UNIVERSITY OF THESSALY

Department of Ichthyology and Aquatic Environment,

MSc THESIS

HosMic

“Host - microbe interactions”

Looking into the evolution mechanism driving, the rare in the bacterial world, specialization of Paenarthrobacters in the degradation of the fungicide iprodione

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ABSTRACT

Iprodione is a fungicide with potential carcinogenic effects for humans and ecotoxic effects for receiving environments. An attractive approach for treating iprodione residues is that of bioaugmentation. We have previously isolated *Paenarthrobacter*/*Arthrobacter* strains with iprodione degrading ability and potential use in such bioremediation strategies. Known *Panaerthrobacter* mediated iprodione degradation involved three steps controlled by the *ipaH, ddaH* and *duaH* genes. There is a background characterization of these strains and gaining insights concerning underlying iprodione biodegradation mechanisms. The sequenced genomes of the iprodione-degrading isolates derived from soil (*Paenarthrobacter* sp. and *Microbacterium* consortium TA1.6 and *Arthrobacter* sp. C1) led to comparative genomic analysis with other iprodione-degrading paenarthrobacters of previous studies. Our isolates carry the catabolic genes with high homology. Comparative genomics with other iprodione-degrading *Paenarthrobacter* strains YJN-5 and YJN-G showed that the transformation pathway of iprodione in the different *Paenarthrobacter* isolates occurred at different evolutionary stages. Possibly, *Paenarthrobacters* employ gene duplication from the ancestral *ipaH* and *ddaH*, and genetic rearrangements for pathway optimization. This thesis enlightens for this specialization of iprodione degradation which is rarely observed in the bacterial world. To validate our computational biology outcomes, we characterized the growth of these isolates and compared the contribution of every gene set, at RNA level. The RTqPCR analysis of C1 gene set proves the correlation of the transformation pathway from the previously analyzed Paenarthrobacters to be the same and being responsible for iprodione degradation to our strain as the aforementioned genomic analysis suggested.

KEYWORDS

Paenarthrobacter sp., iprodione, Iprodione-degrading bacteria, soil, phyllosphere, biodegradation

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1. INTRODUCTION

1.1. The importance of microbial ecology

Microbial ecology is the ecology of microorganisms: how they interact with their surroundings and with each other. It includes almost all living things, Microbiology, Ecology, and Ecosystem science are all being impacted by the current revolution in microbial ecology (Prosser et al., 2007). Numerous uncultivated microbial groups, great diversity, and novel microbial functions are being revealed by the quick accumulation of research data. Microorganisms are the base of all ecosystems, but even more into places that are not called ecosystems. The nitrogen cycle, the phosphorus cycle, the sulfur cycle and the carbon cycle all rely on microorganisms in some way. Together, the cycles control microorganisms in specific processes. For instance, most organisms are unable to utilize the nitrogen gas that makes up 78% of the Earth's atmosphere until it is transformed into a form that is suitable for their needs through the microbial process known as nitrogen fixation. In contrast to the nitrogen and carbon cycles, the phosphorus cycle in the environment does not result in the creation of stable gaseous species. Phosphate is solubilized by microorganisms, which enhances soil health and plant growth. Due to the high rates of horizontal gene transfer between microbiota, microbial ecology plays a major role in evolution (Smets & Barkay, 2005). Microbial ecology contributes to global evolution in a variety of ways. For instance, various microbial species evolved CRISPR dynamics and functions, which improved our knowledge of human health (Westra et al., 2019).

1.2. The relevance of pesticides, problems and biodegradation

Pesticides are substances or solutions that mainly kill, repel or control any living creature that is undesirable due to a negative trait or behavior. Pesticide application and consumption have increased, because of shifting regional climates and rising demand for agricultural products (Shetty et al. 2008). The amount of agricultural land determines the pesticide consumption rates in different nations. Several nations had higher overall pesticide consumption when compared to their agricultural areas. The top 10 countries-consumers use an average of 60,000 tons per year of pesticides (Verma et al., 2014).

Pesticides are sorted into different types, based on toxicity, physicochemical properties and target application. The most common approach of pesticide classification is by their chemical properties and nature of the target. Based on the targets, pesticides are split to herbicides, insecticides, anthelminthics, rodenticides, fungicides, algicides, avicides, bactericides, lampricides, acaricides, molluscicides, virucides and plant growth regulators (EPA, 2017).

Organophosphates, organochlorines, carbamates and pyrethroids have faded from the market in the past decades. Novel pesticides are mostly organic and in the contrary with the aforementioned they are effective at an extremely low doses, they are rapidly degradable and result in less residual in the environment and are defined by high selectivity (Umetsu & Shirai, 2020).

Focusing on the novel fungicides, they are classified from their mode of action. Anilide fungicides like Flubeneteram (Ji et al., 2023) and inpyrfluxam (Adachi et al., 2021) have SDHI (succinate dehydrogenase inhibitor) action. Some pyrazole carboxamides have SDHI mode of action as Isopyrazam Sedaxane and Solatenol (Walter et al., 2015) developed by Syngenta. Another fungicide mode of action is by Inhibition of the mitochondrial electron transport chain complex III. Those are split into QoI (quinone outside inhibitors), and QiI (quinone inside inhibitors). Some of the QoI fungicides are the strobilurins (Bartlett et al., 2002) like Picoxystrobin and Mandestrobin (Zhang & Fernando, 2023). Florylpicoxamid (Li et al., 2022) is a neopicolinamide fungicide with QiI mode of action. DMI (demethyl ation inhibitors) is another mode of action for fungicides like Mefentrifluconazole (Li et al., 2021) which is the first isopropanol azole fungicide and Pyrisoxazole (Duan et al., 2018) a pyridine-type DMI. Also, there are improved organochlorines as diflumetorim (Wang et al., 2021), which has a unique mode of action targeting the mitochondrial complex I–NADH oxido-reductase. Other novel fungicides have mode of action the inhibition of laccase, an example is 4-chlorocinnamaldehyde thiosemicarbazide (Wang et al., 2020).

Soil microorganisms play a significant role in litter degradation (Schneider et al. 2010), boosting plant development (Hayat et al. 2010), nutrient cycling (Van Der Heijden et al. 2008) and biodegradation (Pino and Penuela 2011; Zhao et al. 2009). The processes of biomineralization, bioaccumulation and cometabolism are some of the ways that pesticide dissipation can occur (Shakoori et al. 2000; Park et al. 2003; Finley et al. 2010). Microbial degradation can also be defined as the process of breaking down toxic pesticides into nontoxic compounds through microbial enzymatic mechanisms. This process can occur in contaminated sites such as soil, ground water, sludge, industrial water systems, and gas (Wood 2008; Finley et al. 2010; Porto et al. 2011). Microbes that are particularly important for the *in situ* removal and detoxification of these toxicants from the affected environment are highly valued. They convert waste materials into harmless compounds, these microorganisms are referred to as nature's biodegraders, because of their remarkable ability to use a wide range of xenobiotics as their only source of energy (Siddique et al. 2003).

1.3. Iprodione and Dicarboximide fungicides

Vinclozolin, iprodione, and procymidone are members of the dicarboximide or dicarboxamide family of agricultural fungicides. These fungicides rapidly transform to 3,5-dichloroaniline (3,5-DCA) in the soil (Copping, 1998). It is thought that dicarboximides prevent triglyceride biosynthesis in fungi that form sclerotia, such as *Botrytis cinerea.* Iprodione causes a build up of 4,4-dimethylsterol precursors and a decrease in 4-demethyl compounds in *Botrytis cinerea*, indicating a probable inhibition of ergosterol biosynthesis (Pappas & Fisher, 1979).

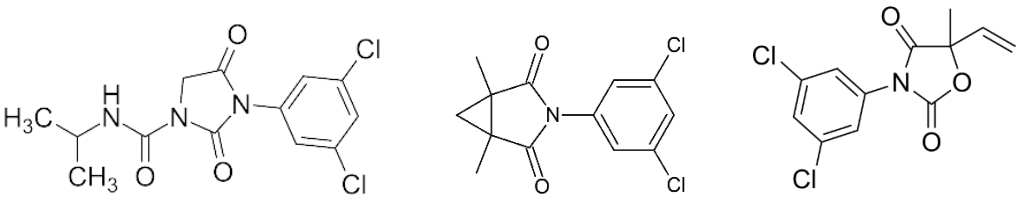


Figure 1: The chemical structures of the dicarboxamide pesticides. Depicted from left to right Iprodione, Procymidone, Vinclozolin

Dicarboximides are considered as endocrine disruptors being demonstrated to carry antiandrogenic effects, or to lower levels of male hormones. Because procymidone and vinclozolin are androgen receptor antagonists that block the expression of androgen-activated genes, they cause aberrant reproductive development in animals. Male rats showed developmental effects such as induction of areolas and reduced anogenital distance even with low doses of antiandrogenic pesticides (Gray, 2001 and Curtis, 2001).

Iprodione is registered for use in a variety of field, fruit, and vegetable crops, such as almonds, grapes, peaches, potatoes, rice, berries, onions, peanuts, lettuce, golf courses, lawns, and ornamentals. Iprodione comes in liquid, dry, granular, and wettable powder forms. Further studies from the United States Environmental Protection Agency (EPA, 1998) categorized iprodione as a Group B2, or "likely" human carcinogen, based on additional research conducted on rats. This classification is based on evidence of tumors in the livers of both sexes of mice and the male rat (Leydig cell). The Leydig cell was used to estimate carcinogenic risk, and the Q* value was 4.39 x 10^{-2}. Developmental and reproductive effects are the basis for the endpoint selection for both the acute risk assessment (reduced anogenital distance, or AGD) and the chronic risk assessment (histopathology of the male reproductive system). Iprodione and its metabolites have been detected in drainage water (Ludvigsen et al. 2004) and surface water (Sequinatto et al. 2013). It's been commercially available in the market under the Bayer CropScience brands "Rovral" and "Chipco green".

Figure 2: The most known iprodione containing products from Bayer CropScience.

The two main dissipation processes ruling iprodione's environmental fate are microbial degradation in both aerobic and anaerobic settings, and abiotic hydrolysis in neutral and alkaline environments (half-life pH 7 = 4.7 days; pH 9 = 27 minutes). Overall, these dissipation mechanisms seem to point to iprodione having low to intermediate environmental persistence. Results from field dissipation studies support the hypothesis of iprodione's low persistence ($t_{1/2}$ = 3–7 days) (Walker et al. 1986; Walker, 1987).

The Commission Implementing Regulation requested that the European Food Safety Authority (EFSA) conduct a reevaluation of maximum residual levels (MRLs) for iprodione based on the updated toxicological reference values. Plant protection products containing iprodione had a grace period ended on June 5, 2018, as the Commission Implementing Regulation (EU) 2017/2091 did not renew iprodione's approval. The European Commission consulted of granting transitional measures for iprodione, as well as for which commodities (EFSA, 2018).

1.4. Iprodione degrading bacteria

Chemical alkaline hydrolysis causes dissipation of iprodione (Belafdal et al., 1989). Nonetheless, certain research indicates that the principal dissipation process of iprodione in soil is microbial degradation. By enrichment techniques, the first pure culture strain MA6, identified as *Arthrobacter* sp., that used iprodione as its only source of carbon and nitrogen was isolated in 1995 from a soil that showed accelerated degradation of iprodione (Athiel et al., 1995). In Table 1 we list all microorganisms reported as efficient degraders of iprodione. mostly belonging to *Paenarthrobacter/Arthrobacter* and a few to the genus *Microbacterium*.

Table 1: All iprodione-degrading microorganisms isolated from the soil environment.

Strain	**Species**	**Origin**	**Reference**
MA6	*Arthrobacter sp*	Fast iprodione degrading soil	Athiel et al., 1995
C1	*Paenarthrobacter sp.*	Pristine soil	Campos et al., 2015
YJN-G	*Microbacterium sp.*	From Henan, China	Yang et al., 2017
CQH-1	*Microbacterium sp.*	Soil in Gansu, China with previous exposure to iprodione	Cao et al., 2018
YJN-5	*Paenarthrobacter sp.*	Iprodione-treated vineyard soil Hebei, China	Yang et al., 2018
TA1.6	*Paenarthrobacter sp* and *Microbacterium sp.*	Soil treated with iprodione, Greece	Katsoula et al., 2020
LP13.7	*Paenarthrobacter sp* and *Microbacterium sp.*	Phyllosphere of pepper plants treated with iprodione,	Katsoula et al., 2020
YJN-D	*Paenarthrobacter sp.*	From Hebei, China	Zhang et al. 2021

1.5. The genus *Paenarthrobacter*

The genus *Paenarthrobacter* was recently formed after the reclassification of strains belonging to the *Arthrobacter aurescens* subgroup (Busse, 2016). The word *Paenarthrobacter means* "paene": almost/nearly, therefore almost *Arthrobacter.*

Arthrobacter species are among the most commonly isolated, native, aerobic bacterial genera identified in soils (Hagedorn & Holt, 1975; Soumare & Blondeau, 1972). They were initially isolated from soils in the 19th century (Koch et al., 1995). In younger cultures, these bacteria usually take the form of Gram-negative rods, but in older cultures, they show as Gram-positive cocci (Lowe & Gray, 1972). Many types of soils, including ordinary soils, the deep subsurface, polar ice, chemically contaminated locations, and radioactive settings, have been reported to harbor *Arthrobacter* sp. (Fong et al., 2001; Hirsch, 1986; Ryan & Shapiro, 2003). At the Department of Energy plant in Hanford, Washington, in the United States, strains of *Arthrobacter sp.* Were reported to be one of the most common genera of bacteria recovered from beneath leaking radioactive storage tanks (Fredrickson et al., 2004).

