected tissue and the pathogenic strain** (Krzyzanowska et al., 2012b) (***publication from Ph.D.***), and **I also developed a method for preparing preparations of roots colonized by fluorescently labeled bacterial strains**. The latter approach allows the observation of microcolonies on large sections of roots using a confocal microscope (Krzyzanowska et al., 2012a)(***publication from Ph.D.***). **During my post-doctoral research, together with my colleagues, we obtained the closed (complete) genome sequence of the P482 strain, we compared it with the draft version of the genome obtained a few years earlier, updated the gene annotations, as well as used the current *in silico* tools to reveal the presence of clusters encoding various secondary metabolites, TonB-type receptors, and prophage-like elements** (**PUBLICATION 3**). Next, I examined the P482 strain for features that enable this bacterium to colonize the roots of various plant species. **As a result of the analysis of the transcriptomic response (RNAseq) of the P482 strain to the presence of maize and tomato exudates, I identified a number of metabolic pathways that may be important for the formation of a stable association of P482 with various plant species and/or in various physiological states** (**PUBLICATION 4**). **Research of this type is crucial for understanding the mechanisms of recruitment of specific microflora by plants, and their results are extremely important for the development of the research area I represent.** The fact that I conducted the above-mentioned transcriptomic analyzes for a strain of the *Pseudomonas* genus has the additional value that many strains from this group have PGPR properties and are, therefore, of interest to many researchers.

The application of methods based on Next Generation Sequencing (NGS) and *in silico* data mining played an important role in my research. This fact is an additional common denominator that brings coherence to the series of articles I put forward as my main achievement. The period of my scientific activity coincided with a significant increase in the availability of DNA sequencing services (Pervez et al., 2022), as well as significant progress in the field of bioinformatics. Over the course of my career, I transitioned from the problem of data being too scarce (low availability of genome sequences for environmental isolates) to the challenge of dealing with excessive data (transcriptomic analyses). I am looking forward, with considerable interest, to the solutions that will be brought to the analysis of "omics" data by tools based on artificial intelligence. What in my opinion what would be a real game-changer is the development of tools that would facilitate the holistic interpretation of data based on the growing volume of literature.

4.3.2. Detailed presentation of articles that make up the series

RESEARCH ARTICLE 1

Krzyżanowska D.M., Maciąg T., Ossowicki A., Rajewska M., Kaczyński Z., Czerwicka M., Rąbalski Ł., Czaplewska P. Jafra S.; ***Ochrobactrum quorumnocens* sp. nov., a quorum quenching bacterium from the potato rhizosphere**. ***PLoS ONE* 2019**, 14(1): e0210874. DOI: 10.1371/journal.pone.0210874 (IF 2,740; MEiN 100)

Introduction

Strain A44 is an isolate from the rhizosphere of potato capable of inactivating N-acylhomoserine lactones (AHL) (Czajkowski i Krzyżanowska et al., 2011a). After its isolation, the A44 strain was assigned to the genus *Ochrobactrum,* but without precise species identification (Jafra et al., 2006). The genus *Ochrobactrum* belongs to the class of Alphaproteobacteria and is closely related to the genera *Brucella*, *Agrobacterium* and *Rhizobium*. The best known representatives of *Ochrobactrum* spp. are *O. anthropi* and *O. intermedium* – strains that can cause opportunistic infections in humans (Velasco et al., 1998, Holmes et al., 1988). Meanwhile, bacteria of the *Ochrobactrum* genus inhabit various environments and have been isolated, among others, from soil (Lebuhn et al., 2000), from sewage (Kämpfer et al., 2008), from plant samples (Kämpfer et al., 2008, Trujillo et al., 2005) and from nematodes and larvae (Dirksen et al., 2016, Huang et al., 2012). The ability of these microorganisms to metabolize xenobiotics makes them a potential source of enzymes for use in bioremediation and industry (e.g. (Qiu et al., 2006, Pozo et al., 2002)). *Ochrobactrum* spp. are an interesting object of research also due to their interactions with plants (Sumayo et al., 2013). Some strains such as *O. lupini* LUP21^T (Trujillo et al., 2005) and *O. cytisi* ESC1^T (Zurdo-Piñeiro et al., 2007) are capable of nodulating roots and fixing atmospheric nitrogen, similar to the related *Rhizobium* spp.. At the time of publication of the described manuscript, the genus *Ochrobactrum* included 18 species (Li et al., 2016), but the information available for most strains was limited to a report on their isolation and species establishment. Genetic diversity within *Ochrobactrum* spp. at the level of whole genomes has previously been analyzed only within a single species, *O. intermedium* (Aujoulat et al., 2014), and cross-species analyzes have been lacking.

Aim of the study

The aim of the study was to determine the exact taxonomic position of the *Ochrobactrum* sp. A44, to obtain genomic sequences for A44 and related strains, as well as to determine differences and similarities within the studied group of strains by biochemical analyzes and comparative genome analysis. Additionally, we planned to link selected phenotypic differences between strains with their genetic background.

Description of the results and their contribution to the field

To determine the phylogenetic position of *Ochrobactrum* sp. A44, me and my colleagues used several approaches providing an increasing level of taxonomic resolution. First, we analyzed the 16S rRNA gene sequence for A44 and 18 type strains for all *Ochrobactrum* species known at that time. Based on this analysis, the group of strains most closely related to A44 was narrowed down to *O. thiophenivorans* DSM 7216^T, *O. pseudogrignonense* CCUG 30717^T, *O. grignonense* OgA9a^T, *O. rhizosphaerae* PR17^T, and *O. pituitosum* CCUG 50899^T. To obtain higher phylogenetic resolution within this group, we used Multilocus Sequence Analysis (MLSA) based on the combined 16S rRNA, *gyrB* and *groEL* gene sequences. The obtained results suggested that the closest relative of A44 is *O. rhizosphaerae* PR17^T (Figure 1). The third phylogenetic method used was based on the analysis of genomic sequences. I calculated ANI (Average Nucleotide Identity) and GGDC (Genome-To-Genome Distance Calculator) parameters for the comparisons between A44 and related strains. ANI is a measure of genomic similarity at the nucleotide level between the coding regions of two genomes, while GGDC is a tool for performing *in silico* hybridization of two genomes. The highest ANI and GGDC similarity values were obtained for comparisons between A44 and *O. pituitosum* CCUG 50899^T (ANI=87.31%, GGDC=35.6%), followed by *O. rhizosphaerae* PR17^T (ANI=86,80%, GGDC=34,3%), *O. grignonense* OgA9a^T (ANI=86.30%, GGDC=33.6%), *O. pseudogrignonense* CCUG 30717^T (ANI=82.23%, GGDC=26.5%), *O. thiopenivorans* DSM 7216^T (ANI=81.04%, GGDC=25.3%), O. anthropi ATCC 49188T (ANI=77.51%, GGDC=23%), and *O. intermedium* LMG 3301^T (ANI=77.26%, GGDC =22.5%). None of the obtained values exceeded the similarity threshold required for classifying two strains into a single species. The commonly accepted threshold values are 95% for ANI and 70% for GGDC. This indicated that A44 is a separate species. **Based on the obtained results, we proposed the establishment of a new species, *Ochrobactrum quorumnocens*, with the A44^T as the type strain.** The species name we proposed - *quorumnocens* - in Latin refers to the ability of the strain to disrupt quorum sensisng. In accordance with formal requirements, the cultures of the new type strain were deposited in international collections (= LMG 30544^T = PCM 2957^T).

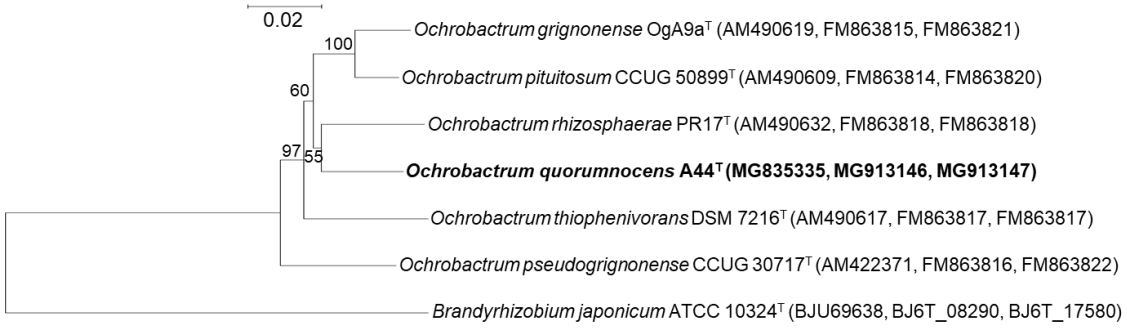


Figure 1. Phylogenetic tree for strain A44 and related *Ochrobactrum* species, obtained based on MLSA analysis for three genes: 16S rRNA (1335 bp), *groEL* (1165 bp) and *gyrB* (1012 bp).

At the time the described research was conducted, genomic sequences were available only for 2 out of 18 described type strains of *Ochrobactrum* spp., namely stains strain *O. anthropi* ATCC 49188^T and *O. intermedium* LMG 3301^T. **This constituted a barrier to for the application of genome-based phylogenetic methods for taxonomic studies within this genus, and also made comparative genome analyses impossible. Therefore, as part of the described research, we obtained the genome sequence of the A44^T strain and five of the above-mentioned closely related strains**. The complete genome sequence of A44^T was obtained by hybrid assembly of data obtained with Illumina HiSeq2500 and PacBio RS (BaseClear B.V., The Netherlands). For the remaining strains, sequences were obtained in the form of drafts (WGS - whole genome shotgun).

The complete genome of A44 consists of four replicons: the main chromosome (2585.393 kbp;) with the classical replication system, the chromid (2008.185 kbp;) carrying the plasmid-type replication system, and some of the genes necessary for basic metabolism, and two plasmids: pOqn1 (1032.012 kbp) and pOqn2 (19,701 kbp). The presence of many replicons, including chromids and megaplasmids, is common among bacteria from the class of Alphaproteobacteria (Dziewit and Bartosik, 2015).

To verify whether the NCBI database contains data for other strains that could qualify for *O. quorumnocens* rescue, **I performed a comparative analysis (ANI parameter) between the genome of A44^T and 65 WGS sequences available at that time (June 2018) for various strains declared by the submitters as belonging to genus *Ochrobactrum***. The conducted analysis did not reveal any other *O. quorumnocens* strains in this group. The highest ANI score (87.54%) was obtained between A44 and the SJY1 strain, designated as *O. rhizosphaerae*. Additionally, we found that the ANI value for *O. rhizosphaerae* SJY1 and PR17^T - a type strain of the *O. rhizosphaerae* species - is 86.10%, and is therefore below the threshold for a single species. Based on the latter we suggested that the SJY1 strain should be classified not as *O. rhizosphere* but as *O. pituitosum* (ANI=96.97%). **The data we have published in this work will be helpful in assigning many isolates to the appropriate species based on rapid *in silico* analysis**.

Obtaining the genome sequences of A44 and the 5 related strains allowed me to perform comparative genome analysis using EDGAR (Blom et al., 2009). **My analysis showed that the core genome for the analyses group, that is the pool of genes shared by all 6 strains, consists of 2,731 coding sequences, which constitutes between 50-66% of the content of individual genomes** (Figure 2), **and 27% of the content of the pangenome (10,296 CDS). The fraction of genes unique to individual strains ranged from 8–15%.** The fact that each of the analyzed strains contributed new genes to the pangenome suggests that the **pangenome of *Ochrobactrum* spp. is "opened" according to the definition of Guimarães et al.** (Carlos Guimaraes et al., 2015).

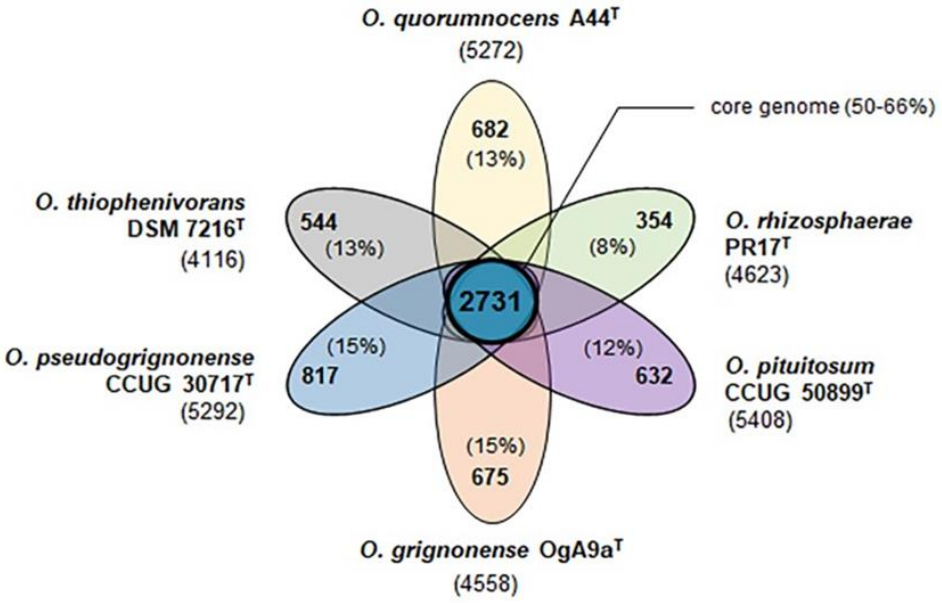


Figure 2. The core genome (in the center) and genes unique to individual strains (shown in the "petals") for the group of six analyzed *Ochrobactrum* spp. strains. This figure is Fig. 2 in the discussed publication.

The standards in force in bacterial taxonomy require that an accepted taxonomic identification with the proposal of a new species cannot be based solely on *in silico* data. Therefore, we compared A44 and the related strains in terms of chemotaxonomic markers: Fatty Acid Methyl Ester (FAME) profiles, and protein profiles for whole cells obtained by mass spectrometry. We also analyzed the morphological characteristics of the cells and cultures, tested numerous biochemical features, investigated the range of temperatures and pH values permissive for growth, as well as the motility of the strains in several conditions. The obtained data have been published, in large part, in supplementary materials of the presented publication.

In the discussed work, I also attempted to link selected biochemical features of the analyzed strains (or more precisely, the differences observed between these strains) with their genetic background. Not only did I find this interesting, but also **the attempt addressed a complaint about genomic analyses that was very common at the time, implying that such analyses often limited to presenting "dry" sets of numerical data. In this work, we went beyond this scheme** by analyzing the genetic background of the observed differences when it comes to two differentiating phenotypes: **urease production and motility**.

Production of ureases

The results of previous studies showed that urease activity may be different for individual *Ochrobactrum* strains (Teyssier et al., 2005). In the presented work, me and my colleagues showed that the A44 and the type starins of *O. pseudogrignonense*, *O. rhizosphareae*, *O. grignonense*, and *O. pituitosum* give negative results in a commonly used diagnostic test for urease production, while the *O. thiophenivorans* DSM 7216^T strain , for which no previous data on urease activity were available, tested positive.

Ureases are enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. Bacterial ureases are complex enzymes whose synthesis requires three structural genes, *ureABC*, encoding the gamma, beta, and alpha subunits, respectively, and four accessory genes, *ureDEFG* (Hausinger et al., 2001). **I have analyzed the presence and the genetic context of the *ureABCDEFG* genes in the six newly sequenced *Ochrobactrum* spp. genomes, and showed that all analyzed strains, regardless of whether they were urease positive or urease negative, carried homologs of the urease operon. However, the organization and genomic context of these genes showed considerable diversity** (Figure 3).

