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Redundant salicylate 1-hydroxylase genes of the *Pseudomonas pseudoalcaligenes* C70

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Redundant salicylate 1-hydroxylase genes of the *Pseudomonas pseudoalcaligenes* C70

Abstract:

Naphthalene, salicylate and phenol-degrading *Pseudomonas pseudoalcaligenes* C70 has two redundant salicylate 1-hydroxylases genes, *nahG* and *nahW*. The comparison of the deduced amino acid sequence of *nahW* and *nahG* of C70 showed that they are almost identical (identity 99.8-100%) with those of the *P. stutzeri* strain AN10. NahWs of the C70 and AN10 formed separate group on phylogenetic tree and they were phylogenetically different from classical NahG sequences. In the mid-logarithmic growth phase the *nahW* expression level was higher than *nahG,* but both *nahW* and *nahG* were expressed at the similar level in the early stationary growth phase. The lag-phase length of C70 did not change significantly on used salicylate concentrations (1-3 mM). Strain C70 was able to tolerate salicylate concentrations up to 25 mM, although concentrations higher than 17 mM were stressful for cells. There is a strong possibility that the degradation of the salicylate is enhanced due to the presence of two salicylate hydroxylases.

Keywords: *Pseudomonas*, biodegradation, salicylate, salicylate 1-hydroxylase, redundant gene

CERCS: B230 BIOMEDICAL SCIENCES Microbiology, bacteriology, virology, mycology

Redundantsed salitsülaadi 1-hüdroksülaasi geenid *Pseudomonas pseudoalcaligenes* tüves C70

Lühikokkuvõte:

Naftaleeni, salitsülaati ja fenooli lagundav *Pseudomonas pseudoalcaligenes* tüvi C70 omab kahte redundantset salitsülaadi 1-hüdroksülaasi geeni, *nahG* ja *nahW*. Tüve C70 *nahW* ja *nahG* ennustatud aminohappeliste järjestuste võrdlus näitas, et need on väga sarnased *P. stutzeri* tüve AN10 vastavate järjestustega (identsusprotsent 99,8-100%). Tüvede C70 ja AN10 NahW järjestused moodustasid fülogeneetilisel puul eraldi rühma ja need olid fülogeneetiliselt erinevad klassikalistest NahG järjestustes. Logaritmilises kasvufaasis oli *nahW* ekspressioonitase kõrgem kui *nahG*, samas kui nii *nahW* kui *nahG* on ekspresseeritud sarnasel tasemel varajases statsionaarses kasvufaasis. Tüve C70 lag-faasi pikkus ei muutunud

oluliselt kasutatud salitsülaadi kontsentratsioonidel (1-3 mM). Tüvi C70 oli võimeline taluma kuni 25 mM salitsülaadi kontsentratsiooni kasvukeskkonnas, kuigi kontsentratsioonidel üle 17 mM oli rakkude kasv häiritud ning söötmesse tekkisid rakkude kogumikud. Tõenäoliselt aitab tüvel C70 kõrgeid salitsülaadi kontsentratsioone taluda kahe salitsülaadi 1-hüdroksülaasi olemasolu.

Märksõnad: *Pseudomonas*, biodegradatsioon, salitsülaat, salitsülaadi 1-hüdroksülaas, redundantne geen

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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Abbreviations

Ap - ampicillin

Ap^r - ampicillin resistant

C12O - catechol 1,2-dioxygenase

C23O - catechol 2,3-dioxygenase

Km - kanamycin

Km^r - kanamycin resistant

LB - Luria Bertani medium

mQ - "ultrapure" water

PAHs - polycyclic aromatic hydrocarbons

PCR - polymerase chain reaction

qRT-PCR - quantitative reverse transcription-PCR

sal operon - naphthalene degradation lower operon

salicylate - sodium salicylate in the growth medium

Introduction

The environmental pollution, mainly due to industrialization, is a world-wide problem. The researchers are focused on finding solutions to minimize harmful effects caused by pollutants. Bioremediation is eco-friendly and cheap method to resolve the problem. Some microorganisms have evolved pathways for degradation of toxic compounds and therefore have a good potential for bioremediation. The process bases on the break down of toxic compounds to the less toxic ones. The effectiveness of biodegradation depends not only on the complexity of the pollution to be removed, but also on genetic factors of microorganism, for example, from the structures of operons containing gene clusters involved in degradation.