Arthrobacter's ability to endure extended periods of harsh conditions brought on by starvation, fluctuations in temperatures, ionizing radiation, oxygen radicals, and toxic compounds may contribute to their environmental ubiquity (Boylen, 1973; Boylen & Ensign, 1970; Labeda et al., 1976; Zevenhuizen, 1966). The recovery of *Arthrobacter* sp. From arid Antarctic soils after three years of drying (Cameron and Conrow, 1971) is an example of this amazing ability to survive (Germida & Casida, 1980; Ensign, 1970). According to this research, the morphogenesis of *Arthrobacter* from rod to coccus—with the tiny, coccoid-like state being regarded as the most stable form—has been linked to the bacterium's capacity to withstand stressors. Manganese has been shown to be necessary for the transition to this coccoid-like form, and its buildup in the bacterial cytoplasm has been connected to *Deinococcus radiodurans*' ability to withstand radiation stress (Germida & Casida, 1980).

Arthrobacter/Paenarthrobacter sp. Are metabolically diverse and have been isolated for their ability to biodegrade a wide range of environmental pollutants, including glyphosate, methyl tert-butyl ether, 2,4-dichlorophenoxyacetate (2,4-D), fluorene, phthalate, nictotine, 4-nitrophenol, dimethylsilanediol, 10ndothall, nitroglycerine, and a range of s-triazine herbicides. *Arthrobacter* has also been proven to be extremely resistant to certain harmful heavy metals and the chromate anion (Eaton 2001, Gil 2000, Jensen 1964, Liu et al. 2001, Marshall & White 2001, Strong et al. 2000b, Strong et al. 2002). Many strains formerly described as *Arthrobacter* that can breakdown other organic pollutants such as atrazine (Strong et al., 2000a), nicotine (Kodama et al., 1992), and 4-nitroguaiacol (Kotouckova et al., 2004) are now reclassified as *Paenarthrobacter* spp. *Paenarthrobacter aurescens* strain TC1 (isolated from a South Dakota spill site containing 1,000 lb of atrazine) has been shown to metabolize over 23 different s-triazine compounds, the most s-triazine compounds catabolized by a single organism to date. Furthermore, metabolic and genetic investigations indicate that *Paenarthrobacter aurescens* TC1 can catabolize more than 500 structurally different s-triazine molecules (Shapir et al., 2007).

In addition, Katsoula et al., (2020) isolated three iprodione-degrading *Paenarthrobacter* bacteria TA1.6, TA1.8 and LP13.7 from the soil and the leaves of pepper plants repeatedly

treated with iprodione. Beyond Paenarthrobacters, two *Microbacterium* strains capable of degrading iprodione have been identified from soils, though their metabolic capacities are unknown (Cao et al., 2018), and they do not appear to complete the iprodione transformation process (Yang et al., 2017).

Repeated applications of iprodione in soil have been shown to increase biodegradation and reduce biological efficacy, as previously mentioned. Studies in soils with rapid biodegradation of the fungicide resulted in the isolation of iprodione-degrading bacteria. (Athiel et al. 1995; Campos et al. 2015; Yang et al. 2018). They transformed iprodione to 3,5-dichloroaniline (3,5-DCA), producing two temporary metabolic products: 3,5-dichlorophenyl-carboxiamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II).

On the contrary, soil application of iprodione has been found to have a negative impact on the abundance of soil bacteria (Zhang et al. 2017c), the abundance and variety of soil fungi (Zhang et al. 2017a), and microbial processes involved in N cycling (Zhang et al. 2017b, 2018). Vasileiadis et al. (2018) discovered that 3,5-DCA, and not iprodione, was responsible for the inhibitory effects reported on ammonia-oxidizing bacteria in soil and the overall microbial activity. Mongodin et al., (2006) investigated the genome of a *Paenarthrobacter aurescens* strain that degrades atrazine and concluded that Arthrobacter-like bacteria optimize their catabolic capacity through mutation and duplication events.

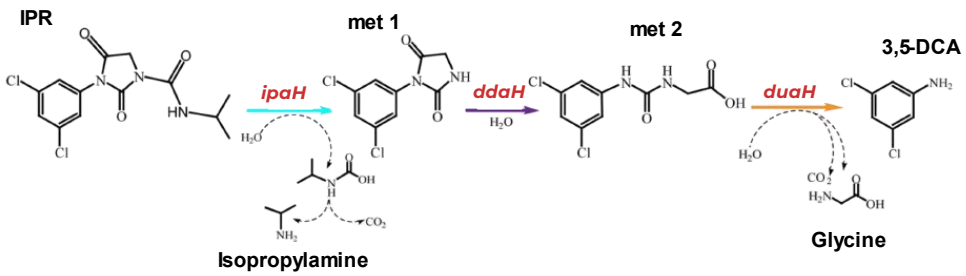
1.6. Iprodione microbial transformation pathway

In Figure 1 the transformation pathway of iprodione is shown. Iprodione (3-(3,5-Dichlorophenyl)-2,4-dioxo-N-(propan-2-yl)imidazolidine-1-carboxamide) is hydrolyzed by an enzyme encoded by the *ipaH* to metabolite 1 or 3,5-dichlorophenyl-carboxamide or N-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine. Metabolite 1 is subsequently hydrolyzed by DdaH to metabolite 2 or 3,5-dichlorophenylurea-acetate. At the end metabolite 2 is converted to 3,5-DCA or 3,5-dichloroaniline by DuaH that cleaves the urea remaining residue.

***ipaH*:** This gene encodes for an acylamidase that controls the first step of the iprodione catabolic pathway. This IpaH is responsible for hydrolyzing the N1 amide bond of iprodione.

***ddaH*:** This gene encodes for a deacetylase and controls the second step in the iprodione catabolic pathway. DdaH is proposed to cleave the hydantoin and cyclic imide moiety of metabolite 1.

***duaH*:** This gene encodes for Hippurate hydrolase or carboxypeptidase that controls the third step of the iprodione catabolic pathway. DuaH is responsible for the cleavage of the urea side chain of metabolite 2.



ipaH: acylamidase
ddaH: polysacharide deacetylase
duaH: hippurate hydrolase

YJN-5 (Yang et al., 2018)

Validation by KOs and heterologous expression isolate YJN-D from China (Zhang et al., 2021)

Figure 3: The transformation pathway of iprodione (IPR).

As shown in Table 1, an *Arthrobacter sp*., strain MA6 was isolated in 1995 from a soil that degraded iprodione rapidly via enrichment culture and it was able to use iprodione as its only source of carbon and nitrogen (Athiel et al., 1995). The same group later (in 1997) isolated a consortium composed of an Arthrobacter strain (strain 1) and three Pseudomonas strains (strains 2, 3, and 4) that was able to transform iprodione into 3,5-dichloroaniline (3,5-DCA) when they were combined (Mercadier et al., 1997). In 2015, an Arthrobacter sp. strain C1 was isolated from an acidic pristine soil, capable of transforming iprodione into isopropylamine (Campos et al., 2015). In 2017, two strains of *Microbacterium* sp. (YJN-G and CQH-1) capable of degrading high concentrations of iprodione as the only carbon intake were isolated and characterized (Yang et al., 2017; Cao et al., 2018). In 2018, *Paenarthrobacter* sp. strain YJN-5, which can degrade iprodione, was isolated and its *ipaH* gene was heterologously expressed. This strain can use iprodione as its sole carbon and nitrogen source. In 2020, three iprodione-degrading bacteria all belonging to the genus *Paenarthrobacter* were isolated from the soil and the phyllosphere of pepper plants (Katsoula et al., 2020), while later genomic analysis showed the presence in those cultures of a *Microbacterium* sp.. A common catabolic pathway has been hypothesized, as shown in Figure 1, as identical intermediates have been discovered during the iprodione-degradation process in diverse species. In this process, iprodione undergoes a three-step sequential hydrolysis reaction to produce 3,5-dichloroaniline and the capacity of each of these isolates in performing the different steps of the transformation process is described.

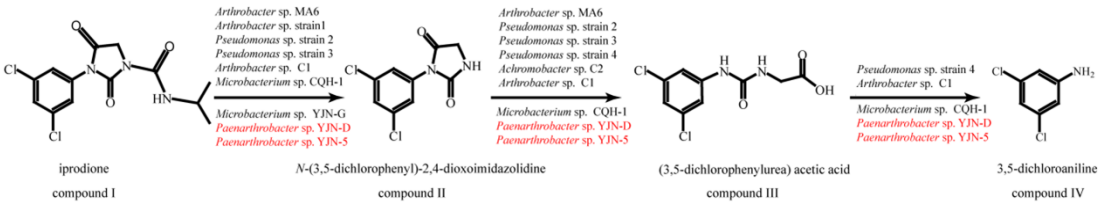


Figure 4: Proposed iprodione-catabolic pathway and the contribution of each of the isolated iprodione degrading strains in the different steps of the pathway, as reported in the literature. Arthrobacter sp. MA6 (Athiel et al., 1995);

Arthrobacter strain 1, Pseudomonas sp. (strain 2, strain 3, strain 4) (Mercadier et al., 1997); Arthrobacter sp. C1, Achromobacter sp. C2 (Campos et al., 2015); Microbacterium sp. YJN-G (Yang et al., 2017); Microbacterium sp. CQH-1 (Cao et al., 2018); Paenarthrobacter sp. YJN-5 (Yang et al., 2018).

The above pathway was validated by gene Knock outs and overexpression experiments of the genes above (Yang et al., 2018; Zhang et al. 2021). To validate the proposed degradation pathway, each gene was cloned separately and all three together. They made three different versions of their Paenarthrobacters with each one of the catabolic genes knocked out respectively. The solely cloned genes were used for protein production, purification and for assay testing the *in vitro* enzymatic reactions of the pathway. The Paenarthrobacters having each knockout lost the ability to conduct the step of the iprodione degradation of the missing gene. These stains regained their degrading capacity of the specific step when the plasmid having the missing gene was transformed to the Paenarthrobacters.

Finally, the two plasmids -encoding in an operon the whole gene set- were transformed to two distinct recipient strains (B. subtilis SCK6 and P. putida KT2440). The transformed strains, KT2440 (pBBR1MCS2-ipro) and SCK6 (pP43NMK-ipro), were able to degrade 0.45 mM iprodione in 42 and 48 hours, respectively and they were able to form transparent halos around their colonies on 1/10 LB medium supplemented with 1.0 mM iprodione (Figure 6). When iprodione was broken down, these strains likewise produced the same metabolites as *Paenarthrobacter* strain.

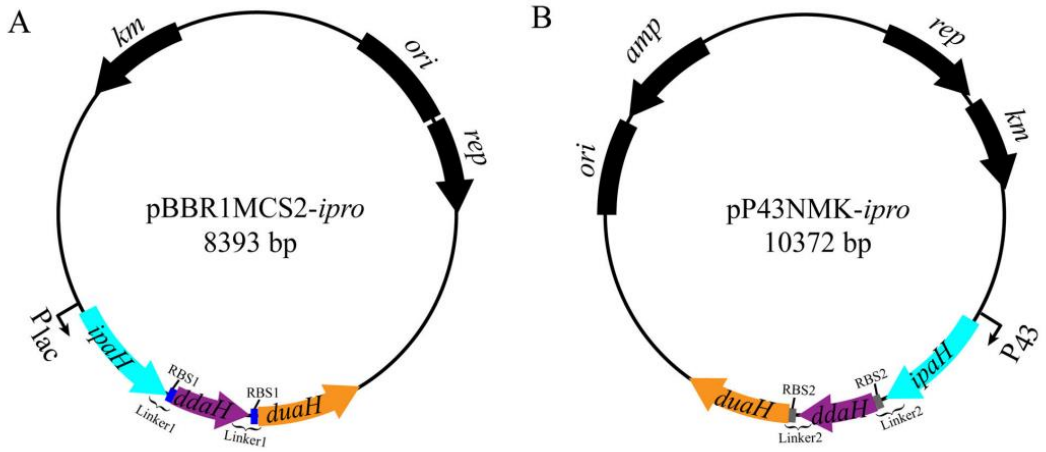


Figure 5: The two plasmids constructed by (Zhang et al. 2021) for the heterologous overexpression of the IPR catabolic pathway in P. putida KT2440 and B. subtilis SCK6 respectively. Vector pBBR1MCS-ipro constructing schematic diagram. B) Vector pP43NMK-ipro constructing schematic diagram. The Plac and P43 promoters are represented, respectively, by Plac and P43. The sequences of the ColE1 replication origin, replicase, ampicillin resistance marker, and kanamycin resistance marker are denoted by the letters ori, amp, rep, and km respectively. Ribosome binding site (RBS) representation. Linker1 (containing RBS1): the region located 69 base pairs downstream of the pET29a(+) T7 promoter. Linker2 is the sequence starting 41 bp downstream of the P43 transcription point. The direction of transcription for each gene is shown by an arrow.

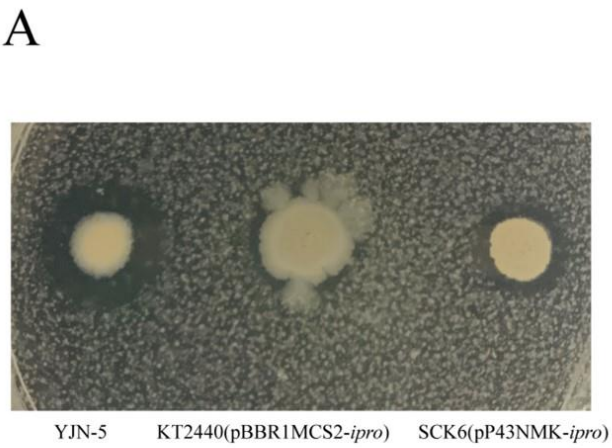


Figure 6: Iprodione breakdown in several strains is shown on 1/10 LB plates with 1.0 mM iprodione, the transparent halo created by strains YJN-5 (unmodified Paenarthrobacter), KT2440 (pBBR1MCS2-ipro), and SCK6 (pP43NMK-ipro).