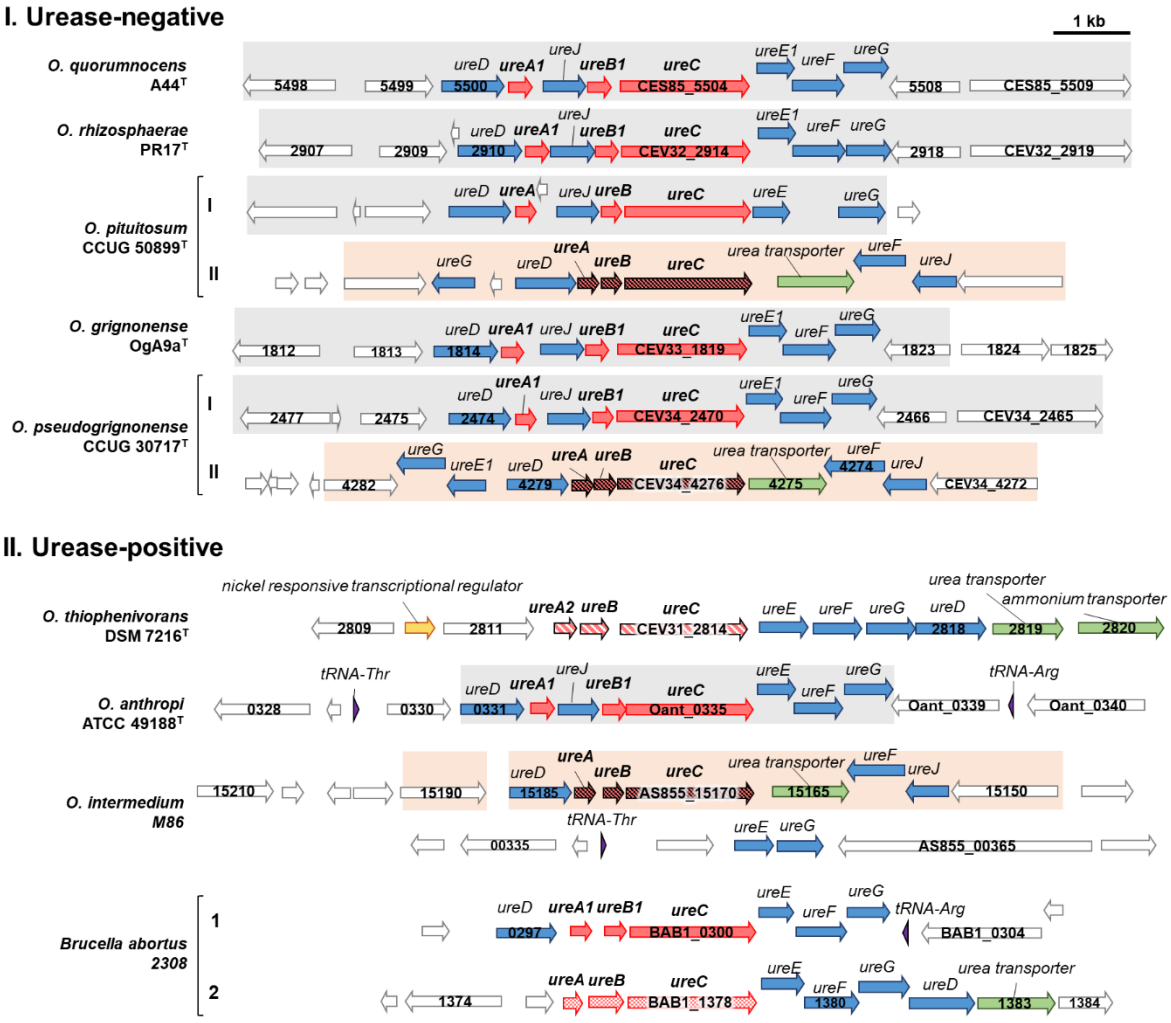


Figure 3. Clusters encoding urease gene homologs in the genome of *O. quorumnocens* A44, closely related *Ochrobactrum* spp.type strains, as well as *O. anthropi* ATCC 49188^T, *O. intermedium* M86 and *Brucella abortus* 2308. The clusters were divided into two groups depending on whether they were identified, based on a diagnostic test, as urease-negative strains (I) or urease-positive strains (II). Individual ORF colors represent: structural genes (red), accessory genes (blue), transport genes (green), genes related to nickel metabolism (yellow), tRNA coding regions (purple). Alleles of the structural genes *ureC*, *urea,* and *ureB*, depending on the degree of similarity, are distinguished with matching or different patterns. Similarity considered in the scale of the entire gene cluster is marked with a similar background color. This figure was presented as Fig. 6 in the discussed publication.

Among the urease-negative strains, *O. quorumnocens* A44, *O. rhizosphaerae* PR17^T, and *O. grignonense* OgA9a^T possess a single urease cluster (hereinafter referred to as type I), showing similar gene organization. In turn, the genomes of *O. pituitosum* CCUG 50899^T and *O. pseudogrignonense* CCUG 30717^T carry two urease clusters - one of type I and another, encoded by a separate operon (type II**). I also showed that for the structural proteins (UreA, UreB and UreC) there is a higher level of identity for homologs originating from the same type of cluster, despite from different strains (95-98%), than the identity observed between homologs from different types of clusters within a single strain (66-71 %).**

Additionally, I analyzed the organization of urease clusters in three urease-positive clinical isolates: *O. anthropi* ATCC 49188^T, *O. intermedium* M86, and *Brucella abortus* 2308. *O. anthropi* ATCC 49188^T contains a single cluster resembling type I, but placed in a different genetic context, and the *ure* operon from *O. intermedium* M86 is similar to the type II operon of *O. pituitosum* and *O. pseudogrignonense*. Neither the type I nor type II clusters identified in the analyzed *Ochrobactrum* strains resembled either of the two urease clusters in *B. abortus* 2308.

The only urease-positive *Ochrobactrum* strain in the group most closely related to A44 was *O. thiophenivorans* DSM 7216^T. **A very interesting discovery was that the *O. thiophenivorans* genome encodes a single urease cluster, but of a completely different type than those found in the other analyzed *Ochrobactrum* spp.** (Figure 3). Unexpectedly, the UreA protein from *O. thiophenivorans* DSM 7216^T showed a high level of identity to homologs occurring in the group of Alphaproteobacteria strains isolated from seawater: *Pseudoruegeria* sp. SK021, *Martelella mediterranea* and *Nitratireductor* sp. OM-1, with much lower identity to homologs from other *Ochrobactrum* spp.. **Further analysis showed that a fragment of almost 7 kb containing the urease cluster from *O. thiophenivorans* shows 91% coverage and 78% identity at the nucleotide sequence level with the analogous cluster from *Pseudoruegeria* sp. SK021 - a strain isolated from North Sea sediments (Pohlner et al., 2017). It is therefore probable that the discussed urease cluster, unique in the *Ochrobactrum* spp. group, was acquired by *O. thiophenivorans* by horizontal gene transfer.**

In this work, I could only speculate that *O. quorumnocens* A44 and related strains are urease-negative despite having urease-encoding operons due to point mutations, frameshifts, or deletions/insertions that have accumulated in gene clusters. Reasons such as activation of gene expression in very specific conditions also cannot be ruled out. As mentioned above, ureases are enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. The production of ammonia as a result of urease activity increases the pH of the environment. In the case of human pathogens such as *Helicobacter pylori*, *Yersinia enterocolitica* and *Brucella* sp., urease activity increases the survival of these bacteria in the acidic environment of the stomach (Sangari et al., 2007). An analogous function has been proposed for ureases produced by clinical isolates of *Ochrobactrum* spp., which, like *H. pylori*, can also be detected in biopsies from this organ (Kulkarni et al., 2013). However, non-pathogenic soil microorganisms, including *Ochrobactrum*-related strains of *Rhizobium* spp. (Toffanin et al., 2002), also produce ureases. The reason for this may not be limited to the need to change the pH of the environment immediate to the cell. Gram-negative bacteria can obtain nitrogen both from organic compounds, such as amino acids, and from inorganic ammonia - a product of decomposition of urea. From the point of view of the nitrogen cycle occurring in nature, the

mineralization of nitrogen from urea by soil microflora, possible thanks to the activity of uereases, makes the nitrogen more available to plants and is necessary to complete the nitrogen cycle. This applies to urea both naturally occurring in the environment and the urea derived from urea-based fertilizers - the most commonly used nitrogen fertilizers in agriculture (http://faostat.fao.org).

Motility

We compared the motility of strain A44 and the closely related strains. **Together with my colleagues, I showed that the motility of strains within the analyzed group is diverse and depends on the composition of the culture medium (environmental conditions)**. Strain A44 was motile under all conditions tested, similar to *O. rhizosphaerae* PR17^T and *O. grignonense* OgA9a^T. However, *O. pseudogrignonense* CCUG 30717^T was motile only in the presence of casamino acids, and *O. thiophenivorans* DSM 7216^T was unmotile regardless of the conditions tested. Strain *O. anthropi* ATCC 49188^T was motile only in the presence of glucose (both with and without casamino acids), but not when the main carbon source was glycerol, unless the medium was supplemented with casamino acids.

The lack of motility we have demonstrated for *O. thiophenivorans* DSM 7216^T was consistent with the observation included in the first report on this species (Kampfer et al., 2008). Curious about the genetic background of this phenotype, I searched the genomes of all six *Ochrobactrum* strains for genes involved in the formation of flagella. We detected a total of 25 such genes in the poll of the tested strains, 24 of which were present in each motile strain. **I showed that the non-motile strain *O. thiophenivorans* DSM 7216^T lacked as many as 12 of the 25 genes involved in flagella formation, of which at least 24 were present in other analyzed *Ochrobactrum* strains**. The missing genes include *fliC*, which encodes flagellin, as well as *flgE, flgK, fliF, fliQ*, and *fliR*. **In motile strains, the mentioned 11 genes appear in the form of a cluster (neighboring genes), which indicates that the loss of motility by *O. thiophenivorans* is caused by the deletion of a large DNA region involved in the formation of flagella.**

An additional analysis that I carried out and described in the discussed publication was the determination of the ability of the tested *Ochrobactrum* spp. strains to inactivate AHL-type signal molecules, namely the C6-HSL molecule. The rationale for the experiment was that the A44 strain had previously been studied primarily for its ability to inactivate signaling molecules. Using the *E. coli* pSB401 AHL biosensor (Winson et al., 1998) and an assay previously described by Jafra and van der Wolf (Jafra and van der Wolf, 2004), **I showed that all tested strains were able to inactivate C6-HSL, with the exception of *O. rhizosphaerae* PR17^T** (Figure 4A). Consistent with this finding, *O. rhizosphaerae* strain PR17^T showed a significantly lower potential to reduce soft rot symptoms caused by *P. parmentieri* SCC3193 compared to A44 (Figure 4B). This is even more interesting due to the fact that the *O. rhizosphere* strain is a type strain very closely related to A44 and, alike A44, originates from the potato rhizosphere. **The abovementioned observation was not analyzed in terms of its genetic background in the discussed work, but it became a starting point for further molecular research, conducted as part of a master's thesis carried out under my supervision. The results of these studies, after completing the pool of additional experiments, will be published in a separate publication.**

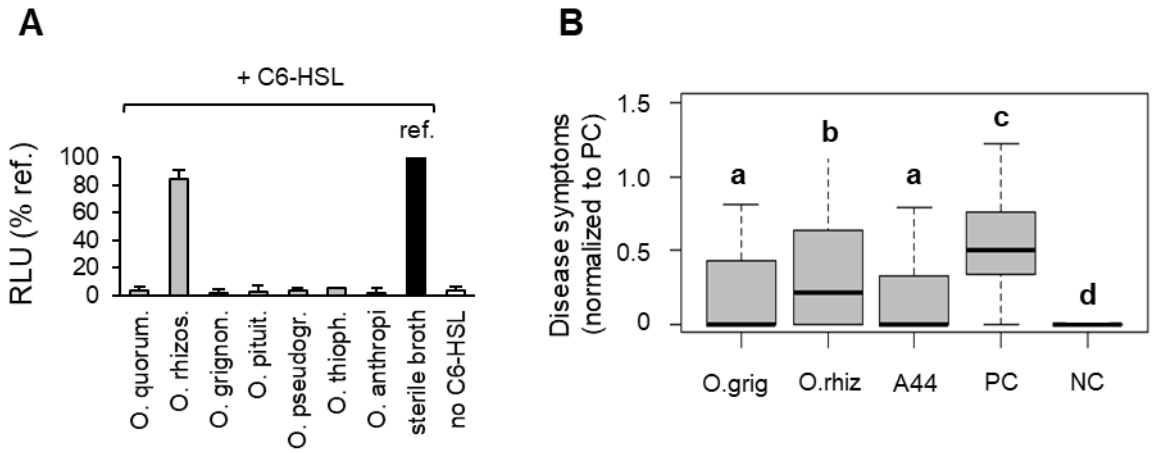


Figure 4 Inactivation of AHL (C6-HSL) by the *O. quorumnocens* A44 strain and related strains (A) and the scale of soft rot symptoms on potato tuber slices co-inoculated with the pathogen *P. parmentieri* and A44 or one of the other analyzed strains (B). Differences in results for data groups marked with different letters indicate statistical significance (significance level 0.05). PC – positive control for the development of disease symptoms (the pathogen itself). NC – negative control (no pathogen). In the discussed publication panels A and B are presented as Fig. 4 and 5, respectively.

To sum up, in the presented work, based on *in silico* sequence analyzes and phenotype assessment, me and my colleagues proposed the establishment of a new species, *Ochrobactrum quorumnocens*, with the A44 strain as its type strain. The A44 strain has a multi-replicon genome not unusual for this group of microorganisms. Before the publication of our study, genome sequences were available for only 2 out of 18 *Ochrobactrum* spp. type strains. Therefore, our release of the genome of A44 and five related type strains has significantly contributed to facilitating taxonomic research based on modern *in silico* tools within the genus *Ochrobactrum*. To my knowledge, this was also the first publication in which a comparative analysis of the genomes of representatives of different *Ochrobactrum* species was carried out. Moreover, the analysis of phenotype-genotype relationship I carried out in the presented work revealed the genetic basis of the lack of motility in *O. thiophenivorans* DSM 7217^T and showed differences in the organization of urease clusters in the studied group of *Ochrobactrum* spp. Additionally, the published research became a starting point to subsequent research on the basis of the loss of the ability of strain *O. rhizosphaerae* PR17^T to inactivate AHLs (*research in progress*).