Pseudomonas species are one of the most widely used bacteria for treating pollution. The research object of the current work is *Pseudomonas pseudoalcaligenes* strain C70. This strain possesses in the genome two phylogenetically different catechol 2,3-dioxygenases, *pheB* and *nahH*, which give to the strain advantage over the strains having only one copy of gene in the degradation of aromatic compounds (Jõesaar *et al.*, 2017). Strain C70 harbours also two salicylate 1-hydroxylases, *nahG* and *nahW,* in the naphthalene degradation lower operon. The present study was designed to determine the physiological role of two redundant salicylate 1-hydroxylases. We hypothesise, that the presence of additional gene is an advantageous for microorganism in biodegradation of toxic compounds, particularly of naphthalene/salicylate.

The first part of the study gives a overview of the aerobic bacterial degradation of aromatic compounds, redundant genes and naphthalene/salicylate degrading reference strains. The aim of the practical part is to test our previously stated hypothesis by analyzing sequences and determination of the expression levels of the salicylate 1-hydroxylases, evaluation of the growth parameters and tolerance of the strain in C70 and reference strains to salicylate. The construction of the mutant strain C70Δ*nahG* was started, but within the limited time frame we were not able to isolate the correct transconjugant.

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1. LITERATURE OVERVIEW

1.1 Bacterial aerobic degradation of aromatic compounds

Aromatic compounds found in environment have natural or man-made origin and they have a range of various structures. The special character of aromatic compounds is a presence of at least one benzene ring that makes these compounds biochemically inert (Seo *et al*., 2009; Fuchs *et al.,* 2011). Aromatic compounds exist in all living organisms as proteins contain beside other amino acids also three aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Second natural source is lignin - an aromatic polymer from plants - and its degradation products. Plants emit different aromatic compounds, however, with the inability of recycling carbon from these compounds. Benzene, toluene, ethylbenzene and xylene (BTEX) are well-known environmental pollutants that are obtained from crude oil (Fuchs *et al.*, 2011).

Biodegradation of aromatic compounds is carried out by anaerobic and aerobic microorganisms. Degradation extent of aromatic compounds depends on the number of benzene rings and on the type of substituents. Some substituents may remain intact or are eliminated or transformed before the ring cleavage, it depends on bacterial species and respective substituent. (Harwood and Parales, 1996; Seo *et al*., 2009; Fuchs *et al*., 2011). Aromatic compounds have low level of chemical reactivity and commonly the aromatic ring is attacked by oxygenases, such as monooxygenases and dioxygenases, which respectively insert one or both atoms of oxygen of O_2 into organic substrate (Gibson and Parales, 2000). As result of hydroxylation, central *o*-hydroxylated intermediates such as catechol, protocatechuatic acid and gentisic acid are formed that are further cleaved by dioxygenases (Fig. 1). *Ortho* positioned cleavage occurs between the two hydroxyl group, and in the *meta* positioned cleavage beside one of the hydroxyl group. The ring-cleavage intermediates, *cis, cis*-muconic acid and 2-hydroxymuconic semialdehyde, respectively, are then channeled to subsequent central pathways leading to the formation of Krebs cycle intermediates (Vaillancourt *et al*., 2006; Fuchs *et al*., 2011).