1.7. Background on the iprodione-degrading bacteria used in this thesis

Genomic analysis of iprodione-degrading *Paenarthrobacter* strains of our lab (C1, TA1.6 and LP13.7) was performed with a combination of long-read and short-read sequencing, for optimum assembly of the genomes as part of the BSc thesis work of Alexandros Zafranas (2021). The sequencing data for strain TA1.6 and LP13.7 resulted in a similar assembly of a circular genome of 4.905.492 and 4.905.313 bp respectively, a second non fully assembled potential genome of 3.941.448 and 3.941.628 bp respectively and three circular replicons of the exact size 95449, 57127 and 29435 bp as shown in Figure 7. For the strain C1 a large circular replicon of 4.697.086 bp was assembled, which was presumed to be of chromosomal origin, a linear replicon of 517.771 bp and two mobile circular replicons of 136.303 bp and 5.386 bp, all presumably being of plasmid origin.

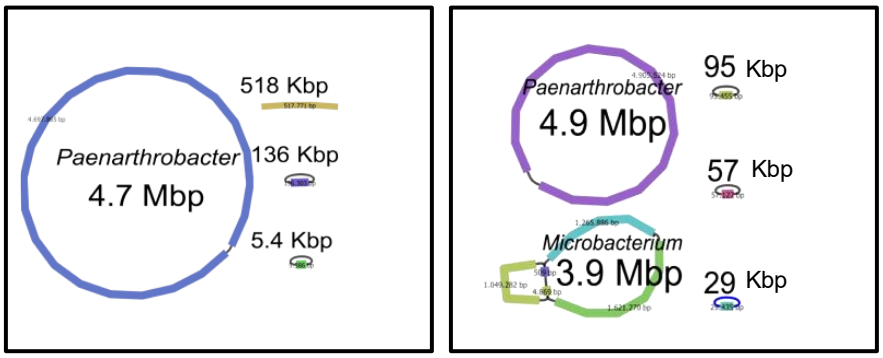


Figure 7: An overview of the genome assemblages of strains C1 on the left and TA1.6/LP13.7. The two last ones being almost the same strain.

Phylogenomic analysis of the proposed bacterial chromosomes of the strains TA1.6, LP13.7 and C1 showed that the strain C1 was clustered nearest to *Paenarthrobacter nicotinovorans* and the iprodione degrading *Paenarthrobacter* strain YJN-D. The *Paenarthrobacter* of the cultures TA1.6 and LP13.7 were identical and clustered together with a *P. nitroguajacolicus* and a *P. aurescens* strain.

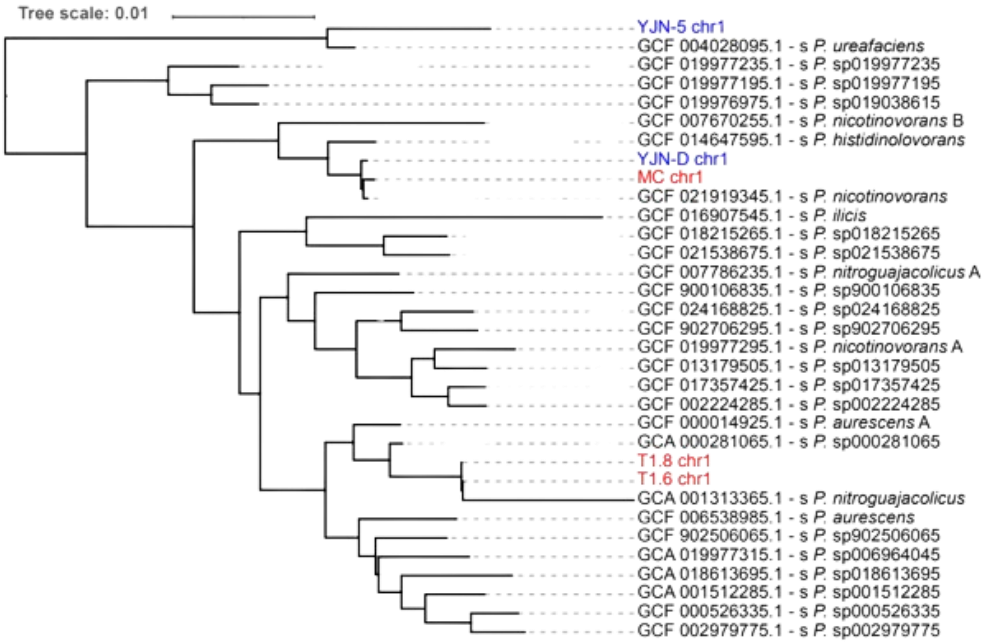


Figure 8: The phylogenomic tree showing the clustering of 5 Paenarthrobacters included in the analysis. YJN-5 chr1, YJN-D chr1, C1 chr(depicted as MC chr1) TA1.6 chr1 and LP13.7 chr1 (depicted as TA1.8 chr1)

The same analysis for the partial *Microbacterium* chromosomes present in the TA1.6/LP13.7 cultures is shown in Figure 9. The *Microbacterium* chromosomes clustered together and they were closely associated with the *Microbacterium maritypicum* A strain.

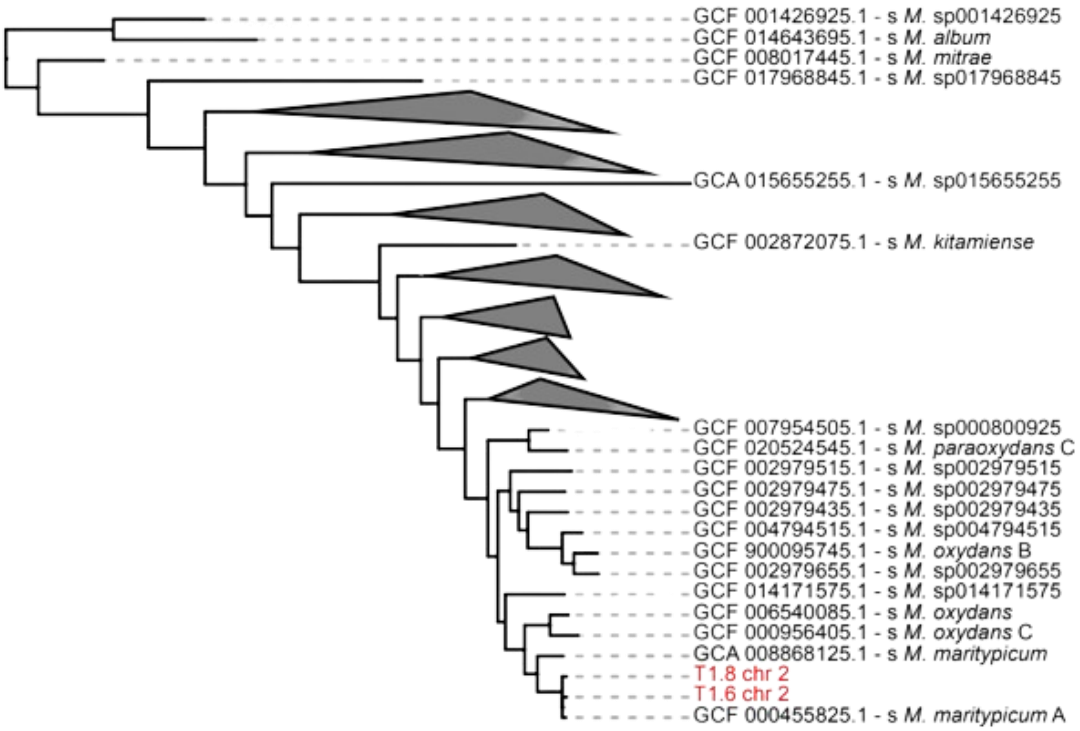


Figure 9: The phylogenomic tree showing the clustering of the chromosomes of the 2 Microbacterium strains present in the cultures TA1.6 and LP13.7 (depicted as TA1.8 chr2)

The Microbial Genome Atlas (MiGA) (Rodriguez et al., 2018) was utilized to decipher whether we had a new species. The ANI (Average Nucleotide Identity) measures to phylogenetically assign bacterial isolates against reference genomes showed higher than 97% identity as sown in Table 2, suggesting that the iprodione-degrading isolates did not constitute new species and grouped to the nearest relative of the phylogenomic trees above.

Table 2: The table of all chromosomes included in the MIGA analysis showing over 97% ANI (Average Nucloetide Identity)

STRAIN	ANI % IDENTITY
YJN-5 chr	97.30
YJN-D chr	98.82
C1 chr	98.85
TA1.6 chr1	97.05
TA1.6 chr2	98.53
LP13.7 chr1	96.98
LP13.7 chr2	98.53

The genomes were scanned for the three responsible genes of iprodione degradation and the results are summarized in Figure 10. The *ipaH* was identified via BLASTN, annotated as acylamidase, in plasmid 2 of TA1.6 strain with 97% homology at the amino acid level compared to IpaH of the *Paenarthrobacter* strain YJN-5. The enzyme encoded according to Pfam-A has 3 action-site motifs of amidase. Further search of the enzyme showed highest homology to the

IpaH of *Microbacterium paraoxydans* (99.8%) and of *Paenarthrobacter* strain YJN-5 (97.5%) in the non-redundant database of NCBI. The chromosomes of the TA1.6/LP13.7 has two ORFs both in the *Paenarthrobacter* and the *Microbacterium* components that showed 27.7% and 28.1% homology respectively compared to the reference IpaH. Regarding strain C1, an acylamidase with 29% homology was identified. Again this ORF in Pfam has 3 putative amidase active sites. Moreover in the non-redundant protein database showed high homology (>99%) to other amidases in *Paenarthrobacter* strains.

The DdaH identified by BLASTN in the TA1.6/LP13.7 genome was annotated as deacetylase in plasmid 1 with 99% homology compared to the reference DdaH of strain YJN-5. This ORF in Pfam has active sites of deacetylase. In the non-redundant protein database showed high homology to the deacetylase of strains YJN-5 (100%) and YJN-D (99%). An extra copy of DdaH was found in the chromosome of TA1.6/LP13.7 having low homology (38.4%) to the reference DdaH. Regarding strain C1, we found an ORF with unknown function in the chromosome that showed 39% homology with the DdaH from strain YJN-5. This ORF in the Pfam database suggested a function of deacetylase, in line with the functional annotation of the reference DdaH. In the non-redundant protein database it showed 100% homology with a polysaccharide deacetylase of *Paenarthrobacter nicotinovorans*.

The DuaH indentified by BLASTN in the TA1.6/LP13.7 genome an ORF in the Paenarthrobacter chromosome annotated as hippurate hydrolase with 88% homology with the reference DuaH. There were also two other ORFs in the chromosome of the *Microbacterium* genome found in the cultures TA1.6/LP13.7 having 51.3 and 28.4% homology. Search against the Pfam database showed that the protein has two peptidase active sites. Search in the non-redundant database showed highest homology to amidohydrolase of other *Arthrobacter/Paenarthrobacter* strains (100%). Referring to C1, an ORF in the chromosome showed 99% homology with the reference DuaH of strain YJN-5. Pfam analysis of the putative protein showed two motifs of active sites of peptidases. Search in the non-redundant database showed highest homology to an amidohydrolase of *Arthrobacter* strain 31Cvi3_1E (99.3%).

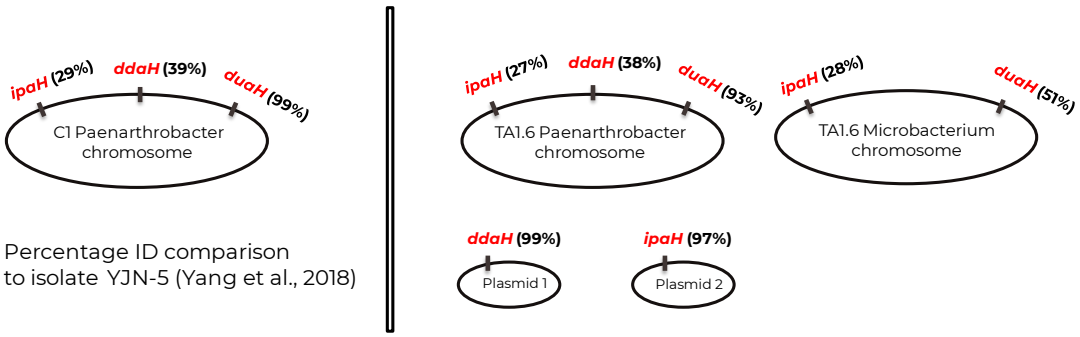


Figure 10: The organization of genes (ipaH,ddaH,duaH) in the genomic regions of strains C1 on the left and TA1.6/LP13.7 on the right. Showing the location of the genes and the percentage homology to reference YJN-5 genes.