RESEARCH ARTICLE 2

Krzyżanowska D.M., Supernat A., Maciąg T., Matuszewska M., Jafra S.; **Selection of reference genes for measuring the expression of *aiiO* in *Ochrobactrum quorumnocens* A44 using RT-qPCR**. ***Scientific Reports,* 2019**, 9, 13129 (IF 3,998; MEiN 140)

Introduction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is the most commonly used approach for gene expression analysis (Wagner, 2013). This method is extremely useful, but it involves many experimental steps, as well as many data processing steps, hence its correct use requires a considerable level of awareness to obtain correct results. One of the key decisions to be made when conducting an RT-qPCR experiment is the selection of reference genes with stable expression in order to later normalize the data to obtain expression values for the target genes. In the case of model organisms or other well-known organisms, the selection of appropriate reference genes is facilitated by the availability of the results of previous research. When it comes to bacteria, literature data can serve to create a list of candidate reference genes, with potentially general application in the analysis of gene expression in bacteria due to the occurrence of their orthologs in many bacterial species. The most popular of those genes include *rho, 23S rRNA, rpoD, gyrB, recA, 16S rRNA, dnaK, rpoB, groEL, gyrA*. However, the above-mentioned genes do not perform equally well for all microorganisms carrying those genes and under all experimental conditions. It is also known that including inappropriate genes in the normalization factor can significantly affect the results (DeLorenzo and Moon, 2018). Due to this fact, the stability of expression of a set of candidate reference genes should be verified when using RT-qPCR for the first time in given (micro)organisms (Rocha et al., 2015, Kałużna et al., 2017). In 2009, a set of MIQE guidelines (Minimum Information for publication of Quantitative real-time PCR Experiments) was published (Bustin et al., 2009). The aim of those guidelines was to standardize the quality of RT-qPCR results published in scientific journals, primarily in medical studies. At the time of publication of this work, the pursuit to meet MIQE standards in research involving gene expression in Procaryota was much weaker than in medical research. Despite notable exceptions, in many studies the expression of studied prokaryotic genes was normalized to arbitrarily selected reference genes, without validation as to the stability of their expression, or even without a clear justification why the given genes were used as reference genes.

One bacterial genus for which no data on the appropriate reference genes was available was the genus *Ochrobactrum*. In the described publication, together with my colleagues, I focused on the A44 strain of the species *Ochrobactrum quorumnocens* (Krzyżanowska et al., 2019a) - an isolate from the potato rhizosphere (Jafra et al., 2006). As a result of the activity of AiiO hydrolase, cells of A44 strain inactivate a wide spectrum of bacterial signaling molecules from the group of N-acylhomoserine lactones (AHLs) (Czajkowski i Krzyżanowska et al., 2011a). Numerous enzymes that inactivate AHLs have been described in literature (Czajkowski and Jafra, 2009, Grandclément et al., 2016). The discovery of many of them, including AiiO from strain A44, was the result of screening aimed at selecting new factors interfering with quorum sensing. Interestingly, it is not clear whether the inactivation of AHL

by bacteria is the primary function of the enzymes involved in the inactivation reaction (Roche et al., 2004). For some of these enzymes, additional functions unrelated to AHL inactivation have been identified in the source microorganisms, which indicates that AHL inactivation may be the result of the catalytic promiscuity of these enzymes (Grandclément et al., 2016)(Roche et al., 2004). For example, BlcC (AttM) from *Agrobacterium tumefaciens* has been shown to be involved in the metabolism of gamma-aminobutyrate (Khan and Farrand, 2009), and the PvdQ enzyme from *Pseudomonas aeruginosa* is essential for the formation of the pyoverdine siderophore (Nadal Jimenez et al., 2010). Even if we would assume that the main function of some of the AHL-inactivating enzymes is indeed the inactivation of AHLs, the expected biological purpose of this activity will not be uniform. Depending on whether the AHL-inactivating strain produces, at the same time, its own AHL(s), the potential biological functions of AHL inactivation may include, but are not limited to, self-regulation of its own QS, utilization of AHLs as a nutrient source, and providing the strain with an environmental advantage over the AHL-producing competitors (Grandclément et al., 2016). Also in the case of *O. quorumnocens* A44, the role of the AHL-degrading enzyme (AiiO) in the metabolism and environmental competitiveness of this microorganism remains unclear.

Aim of the study

The first goal of the presented work was to select *O. quorumnocens* A44 genes that are stably expressed in many environmental conditions and in various growth phases, so that these genes could serve as reference genes in the analysis of gene expression by RT-qPCR in the A44 strain. The second goal was to use RT-qPCR to measure changes in the expression level of *aiiO* in the A44 strain in response to AHLs and other environmental factors, such as growth phase, temperature, pH and the addition of potato root extract. The second objective was part of our broader investigation concerning the role of AiiO in the metabolism and environmental competitiveness of A44.

Description of results and their contribution to the field

As part of the presented publication, we developed an RT-qPCR test to examine the expression level of the *aiiO* gene in *O. quorumnocens* A44. To select appropriate reference genes (RG), we tested the expression stability of 11 candidate genes. In an optimal setup, the expression stability of a potential reference gene is verified under all experimental conditions for the target experiment (Gomes et al., 2018). For A44, in a pilot test, we assessed the stability of expression of 11 genes under 10 different culture conditions, on samples collected from a single experimental run. In the next stage, we verified the performance of the narrowed-down pool of the best candidate genes in an experiment with 11 conditions, in three biological replicates. The strategy of using a pilot test to select the most promising candidates is in line with the MIQE guidelines (Taylor et al., 2010).

The expression stability of candidate genes was assessed using geNorm (Vandesompele et al., 2002) and two other popular algorithms, NormFinder (Andersen et al., 2004) and

BestKeeper (Gomes et al., 2018), each based on a different statistical approach (Figure 5). According to geNorm, the most stable RG candidates in A44 were *rho*, *gyrB*, and *rpoD*. These genes were also among the top five genes according to NormFinder. The gene stability ranking suggested by Bestkeeper was different from the ones uggested by the other two algorithms. This method also yielded the lowest resolution, attributing good performance to most of the tested genes. Interestingly, *recA*, listed among the top 4 genes by geNorm and top 3 genes by NormFinder, was among the two ranked worst by Bestkeeper. The discrepancy between the results from BestKeeper and the results from both gNorm and NormFinder can also be observed in other studies (Martínez-Giner et al., 2013). The reason for this is the different input data (raw Cq values for BestKeeper vs. relative expression values for geNorm and NormFinder) and the different stability criteria considered by the three programs.

Based on a summary of results from genNorm, NormFinder, and BestKeeper, my colleagues and I selected the *rho*, *rpoD*, and *gyrB* genes as the most promising candidates for use in normalizing RT-qPCR data in *O. quorumnocens* A44. Importantly, these genes do not belong to a common functional group, which reduces the chance that they are coregulated. Our **study also provides an important example that the 16S rRNA gene, often used in bacteria to normalize expression without proper validation, is not suitable as a reference gene for all species.** Our experiments also showed that it made little difference to our results whether 2 or 3 reference genes were used to normalize expression.

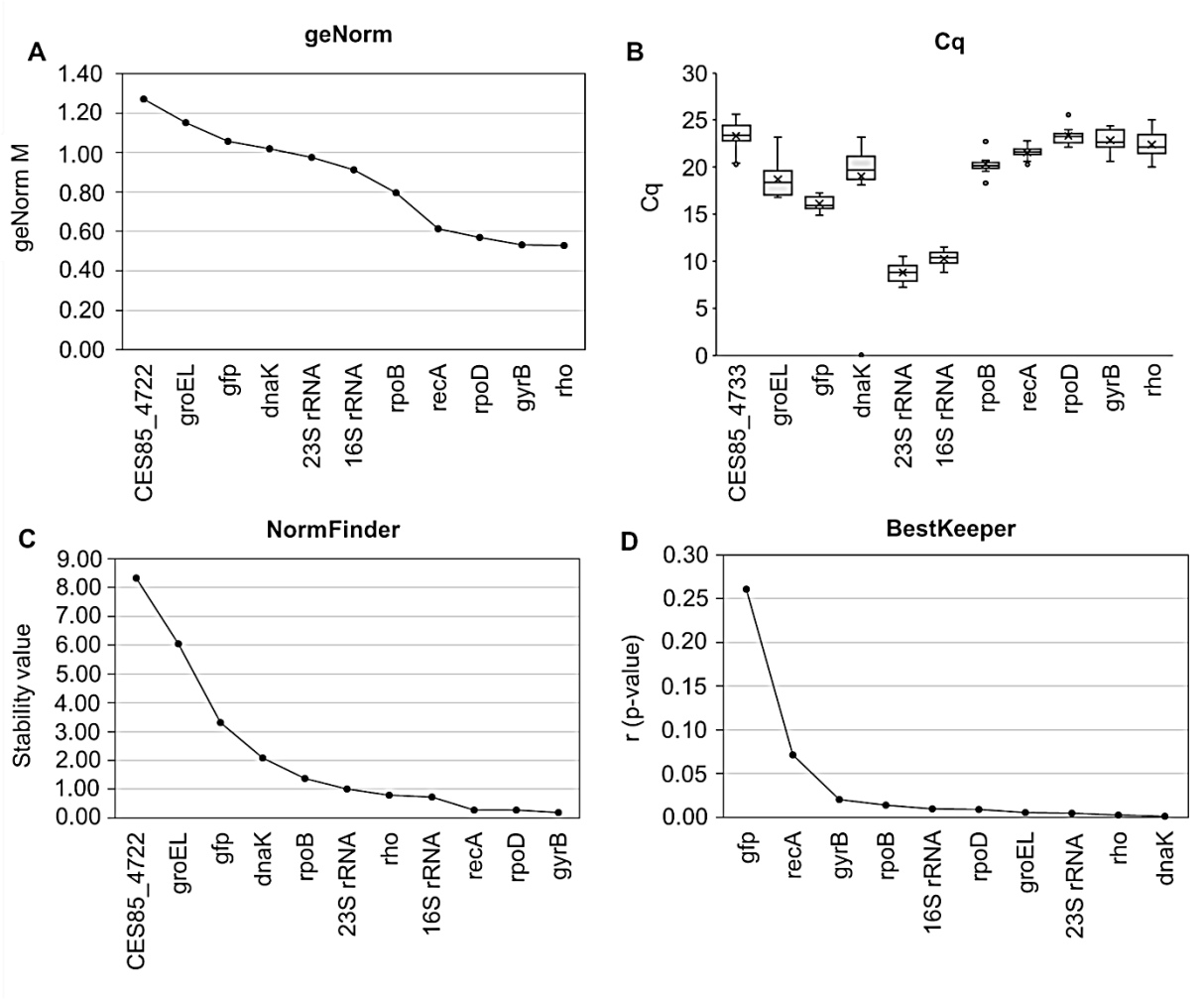


Figure 5 Expression stability of candidate reference genes of *O. quorumnocens* A44, determined using the following algorithms: geNorm (A), NormFinder (C) and BestKeeper (D). Panel B shows Cq values indicating the baseline expression level of the studied genes. Expression stability was tested under 10 different culture conditions. The figure was presented as Fig. 1 in the discussed publication.

In the presented work, as part of broader research on the role of AiiO in the metabolism and environmental performance of the A44 strain, me and my colleagues used RT-qPCR with newly established reference genes to examine whether the expression of AiiO in A44 is changed after exposure to AHLs and whether the level of this expression depends on other selected culture conditions, such as growth phase, temperature, pH and the addition of potato root extract. It is known that the expression of some enzyme-encoding genes can be significantly increased in the presence of the target substrate(s). Demonstrating such a relationship is a convincing argument for link between a given compound (substrate) and the protein's function. For example, it has been shown that the expression of *pelD*, a pectate lyase, can be induced more than 10-fold in the stationary phase when its substrate polygalacturonate is present (Hommais et al., 2011).

The use of RT-qPCR allowed to demonstrate that the expression of *aiiO* in *O. quorumnocens* A44 is not induced after supplementation with synthetic AHLs, specifically C6-HSL or 3OC12-HSL, nor in the presence of naturally produced 3OC8-HSL and 3OC6-HSL and other compounds secreted into medium by *P. parmentieri* SCC3193 (Figure 6). For comparison, there are reports in literature showing no induction after the addition of AHL also for *aiiB* and *blcC* from *A. tumefaciens,* both of which genes encode two different AHL-inactivating enzymes from the lactonases group (Khan and Farrand, 2009, Haudecoeur et al., 2009). Contrary, the induction of expression of these enzymes was observed in the presence of compounds of plant origin: succinic semialdehyde, gamma-hydroxybutyrate, gamma-butyrolactone, gamma-aminobutyrate, and salicylic acid in the case of *blcC,* and plant extracts enriched in agrocynopines in the case of *aiiB* (Khan and Farrand, 2009, Haudecoeur et al. al., 2009, Lang and Faure, 2014). In our study, as part of one of the experimental conditions, we assessed the expression of *aiiO* in the presence of potato root extract, but this supplement had no effect on the expression of *aiiO*.

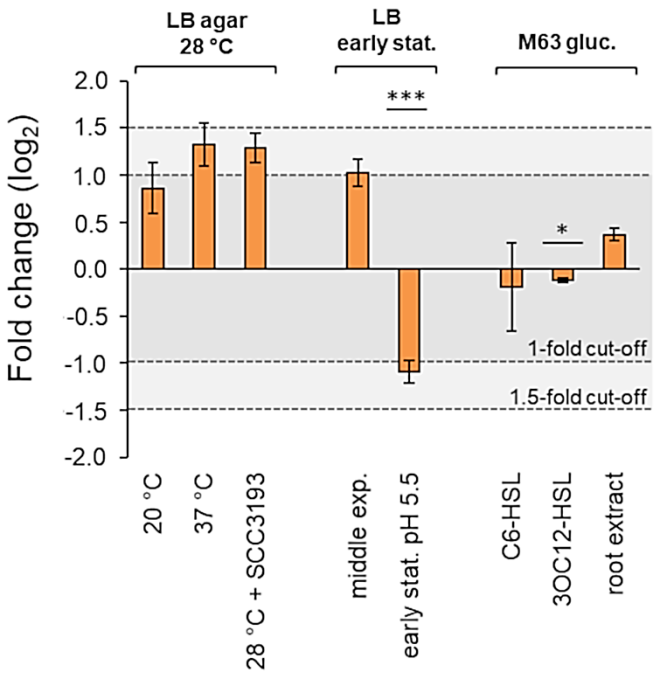


Figure 6 Differences in the expression level of the *aiiO* gene under different experimental conditions. *rho, gyrB,* and *rpoD* were used as reference genes. The change in the expression level was calculated for each of the media separately (LB agar, LB and M63 0.4% glucose). SCC3193 - growth in the presence of AHL and other compounds secreated by *P. parmentieri* SCC3193; early stat. – early stationary growth phase; middle exp. – the phase of exponential growth; C6-HSL, 3OC12-HSL – after 90 min. exposure to synthetic AHLs (50 μM·mL-1); root extract – water extract from potato roots 25%. *p < 0.05 ***p < 0.0005. The figure was presented as Fig. 4 in the discussed publication.

Although the expression of *aiiO* was not significantly altered under any of the conditions included in this study, we cannot rule out that such conditions exist. Some genes may be conserved in bacterial genomes, just as *aiiO* homologs appear to be present in the genomes of various *Ochrobactrum* spp. (Czajkowski et al., 2011a, Mei et al., 2010), even though the conserved genes are useful only in a very specific niche or only in the presence of some environmental stress (Rohmer et al., 2011). Strains of the genus *Ochrobactrum* have been isolated from various environments, including soil, plants, and soil-dwelling organisms such as the nematode *Caenorhabditis elegans* and larvae of *Holotrichia parallela* (Huang et al.,

2012). Some *Ochrobactrum* species can cause opportunistic infections in humans, and one strain has been shown to cause disease in fungi (Wu et al., 2016). It is possible that the induction of *aiiO* expression occurs in a very specific niche/host or in the presence of a specific xenobiotic. Functional and structural similarities between enzymes degrading bacterial signaling molecules and enzymes degrading xenobiotics/antibiotics have been previously indicated in several studies (Elias and Tawfik, 2012, Tannières et al., 2013).