Intradiol dioxygenases, catechol 1,2-dioxygenase (C12O, EC 1.13.11.1) and protocatehcuate 3,4-dioxygenase (PC34O, EC 1.13.11.3), contain Fe^{3+} in catalytic centre and cleave *o*-dihydroxylated aromatic compounds via *ortho* pathway to the production of

cic,cis-muconic acid or its derivatives (Fig. 1). While extradiol dioxygenases, catechol 2,3-dioxygenase (C23O, EC 1.13.11.2) and protocatechuate 4,5-dioxygenase (PC45O, EC 1.13.1.18), which contain Fe^{2+} in catalytic centre cleave *o*-dihydroxylated aromatic compounds via *meta* pathway (Fig. 1; Nogales *et al.*, 2005). Extradiol dioxygenases differ from the intradiol dioxygenases by the ability to cleave also methyl-substituted catechols (Vaillancourt *et al.*, 2006; Müller *et al.*, 1996). Degradation of methylated aromatic compounds via *ortho* pathway leads to the accumulation of dead-end product, 4-methylmuconolactone (Chari *et al.*, 1987). The so far described *meta* cleavage pathways involved in the degradation of phenol, toluene and naphthalene are mostly plasmid-encoded (Harwood and Parales, 1996).

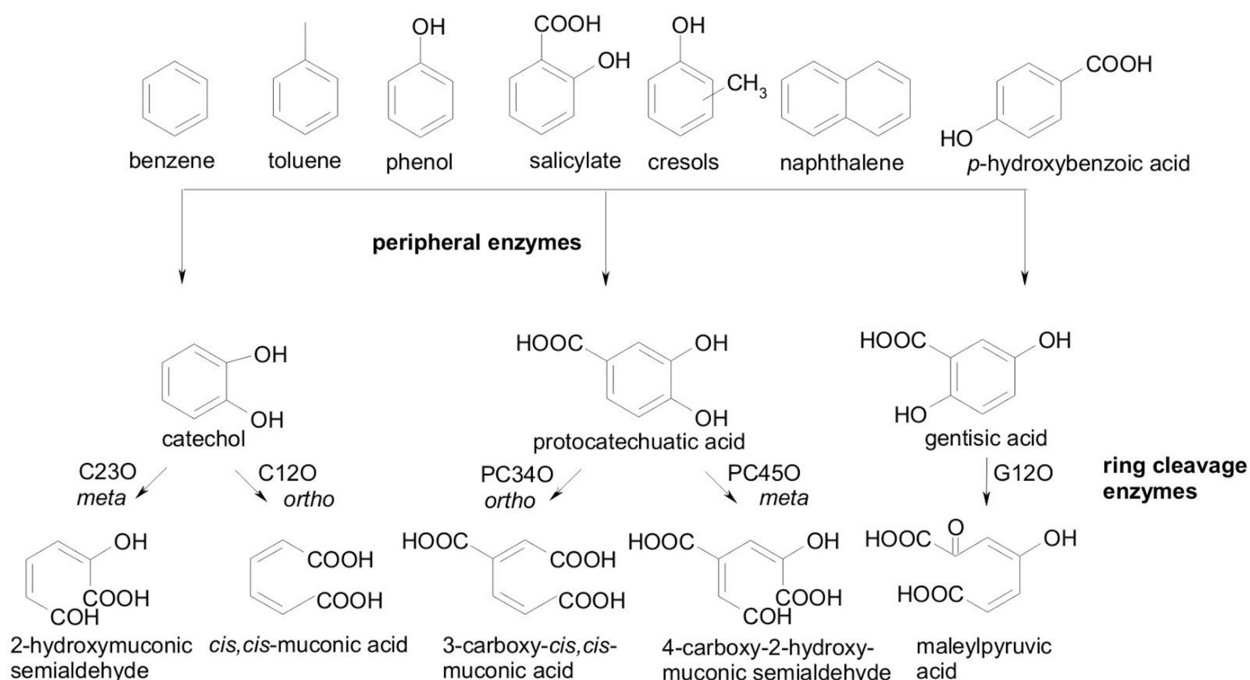

Figure 1. Aerobic routes of aromatic ring cleavage (Harwood and Parales, 1996).