The genetic conservation of the metabolic pathway of iprodione was explored among *Paenarthrobacter* strains. Different conservation levels were observed in the organization of the genomic regions harboring the genes of interest. There is high level of conservation between Paenarthrobacters about the harboring regions of *ipaH* and *ddaH*. An exception to this was the strain C1, whose relevant genes are located in the chromosome and showed no synteny with the corresponding regions in the genomes of other iprodione-degrading strains. The genomic regions hosting duaH were highly conserved among all Paenarthrobacters included. This region composed of an amidase, a transcription regulator (AsnC) and an alkaline phosphatase D (APaseD) upstream of duaH, and downstream, an acetyl-coenzyme A carboxylase carboxyl transferase (ACCase) and an CoA-transferase

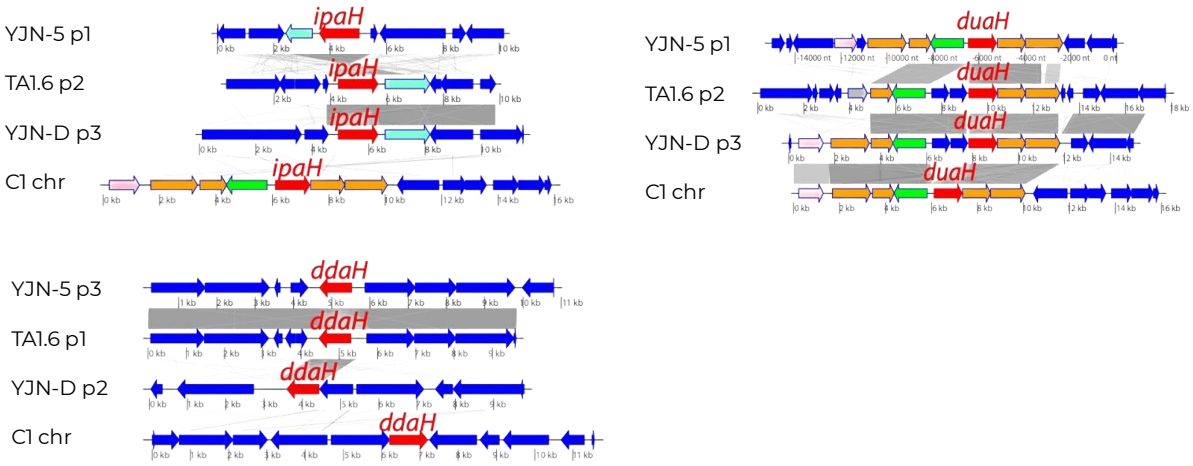


Figure 11: Synteny analysis of the genomic regions harboring the genes, chromosomal (chr) or plasmid (p) of iprodione-degrading Paenarthrobacter strains YGN-5, TA1.6/LP13.7, YGN-D and C1.

To answer how these genes were aquired, an IS analysis for our Paenarthrobacters showed in plasmid 2, 4000 bp upstream of *ipaH* an ISMlu14, however no terminal ISs were found. Similarly, in plasmid 1, 7000 bp upstream of *ddaH* an ISArsp14 was found. A further search in the chromosome of TA1.6/LP13.7 identified several putative IS belonging to other IS families. However, none of these IS were flanking the *duaH* gene. Finally, a search in the strain C1, identified an ISAcl1 belonging to the IS30 family directly upstream of *ddaH*, the rest ISs were not flanking any of the other genes as seen in Figure 12.

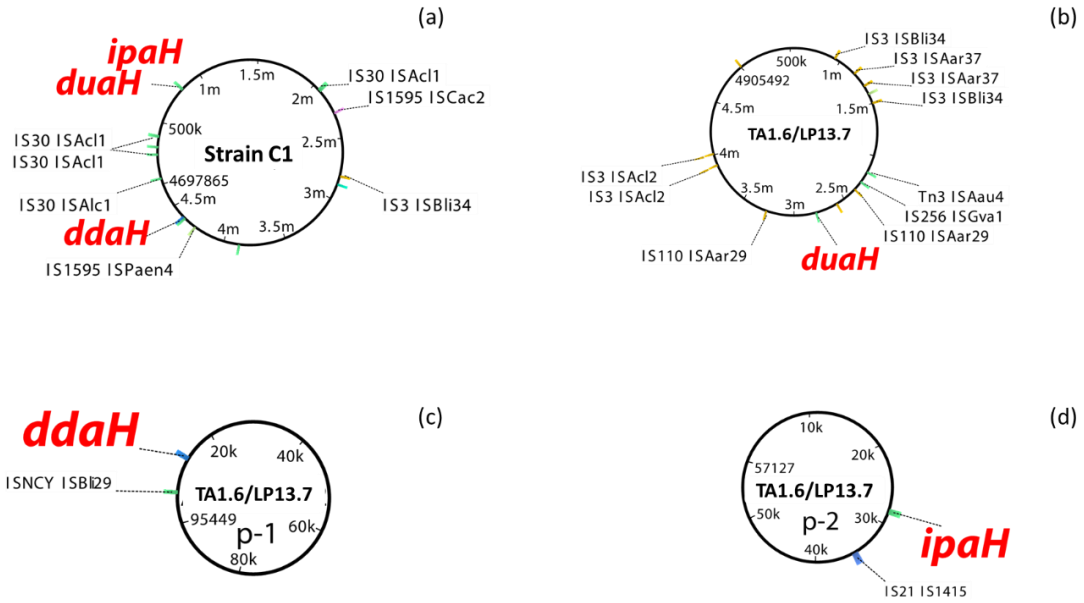


Figure 12: Analysis of the localization of putative ISs in the chromosomal replicon of C1 (a), the chromosomal replicon of strains TA1.6/LP13.7 (b) the plasmids 1 (c) and plasmid 2 (d) of TA1.6/LP13.7 hosting genes ddaH and ipaH.

Core genome analysis identified 657 orthologue proteins that were shared amongst all *Paenarthrobacter* genomes analyzed and constituted the core genome of this genus (Figure 13). On the other hand, we identified 10226 orthologue proteins that constitute the pangenome of the genus *Paenathrobacter*. When we looked for the position of the *ipaH, ddaH* and *duaH* in the core genome and the pangenome of Paenarthrobacters we observed that *ipaH* and *duaH* were part of the core genome, while *ddaH* was part of the pangenome. This finding provided a first explanation for the high specificity of Paenarthrobacters in the degradation of iprodione.

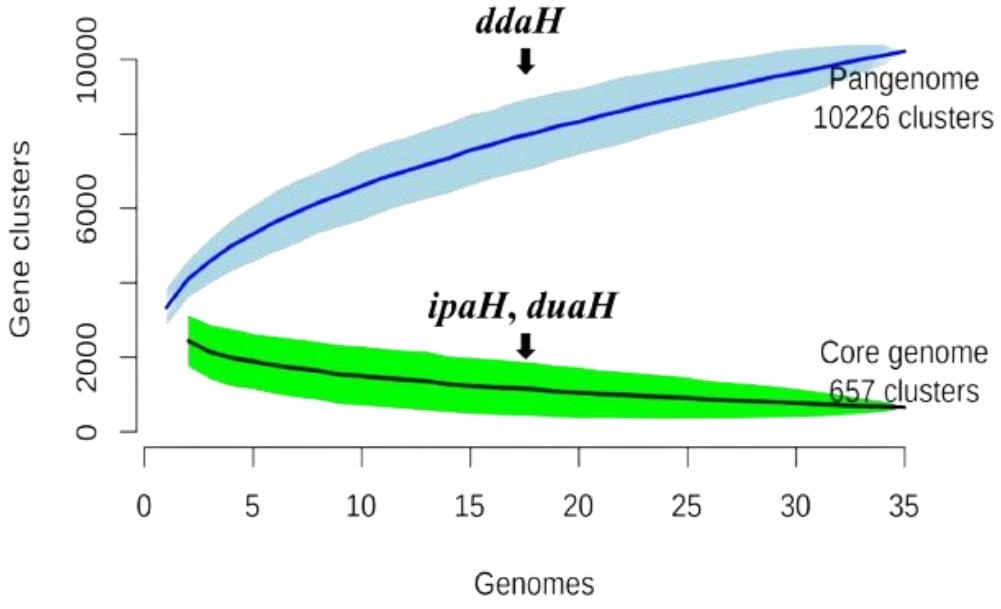


Figure 13: Localization of ipaH, ddaH and duaH genes in the pangenome and the core genome of the genus Paenarthrobacter, after analysis of the 33 available Paenarthrobacter genomes including the TA1.6/LP13.7 and C1. Showing the ddaH gene in the pangenome and ipaH and duaH in core genome.

1.8. Aims of the thesis

The aims of this thesis were (a) in the short term, to verify the role of *ipaH, duaH, ddaH*, encoded in the genome of C1 strain, in the transformation of iprodione via RT-q-PCR and (b) in the longer term, to identify the contribution of the different copies of the *ipaH, duaH* and *ddaH* found in the *Paenarthrobacter* and *Microbacterium* components of the TA1.6 culture on the transformation of iprodione via metatranscriptomics. The short-term aim was accomplished and it is presented in the thesis while the longer term aim is not completed since the results of the transcriptomic analysis are pending.

2. MATERIALS AND METHODS

2.1. Chemicals

Carbenicillin (C1389) for bacterial clone selection, succinic acid (398055) as substrate for control cultures, iprodione (36132) for the HPLC and as a substrate for the cultures, 3-5 dichloroaniline (36704), acetonitrile (900686) and methanol (34860) for the HPLC analysis were all purchased by Merck.

2.2. Culture media and strains

The bacteria used in our study were the following (a) an *Arthrobacter* isolate C1, clustered later in the genus *Paenarthrobacter* (Campos et al., 2015), (b) isolate TA1.6 which based on the genomic analysis was composed of a *Paenarthrobacter* showing highest homology to *Paenarthrobacter nitroguajacolicus* and a *Microbacterium maritypicum* (Katsoula et al., 2020). These bacteria were routinely cultured in LB Broth Miller (L3522) with or without the addition of Iprodione. These bacteria are also cultured in selective medium MSM and MSMN (Karpouzas and Walker, 2000) (APPENDIX 3) with the addition of Iprodione (10 μg/ml), as the only carbon source and nitrogen or carbon source respectively. All bacterial cultures were incubated at 25˚C/210 rpm until full degradation of Iprodione. The consortium was also cultivated (as control) with succinate as the sole carbon source. The aim was to use a concentration (4 μg/ml) of succinate which provides to the bacterium an equivalent concentration of carbon (10 μg/ml) relatively to IPR.

E. coli DH5a cells were used for the cloning. For the liquid cultures of DH5a LB Broth Miller (L3522) was used. Cultures of 5 ml and incubation overnight at 37˚C/210 rpm until saturation (10^9 cells/ml) and LB agar plates (L3147) overnight incubation at 37˚C without shaking.

2.3. Experimental Setup

2.3.1. *Arthrobacter* strain C1 experiment

Triplicates flasks of MSMN + iprodione and LB + iprodione were inoculated with fresh inoculum of the strain C1, originated from C1 MSMN and LB cultures respectively, corresponding to 10^6 cells/ml. Triplicate flasks containing only MSMN + iprodione and LB + iprodione without inoculum were used as controls to determine the abiotic degradation of iprodione during the experiment. Triplicate flasks containing only MSMN + succinate + inoculum and LB + inoculum without iprodione were also included in the experiment. Samples of the liquid cultures were removed from all bacterial cultures at frequent time points (selection was based on the degradation pattern of these specific cultures post experiment via HPLC) to ensure that enough timepoints were sampled to choose the desired stages. The collected samples were centrifuged, the pellets collected were used for DNA and RNA analysis and downstream RT-q-PCR of selected genes as it is described below.

2.3.2. Culture TA1.6 experiment

Triplicates flasks of MSMN + iprodione and MSMN + succinate were inoculated with fresh inoculum of TA 1.6 corresponding to 10^6 cells/ml. Triplicate flasks containing only MSMN + iprodione without inoculum were used as controls to determine the abiotic degradation of iprodione during the experiment. Samples of the liquid cultures were removed from both iprodione, and succinate amended bacterial cultures at specific time points (selection was based on preliminary growth curves monitored via q-PCR) to ensure that the bacteria in the two cultures were in corresponding phases of their growth. The collected samples were centrifuged, the pellets collected were used for DNA analysis (growth curve) and RNA analysis (metatranscriptomics).

2.3. Quantification of iprodione and 3,5-DCA via HPLC

Iprodione and 3,5-DCA were extracted from the liquid culture samples by mixing 300 µl of culture with 600 µl of acetonitrile. Samples were vortexed for 30 sec and then centrifuged (1 min at max g) or filtered through hydrophobic 0.22 nm pore syringe filters.

The routine check of the degrading capacity of the isolates was determined with HPLC measurement of the levels of iprodione and 3,5-DCA. A SHIMADZU HPLC equipped with a column RP-C18 was used for the analysis of the residues of iprodione and 3,5-DCA. Detection was done for both substances at 229 nm with a mobile phase composed of 65% acetonitrile and 35% H_2O by volume. Under these chromatographic conditions the retention times of 3,5 DCA and iprodione were 3,5 and 6 min respectively.

2.4. DNA extraction

A sample of the actively growing bacterial cultures (2 ml) was sampled at each time point and centrifuged at max speed for 10 mins. The pellet derived was used for DNA extraction using the MN nucleospin kit (740952). Instead of the T1 buffer, preincubation with buffer including lytic enzyme was employed to maximize extraction efficiency considering the phylogeny of the studied strains (gram positive bacteria). The pellet was resuspended in 20 mM Tris/HCl, 2

mM EDTA, 1 % Triton X-100 at pH 8, supplemented with lysozyme to a final concentration of 20 mg/ml and incubated for 60 mins at 37 °C. Subsequently 25 µL Proteinase K were added and incubated at 56 °C overnight, until complete lysis was obtained. The extraction protocol was subsequently followed as described and DNA was eluted in 100 µl elution buffer. DNA was quantified by the Quant-iT™ dsDNA-HS kit & Qubit v2 fluorometer (Invitrogen by Thermo Fisher Scientific).

2.5. q-PCR analysis

The growth of the bacteria during cultivation was monitored via q-PCR quantification of the number of copies of the 16S rRNA gene. 2 µl of bacterial DNA extracts were used as template for the qPCR. The 16S rRNA gene was amplified with universal primers Eub 338 and Eub 518 (Fierer et al., 2005) targeting the V3-V4 region of the gene. Quantification was achieved through quantification of serial dilutions of plasmid containing the target gene. The concentrations of the different reagents in the q-PCR and the thermocycling conditions utilized are given in Tables 3 and 4.