In conclusion, in the described work we have shown that *rho, gyrB,* and *rpoD* are suitable reference genes for gene expression analyzes in *O. quorumnocens* A44. To our knowledge, this was the first study to identify reference genes for RT-qPCR analyses in the genus *Ochrobactrum*. Our selection of stable reference genes may be a great help during selection of reference genes in the related bacteria, and the detailed description of the adopted step-by-step strategy (methodology) for the selection of reference genes in bacteria may be helpful in selection of such genes also in other bacterial species. As a result of the conducted research, it was also found that the expression of *aiiO* is not significantly induced by the presence of AHLs. This makes it more probable that AHLs are not the main substrate of AiiO, and therefore their inactivation (QS silencing) is not the main function of this enzyme.

RESEARCH ARTICLE 3

Krzyżanowska, D.M., Iwanicki, A., Czajkowski, R., Jafra, S.; **High-quality complete genome resource of tomato rhizosphere strain *Pseudomonas donghuensis* P482, a representative of a species with biocontrol activity against plant pathogens**. ***Molecular Plant-Microbe Interactions,* 2021**, 34:12, 1450-1454 (IF 3,422; MEiN 140)

Introduction

Pseudomonas spp. are a group of Gammaproteobacteria known for their ability to adapt to a variety of environments, metabolize multiple carbon sources, and produce a wide range of secondary metabolites (Gross and Loper, 2009). Although the genus *Pseudomonas* includes some known pathogens, such as *P. aeruginosa* (a pathogen of humans and animals) or *P. syringae* (a plant pathogen), the majority of *Pseudomonas* spp. are organisms that are harmless or beneficial from the point of view of humans (Peix et al., 2018). Many strains have been shown to promote plant growth and/or protect their plant hosts against the harmful activity of pathogens, which classifies them as plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009). Importantly, the mechanism by which a given representative of *Pseudomonas* sp. exhibits PGPR activity may vary depending on the species or even the strain tested. This is related to the fact that *Pseudomonas* spp. have relatively large genomes, a significant part of which are genes that are not shared (do not have their orthologues) in all representatives of the entire group (Silby et al., 2011, Loper et al., 2012). *Pseudomonas* spp. strains classified as PGPR are of interest due to their potential for the application in biological plant protection (Mercado-Blanco, 2015).

At the time of publication of this work, the species *P. donghuensis* included four strains: P482, HYS^{T}, SVBP6 and 22G5 (Gao et al., 2015, Agaras et al., 2018, Krzyzanowska et al., 2016, Tao et al., 2020). Antagonism towards plant pathogens was demonstrated for each of these strains. The antimicrobial properties of *P. donghuensis* strains are mainly, but not exclusively, attributed to the production of the antimicrobial and iron scavenging compound 7- ydroxytropolone (7-HT). 7-HT is not a typical metabolite for the *Pseudomonas* spp. group. The *nfs* gene cluster necessary for its production has then been detected only in *P. donghuensis* (Yu et al., 2014, Krzyzanowska et al., 2016, Muzio et al., 2020, Tao et al., 2020) and two closely related species: *P. qingdaonensis* and *P. wadenswilerensis* (Muzio et al., 2020). Previously, the production of this metabolite was described in *Pseudomonas lindbergii* ATCC31099 (Korth et al., 1981) and *Streptomyces neyagawaensis* (Kirst et al., 1982). In the latter case, the 7-HT was tested for the ability to reduce microbial resistance to aminoglycoside antibiotics by inhibiting aminoglycoside adenyltransferase (Allen et al., 1982).

In 2014, we obtained and published a draft version (WGS) of the genome of the *P. donghuensis* P482 strain (Krzyzanowska et al., 2014) (***publication not included in the series***). At that time, the quality of data and the available software did not allow combining the sequencing reads into a single molecule (chromosome), hence the draft version consisted of 69 contigs. Genome sequencing of the three remaining *P. donghuensis* strains known at that time (HYS^{T}, SVBP6, and 22G5), performed by other groups, also failed to produce a closed chromosome (Table 1).

Table 1. Strains of *P. donghuensis* and the level of assembly of genomic data for these strains at the time of publication of the discussed work (2021).

Strain	Origin	Assembly level (contig no.)	GenBank accession no.	Size (bp)	G+C %	ANI %[A,B]	Reference (genome)
P482	Tomato rhizosphere, Poland	Complete (1)	CP071706	5 656 185	62.36	99.47[B]	This work
		WGS (69)	JHTS00000000.1	5 623 997	62.38	99.47	(Krzyzanowska et al., 2014)
HYS^{T}	Lake water, China	WGS (231)	AJJP00000000.1	5 646 028	62.42	100	(Gao et al., 2012)
SVBP6	Farmland soil, Argentina	WGS (43)	NWCB00000000.1	5 701 342	62.37	99.45	(Agaras et al., 2018)
22G5	Rhizosphere of weeds, China	WGS (3185)	RWIB00000000.1	6 546 541	60.74	99.41	(Tao et al., 2020)

[A] ANI calculated relative to the HYS^{T} type strain using ChunLab's online Average Nucleotide Identity (ANI) calculator (https://www.ezbiocloud.net/tools/ani) (Yoon et al., 2017)
[B] ANI calculated between the complete version and the 2014 WGS was 99.98%
[T] strain typical of the species

Aim of the study

The main goal of the work was to obtain a closed genome (complete chromosome) for the *P. donghuensis* strain P482, along with annotation. An additional aim of the study was to determine, using *in silico* methods, the potential of the P482 strain to produce secondary metabolites, the presence of receptors responsible for iron acquisition in the genome and the presence of prophages.

Concise description of the results

As part of the described work, together with my colleagues, I obtained a closed chromosome sequence of the *P. donghuensis* strain P482. This was the first complete genome sequence for a strain of the *P. donghuensis* species. Genomic DNA sequencing was outsourced to Oligo.pl. The service included the use of two platforms: Illumina MiSeq (69 × coverage) and Oxford Nanopore GridION (109 × coverage), followed by hybrid data assembly. Sequence ambiguities and errors were eliminated using PCR and Sanger sequencing. The obtained complete genome of the P482 strain contains one circular chromosome with a length of 5,656,185 base pairs, with an average G+C pair content of 62.36%. No plasmids were detected. We annotated the sequence using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), version March 2021 (Li et al., 2021). We found that the genome contains 5,258 ORFs, including 5,158 CDSs (coding sequences), 74 tRNAs, 22 rRNAs, 3 ncRNAs and 1 tmRNA. **Obtaining a closed version of the genome was important due to the fact that draft versions (WGS) (including that obtained for P482 in 2014), in which the genomic information is fragmented and present in a form of numerous contigs, although very helpful in research on a given microorganism, have their limitations. In the WGS versions, the orientation (linear arrangement) of part of the gens is unknown. Regions near the ends of contigs are particularly problematic. What was also important from my perspective, only closed genomes are systematically downloaded to useful databases (including KEGG metabolic pathways browser, BioCyc), allowing the use of additional tools during studies on a given strain.**

The new annotation performed on the complete genome of P482 gave us the benefit of an up-to-date annotation of protein functions. However, the downside of re-annotating a genome is that you obtain a new set of locus tags for all genes. **I had in mind that some already published works, as well as research in progress at that time, were based on loci designations from the earlier draft version of the genome. To avoid confusion regarding gene designations, we performed CDS cross-mapping between the 2014 WGS version and the newly obtained complete genome of P482.** Mapping was performed at the protein level using BLASTP 2.11.0+ (Altschul, 1997). The threshold for considering sequences to be identical was set at E value ≤0.001 and identity ≥96%. As a result, it was possible to transfer locus designations from the “old” version to the new one for 4942 protein-coding genes (95.8%). Proteins encoded by 4433 mapped CDSs showed 100% identity between the two versions, 487 showed 99% identity, and 22 showed identity between 96-98%. All remaining genes, including non-protein-coding genes, were annotated with new five-digit locus tags from the new annotation.

My comparison of the complete genome of the *P. donghuensis* P482 with the draft version revealed that the complete chromosome contains 32,188 base pairs more than the WGS version. Figure 7 presents the order and the orientation of the genome content in the closed chromosome relative to the draft version. The tool that I used for the analysis was the ProgressiveMauve (Darling et al., 2010). The same program revealed 519 single-nucleotide polymorphisms (changes in single base pairs) between the full genome and the draft. Cross-mapping of the loci revealed 188 protein-coding sequences in the WGS version that could not be mapped to the closed chromosome P482. The majority of unmapped genes encoded hypothetical or putative proteins (92% in total), and only 4 (2.1%) of the remaining loci could be assigned a function by KEGG using BlastKOALA (Kanehisa et al., 2016). On the other hand, 118 protein-coding sequences revealed in the new closed genome annotation could not be mapped to the WGS version. Of this set, 11 loci (13%) were assigned the KEGG KO ID. The latter included genes such as *recC, recD* and *pqqA*. Differences in the detected ORFs between the closed genome and the WGS version may have various causes, including small differences in the obtained sequences, the fact that some ORFs were not identified in the WGS version due to the fragmentation of the genome into 69 contigs, and the fact that a different algorithm was applied for gene annotation.

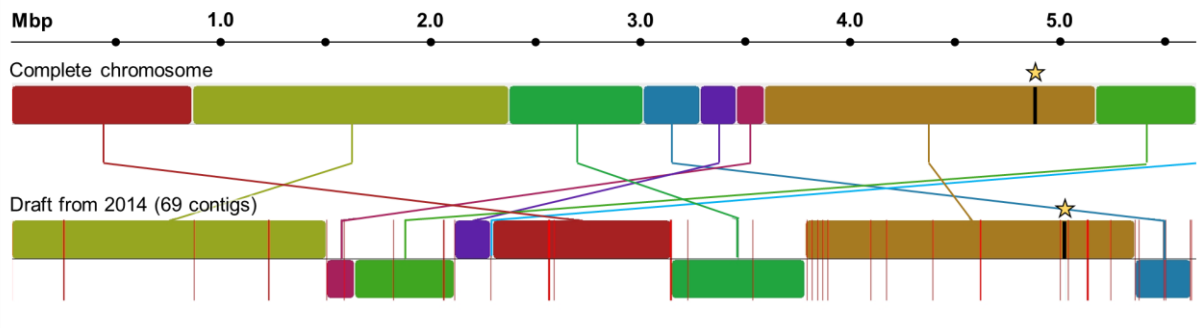


Figure 7. Order and orientation of the genome content between the closed chromosome of *P. donghuensis* P482 (this work) and the earlier version of WGS (64 contigs). Each block represents a region in the closed genome that has an equivalent in the WGS version. Red vertical lines mark the boundaries of the contigs. Asterisks indicate the genomic location of the *nfs* gene cluster homolog encoding the production of 7-hydroxytropolone, an important antimicrobial compound produced by *P. donghuensis* strains. The figure was presented as Fig. 1 in the discussed publication.

During my Ph.D., I analyzed the WGS version of the P482 genome sequence for the presence of genes encoding the production of secondary metabolites using antiSMASH 2.0 (Blin et al., 2013, Krzyzanowska et al., 2016). At that time, when the data mining tool was a new thing, it revealed the presence of 23 clusters of genes that may be involved in the synthesis of secondary metabolites, including only 5 with any suggestion as for their function: two non-ribosomal peptide synthases ("NRPS"), one "bacteriocin" and two clusters annotated as "Other", as well as 18 putative (hypothetical) clusters. However, in the field of *in silico* tools for the prediction of gene function, significant progress has been made between the acquisition and analysis of the P482 draft genome and the acquisition of the full genome. **Therefore, as part of the discussed work, I analyzed the P482 genome using antisMASH 6.0 beta (Blin et al., 2021). The newer version of antiSMASH, when using the closed genome of**

P482 as input data, recognized regions in the P482 genome similar to those known to be involved in the synthesis of compounds such as pseudopyronin (62% cluster similarity), arylpolyene Vf (40%), fragin (37%), lipopolysaccharides (27%, 5%), O-antigens (19% and 14%), pyoverdine (17%, 11%, 6%), lankacidin C (13%) and chejuenolide (7%) . Interestingly, although these results were much more specific than for antiSMASH 2.0 analyses, antiSMASH 6.0, even with the most relaxed search parameters (i.e. those returning the largest number of hits, including hypothetical clusters), did not predict in the genome of P482 the cluster which we have previously found to encode the synthesis of the antimicrobial 7-HT. This particular cluster, although without an assigned function, had been found among hypothetical clusters by the older version of the software (Krzyżanowska et al., 2016). It is also physically present in the closed version of the genome (Figure 7).

Pseudomonas bacteria have a system to uptake iron from the environment based on TonB-dependent outer membrane receptors that recognize, as ligands, siderophores bound to iron or heme (Cornelis, 2010). Our analysis of the P482 genome revealed the presence of 43 genes encoding TonB-dependent receptors, 21 of which were described as TonB-dependent siderophore receptors, and 3 as TonB-dependent hemoglobin/transferrin/lactoferrin family receptors. Iron is a microelement necessary for the functioning of the cells of living organisms. In many environments, iron in its bioavailable form is relatively scarce, limiting cell growth. Therefore, possessing numerous Ton-B-dependent receptors may give *P. donghuensis* P482 an advantage in colonizing the occupied ecological niche (e.g. plant rhizosphere or soil) and increases the competitiveness of the strain in an iron-poor environment.

We also searched the P482 genome for the presence of prophage sequences using the Prophage Hunter tool (https://pro-hunter.genomics.cn). **The analysis resulted in the discovery of the sequence of a single, presumably intact (active) prophage with a length of 57,737 bp, located between positions 2812547 and 2870283.** The genome of the discovered prophage contains genes necessary for the functioning of bacteriophages, such as integrase, transcription factors regulating the transition between the lytic and lysogenic life cycle, genes encoding holin and lysine, and structural proteins. According to PHASTER (Zhou et al., 2011), this prophage is phylogenetically most similar to the phage from *Pseudomonas* sp. YMC/01/01/P52_PAE_BP (Jeon et al., 2012). We predict that the detected prophage belongs to the order *Caudovirales* and the family *Siphoviridae*. The role of the presence of this prophage in the genome of P482, the fact whether it is functional and can infect other cells (host spectrum), remains an interesting topic for future exploration.

RESEARCH ARTICLE 4

Krzyżanowska D.M., Jablońska M., Kaczyński Z., Czerwicka-Pach M., Macur K., Jafra S.; **Host-adaptive traits in the plant-colonizing *Pseudomonas donghuensis* P482 revealed by transcriptomic responses to exudates of tomato and maize**. ***Scientific Reports***, **2023**, 13(1), 9445 (IF 4,6; MEiN 140)

Introduction

The microflora of the root zone of plants (the rhizosphere) is much more rich than the microflora of bulk soil. Behind this phenomenon is the fact that plants secrete mixtures of organic compounds through their roots, therefore providing nutrients for microorganisms. The root-secreted compounds, known as root exudates, contain primary metabolites such as organic acids, amino acids, sugars, as well as secondary metabolites with bioactive or signaling properties. The exact chemical composition of the exudates depends on the plant species and the physiological state of the plant, the latter of which depends on numerous factors, including the stage of development, nutrient availability, and the presence of stress factors (Chaparro et al., 2014). The composition of root exudates and the plant's innate immunity are of key importance in the process of shaping the composition and activity of root microflora (Bever et al., 2012).