Third common intermediate in aromatic compounds degradation pathways is gentisic acid (Fig. 1), that is cleaved by intradiol dioxygenase, gentisate 1,2-dioxygenase (G12O, EC 1.13.11.4), to maleylpyruvic acid (Lack, 1959).

1.2 Degradation of naphthalene

Polycyclic aromatic hydrocarbons (PAHs) are high priority organic pollutants due their toxic, genotoxic, mutagenic and/or carcinogenic properties and poor biodegradability (Cerniglia, 1992; Ghosal *et al.*, 2016). Naphthalene is the simplest, most volatile and least toxic of the PAHs, which consists of two benzene rings, and is used by microorganisms as a source of carbon for growth maintenance (Pathak, 2009). Naphthalene is widespread pollutant and therefore it has been used as a model substrate for the studying of the biodegradation of PAHs (Di Gennaro *et al.*, 2009). Other well studied PAHs are phenanthrene and anthracene that consist of three benzene rings (van der Meer *et al.*, 1992).

Aerobic degradation of naphthalene occurs gradually, the first benzene ring is cleaved by the multicomponent naphthalene dioxygenase to the central intermediate salicylate (Fig. 2) and it is called upper pathway. To cleave the last benzene ring salicylate is decarboxylated to catechol by salicylate 1-hydroxylase (Seo *et al.*, 2009) or converted to gentisate by salicylate 5-hydroxylase (Fuenmayor *et al.*, 1998), in both cases it is called naphthalene degradation lower pathway. The genes encoding the enzymes responsible for the degradation of the naphthalene are located on chromosome or in plasmid.

Most PAHs degrading strains have been isolated from polluted sediments or soil as higher molecular mass PAHs have low solubility in the water. These strains belong to different phylogenetic genera, for example *Pseudomonas, Mycobacterium, Haemophilus, Rhodococcus, Paenibacillus* etc. (Haritash and Kaushik, 2009)

The bacterial degradation of naphthalene has been well characterized in *Pseudomonas putida* G7, where the catabolic operon for naphthalene degradation is located in the plasmid NAH7. This operon consists from two operons: the genes locating in *nah* operon encode