Table 3: The 10 µl reaction of the KAPA SYBR qPCR

COMPONENT	10 µl REACTION	FINAL CONCENTRATION
KAPA SYBR 2X mix	5 µl	1X
BSA	0,2 µl	1X
Forward primer (10 µM)	0.1 µl	0.1 µM
Reverse primer (10 µM)	0.1 µl	0.1 µM
Template DNA	2 µl	10 ng/µl
Nuclease-free Water	2,6 µl	

Table 4: The thermocycler conditions of the KAPA SYBR qPCR

STEP	TEMP	TIME	CYCLES
Initial Denaturation	95°C	1 minute	1
Denaturation Annealing/Extension	95°C 60°C	5 seconds 30 seconds** (+ plate read)	40
Melt Curve	60-95°C	Every 0,5 seconds	1

2.6. RNA extraction

RNA extraction from growing bacterial cultures was performed for downstream RT-q-PCR analysis of the specific iprodione-degrading genes but also for the transcriptomic analysis (not part of this thesis). Regarding the cultures of C1 strain in LB, 2ml of the liquid medium was collected and centrifuged at max speed for 10 minutes. When cultures C1 and TA1.6 were cultivated in MSMN, 100-150 ml were harvested at each time point and pelleted by centrifugation for 15 minutes at max speed. RNA was extracted using the MN RNA nucleospin mini kit (740955) according to the manufacturers protocol. Briefly, pelleted cells were homogenized with the TissueLyser II by QIAGEN using 4mm stainless steel beads during three

rounds of beating for 3 mins at 17 Hz, with subsequent spin down and snap freeze in liquid nitrogen. The protocol was followed without the use of β-mercaptoethanol. Also using 600µl of RA1 and 70% EtOH, instead of 350µl. Elution in 100µl elution buffer. Samples stored at -80^{o}C.

2.7. RT-q-PCR analysis

Primers targeting the three genes involved in the transformation of IPR, *ipaH, ddaH* and *duaH* were designed and tested with the tool Primer Blast NCBI (US NCBI, 2024), see the appendix for primer sequences. The specificity of the primers was also verified *in vitro* by amplification of the target genes, cloning, transformation and Sanger sequencing which verified that the specific primers amplified the desired genes.

The cDNA synthesis and the subsequent qPCR was performed in one step by the Luna® Universal One-Step RT-qPCR Kit from NEB (E3005). The relative expression analysis was performed following the instructions by (Livak & Schmittgen, 2001) using the 2− ΔΔCT method. The p value for the statistical significance was performed using the T test (Mishra et al., 2019). The reagents and the thermocycling conditions used in RT-q-PCR analysis are given in the two Tables below.

Table 5: The 10 µl reaction of the LUNA RTqPCR.

COMPONENT	10 µl REACTION	FINAL CONCENTRATION
Luna Universal One-Step Reaction Mix (2X)	5 µl	1X
Luna WarmStart RT Enzyme Mix (20X) 1 µl 1X	0,5 µl	1X
Forward primer (10 µM)	0.4 µl	0.4 µM
Reverse primer (10 µM)	0.4 µl	0.4 µM
Template RNA	2 µl	10 ng/µl
Nuclease-free Water	1,7 µl	

Table 6: The thermocycler conditions of the LUNA RTqPCR.

STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation Extension	95°C 60°C	10 seconds 30 seconds** (+ plate read)	 45
Melt Curve	60-95°C	Every 0,5 seconds	1

3. RESULTS

3.1. Degradation of iprodione

3.1.1. Degradation of iprodione by *Arthrobacter* sp., strain C1

Prior to further analysis the cultures had to be tested for growth and their capacity to degrade iprodione. Hence strain C1 was cultured in LB and MSM at pH 5.0 to avoid abiotic degradation of iprodione. The strain C1 fully degraded iprodione at 5 days resulting in the formation of small amounts of 3.5 DCA (Figure 14). No appreciable degradation of iprodione and formation of 3,5-DCA was evident in the abiotic controls.

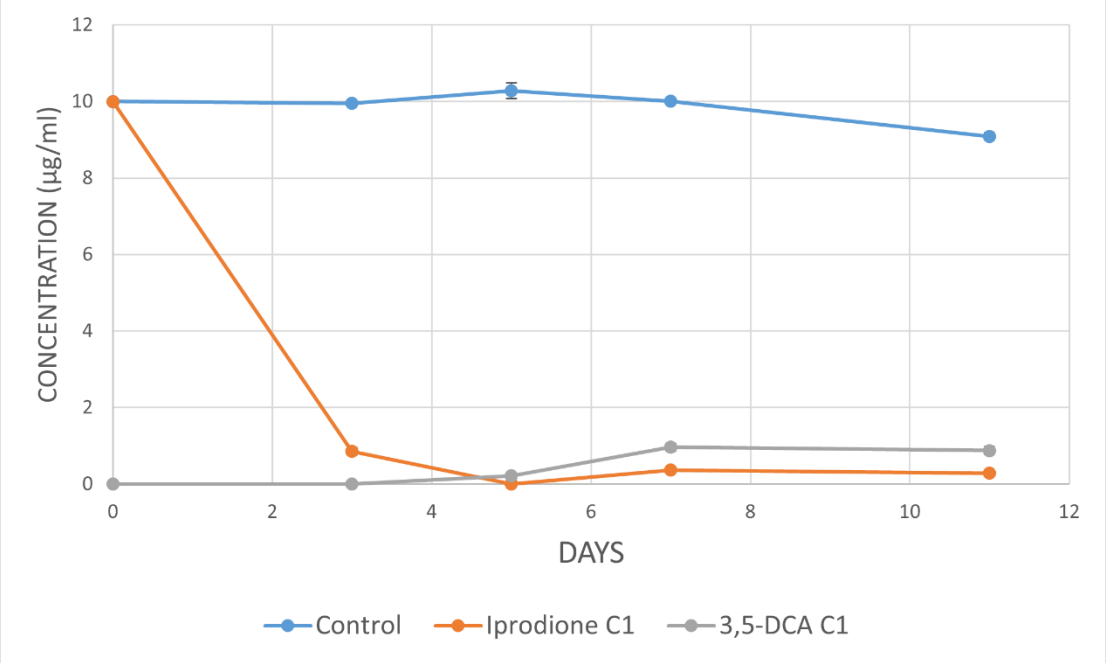


Figure 14: The degradation pattern of iprodione and the formation of 3,5-DCA in LB inoculated with the strain Arthrobacter sp. C1. The degradation of iprodione in the abiotic controls (non-inoculated) is also presented. Each value is the mean of triplicates ± the standard deviation.

When the same strain was cultured in MSM we noted rapid degradation of iprodione but no formation of 3,5-DCA (Figure 15). Furthermore, no degradation of iprodione and formation of 3,5-DCA was observed in the abiotic controls.

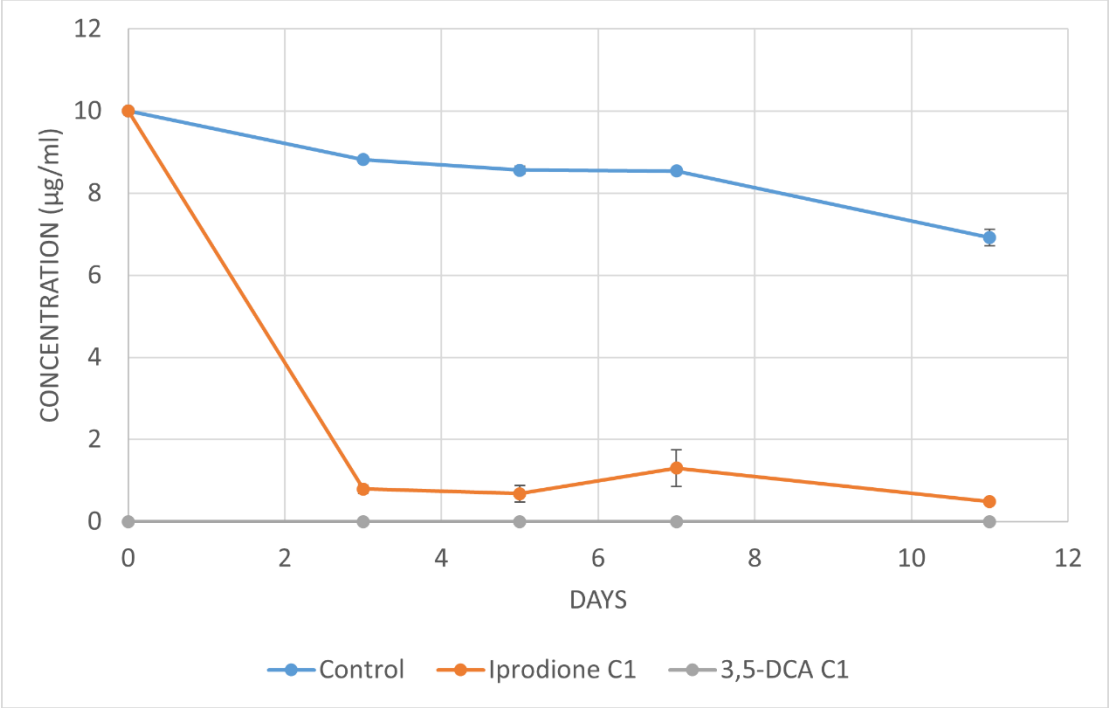


Figure 15: The degradation pattern of iprodione and the formation of 3,5-DCA in MSM inoculated with the strain Arthrobacter sp. C1. The degradation of iprodione in the abiotic controls (non-inoculated) is also presented. Each value is the mean of triplicates ± the standard deviation.

3.1.2. Degradation of iprodione by culture TA1.6

The culture TA1.6 was grown in MSMN and achieved complete degradation of iprodione in 12 hours with concurrent formation and accumulation of 3,5-DCA (Figure 16).

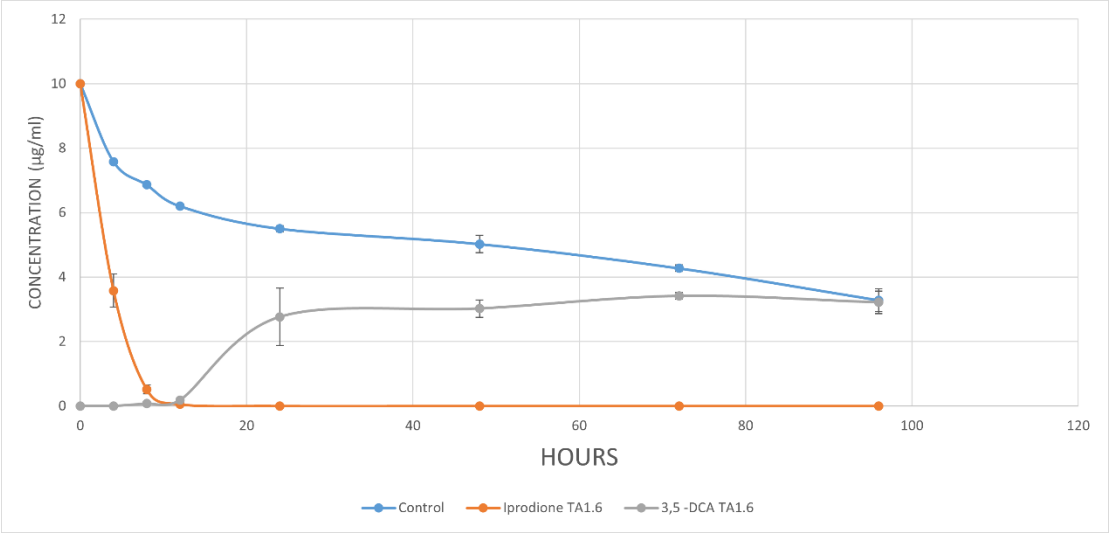


Figure 16: The degradation pattern of iprodione and the formation of 3,5-DCA in MSMN inoculated with the TA1.6 consortium. The degradation of iprodione in the abiotic controls (non inoculated) is also presented. Each value is the mean of triplicates ± the standard deviation.

3.2. RNA extraction and preparation of strain *Arthrobacter* C1

The primers designed for the amplification of the three iprodione degrading genes plus the 16S rRNA and *gyrB* as reference genes for expression analysis were tested for their specificity at different T_m of 56, 58 and 60°C (Figure 17). All primers showed higher specificity in their amplification at 60°C providing a band of the expected size. The amplicons derived for these genes were excised from the gel and sequenced, and the sequencing analysis verified the amplification of the target genes.

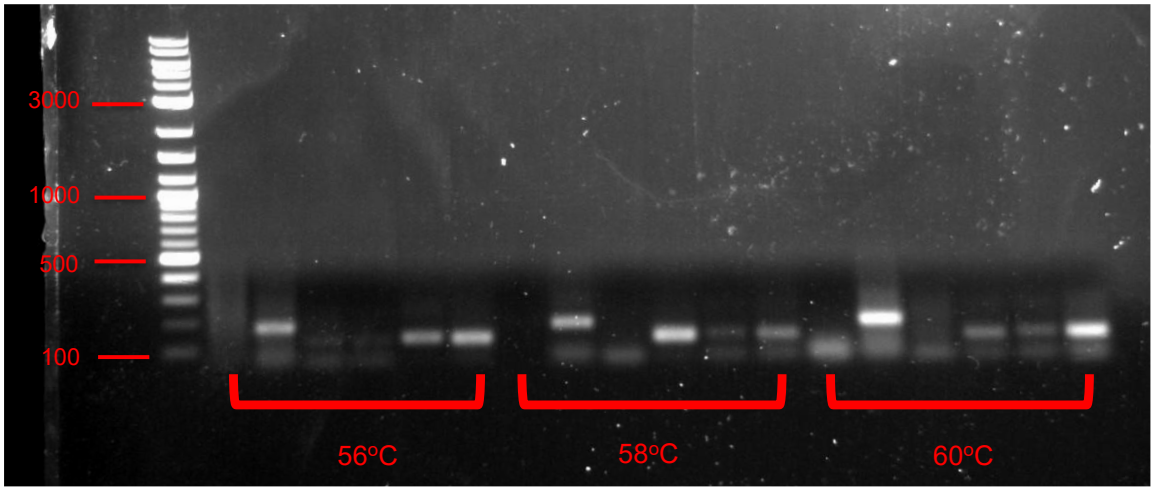


Figure 17: Agarose gel electrophoresis showing the amplification of genes 16S rRNA, ddaH, ipaH, duaH, gyrB at three different Tm. For each temperature the lanes show amplicons in negative control, 16S rRNA, ddaH, ipaH, duaH, gyrB.