Bacteria of the *Pseudomonas* genus can inhabit various ecological niches, including the roots of plants. This ability to survive in a variety of environments is attributed to the metabolic flexibility of *Pseudomonas* spp. and their ability to produce a wide range of secondary metabolites, including antimicrobial compounds and iron-scavenging compounds (siderophores) (Loper et al., 2012). Many plant-associated *Pseudomonas* spp. have a beneficial effect on plant growth (Haas and Défago, 2005). However, there is no comprehensive research on the spectrum of plant species that given *Pseudomonas* strains can colonize. However, it is known that some strains have proven effective as biological plant protection agents when applied on plant species other than those of their origin, suggesting that single strains of *Pseudomonas* spp. can colonize different plant hosts (Dekkers et al., 1998).

Different plant species have been shown to recruite different populations of microbiota, and phylogenetically distant hosts are thought to recruit microbial communities with more diverse compositions (Imam et al., 2016, Bouffaud et al., 2014). Therefore, the fact that some microorganisms, such as *Pseudomonas* spp., can colonize different plant hosts raises questions about the metabolic adaptations that such strains must undergo to colonize a given host, or to persist in the host plant's microbiome despite the physiological changes taking place in the plant. These questions are difficult to answer on the basis of a meta-analysis of available data due to the fact that most publications describe strain-host interactions in systems of one host-one strain or one host and a larger number of strains. Moreover, while the determinants of specificity in bacteria-plant host interactions have been thoroughly studied in the case of symbiotic bacteria of the *Rhizobium* genus, so far little attention has been paid to this topic in the case of microorganisms forming less "intimate/close" relationships with plants (Drogue et al., 2012).

Pseudomonas donghuensis P482 is a strain from the collection of the Laboratory of Plant Microbiology (IFB UG and GUMed). The strain has a potential potential for being used in biocontrol as it inhibits the growth of several bacterial and fungal plant pathogens (Krzyzanowska et al., 2012b, Krzyzanowska et al., 2016, Ossowicki et al., 2017). These bacteria, although originally isolated from the rhizosphere of tomato (*Solanum lycopersicum*

L.), can also colonize the rhizosphere of potato (Krzyzanowska et al., 2012a) and, as shown in the studies described below, the roots of phylogenetically distant maize. This made strain P482 a promising model for studying the mechanisms of adaptation of host promiscuous bacteria to specific plant hosts.

Aim of the study

The aim of the study was to identify metabolic pathways involved in the adaptation of *Pseudomonas* spp. to various plant hosts by identifying genes constituting the differential (host-specific) and shared (host-independent) transcriptomic response of the *P. donghuensis* strain P482 to the root exudates of two phylogenetically distant plant species: tomato (dicotyledonous plant) and maize (monocotyledonous plant).

Description of the results and their contribution to the field

Our notion that strain P482, apart from being able to colonize the rhizosphere of tomato, can also colonize the roots of maize, came from experiments on *in vitro* grown plants (M. Rajewska, unpublished data). Therefore, as part of preliminary research, I conducted a pot experiment in which I verified the ability of P482 to colonize soil-grown tomato (*Solanum lycopersicum* L., cv. Saint Pierre) and maize (*Zea mays* L., cv. Bejm). The plants were inoculated with P482 by seed bacterization (coating). To enable selective re-isolation of P482 from roots, a spontaneous rifampicin-resistant variant of the P482 strain was used in the study. We showed that P482 is present on the roots of 18-day-old plants of both tomato and maize, although the mean population size on tomato roots was higher than that for maize (1.54×10^7 CFU g^{-1} and 2.35×10^6 CFU g^{-1}, respecively)[1].

To compare the transcriptomic response of the tested strain to the exudates of both plants, I cultured cells of the *P. donghuensis* P482 in mineral medium containing a single carbon source and supplemented with maize or tomato exudates, or without the addition of exudates (control). Next, total RNA I have isolated form the samples was sequenced as part of an outsourced service. **Differential gene expression analysis showed that the addition of tomato exudates caused a change in the expression of 413 genes (8%), while maize exudates caused a change in the expression of 181 genes (3.5%) when compared to the unsupplemented medium. The fact that a greater number of genes showed an altered expression in the presence tomato exudates suggests that the response of P482 to tomato-derived compounds was broader than that to compounds from maize**. However, it remains an open question whether this is related to the fact that this strain was isolated from tomato and not maize. **Based on the analyzed data, I also showed that the response that differentiates between the two types of exudates is broader (it involves more genes) than the shared response, with the latter understood a response common to root exudates regardless of their source of origin.** The set of differential response genes consisted of 278 genes (5.38% of the

[1] CFU – colony forming units

transcriptome), and the set of shared response genes consisted of 63 genes (1.22% of the transcriptome) (Figure 8).

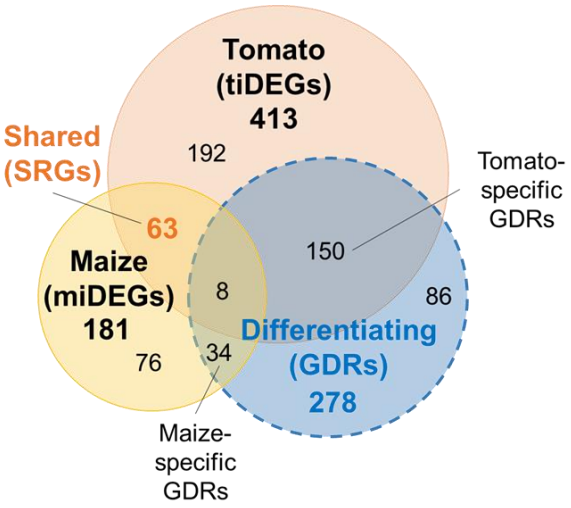


Figure 8. Venn diagram showing subsets of genes the expression of which was altered in *P. donghuensis* P482 transcriptome in response to tomato exudates (423) and maize exudates (181), superimposed with genes that differentiate between both responses (278). Shared response genes (63) are genes the expression of which was changed in a similar way by both types of exudates, regardless of their source. Superimposition also revealed additional subsets: differentiating genes specific for tomato (150) and specific for maize (34). The latter groups simultaneously belong to differentiating genes and those whose expression was changed only by one type of exudates. Criteria used to consider a difference in expression significant: $p<0.05$ (padj/FDR; Benjamini-Hochberg correction); $\log_2 FC>1.5$. The figure was presented as Fig. 1 in the discussed publication.

Next, I have analyzed the pools of differential response genes and the shared response genes in terms of enrichment of metabolic pathways (KEGG), enrichment of clusters of orthologous protein groups (COG), and enrichment of gene clusters with assigned function and gene networking (STRING). The collective analysis of the obtained data and an extensive literature review regarding possible connections between the selected metabolic pathways allowed me to characterize the transcriptomic response of P482 to the exudates of the tested plants. Below I present a brief description of the obtained results, divided into section dedicated to the shared response (analogous for the two types of exudates,) and responses specific to tomato and maize exudates. A diagram summarizing the most important results can be found in Figure 9. Additionally, as part of the described work, chemical analysis of the composition of both types of exudates was carried out using gas chromatography (GC-MS), nuclear magnetic resonance (NMR), and liquid chromatography with selective monitoring mass spectrometry (LC-SRM), aim at trying to link the presence of some primary metabolites in the exudates to the transcriptomic response of P482.

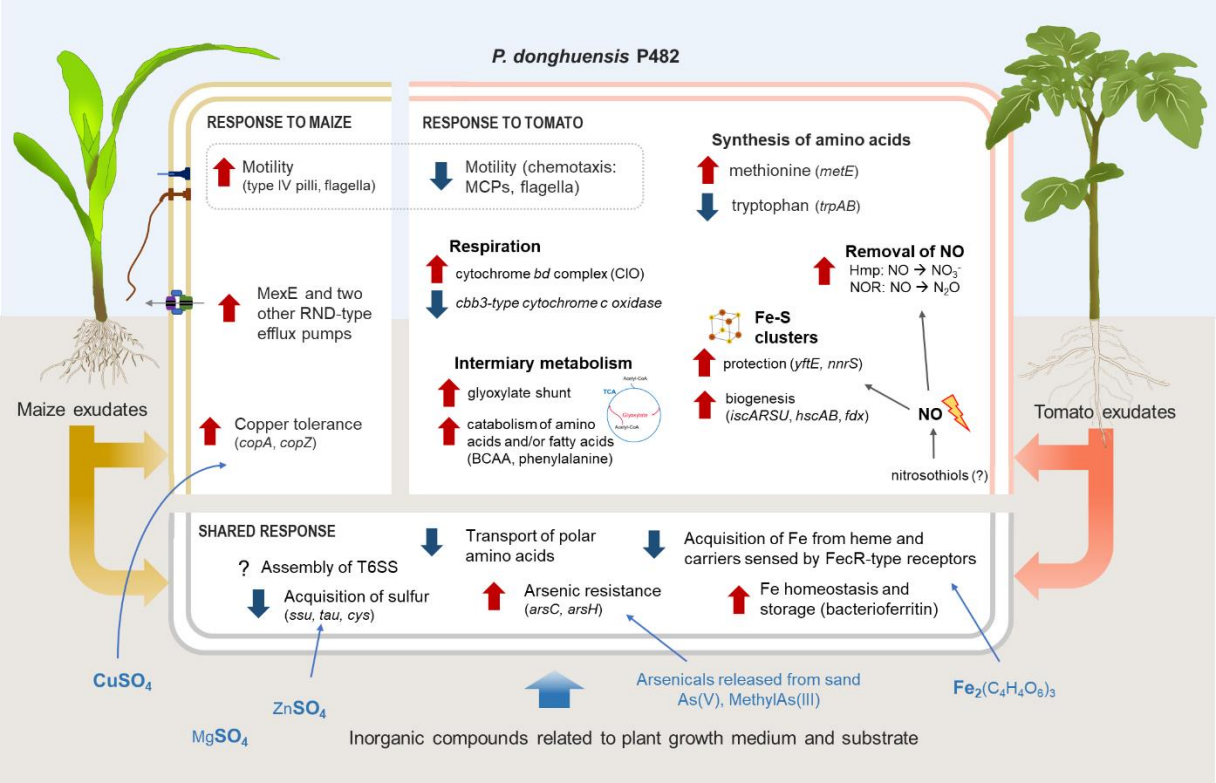


Figure 9. Aspects of the transcriptomic response of the *P. donghuensis* P482 to root exudates of tomato and maize - shared and host-specific responses. Red arrows pointing up indicate increased expression of genes involved in a given metabolic pathway, while blue arrows pointing down indicate decreased expression. The figure was presented as Fig. 4 in the discussed publication.

Shared response

I have shown that the shared response of P482 to both types of exudates includes such aspects of metabolism as sulfur assimilation, arsenic metabolism, iron acquisition from xenosiderophores and heme compounds, iron homeostasis, and regulation of the assembly of the type six secretion system (T6SS). Even though the roots of our test plants were rinsed in water before exudate harvest, the shared response, at least in part, may be due not to the exudates themselves but to the plant growth conditions we have used in our experiment. In particular, I consider the potential impact of the transfer of microelements from the culture medium (Hoagland's salts) and the substrate (coarse-grained aquarium sand) onto/into the roots and to the exudates.

Supplementation of the growth medium with exudates, regardless of their source, resulted in decreased gene expression within the overlapping pathways of sulfur metabolism and ABC transporters. This included both genes involved in the acquisition of sulfur from inorganic sources and cysteine (*cysW* and *cysA*) and genes involved in the assimilation of alternative organic sources of sulfur, such as alkanesulfonates, including taurine (selected genes of the *ssu* and *tau* operons). A reduction in the expression level of the *ssu* genes or both

ssu and *tau* was also observed for 6 out of 8 *Pseudomonas* spp. exposed to root exudates of the grass *Brachypodium distachyon* (Mavrodi et al., 2021).

The addition of exudates increased the expression level of the *arsC* and *arsH* genes in P482. Both genes are linked to the metabolism of arsenic, a highly toxic metalloid. Arsenic is ubiquitous in the environment, including soil and sand. Therefore, bacteria have developed various mechanisms for its processing. In an attempt to explain the increased expression of *ars* genes in P482 as part of the shared response, I formulated a hypothesis that the increased expression of *arsC* and *arsH* in P482 could have been caused by arsenate and organic arsenic compounds transferred to the roots from the sandy substrate used for growing tomatoes and maize.

One of the COG categories enriched in the shared response of P482 to exudates was the "Inorganic Ion Transport and Metabolism/Signal Transduction", represented by seven *fecR*-like genes. Expression of all of those genes was downregulated. FecR proteins are signal-transducing transmembrane sensors, involved in bacteria in the uptake of iron-binding molecules such as citrate (ferric citrate), but also other compounds, including molecules produced by competing microorganisms to obtain iron from the environment (so-called xenosiderophores)(Visca et al., 2002). Another source of iron that *Pseudomonas* spp. can use are heme molecules released from hemoproteins. In exudate-treated P482, we observed downregulated expression of numerous genes involved in heme uptake and metabolism (BV82_0056, *hasR, hemO, hmuU, hmuV, hasD*). In parallel, exudates did not affect the expression of genes involved in the synthesis of pyoverdine and 7-HT siderophores in P482. No increase in the expression of pyoverdine-related genes was also observed for eight *Pseudomonas* strains exposed to exudates of *B. distachyon* (Mavrodi et al., 2021). In the cited work, an increase in the expression of any genes related to iron acquisition was observed only in half of the investigated strains. In another work, the expression of genes related to heme acquisition and pyoverdine synthesis in *P. protegens* CHA0 was relatively low in response to wheat-derived compounds (plant origin) compared to the expression of these genes observed during insect infection by *P. protegens* CHA0 (Vesga et al., 2020).

In P482, in contrast to the genes involved in iron acquisition, the *bfr* gene involved in intracellular iron homeostasis was upregulated in response to exudates. Iron, when present in excess in a free form, can cause oxidative stress in the cells by producing reactive oxygen species. Bfr acts as an iron storage protein. The increase in the level of Bfr synthesis in P482 is another indication that the root exudates themselves, collected from plants grown in gnotobiotic conditions, do not limit the availability of iron for P482. It is worth emphasizing, however, that in our experimental conditions and those used by Mavrodi et al., who observed a similar tendency for some *Pseudomonas* spp., the strains were cultivated in the form of monocultures. It is highly likely that P482 would have to use its extensive arsenal of methods to obtain iron in the presence of competing microbes.

After exposure of P482 to root exudates, we observed increased expression of *tagQ*, *pppA*, and *crp*. In *P. aeruginosa*, the *tagQ* and *pppA* genes are involved in a regulatory cascade controlling T6SS activation, with *pppA* considered to be a negative regulator of the T6SS (Silverman et al., 2011). The third gene, *crp*, encodes the cAMP receptor. Reports on the role of Crp in the regulation of T6SS are scarce and concern *Vibrio cholerae*, where Crp was

suggested to be a positive regulator of T6SS (Ishikawa et al., 2009). To sum up, the results obtained by me and my colleagues suggest that the presence of root exudates has some influence on T6SS in P482, but the direction of this influence requires further research. It can also be assumed that the expression of T6SS-related genes would be different in the presence of competing (micro)organisms. The T6SS allows bacteria to inject proteins into other prokaryotic or eukaryotic cells, and the system itself is known for its role in interbacterial competition (Boak et al., 2022).