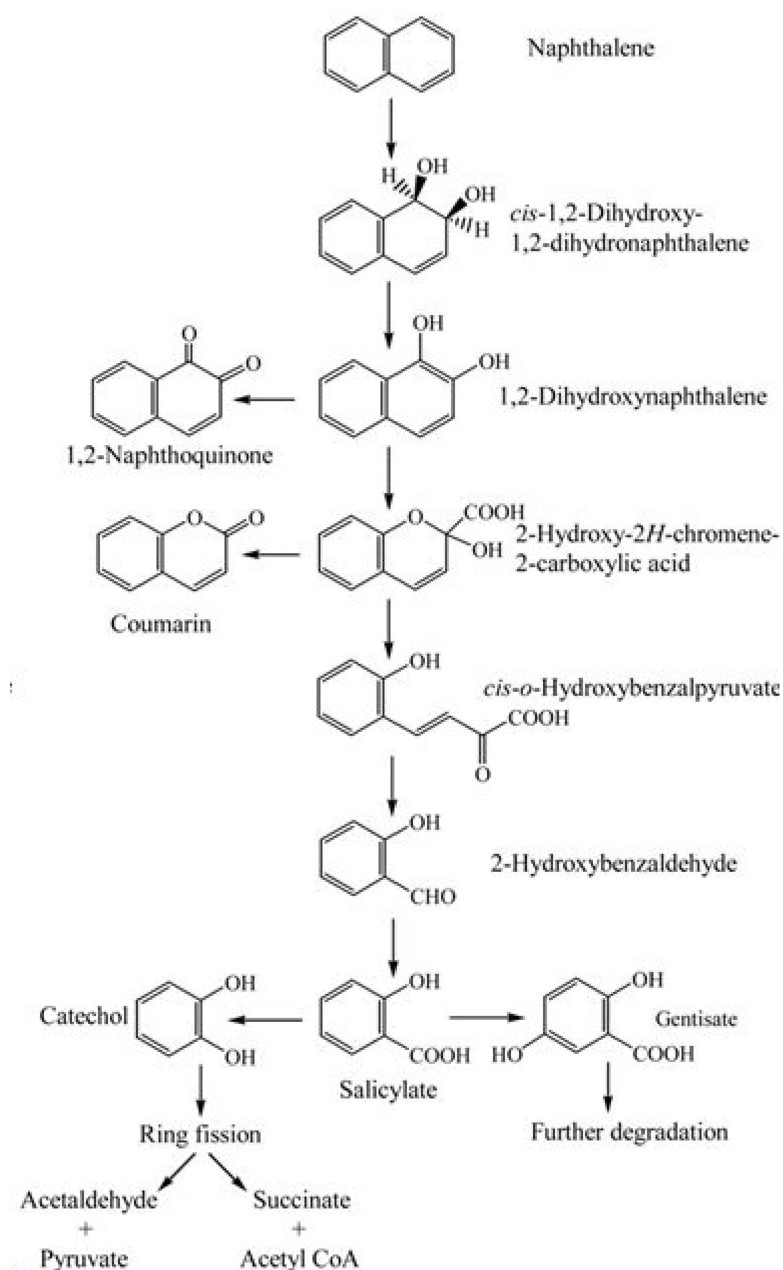

Figure 2. Degradation pathway of naphthalene (Seo *et al*., 2009).

enzymes responsible for transformation of naphthalene to salicylate (upper pathway), while the genes in *sal* operon encode enzymes for conversion of salicylate over catechol to pyruvate and acetaldehyde (lower pathway) (Fig. 3). Both operons are under control of positive transcriptional regulator NahR (LysR type) (Harayama *et al*., 1987; Cebolla *et al*., 1997; Ghosal *et al*., 2016), that is located between the two operons and is induced by salicylate leading to high-level expression of the *nah* genes (Peng *et al*., 2008). Similar naphthalene degradation operon has been described also in the *Pseudomonas putida* NCIB 9186-4 plasmid pDTG1 (Dennis and Zylstra, 2004) and *Pseudomonas fluorescens* PC20 plasmid pNAH20

(Heinaru *et al*., 2009). There is only one difference between the structures of the lower operons in the plasmids NAH7 and pDTG1, last one has an insertion of the IS*Pre1*-like element between the first and second gene of the *sal* operon. Insertion of IS element produces a stem-loop structure, preventing the expression of the downstream genes. According to this complete naphthalene degradation in the strain NCIB 9816-4 is carried out over *ortho*-cleavage pathway instead of *meta* pathway (Park *et al.*, 2002; Dennis and Zylstra, 2004).

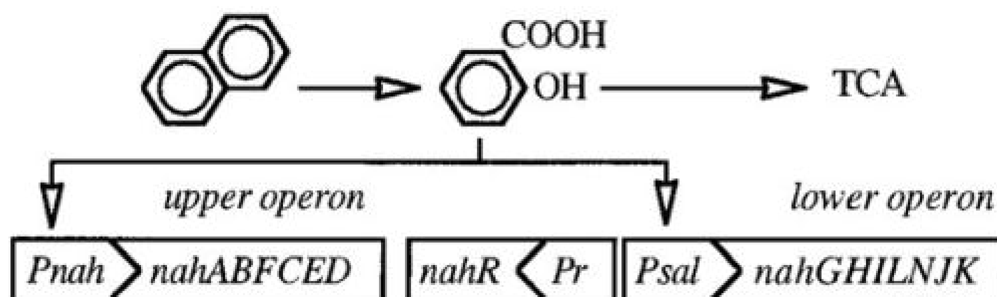

Figure 3. Operon of the naphthalene catabolism in the *Pseudomonas putida* strain G7 plasmid NAH7 (Cebolla *et al*., 1997).