After primer validation, RNA was extracted from different time points along the degradation of iprodione and in LB (Figure 18) and MSMN (Figure 19).

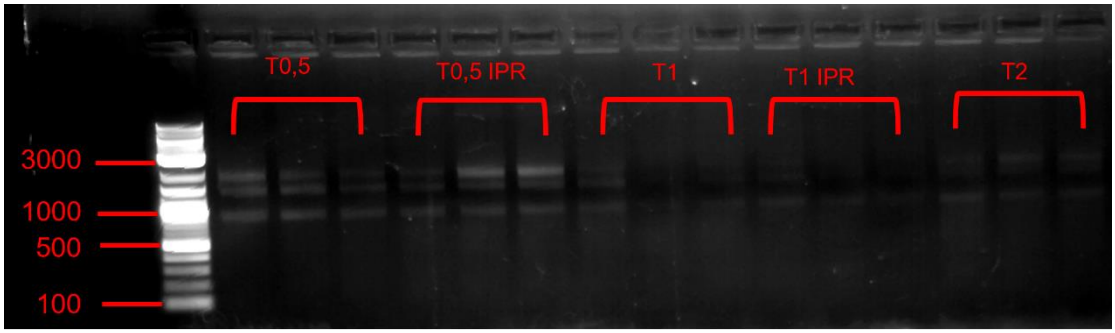


Figure 18: Agarose gel electrophoresis showing RNA extracts, without degradation, from the strain C1 in LB. All wells contain a variety of 50 to 100 ng RNA of the time points-in triplicates- 12 hours, 1 day, 2 days, 4days and 6 days

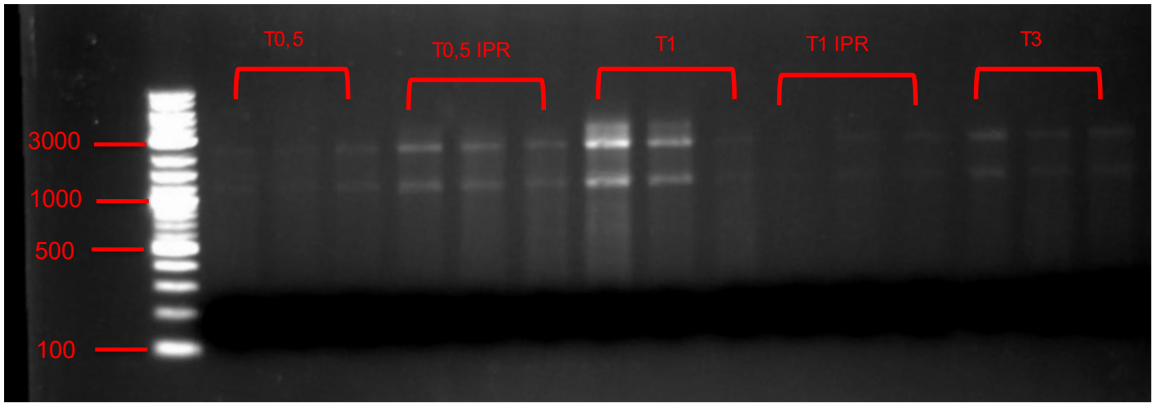


Figure 19: The gel showing no degradation of the isolated RNA from the C1 in MSMN cultures.
All wells contain a variety of 50 to 150 ng RNA of the time points-in triplicates- 12 hours, 1 day, 3 days and 5 days

RNA extraction was followed by RT-q-PCR using a one-step RNA amplification approach. DNAse treatment of RNA was effective in removing DNA contamination as denoted by the lack of any amplification of the 16S rRNA gene (see Figure 20).

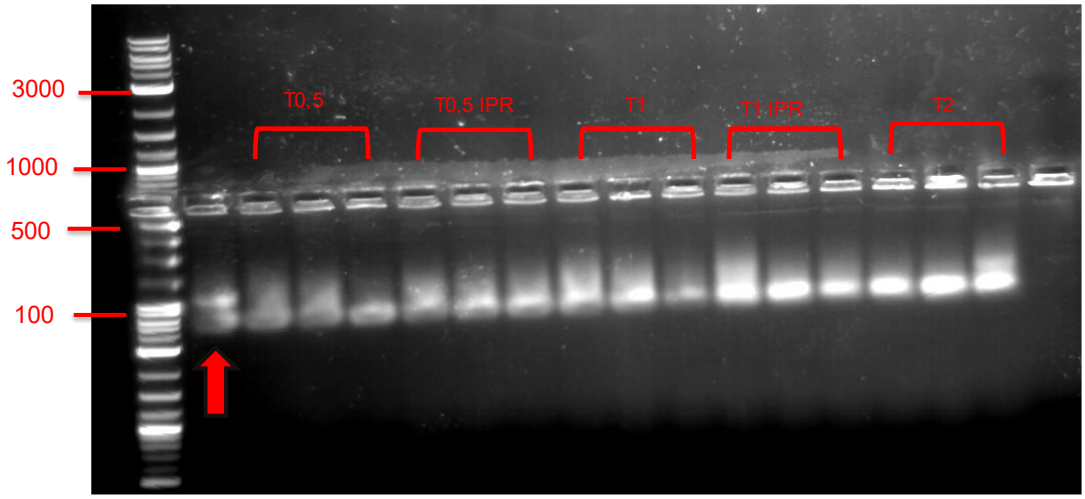


Figure 20: Agarose gel electrophoresis showing no amplification when RNA from the C1 strain was used as a template. The arrow indicates the positive control, where amplification of the 16S RRNA gene was achieved. This gel had two rows of samples, the gel was ran extensively in order to separate the primer dimers from the DNA amplification. Except the first well with the arrow, the rest are the RNA from the figures above.

3.3. RT-q-PCR analysis of the strain *Arthrobacter* C1

RT-q-PCR analysis of the relative expression of the genes *ipaH, ddaH*, and *duaH* in *Arthrobacter* strain C1 when cultivated in MSMN and LB are shown in Figures 21 to 23 and 24 to 26 respectively. For all three genes we noted in most time points a significantly higher expression in the presence compared to the absence of iprodione grown cultures. This was particularly the case at the 12-hours collected samples, suggesting a rapid response of the catabolic machinery of the bacterium to iprodione.

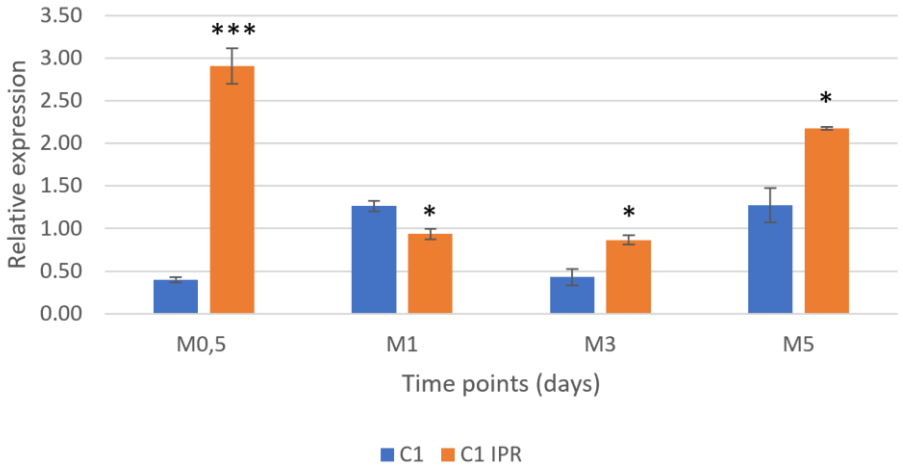


Figure 21: The relative expression of ipaH gene in the cultures of the Arthrobacter strain C1 when grown in MSMN in the presence or absence of iprodione. At each time point, significant differences in the expression levels of ipaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

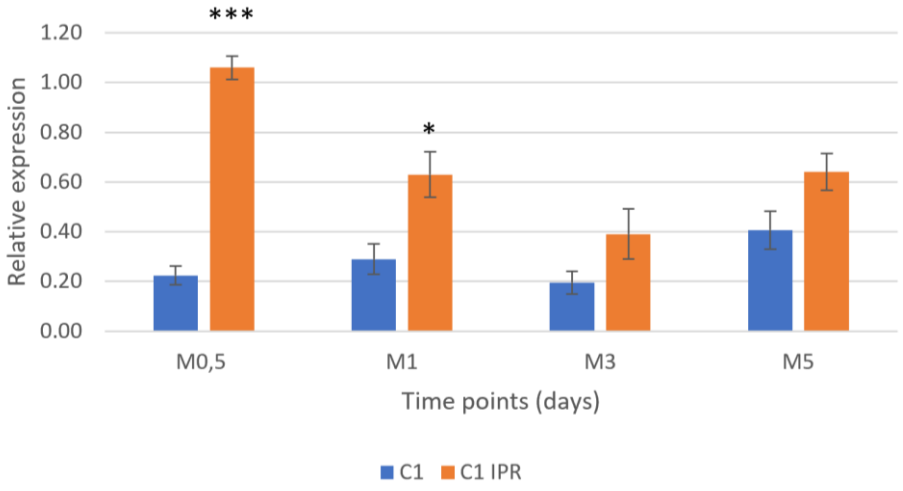


Figure 22: The relative expression of ddaH gene in the cultures of the Arthrobacter strain C1 when grown in MSMN in the presence or absence of iprodione. At each time point, significant differences in the expression levels of ddaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

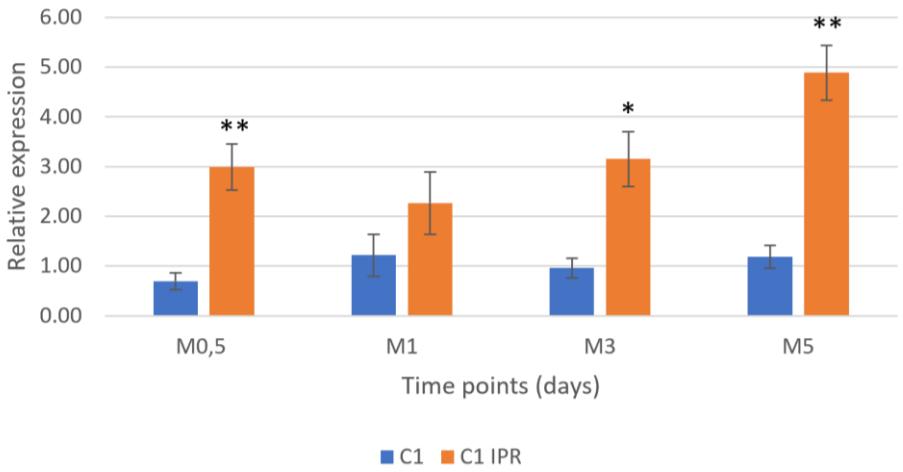


Figure 23: The relative expression of duaH gene in the cultures of the Arthrobacter strain C1 when grown in MSMN in the presence or absence of iprodione. At each time point, significant differences in the expression levels of duaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

When the relative expression of the three iprodione-degrading genes was determined in cultures of the strain *Arthrobacter* C1 grown in LB we noted different temporal expression patterns. Hence for all genes we observed a delayed response with the expression of *ipaH, ddaH* and *duaH* showing significantly higher levels in the presence of iprodione at 2, 1 and 4 days respectively.