Tomato-specific response

Based on the conducted analyses, I showed that the P482 strain treated with tomato exudates shows symptoms of stress caused by nitric oxide (also known as nitrosative stress). Nitric oxide is a radical that has a toxic effect on cells. To counter nitrosative stress, bacteria have several pathways to convert NO into less reactive molecules such as N_2O, NO_3, or ammonia (Vine and Cole, 2011). When P482 was exposed to tomato exudates, we observed a significant increase in the expression of the *hmp* gene (6.13 log_2FC). Under aerobic conditions, Hmp catalyzes the reaction of NO with oxygen, resulting in the formation of nitrate ion (NO^{3-}) (D'Autréaux et al., 2002). We also observed increased expression for *norD, norC* and *norB*, encoding nitric oxide reductase (NOR). This membrane-integrated enzyme catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O) (Shiro, 2012). Hmp and NOR are well-documented components of the mechanism responsible for NO detoxification in bacteria.

Nitric oxide acting on a bacterial cell may come from the environment or be produced endogenously by cells through the process of denitrification in cells experiencing limited oxygen availability. **Based on the analysis of the expression of genes involved in denitrification in P482, I concluded that the reason for the activation of NO detoxification pathways in P482 is not denitrification, but rather coping with the toxicity of nitric oxide present in the growth environment.**

The harmful effects of NO on the cell are mainly due to the inactivation of proteins containing iron-sulfur (Fe-S) clusters as cofactors (Shi et al., 2021). Fe-S clusters are common in living organisms and are involved in many basic biochemical processes (Shi et al., 2021). **In P482 cells exposed to tomato exudate, there was a marked increase in the expression of *yftE* (6.36 log_2FC) - a gene known for its role in the metabolism/repair of Fe-S clusters**. Homologues of *ytfE* are present in numerous bacteria, and the induction of this gene expression in response to NO has been previously documented (Bowman et al., 2011). Literature data show that the YftE protein contributes to the survival of *Yersinia pseudotuberculosis* in the spleen after nitrosative stress and contributes to the virulence of this human pathogen (Davis et al., 2019). Another gene the expression of which was significantly induced by tomato exudates was the *nnrS* gene, encoding a protein that has been shown to play a role in protecting the Fe-S clusters against NO in *Vibrio cholerae* (Stern et al., 2013). It is worth noting that in P482, the *yftE* and *nnrS* genes are encoded within one cluster with the genes encoding NOR and two regulators: *norR* and *dnr*. **In parallel, the expression of *iscR, iscS, iscU, iscA, hscB, hscA* and *fdx* genes, encoding proteins related to the biogenesis of Fe-S clusters, was also upregulated** (Figure 10).

Eukaryotic organisms can produce nitric oxide as a defense during infection (Stevanin et al., 2005). In contrast, plants use NO as a signaling molecule in response to biotic and abiotic stress, but also as part of numerous physiological processes such as germination, flowering and senescence (Pande et al., 2021). Because NO is highly reactive, it is stored in plants in the form of S-nitrosothiols (SNO), which act as NO reservoirs *in vivo* (Pande et al., 2021). **NO plays a role in plant responses to pathogens, but is also crucial for establishing symbiotic interactions between rhizobia and legumes (Spiro, 2007). It is also worth noting that although the tomato exudates we tested caused nitrosative stress in P482, their addition to the culture did not negatively affect the growth rate of P482 *in vitro*. This suggests that P482 is well adapted to cope with these type of conditions when it encounters them.**

Bacteria of the *Pseudomonas* genus have a branched respiratory chain. *P. aeruginosa* contains five different oxidases, allowing the electron transport pathway to be optimized for the given environmental conditions (Arai, 2011). **I have shown that in P482, in response to tomato exudates, the expression of the *ccoN, ccoO, ccoQ* and *ccoP* genes encoding the *cbb3* type cytochrome c oxidase subunits is decreased, while the expression of *cioA* and *cioB* genes, encoding the cyanide-insensitive oxidase of cytochrome *bd*, is upregulated (CIO; cyanide insensitive oxidase)** (Figure 10). Microorganisms that can switch to CIO utilization are able to survive in the presence of high concentrations of hydrogen cyanide, which blocks respiration through the cytochrome c oxidases (Quesada et al., 2007). Although the *cio* genes were first described in the context of resistance to hydrogen cyanide, other factors can also modulate their expression. Literature data show that cytochrome *bd* plays a role, among others: in bacterial tolerance to oxidative and nitrosative stress (Giuffrè et al., 2014). Interestingly, in the *Pseudomonas* sp. WCS365 strain, it was reported that the *cioA* genes are involved in avoiding the host defense reaction during interaction with *Arabidopsis thaliana* (Liu et al., 2018).

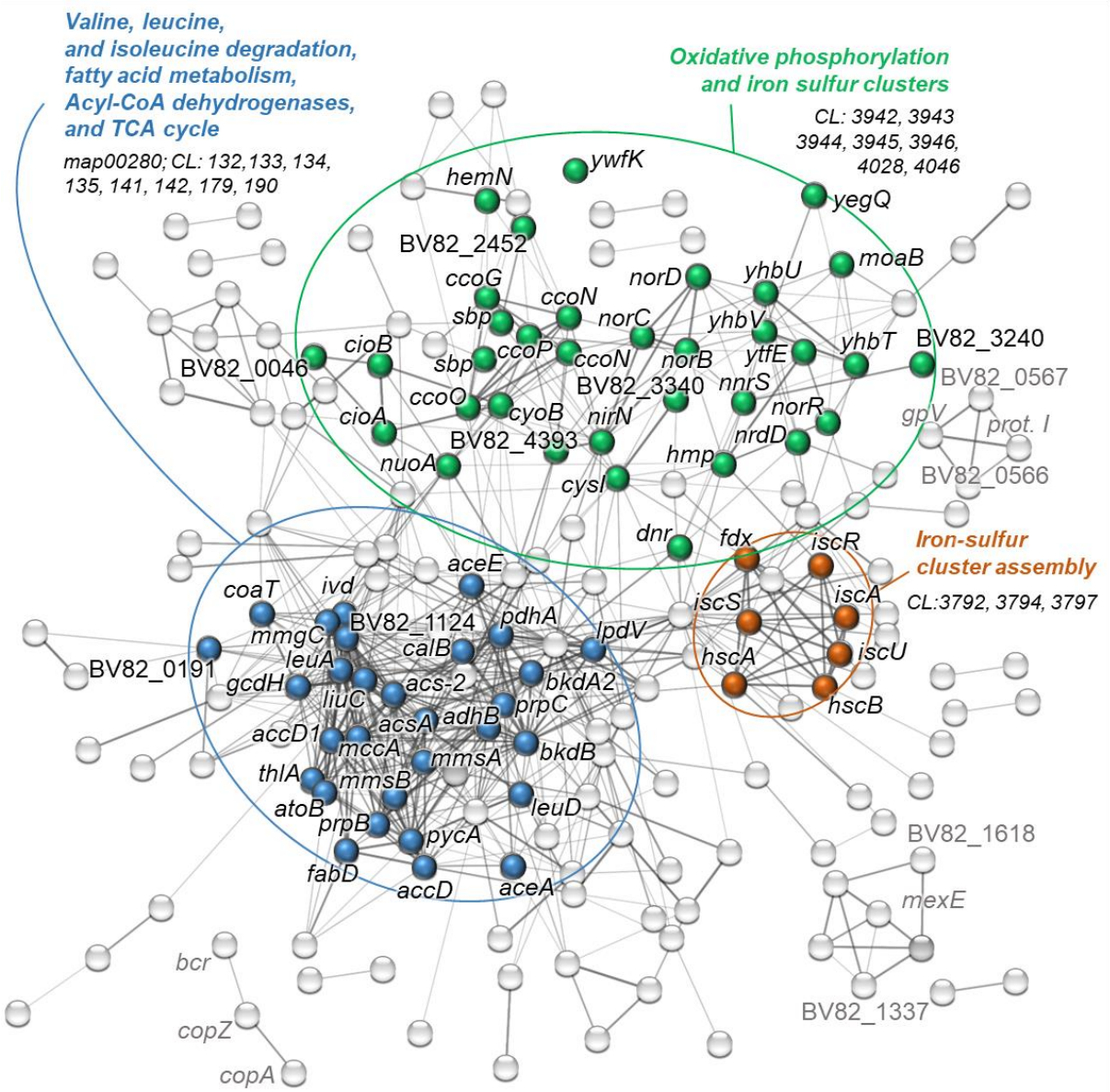


Figure 10. STRING analysis result for 278 P482 transcriptomic response genes differentiating between tomato and maize root exudates. The analysis was performed with the *P. donghuensis* HYS genome as the reference genome (the most closely related microorganism in the database). Each node represents the P482 gene with a name assigned using EggNOG. Filled nodes indicate genes belonging to enriched groups identified by STRING. All available interaction types available for STRING version 11.5 (May 2023) are included. Genes that showed no interactions were not included in the figure, except for those found to be part of the enriched groups. The figure was presented as Fig. 3, panel B, in the discussed publication.

In the described work, I proposed the hypothesis that the switch to cellular respiration involving cytochrome *bd* (CIO) in the P482 strain treated with tomato exudates results from the presence of inhibitors of the cytochrome *bc1* complex and/or nitric oxide in the exudates. The cytochrome *bc1* complex is involved in electron transfer to cytochrome c oxidases such

as *cbb3*, but not to CIO, to which electrons are transferred directly from ubiquinone (Arai, 2011, Ha et al., 2018). Research conducted by another group on the *Mycobacterium tuberculosis* model showed that the bactericidal effect of *bc1* inhibitors was significantly enhanced by the simultaneous addition of NO donors, what these bacteria tried to counteract by switching to the cytochrome *bd* pathway (Zeng et al., 2021).

Based on my analyses, we also showed that tomato exudates have an impact on the primary metabolism of the P482 strain. Within the differential response, there was enrichment for three overlapping KEGG pathways: "pyruvate metabolism," "propanoate metabolism," and "valine, leucine, and isoleucine degradation." The genes involved in these pathways largely overlap with those included in the also enriched COG category "energy production and conversion" (Figures 10 and 11).

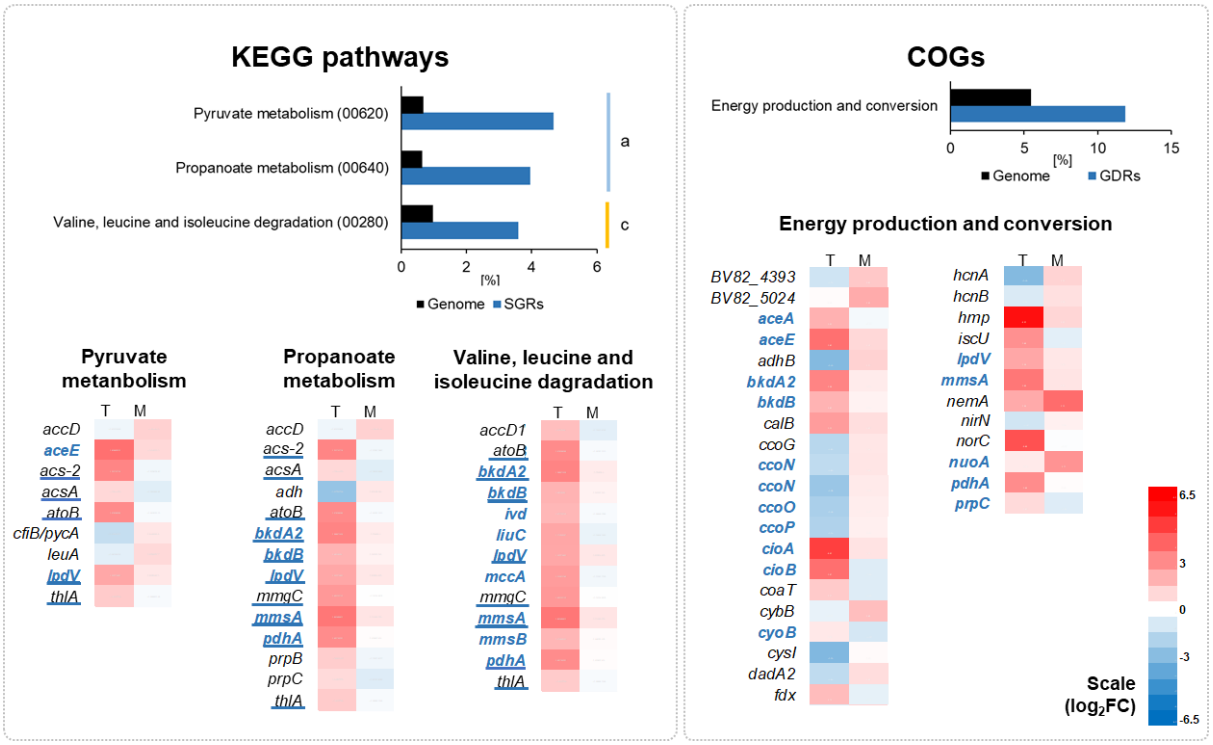


Figure 11. KEGG pathways and COG categories enriched within the differential transcriptomic response of P482 to the root exudates of tomato and maize. For individual genes within pathways: red and blue colors indicate increased and decreased gene expression, respectively; the left column shows expression changes (log_2FC) after the addition of tomato exudates (letter T - tomato), and the right column after the addition of maize exudates (letter M - maize). KEGG categories: a – carbohydrate metabolism, b – environmental information processing, c – amino acid metabolism. Genes shown in bold appear in parallel in enriched KEGG and COG pathways. The underlined genes overlap between different categories within the KEGG pathways. The figure was presented as panels C and D of Fig. 2 in the discussed publication.

As part of the P482 response to tomato exudates, we observed increased catabolism of branched chain amino acids (BCAAs), i.e. valine, leucine and isoleucine. Tomato exudates increased the expression of *lpdV, bkdB, bkdA2*, and *pdhA*. The general function of the complexes encoded by these genes is to convert alpha-keto acids to acyl-CoA and CO_2. Alpha-keto acids can be formed, among others: as a result of oxidative deamination of amino acids and vice versa, they are precursors for their synthesis (Ævarsson et al., 1999). 2- Oxoisovalerate dehydrogenase encoded by *bkdA2* and *pdhA* is an enzyme involved in the catabolism of BCAA (Namba et al., 1969). At the same time, we observed the induction of the *mmsA* and *mmsB* genes, involved in valine metabolism in *P. aeruginosa* (Steele et al., 1992).