In *Ralstonia* sp. strain U2 and *Paraburkholderia aromaticivorans* strain BN5 naphthalene is degraded via gentisate (Fig. 4) (Habe and Omori, 2003; Lee *et al*., 2019). The plasmidial catabolic naphthalene *nag* operon of both strains have highly identical upper pathway genes that are also in the same order as in the classical *nah* operon in the *P. putida* strain G7. While the genes *nagGH,* encoding salicylate 5-hydroxylase, responsible for metabolization of the salicylate to gentisate, are inserted between the genes of the *nah* upper operon (Habe and Omori, 2003;. Lee *et al*., 2019).

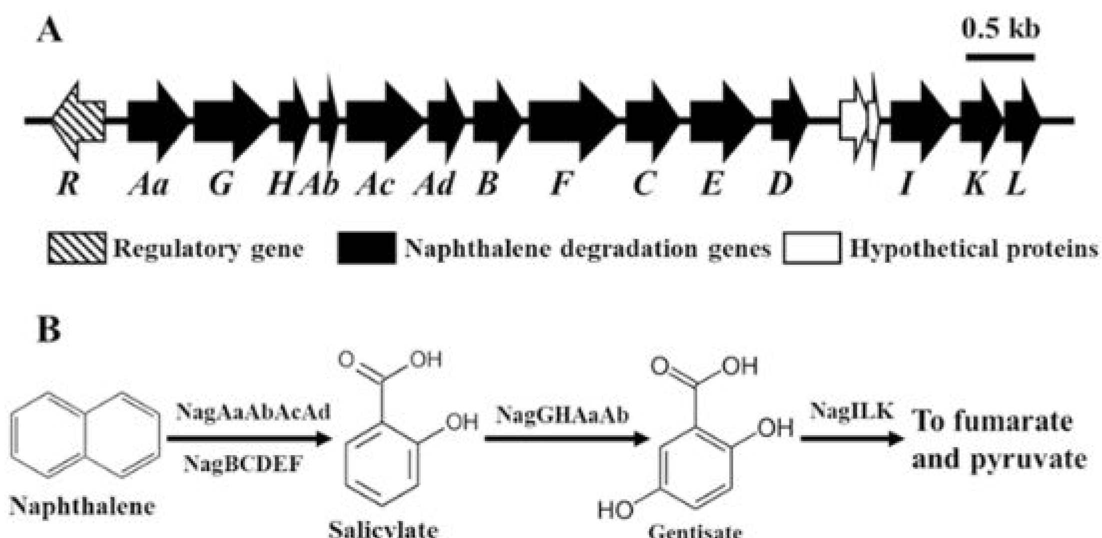

Figure 4. Physical map of naphthalene degradation genes (A) located in the plasmid pBN2 of *Paraburkholderia aromaticivorans* strain BN5 and naphthalene degradation pathway (B) (Lee *et al.*, 2019).

Gram-positive bacteria, for example *Rhodococcus* strains, usually possess for degradation of naphthalene only three genes that are not arranged into a single operon. Their transcription is induced in the presence of naphthalene, but not by salicylate as it is in the case of *Pseudomonas* species. Expression of the naphthalene degradation genes is controlled by two GntR-like transcriptional regulators (Larkin *et al.*, 2005; Ghosal *et al.*, 2016).

1.2.1 Salicylate-1-hydroxylase

Salicylate 1-hydroxylase (EC 1.14.13.1) is a flavoprotein monooxygenase (molecular weight 54 kDa) containing one molecule of FAD as a prosthetic group and it belongs to the class of oxidoreductases. This enzyme catalyzes decarboxylative hydroxylation of salicylate to catechol, CO_2 and H_2O in consumption of the NAD(P)H and O_2 (Fig. 5) (You *et al.*, 1990, 1991; Suzuki *et al.*, 1996; Costa, 2019). The first salicylate 1-hydroxylase was purified from *Pseudomonas putida* S-1, later from *Pseudomonas cepacia*, *Pseudomonas putida* G7 and other microorganisms (Yamamoto *et al.*, 1965; You *et al.*, 1990; Tu *et al.*, 1981). The salicylate 1-hydroxylase catalyzes essential reactions at the junction between the so-called upper and lower operons of polycyclic aromatic hydrocarbons (PAHs) catabolic pathway (Jouanneau *et al.*, 2007). The purified salicylate 1-hydroxylase can convert a broad spectrum of substituted salicylates for example 3-chloro-, 4-chloro-, 5-chlorosalicylate (Balashova *et*