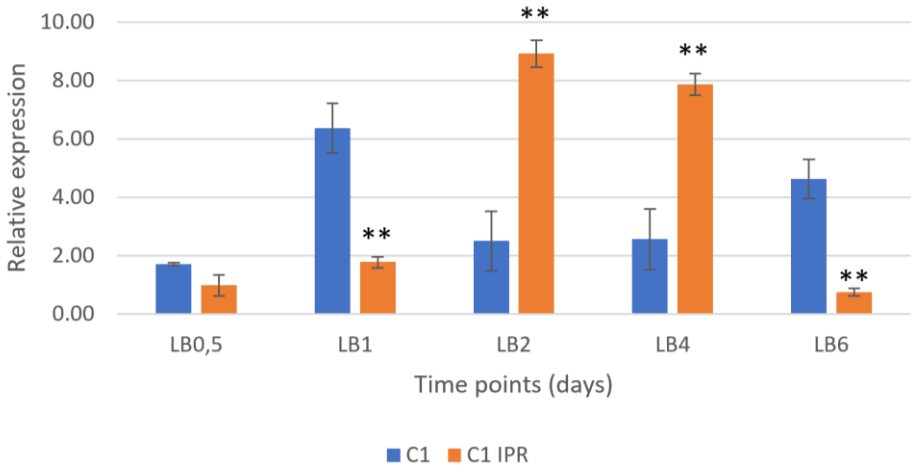


Figure 24: The relative expression of ipaH gene in the cultures of the Arthrobacter strain C1 when grown in LB in the presence or absence of iprodione. At each time point, significant differences in the expression levels of ipaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

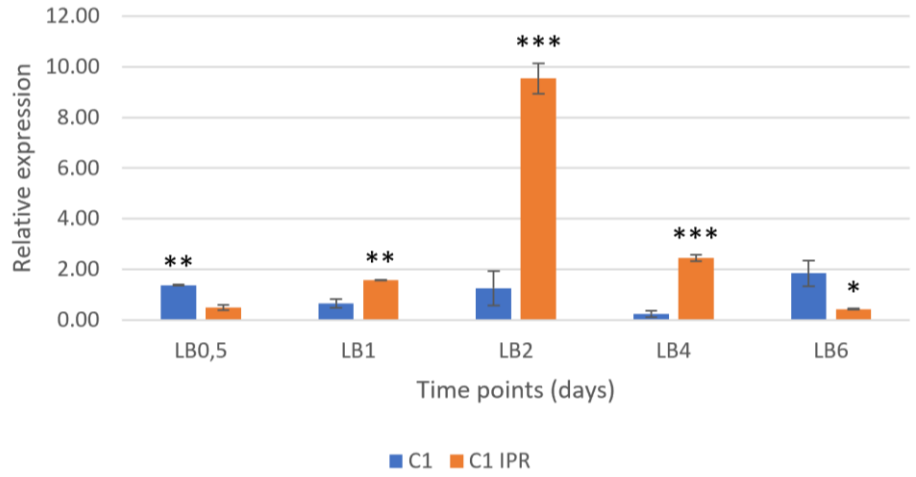


Figure 25: The relative expression of ddaH gene in the cultures of the Arthrobacter strain C1 when grown in LB in the presence or absence of iprodione. At each time point, significant differences in the expression levels of ddaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

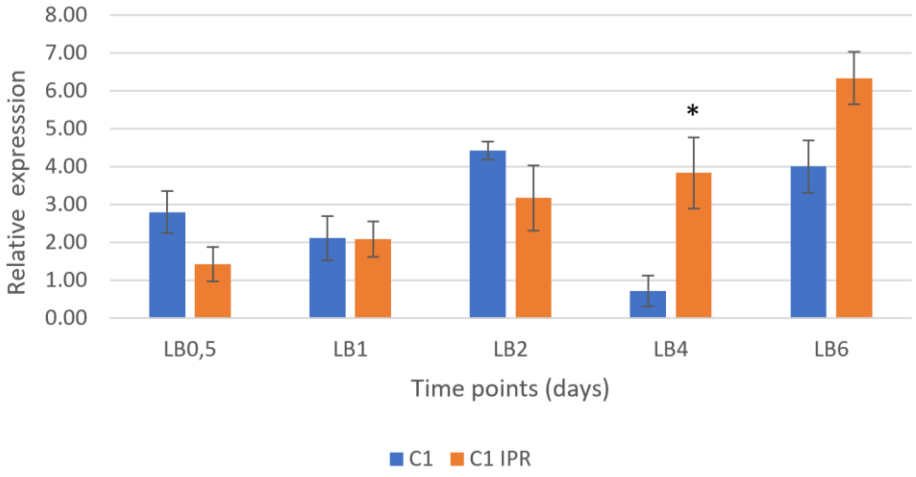


Figure 26: The relative expression of duaH gene in the cultures of the Arthrobacter strain C1 when grown in LB in the presence or absence of iprodione. At each time point, significant differences in the expression levels of duaH between the two treatments are denoted by asterisks (for p<0.05, ** for p<0.01, *** for p<0.001).*

1. Preparation for the transcriptomic analysis of the culture TA1.6

In parallel to the determination of the relative expression of the iprodione-degrading genes in the *Arthrobacter* strain C1, which was axenic, we have also employed a transcriptomic experiment to identify the role of the different copies of the *ipaH, duaH* and *ddaH*, found in the *Paenarthrobacter* and *Microbacterium* components of the culture TA1.6. Similarly to the strain C1, TA1.6 was cultivated in MSMN in the presence of iprodione (10 mg/l) and succinate

(as an alternative carbon source) respectively. Samples were collected from both cultures at three time points (8, 12 and 24 h) and the pellets were used for RNA extraction.

The specific time points were selected after preliminary tests (See Figure 27 below) and q-PCR determination of bacteria growth in order to identify time points where the culture was at similar growth stages (early, mid and late log phase) in the two different growth conditions.

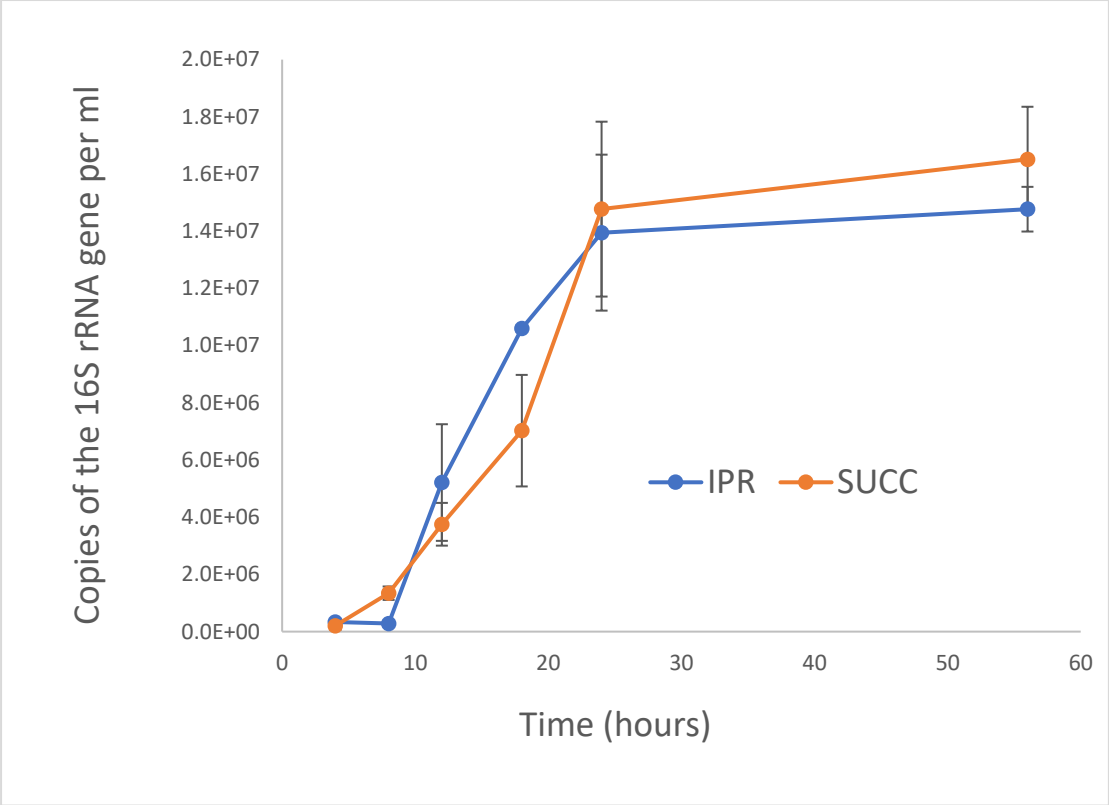


Figure 27: The growth pattern of the TA1.6 culture, as determined via q-PCR of the 16S rRNA gene, when grown in MSMN in the presence of iprodione or succinate. Each value is the mean of triplicates ± the standard deviation.

4. DISCUSSION AND FUTURE WORK

This thesis is a small part of a bigger project on Paenarthrobacters and there are published and unpublished data that bind the whole project. Our lab cultures, *Arthrobacter* strain C1 (Campos et al., 2015) and the TA1.6 culture composed of *Panarthrobacter* and *Microbacterium* (Katsoula et al., 2020) are part of a group of mostly Paenarthrobacters that specialize in iprodione degradation. This group consists of bacteria from 3 different continents (China, Chile, Greece), different habitats (soil and leaves) and different restrain habitats (pristine soil and heavily treated soil). The iprodione transformation pathway to 3,5 DCA encoded in Paenarthrobacters from China was validated in YJN-5 strain by Yang et al. (2018) and in YJN-D strain by Zhang et al. (2021). The genes responsible, *ipaH*: acylamidase, *ddaH*: polysacharide deacetylase, *duaH*: hippurate hydrolase, were heterologously expressed and gave iprodione degrading capabilities to other stains, they were also knocked out from the original strains and lost the degrading ability. Our background lab genomic analysis showed high homology in all genes and revealed some extra sets of genes in plasmids and to other strain's chromosome.

The background work on our lab strains included a synteny analysis that resulted in the hypothesis that C1 is a step back in the evolution of iprodione degradation compared to the other *Paenarthrobacter* degraders. This hypothesis is in line with the thesis data, where C1 in every medium and condition degrades slower and lacks in the turnover of the iprodione to 3,5 DCA. Is also in line with the Chinese (Zhang et al. 2021 and Yang et al., 2018) super-fast evolved degraders.

It is observed that there is no RNA-based analysis in any of the *Paenarthrobacter* degraders. The main achievement of this thesis is that it provides a first validation of the hypothesis that the proposed catabolic gene set is utilized by our C1 strain for the degradation of iprodione on RNA level. This is based on the observation of the increased gene expression of the whole gene set in the MSMN cultures with presence of iprodione. This suggests that the strain C1 relies on the same catabolic genes, as other known iprodione degrading bacteria, for the transformation of iprodione.

The RT-q-PCR results for strain C1 are segregated into MSMN and LB cultures. The quick response of all three genes to iprodione (upregulated at 12 hours post inoculation in MSMN) reveals that C1 could use iprodione as energy source. On the contrary the same strain in LB, a rich growth medium, takes longer to respond (2, 1 and 4 days for genes *ipaH, ddaH* and *duaH* respectively) and upregulate the catabolic genes. It is noteworthy that the HPLC degradation patterns of iprodione from C1 in LB cultures, indicate that the conversion happens from the initiation of incubation. This implies that the enzymes produced, even at low rates, can initiate the degradation process. Our background work on the pangenome analysis included the first and the third gene in the core genome of the Paenarthrobacters, additionally the synteny analysis provided information that these genes are encoded in operons with housekeeping genes. By integrating the RT-q-PCR data, these housekeeping genes are responsible for the iprodione degradation. Consequently, it can be inferred that their upregulation is not necessarily required to initiate iprodione catabolism.

The next step is to decipher the contribution of *Microbacterium*, being present in the TA1.6 culture. Previous studies (Yang et al., 2017 and Cao et al., 2018) have isolated *Microbacterium* strains related to iprodione degradation. The presence of two copies of *ipaH* and *duaH* genes in the chromosome of *Microbacterium* present suggests that it has the potency to contribute to the transformation of iprodione. The fact that the *Paenarthrobacter* and *Microbacterium* strains were not isolated separately might imply a synergism between the two strains. However, the presence of not the full set of iprodione-degrading genes in the genome of *Microbacterium* might be an indication that its contribution to the degradation of iprodione is only partial. The ongoing RNAseq analysis will shed light on the functional conservation and role of the homologues of the different iprodione-degrading genes in the *Paenarthrobacter and Microbacterium* components of culture TA1.6.

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6. APPENDIX

1. How the primers were designed

Table 7: The oligonucleotide sequences of the 4 primer sets targeting the 3 IPR degradation genes and 1 housekeeping gene for the Paenarthrobacter C1

Primer	5'->3' sequence	Tm ºC	GC %	Product length
gyrb1F	TCGACTTCGAGACTCTTCGC	59.56	55	129
gyrb1R	CTACTGCGTCCAGGTCAAGG	60.11	60	129
ipahF	TCGGGATAACGAAGCGTAGC	59.97	55	132
ipahR	GGACCTCATCGAAACCACGG	60.74	60	132
duahF	AGTTCACCCTCAACATCCGC	60.32	55	126
duahR	ACAGCTCTTCGATCTTCGGG	59.54	55	126
ddahF	AGGCACTTGAGATGTGGACC	59.67	55	128
ddahR	TTCGATCAGCCGTTCCAAGG	60.39	55	128

Following the next session of Figures you can have the workflow of the primer design and in silico test.

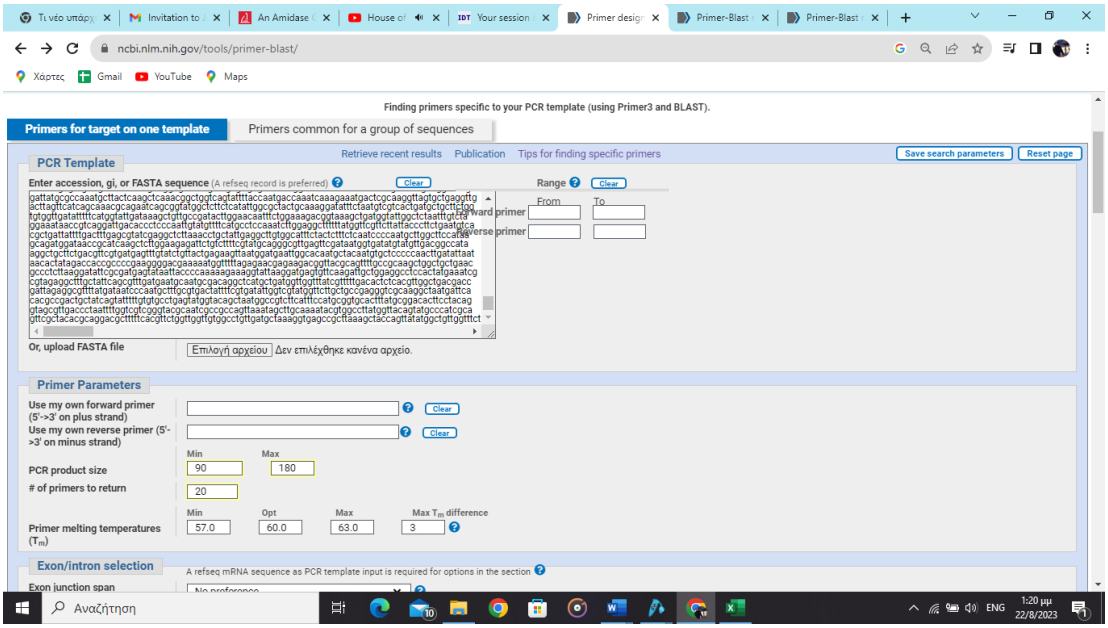

Figure 28: The PCR template is the whole genome sequence of the C1 target strain including the sequence of the mobile elements of C1 . The minimum length of the amplicon was set at 90 bp and the maximum at 180 bp. The Tm between 57°C and 63°C with optimum 60°C.