The gene expression profile of P482 also suggested activation of the catabolism of three other amino acids: phenylalanine (*phhA, phhB* and *phhC* genes), glycine (*gcvH* and *gcvP*) and methionine (*aceE/mdeB, mdeA*). Other metabolic changes in P482 associated with increased catabolism of amino acids and/or fatty acids included an increase in the activity of two dehydrogenases catalyzing α,β-dehydrogenation of acyl-CoA esters (Swigonová et al., 2009). **Moreover, isocitrate lyase encoded by *aceA* was upregulated, suggesting activation of the glyoxylate shunt (GS). Many bacteria activate GS when acetyl-CoA, formed by catabolism of amino acids or fatty acids, constitutes the main carbon source available to the cell** (Ahn et al., 2016). The classical tricarboxylic acid (TCA) cycle cannot assimilate carbon with the formation of citrate when acetyl-CoA is the only available carbon source. This happens due to the loss of CO_2 and the inability to regenerate oxaloacetate. GS allows metabolism to be carried out without the problematic TCA stages. In addition to its important role in primary metabolism, the glyoxylate cycle probably plays a still poorly understood role in defense against stress and in pathogenicity. There are reports that this pathway is crucial for the growth and virulence of *P. aeruginosa* during infection, when the bacterium preferentially uses fatty acids (Crousilles et al., 2018). Moreover, it has been shown that targeting the metabolism towards the use of amino acids increases the resistance of *P. aeruginosa* to antibiotics (Mielko et al., 2020).

At this point, it is worth referring to the analysis of the chemical composition of the exudates used in the described paper, and more precisely to the LC-SRM analysis performed specifically in terms of amino acid content. The content of valine and leucine was approximately 4 times higher in maize exudates than in tomato exudates, and isoleucine content was comparable in both samples. The content of phenylalanine and tryptophan was 4.5 and 2.3 times higher, respectively, in the case of maize compared to tomato exudates. The methionine content in both types of exudates did not differ. Therefore, it is not the case that amino acids the catabolism of which is increased in cells growing in the presence of tomato exudates (including BCAA), are present in higher concentrations in these particular exudates. **Due to this, in the described work I put forward a hypothesis that the switch of P482 to amino acid catabolism in samples treated with tomato exudates was aimed at increasing the resistance of cells to stress factors in a similar way as described for *P. aeruginosa* in human infection models** (Crousilles et al., 2018)·(Mielko et al., 2020)**. The presence of NO, causing damage to Fe-S clusters in the active centers of many enzymes, may disturb some metabolic pathways. In such a case, the observed change in the primary metabolism of P482 would not be related to the preference of this bacterium for specific carbon sources, nor their high availability in the exudates, but rather by the selection of metabolic pathways that are not impaired by the activity of stress factors.** In samples of plant origin, the presence of such

factors (compounds) may be species-specific or related to the physiological state of a given plant. The validity of this explanation for the observed results requires further research.

In the context of the effect of tomato exudates on amino acid metabolism in P482, it is worth mentioning that the activation of certain metabolic pathways may also have less direct/obvious causes. There are reports that in *P. fluorescens,* the enzyme phenylalanine 4-hydroxylase (PAH) is involved not only in the catabolism of phenylalanine, but also in the biosynthesis of melatonin from tryptophan, which has antioxidant properties (Ma et al., 2017, Jiao et al., 2021). It has also been shown that the availability of tryptophan, methionine, tyrosine and phenylalanine, well-known precursors of plant secondary metabolites, can contribute to an enhanced plant immune response (Tünnermann et al., 2022). **In the light of these reports, one could speculate that P482, by decomposing phenylalanine and reducing tryptophan synthesis, may attempt to modulate what it "interprets" as the plant's immune response.**

Maize-specific response

Maize exudates resulted in an increase in the expression of three MFP (Membrane Fusion Protein) subunits for three RND (Resistance-Nodulation-Division) exporters: *mexE*, BV82_1337, and BV82_1618. RND exporters are a family of efflux pumps associated with bacterial resistance and the nodulation process. Efflux pumps from the RND family, together with the Omp proteins, form complexes that enable Gram-negative bacteria to export harmful compounds directly to the outside of the cell and not, as in the case of other efflux systems, to the periplasmic space. This makes RND pumps important determinants of multidrug resistance in bacteria (Nikaido and Takatsuka, 2009). In *P. aeruginosa*, it has been reported that the *mexEF-oprN* operon containing *mexE* confers resistance to quinolones, chloramphenicol, and trimethoprim (Köhler et al., 1999). However, it is likely that resistance to synthetic antibiotics is merely a side effect of resistance to natural secondary metabolites. It is therefore possible that the increased expression of *mexE* in P482 in response to maize exudates is related to the resistance of the bacteria to the presence of certain secondary metabolites specific to this plant host. Importantly, the genes of only selected (not all) RND transporters were induced in the presence of maize compounds. For example the expression level of *czcA* and *mdtA*, also encoding RND pumps, was increased in the presence of tomato exudates but not maize. Both maize and other grasses (*Poaceae*) are known to produce and secrete biocidal benzoxazinoids - compounds proposed to play an important role in shaping the plant microbiome (Cotton et al., 2019, Kudjordjie et al., 2019). The role of RND transporters in the tolerance of some bacteria to this group of compounds seems to be an interesting research topic.

Both tested types of exudates increased the expression of the *copA* and *copZ* genes in P482, but the induction of these genes was significantly higher in response to maize. CopA is a membrane-anchored ATPase responsible for the removal of Cu^+ ions provided by the cytoplasmic protein CopZ. Both proteins are the elements of a well-characterized copper ion tolerance systems. Copper is an essential trace element for all organisms, but excess of it is toxic. Copper in the form of $CuSO_4$ is a component of Hoagland's culture medium, used in the described research to grow both tomato and maize plants. Hence, the fact that the induction

of copper tolerance genes is significantly stronger in response to maize exudates must be due to host plant-dependent factors influencing copper availability or toxicity. Mavrodi et al. demonstrated a significant increase in the expression of one or more copper tolerance-related *cop* genes in 5 of 8 tested *Pseudomonas* spp. treated with *B. distachyon* exudates (Mavrodi et al., 2021). Both maize and *B. distachyon* are grasses, and in the discussed experiments both plants were grown on Hoagland's medium.

At the gene expression level, P482 cell motility was increased by maize exudates and decreased by tomato exudates. Maize exudates increased the expression of the gene encoding the FimV protein, involved in the formation of type IV pili. These structures are involved in adhesion, biofilm formation, but also in specific types of bacterial cell movement. Other studies have shown that type IV pilli are essential for endophytic colonization of rice by the nitrogen-fixing endophyte *Azoarcus* sp. strain BH72 98. Interestingly, the expression of *pilK*, another gene involved in pili synthesis, was downregulated in *P. aeruginosa* in response to exudates of beetroot (Mark et al., 2005), suggesting that beetroot exudates inhibit pili formation in *P. aeruginosa*, in contrast to the stimulating effect of maize exudates on pili formation in *P. donghuensis* P482. Moreover, maize exudates increased the expression of the *fliS* gene, suggesting an increase in the synthesis of flagella responsible for swimming in P482. Flagella are necessary for some strains of *Pseudomonas* to successfully colonize their hosts. The *P. fluorescens* F113 strain with a defective *fliS* gene was immotile and unable to compete with the wild-type strain for the colonization of the alfalfa root tip (Capdevila et al., 2004).

In the context motility of P482, tomato exudates resulted in an increased expression of a gene in the BV82_3459 locus, the latter presumably encoding a negative regulator of the operon involved in the synthesis of flagella. It has been shown that although flagella can give cells an advantage in the environment, their presence can also have negative consequences. Recognition of specific flagellar antigens may trigger a hypersensitivity reaction in the host plant and thus lead to the death of bacterial cells. Reduction of flagella synthesis is considered to be an important mechanism used by bacteria to evade the plant's defense response (Rossez et al., 2015). Additionally, studies conducted on the *P. putida* KT2440 strain showed that a mutant of this strain incapable of producing flagella is characterized by a shorter lag phase during growth and is more resistant to oxidative stress than the wild type strain (Martínez-García et al., 2014). Authors of the cited studies suggested that the lack of metabolic cost associated with the production of flagella may provide cells with surplus energy which they can use for other purposes, including fighting exogenous stress. **Therefore, it appears that in the case of P482 treated with tomato exudates, the advantages of reduced expression of genes related to flagella synthesis outweighthed the potential costs of reduced motility.** It is also worth mentioning that tomato exudate also caused a reduction in the expression level of two genes encoding methyl-accepting chemotaxis proteins (MCPs), which act as chemoreceptors in bacteria and archaea (Salah Ud-Din and Roujeinikova, 2017).

In summary, **maize exudates increased the expression of motility-related genes in P482, while tomato exudates had the opposite effect. It remains to be clarified whether these differences are related to the differential importance of motility for the ability of P482 to colonize these two plants, or whether it is rather the physiological state of the tomato from which the exudates were sampled in our study that resulted in the expression of motility-related genes unfavorable for P482.**

Conclusions and outlook

Understanding plant-microbe interactions is key to fully harness the potential of microbes to support plant health. Studying these interactions in highly complex natural systems remains a technical challenge. Therefore, simplified experimental systems, although flawed, are still helpful in obtaining scientific data by isolating selected interactions (Mavrodi et al., 2021, Yi et al., 2017). As part of the presented research, we identified aspects of the metabolism of *P. donghuensis* P482 that are differentially modulated in the presence of root exudates of two different plant hosts: tomato and maize. The composition of the tested exudates, and, consequently, their impact on P482, most likely results from both the genotype of the tested plants and their physiological condition. **Based on the performed analyzes, I believe that the following aspects of metabolism: catabolism of branched chain amino acids, resistance to nitric oxide, flexibility of the respiratory chain, methionine synthesis and selective activation of RND-type efflux pumps, deserve particular attention in terms of their role in providing *Pseudomonas* bacteria with the ability to colonization of various plant hosts, as well as remaining within the microflora of a given host despite physiological changes occurring in the plant.**

I consider that when it comes to shaping the composition of the microbiome, a lot of attention is paid to positive interactions between plants and microorganisms. In this approach, the plant, through exudates, provides microorganisms with carbon sources and enables a given group of microorganisms to "live comfortably", hence the microbes are somehow "attracted" to the host. Contrary to this, most of the plant host-specific pathways we have described in P482 are related to stress resistance. At the same time, we showed that during *in vitro* growth, the addition of exudates stimulated rather than inhibited the growth of P482. **In my opinion differential resistance of some plant-associated microbes to specific stressors may lead to changes in the composition of plant-associated microbial populations, especially in plants with activated defense systems**. In analogy to human social interactions, tolerance for the negative behavior of our neighbors may be a crucial factor for whether we will remain a member of a given community.

There are still many unknowns considering the mechanisms of plant-microorganism interactions. **The results obtained by me and my colleagues provide great potential for undertaking new research initiatives focusing, in more detail, on the identified aspects of P482 metabolism involved in plant-microbe interactions.**

4.4. Other scientific achievements since obtaining Ph.D.

My scientific career is strongly connected with the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk. Since my doctoral defense (2015), I have been working at the Faculty in various scientific projects as **a contract employee (fixed-term employment) in various positions. The type of my employment played an important role in shaping my achievements over the last 8 years (the period since the defense without deducting official breaks in research work)**. The advantage

of such employment was certainly the opportunity to gain diverse scientific experience by working in more than one research team, as well as administrative and organizational experience (employment as a lab manager). On the other hand, I may have less achievements in the areas where they are often gathered as part of carrying out duties assigned to permanent university employees. My scientific achievements were also influenced by the fact that **I became a mother twice during my professional career - first at the end of my Ph.D. (2013), and then again five years after my Ph.D. (2020).** This was associated with typical periods of absence and some slowdown in my scientific work.

Four **experimental works for which I am the first authors, which works I decided to include to the series of thematically related articles for the purposes of habilitation proceedings, constitute only a part of my post-doctoral research achievements. I also have other accomplishments from that period:** after my Ph.D. defense I became a **co-author of 7 published experimental works that were not included in the series, including one publication being the outcome of my internship abroad**. Additionally, I am a **co-author of a chapter** in a book and **two manuscripts awaiting reviews** in scientific journals. I am also **among the inventors of 3 patented inventions**.

Among the works outside the series there are three works that deserve particular attention. They all concern a **mixture of five antagonistic bacteria, called by us "The Great Five", which effectively inhibits the symptoms of soft rot caused by *Pectobacterium* and *Dickeya* spp. (SRP strains) on potato tubers under storage conditions**. The described research was application-oriented. Together with my colleagues, I tested a set of randomly composed mixtures of microorganisms showing various types of antagonism towards SRPs, and then, by testing against several species of pathogens from this group, we selected an effective protective mixture **(Krzyżanowska et al., 2019b)** (***publication not included in the series***). The strategy that we adopted for composing and testing a consortium for the use in biological plant protection was innovative at that time. Moreover, as part of research carried out with my colleagues, several **formulations of the biopreparation** were developed based on the selected mixture of strains. Then, the shelf life of the preparations was tested for a period of 12 months. **For the most promising formulation, we have proven, in a six-month storage test conducted on a semi-technical scale, that the preparation is effective when used to protect potato tubers against SRP (Maciag et al., 2020)** (***publication not included in the series***). Additionally, we sequenced the genomes of the strains constituting the “The Great Five” consortium and, **through data mining and genomic analyses, we showed that in the genomes of the studied bacteria carry no genes known to produce toxins that could interfere with the use of the above-mentioned consortium in application for the protection of potato tubers (Maciag et al., 2022)** (***publication not included in the series***). The discussed research resulted in three scientific publications. In two of them I am the second author, and in one of them the first author with equal contribution stated for me and Mr. Tomasz Maciąg. The mentioned works were used by Mr. Maciąg in the procedure for awarding a doctoral degree. In addition to the publications, the above-mentioned research on "The Great Five" resulted **in 3 granted patents, including one European patent**. Additionally, I am a co-inventor of one invention in the field of microbial cell preservation (a reagent increasing cell survival during freeze-drying) (patent

application P.428215). Below I provide a detailed description of my contribution to the three above-mentioned publications and a list of granted patents. In the mentioned patents, all co-inventors declared an equal contribution to the creation of protected inventions.

- **Krzyżanowska D.M.***, Maciąg T*., Siwińska J., Krychowiak M., Jafra S., Czajkowski R. Compatible mixture of bacterial antagonists developed to protect potato tubers from soft rot caused by *Pectobacterium* spp. and *Dickeya* spp. ***Plant Disease* (2019)** (IF 3,809; MEiN 70)

  Contribution of the applicant: *I participated in the conceptual work, I took part in all the experiments described in the work (including large-scale pathogenicity tests, which required the cooperation of 2-3 people), I participated in the processing of data, preparation of drawings, writing the manuscript and preparing responses to reviewers.*
  ***equal contribution of co-authors*

- Maciag, T., **Krzyżanowska, D.M.**, Jafra, S., Siwińska J. Czajkowski R. **The Great Five—an artificial bacterial consortium with antagonistic activity towards *Pectobacterium* spp. and *Dickeya* spp.: formulation, shelf life, and the ability to prevent soft rot of potato in storage. *Applied Microbiology and Biotechnology* (2020)** (IF 4,81, MEiN 100)

  Contribution of the applicant: *I participated in the development of the methodology for conducting experiments, I took part in all the described experimental work, I participated in writing the manuscript and in preparing the figures.*

- Maciąg T., **Krzyżanowska D. M.**, Rąbalski Ł., Jafra S., Czajkowski R. **Complete genome sequences of five Gram-negative bacterial strains comprising synthetic bacterial consortium "The Great Five" with antagonistic activity against plant pathogenic *Pectobacterium* spp. and *Dickeya* spp. *Molecular Plant-Microbe Interactions* (2022)** (IF 3,5; MEiN 140)

  Contribution of the applicant: *I participated in the conceptualization and editing of the manuscript.*

Patent 1

No.	Publication date	Region
EP3495510	21.07.2021	Europe

Antagonistic bacterial strains, composition thereof and use for plant protection

Czajkowski Robert, Jafra Sylwia, **Krzyżanowska Dorota**, Maciąg Tomasz, Siwińska Joanna

Patent 2

No.	Publication date	Region
238148	12.07.2021	Poland

Biopreparations to protect plants against bacterial infection

Czajkowski Robert, **Krzyżanowska Dorota**, Maciąg Tomasz, Jafra Sylwia

Patent 3

No.	Publication date	Region
236445	11.01.2021	Poland

A mixture of antagonistic bacterial strains *Serratia plymuthica* strain A294, *Enterobacter amnigenus* strain A167, *Rahnella aquatilis* strain H145, *Serratia rubidaea* strain H440, *Serratia rubidaea* strain H469 and use for the protection or treatment of plant infections caused by *Pectobacterium* and *Dickeya* bacteria

Czajkowski Robert, **Krzyżanowska Dorota**, Maciąg Tomasz, Jafra Sylwia, Siwińska Joanna

Additionally, during the research on the above-mentioned mixture (consortium) of microorganisms called "The Great Five", I became a co-author of a book chapter on the use of microbial consortia in biological control of plat diseases.