al., 2001); 3-amino-, 4-amino-, 5-aminosalicylate (Yamamoto *et al*., 1965), and 3-methyl- and 5-methylsalicylate (Bosch *et al*., 1999). However, the activity of the enzyme depends on the nature of the substituent groups, because they may affect the charge of carboxylic and hydroxylic groups of salicylate (Puntus *et al.*, 2015). It has been shown also that salicylate 1-hydroxylase is responsible for the oxidation of 1-hydroxy-2-naphthoate in phenanthrene-degrading fluorescent pseudomonads (Balashova *et al*., 2001), but it has rather narrow specificity for the substrates like phenolic compounds with carboxyl or carbonyl substituent in the *ortho* position (Suzuki *et al*., 1991).

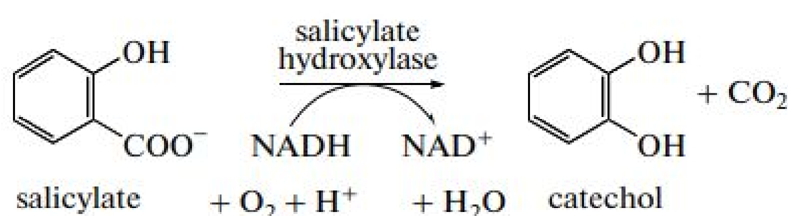

Figure 5. Conversion of salicylate to catechol by salicylate 1-hydroxylase (Puntus *et al.*, 2015).

Differently from pseudomonads that possess single-component salicylate 1-hydroxylase, sphingomonads have three-component Fe-S protein complex (Jouanneau *et al*., 2007). The oxygenase (PhnII) of the *Sphingomonas* sp. CHY-1 is functionally associated with an NAD(P)H oxidoreductase (PhnA4) and a ferredoxin (PhnA3) in a three-component enzyme complex able to oxidize a wide range of two- to five-ring PAHs (Jouanneau *et al*., 2007). The studied salicylate 5-hydroxylase catalyzes the hydroxylation of salicylate to catechol and exhibits similar activity levels with methylsalicylates, but lower activity with substituted salicylates having additional hydroxyl or electron-withdrawing substituents (Jouanneau *et al*., 2007).

1.2.2 Redundancy of salicylate 1-hydroxylases

Genetic redundancy is defined by Nowak *et al*., (1997) as "two or more genes are performing the same function and that inactivation of one of these genes has little or no effect

on the biological phenotype". It is believed that gene redundancy promotes genetic adaptation or creates novel biochemical functions (Jiménez *et al.*, 2014).

Several research groups have shown examples of redundancy of C12Os and C23Os in different bacterial strains (Aoki *et al*., 1984; Keil *et al.*, 1985; O´Donnell and Williams, 1991; Kim *et al*., 1997; Murakami *et al*., 1999; Pessione *et al.*, 2001; Jiménez *et al*., 2014). Furthermore, in naphthalene-degrading *Pseudomonas stutzeri* strain AN10 two genes encoding distinct salicylate-1-hydroxylases, named *nahW* and *nahG*, have been described. Typically, such naphthalene-degrading genes are plasmidial, but in the case of *P. stutzeri* AN10 they are located on the chromosome (Bosch *et al.*, 1999). While the *nahG* of the strain AN10 is similar to the *nahG* (identity 83%) of the strain G7 and it is located also in the lower operon of naphthalene degradation, the *nahW* is phylogenetically different and it is located between IS5-like transposons outside the transcriptional unit. NahG and NahW shared only 23% of amino acid identity (Bosch *et al*., 1999b). Both salicylate 1-hydroxylases of the strain AN10 were expressed at the similar level when cells were incubated in salicylate containing growth medium. These two salicylate 1-hydroxylases had broad substrate specificity, they can degrade also methylsalicylates and chlorosalicylates. The highest specific activities of the NahW were determined when 3-chlorosalicylate was used as a substrate, while in the case of the NahG higher values were obtained with methylsalicylates (Bosch *et al*., 1999, 2000). Lanfranconi with co-workers (2009) showed that possessing of the additional salicylate 1-hydroxylase gives to the strain AN10 advantage, as it is able to degrade higher concentration of the naphthalene and also tolerate higher concentrations of the salicylate.