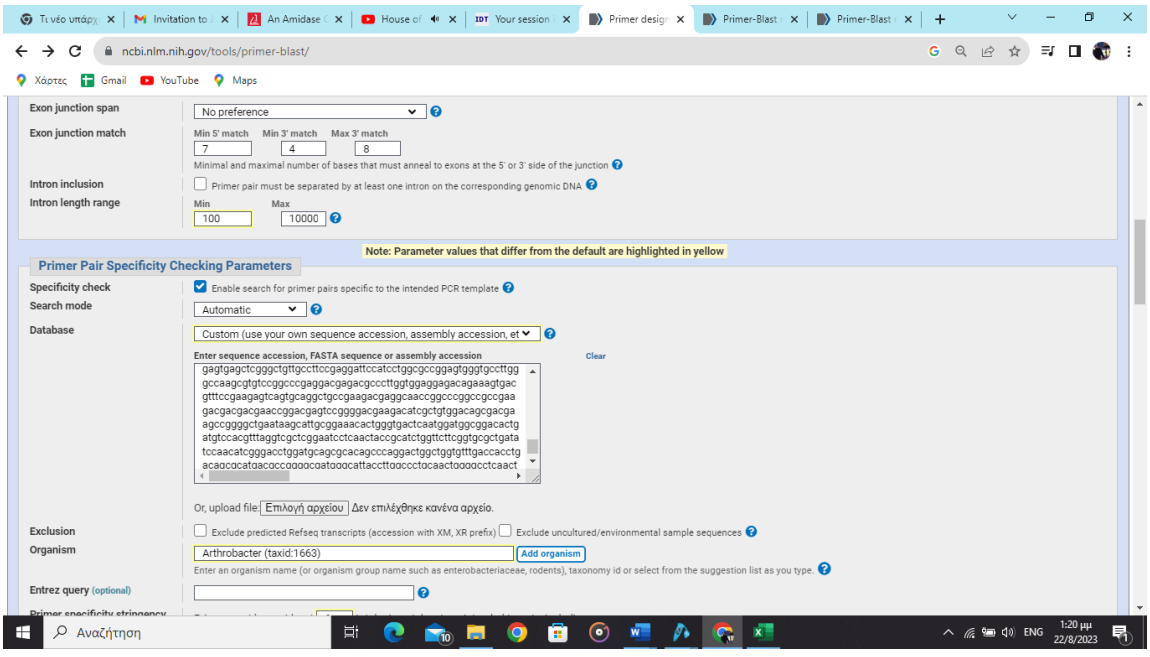

Figure 29: The introns are not of interest as our strain does not have. The PCRs were performed with a template only having extracts of the C1 paenarthrobacter strain, so the database for the primer specificity was again the whole genome of C1. Extra database was used form the Arthrobacters asclosest related species.

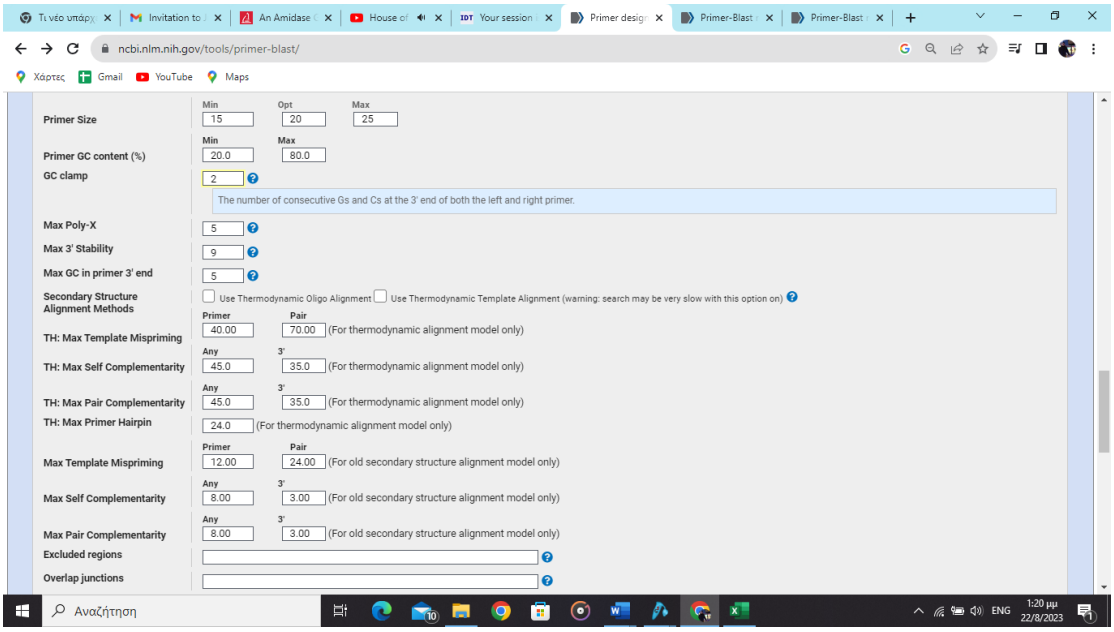

Figure 30: The high primer specificity stringency is key to a successful targeted PCR. So the highest option for primer mismatches at unintended templates..

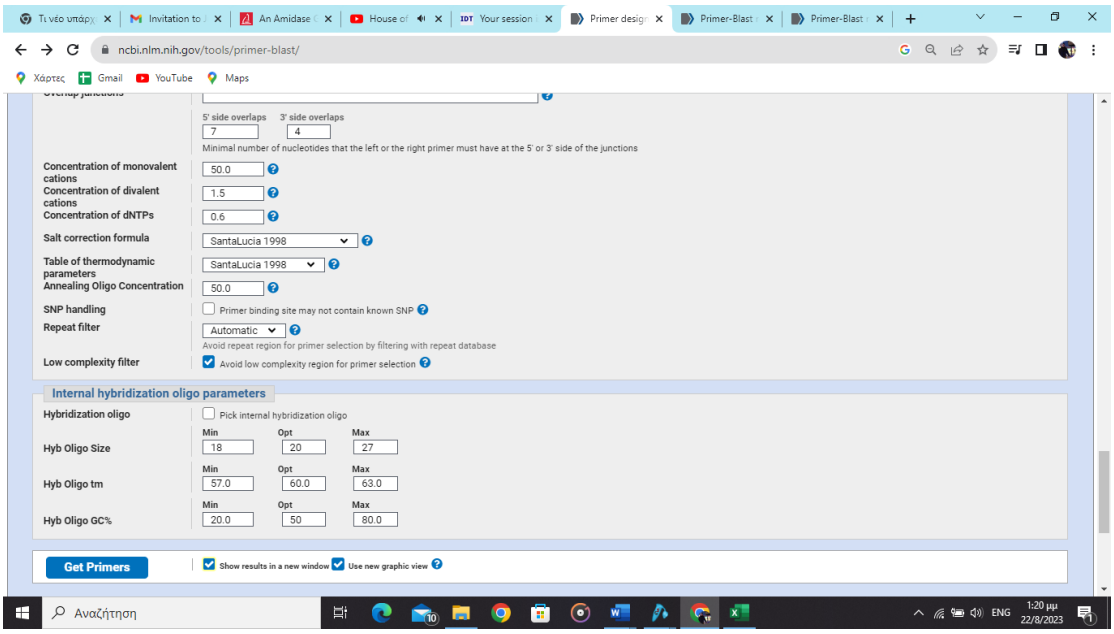

Figure 31: The GC clamp is an empiric measure to increase the primer affinity. Having higher affinity with GC in the 3' end due to the stronger bonding of G and C bases.

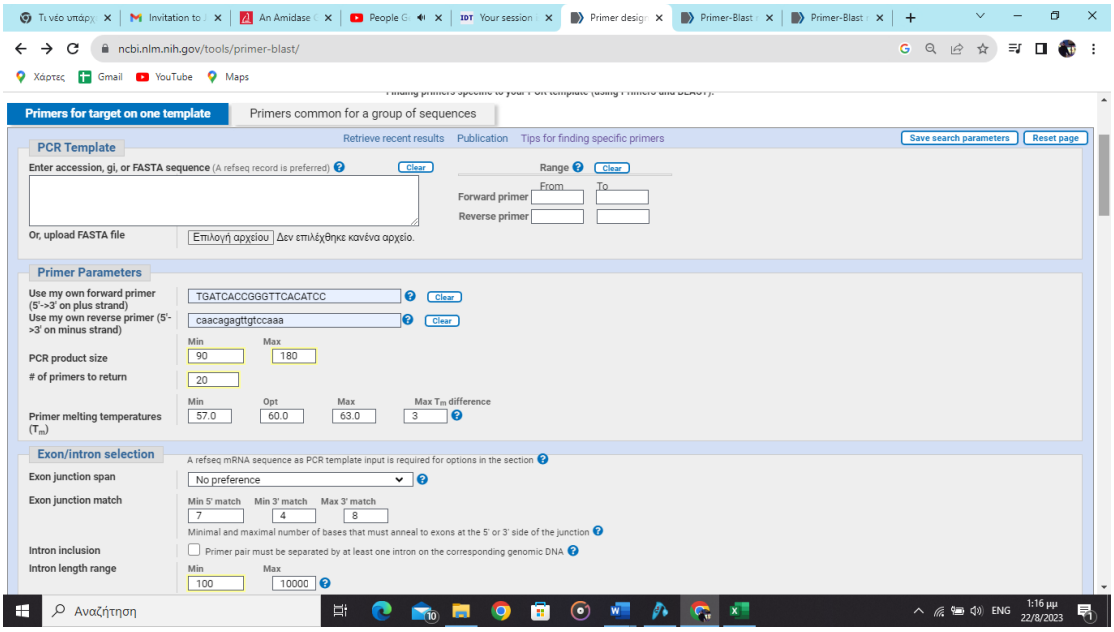

Figure 32: Proceeding to the primer check with the same tool. Importing the chosen primer set.

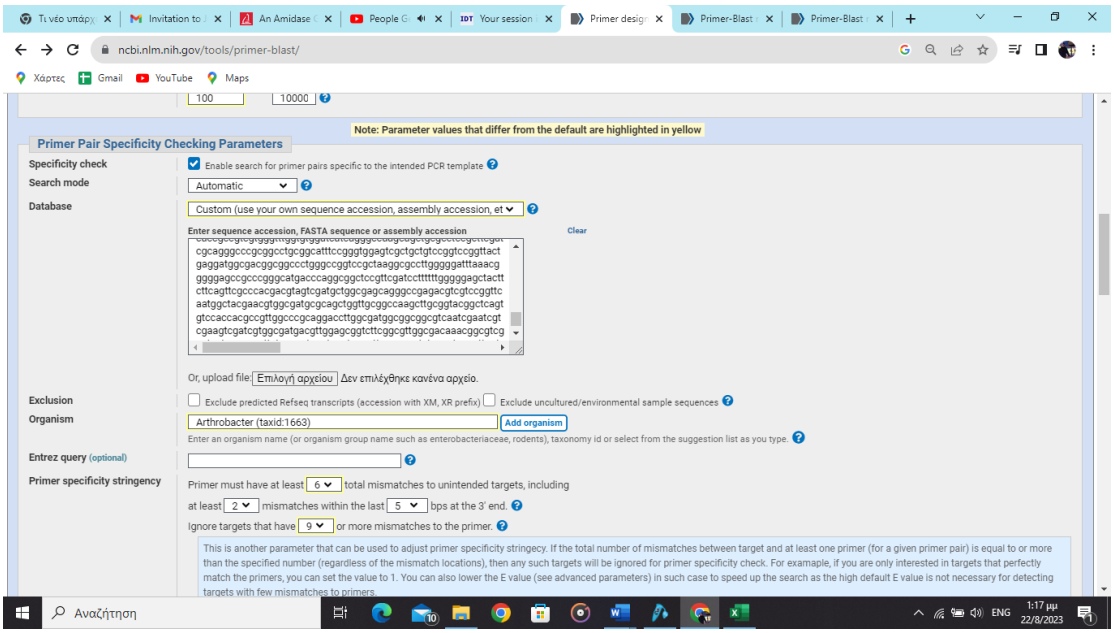

Figure 33: Checking the specificity automatically to the Arthrobacter database and manually towards the C1 whole genome sequence.

3. Preparation of minimal salts medium with nitrogen (MSMN):

Protocol for 1lt:

1) Prepare the following stocks:

Stock1 (x10 concentration)

KH2PO4 22.7 g/lt

Na2HPO4.12H2O 59.7 g/lt

NH4Cl 10.0 g/lt

Stock2 (x10)

MgSO4.7H2O 5.0 g/lt

CaCl2.2H2O 0.1 g/lt

MnSO4.4H2O 0.2 g/lt

Stock3 (x50)

FeSO4.7H2O / FeSO4 0.46/0.25 g/lt

2) Autoclave stock 1 και 2 and filter sterilize stock 3 (check for precipitation)

3) Combine 100 ml from stock 1 with 780 ml sterile ddH2O to produce solution 1.

4) In aseptic condition add the 100 ml from stock 2 and 20 ml from stock 3 in the 880 ml solution 1 crafted before.

5) Add the desired pesticide or other sterile carbon source. In our case iprodione and succinate.

NOTE

In order to make MSM instead of MSMN (without Nitrogen).

Exclude the NH4Cl from stock 1 and procced with the same recipe.