- Robert Czajkowski, Tomasz Maciąg, **Dorota M. Krzyąanowska**, Sylwia Jafra; Title of the chapter: **Biological Control Based on Microbial Consortia–From Theory to Commercial Products;** Title of the book: How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases; **2020,** pp. 183-202, Springer, Cham

  Contribution of the applicant: *describing various strategies for selecting microorganisms when creating consortiums of microorganisms for use in biological plant protection*

As part of my post-doctoral activity, I also co-authored a publication on a method for determining the ability of bacteria to form biofilm in catheters and other medical tubes (Borowicz et al., 2023)(***publication not included in the series***), as well as a review of a manuscript describing the results of research on the relationship between 7-HT production and the production of pyoverdine siderophore in the P482 strain, and the significance of this relationship for the antimicrobial activity of P482 in various types of growth media (Jafra et al., ***manuscript under review***).

- Borowicz M., **Krzyżanowska D.M.** Jafra S.. **Crystal violet-based assay for the assessment of bacterial biofilm formation in medical tubing**. ***Journal of Microbiological Methods*** **(2023)** (IF 2,2; MEiN 70)

  Contribution of the applicant: *I participated in writing the manuscript and conceptual work on its content. I prepared one of the drawings and participated in determining the final appearance of the others*

- Jafra S., Jabłońska M., Maciąg T., Matuszewska M., Borowicz M., Prusiński M., Żmudzisńska W., Theil M., Czaplewska P., **Krzyżanowska D.M**., Czajkowski R.. An iron fist in a velvet glove: **The cooperation of a novel pyoverdine from *Pseudomonas donghuensis* P482 (PVDP482) with 7-hydroxytropolone is pivotal for its antibacterial activity (in review)**

  Contribution of the applicant: *description of genes encoding pyoverdine synthesis in the P482 strain, determination of the antimicrobial activity of the P482 strain and its mutants in CAA medium with different iron availability*

I am currently expanding my research interests to include new topics. Due to the change of the research group in which I am employed, I had the opportunity to become a co-author of a publication on the genes determining the resistance of *Dickeya solani* 2222 (an SRP strain) to infection with phage ϕD5 and the ecological costs of this resistance (Sokolova et al., 2023) (***publication not included in the series***). I was also involved in research on tailocins - high-molecular-weight bacteriocins with a structure resembling phage tails - produced by SRP pathogens. As part of the latter research, a manuscript (***in review***) was created, of which I am a co-author, and whose first author is Mr. Marcin Borowicz, whose doctoral thesis I am co-supervising.

- Sokolova, D., Smolarska, A., Bartnik, P., Rąbalski L., Kosiński M., Narajczyk M., **Krzyżanowska D.M.**, Rajewska M., Mruk I., Czaplewska P., Jafra S., Czajkowski R. **Spontaneous mutations in *hlyD* and *tuf* genes result in resistance of *Dickeya solani* IPO 2222 to phage ϕD5 but cause decreased bacterial fitness and virulence *in planta*. Scientific Reports (2023)** 13, 7534. DOI: 10.1038/s41598-023-34803-7 (IF 4.6; MEiN 140)

  Contribution of the applicant: *I conducted experiments on the isolation and separation of lipopolysaccharides and prepared a figure presenting the above-mentioned results.*

- Borowicz M., **Krzyżanowska D.M**., Narajczyk M., Sobolewska M., Rajewska M., Czaplewska P., Węgrzyn K., Czajkowski R.; **Soft rot pathogen *Dickeya dadantii* 3937**

produces tailocins resembling the tails of *Enterobacteria* bacteriophage P2; (**w recenzji;** available in bioRxiv)

Contribution of the applicant: *I performed a comparative analysis between the cluster encoding tailocin and other available sequences in the database, including the genome of bacteriophage P2, preparaed selected figures and participated in the edition of the remaining ones, I participated in the writing of the manuscript.*

In the coming years, I plan to develop my scientific career, including: continue to engage in work on tailocins, publish results on the molecular basis of the inability of the *O. rhizosphere* PR17 strain to inactivate AHLs (continuation of the research from PUBLICATION 1), and also start detailed research on aspects of the metabolism involved in host-induced response in *P. donghuensis* P482 (continuation of the research from PUBLICATION 4) - in the latter case preferentially after I obtain separate funding for the planned research. I am motivated to apply for a habilitated doctor's degree by, among others, the resulting entitlement to be an independent supervisor of Ph.D. candidates, as well as the opportunity to participate more actively in the functioning of the Faculty thanks to membership in the Faculty Council.

5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

During my professional career, I completed **3 internships in foreign research institutions**. Each of the internships I completed at a different institute, and **each time engaging in scientific work related to the topic of the host group.**

In 2008, thanks to the ERASMUS initiative, I completed a **3-month internship at Plant Research International (currently Wageningen Plant Research, WUR) in the group of Dr. Jan van der Wolf**. During the internship, I participated in research on the isolation and identification of bacterial plant pathogens from the SRP group. This internship was important for me from the perspective of consolidating my research interests, learning useful methods, and creating professional relationships proved valuable for me in later years.

At the end of 2014, a few months before defending my doctorate, I completed a **3-month internship in the group of Dr. Paolina Garbev at the Nederlands Instituut voor Ecologie (NIOO-KNAW). I obtained funds for the internship from the National Science Center**, as a laureate of the **ETIUDA1 scholarship (UMO-2013/08/T/NZ9/01049)**. As part of the internship**, I participated in research on volatile organic compounds (VOCs) produced by bacteria of the *Collimonas* genus and on the impact of these compounds on microorganisms potentially**

coexisting (competing) with *Collimonas* spp. in the soil environment. During the internship, I also took part in many research-oriented seminars taking place at NIOO.

An important outcome of the above-mentioned internship is my co-authorship in a research article (*publication not included in the series*):

Song C., Schmidt R., de Jager V., **Krzyżanowska D.**, Jongedijk E., Cankar K., Beekwilder J., van Veen A., de Boer W., van Veen J., Garbeva P.: **Exploring the genomic traits of fungus-feeding bacterial genus *Collimonas*, *BMC Genomics***, vol. 16, 2015, s. 1-17 (IF 3,867)

> ***Contribution of the applicant:*** *I constructed a plasmid vector enabling heterologous expression of terpene synthetase from Collimonas sp. Ter91 and I assessed the antibacterial activity of a pool of volatile compounds (VOCs). I also described the results of the experiments conducted for the purposes of the manuscript.*

In 2018, I completed **a 1-month internship at the Centro Nacional de Biotechnologia, Consejo Superior de Investigaciones Científicas (CNB-CSIC)**, Madrid, Spain. I **obtained funds for the internship from the Foundation for Polish Science**. They came from an additional travel grant that could be awarded to the **laureates of the START scholarship (2017 edition of the contest)**. During my stay, I participated in research **conducted at the Synthetic Biology Laboratory, led by Prof. Victor de Lorenzo. The research concerned the use of a CRISPR/Cas9-based system to introduce mutations in the bacterium *Pseudomonas putida* strain KT2440 and its derivative strains (i.e. mutants of the KT2440 strain). I also participated in research aimed at explaining the mechanism enabling the functioning of the above-mentioned system in the studied bacterium**. In addition to conducting experiments, I took part in the seminars of the hosting group. This included a seminar I gave myself concerning my research at the IFB UG-MUG. **As one of the results of the internship, I obtained many useful vectors and strains from the SEVA (Standard European Vector Architecture) collection**, which were included in the strain collection of the Laboratory of Plant Microbiology. I was also **recommended by Professor de Lorenzo as one of the participants in the European project under COST Action**. Application for financing of the project entitled Bringing Synthetic Biology standards to end-users (acronym STAND_HARD) was submitted in 2020 by Mireia Alonso-Monasterio Fernández. Unfortunately, the project did not receive funding.

Additionally, during my professional career, I went on **a 5-day study visit** to the Institute of Plant Sciences, Ornamental Horticulture Dep. ARO, The Volcani Center, Bet-Dagan (Israel), to visit the group of Dr. Iris Yedidia (**2015**).

My experience also involves a one-month **internship in a commercial diagnostic laboratory** in Poland (Bruss, ALAB group) (**2007**).

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art.

6.1. Teaching achievements

During my professional career, I was not employed on a permanent position at the University, the latter usually involving obligatory teaching hours. The fact that I was a contract employee for research projects meant that **conducting classes with students was not obligatory for me. However, I consider that sharing knowledge with young people and supporting them on their way to individual development is an important part of the activity of a scientist.** The need to prepare classes is also a good opportunity to obtain a new grasp of one's own knowledge. Due to these reasons, whenever such an opportunity arose, I always undertook teaching tasks, either based on official agreements for conducting classes included in the teaching program or as part of less formalized individual laboratory practice for students. Thanks to this, **despite the lack of obligatory teaching duties, I managed to gather achievements in this area covering a wide range of activities, from conducting classes included in the program of studies in 'Biotechnology', through managing the work of students as part of individual labs, to supervising bachelor's and master's theses, and co-supervision of a Ph.D. candidate currently carrying out his project.** I am also a co-author of script for 'Industrial microbiology' classes.

Supervision of students

- From March 2023 – auxiliary supervisor of the Ph.D. thesis of Mr. Marcin Borowicz (in progress)
- Supervisor of two experimental master's theses: Mr. Paweł Gutowski (2017) and Mr. Paweł Płaszewski (2019)
- Supervisor of two bachelor's theses: Ms. Anna Kocięba and Mr. Marcin Borowicz (both in 2020).
- Reviewer of master's thesis of Ms. Daria Pośpiech (2016)
- Board member for 5 master's exams, three in 2017 and two in 2019
- Person supervising the work of students doing individual laboratory practice or internships, including international exchange students:
    - intern from the USA, Mrs. Lilian Antunes, summer 2012
    - intern from the USA, Ms. Jackie Rocha, summer 2016
    - students working in the laboratory as part of the 'rotating' classes: Mr. Stijn De Moor and Ms. Aleksandra Jurkiewicz (academic year 2015/2016), Mr. Joanna Mancewicz, Alicja Januchowska and Mr. Patryk Mucha (academic year 2018/2019), Mr. Robert Burzyński (2019/2020)
    - master's student Nathalie Lammens, doing a combined master's thesis between Vrije Universiteit Brussel (Belgium) and the University of Gdańsk (academic year 2016/2017)
    - third-year student of the 1st MSU doing individual laboratory practice, Mr. Kamil Moskal, summer semester 2018/2019
    - student of the 1st MSU of the Lodz University of Technology performing summer internships (1 month), Mr. Bartosz Kopka (2019)

- student of the 1st MSU doing individual laboratory practice, Mrs. Joanna Klassa, beginning of 2020 - practice interrupted due to COVID19

Subjects (classes) taught during professional career

- Laboratory classes as part of the subject "Industrial Microbiology" taught at the IFB UG and MUG. Years 2010-2013 (total 205 h), academic year 2021/2022 (45 h)
- Proseminar on the subject "Biomolecles - structure, synthesis and properties" taught at the IFB UG and MUG. Academic year 2019/2020 (30 h)
- Seminar and laboratories in the subject Fundamentals of Biotechnology - Introduction, Section: Model organisms and their applications in science, Scope: bacteria. Academic year 2019/2020 (5 h)

In the academic year 2023/2024, I am scheduled to conduct laboratories, auditorium and lectures for a total of 24 hours.

Co-author of a script for laboratory classes

"Industrial microbiology – materials for laboratory classes" (2014). Collective work edited by Sylwia Jafra; co-authors: Robert Czajkowski, Tomasz Maciąg, Marta Matuszewska, Dorota Krzyżanowska, Adam Ossowicki, Aleksandra Królicka, Magdalena Rajewska.

6.2. Activities that popularize science or art

- Organization of a stand entitled “Fascinating facts from the life of bacteria” at the 11th Baltic Science Festival (2013)
- Giving an interview entitled "Good bacteria save potatoes from soft rot", published on the "Nauka w Polsce" website (2017)
- Supervising a student of the 20th secondary school in Gdańsk as part of the Entrepreneurship Day (20/03/2019)

6.3. Organizational activity

- Preparation of applications for the contained use of GMM category II for the Biological Plant Protection Laboratory and the Laboratory of Biologically Active Compounds, as well as one application for the contained use of GMM category I for a project implemented by students of IFB UG and MUG (iGEM competition)
- Introduction of the FLUICS system in the Laboratory of Biologically Active Compounds, intended for professional management of laboratory resources, enabling cataloging and easy location of reagents and samples, including using a mobile application

- Conducting on-the-job training for students starting their internship in the laboratory and maintaining appropriate documentation (OHS)
- Placing orders and preparing tender documentation for the implementation of selected research projects
- Coordinating service/repair activities for selected equipment in stock at the Institute of Biotechnology

7. Apart from information set out in 1-6 above, the applicant may include other information about his/her professional career, which he/she deems important.

7.1. Awards

- 1st degree team scientific award granted by the Rector of the Medical University of Gdańsk for research on the biology of *Bacillus subtilis* and its practical use (2014)

7.2. Scholarships

- **START scholarship** awarded in a competition by the Foundation for Polish Science (2017). Additionally, funds were allocated for a 1-month internship
- Annual **ETIUDA1 scholarship (UMO-2013/08/T/NZ9/01049)** awarded by the National Science Center (2013/2014). In addition to the scholarship, a 3-month internship was financed
- **Pomeranian Special Economic Zone (PSSE) scholarship**, awarded in a competition based on applications submitted under the 5th edition of the InnoDoktorant program (2013)
- **Annual scholarship as part of the project "We educate the best - a comprehensive program for the development of doctoral students, young doctors and academic teaching staff of the University of Gdańsk"**. Awarded in a competition. Project financed by the European Social Fund under the Human Capital Operational Program (2011/2012)
- Annual **scholarship from the so-called "pro-quality subsidy", awarded to a group of the best doctoral students** doing doctoral projects at IFB-UG and MUG (years 2013/2014, 2012/2013, 2011/2012)
- Annual **scholarship for the best PhD students of SD Chemistry and Biochemistry** at the Faculty of Chemistry of the University of Gdańsk (years 2014/2015, 2013/2014, 2012/2013, 2010/2011)

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(Applicant's signature)

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