Pseudomonas sp. ND6 has also two salicylate 1-hydroxylases, NahG and NahU, but unlike to the strain AN10, the genes encoding the enzymes are in plasmid pND6-1 (Li *et al*., 2004; Lanfranconi *et al*., 2009). Again the NahG of the ND6 is similar with that of the strain G7, but identity between NahG and NahU was 63% and, as in the case of the strain AN10, they are in different transcriptional units. Both, NahU and NahG, possess broad substrate specificities and can degrade salicylate, sulfosalicylate, aspirin, 3-methylsalicylate, 5-methylsalicylate, 3,5-dinitrosalicylate and 5-chlorosalicylate. NahU had higher activities with almost all mentioned substrates, except with sulfosalicylate and 5-chlorosalicylate (Zhao *et al*., 2005).

The NahG type of salicylate 1-hydroxylases have been studied widely, but the mechanisms of other types of salicylate 1-hydroxylases, for example NahU and NahW from

Pseudomonas sp. ND6 and *P. stutzeri* AN10, respectively, are not well understood yet (Costa *et al.*, 2019). Although, it has been shown that the presence of nonhomologous salicylate 1-hydroxylases in one strain provides a broader substrate range and an adaption to the higher concentrations of the pollutants (Puntus *et al.*, 2015), elucidation of the mechanisms behind it is needed.

1.3 *Pseudomonas pseudoalcaligenes* C70

Pseudomonas pseudoalcaligenes strain C70 was isolated by researcher Eeva Heinaru in 2008 from surface seawater of Baltic Sea, strain is deposited in the collection of environmental and laboratory microbial strains (CELMS; http://eemb.ut.ee/celms/main_list.php?qs=EEUT%20C70) with the number EEUT C70. Analysis of the genome of the strain C70 revealed the presence of three different sequences of the 16S rRNA gene similar to sequences of the *P. pseudoalcaligenes* (two sequences) and *P. mendocina* (one sequence). However, the sequence of the *rpoB* gene and phenotypic characteristics were also taken into account for phylogenetic classification of the strain C70. Based on the obtained data strain C70 was determined to *P. pseudoalcaligenes* (Vedler *et al*., 2013).

Strain C70 is not able to grow on benzoate or toluate, but it grows well on phenol and naphthalene (Vedler *et al*., 2013). Phenol is degraded by multicomponent phenol hydroxylase over catechol *meta* pathway, which is similar to that described in *dmp* operon of *Pseudomonas* sp. CF600. For degradation of naphthalene, strain C70 possesses naphthalene (*nah*) upper and lower (*sal*) operons, which are similar to classical *nah* operons of *P. putida* G7 (Jõesaar *et al*., 2017). So, the strain C70 has two catechol *meta* pathways, both of them are chromosomal.

The organization of the genes *nahGTHINLOMKJX* of the *sal* operon of the strain C70 is shown in the Figure 6. *Sal* operon is regulated by LysR-type regulator NahR (Jõesaar *et al.*, 2017). Differently from the classical *nah* lower operon, strain C70 possess additional salicylate 1-hydroxylase NahW as a *P. stutzeri* AN10, which is located between two transposons (Fig. 6). The role of two salicylate 1-hydroxylases of strain C70 in the degradation of the salicylate (naphthalene) has not been studied.

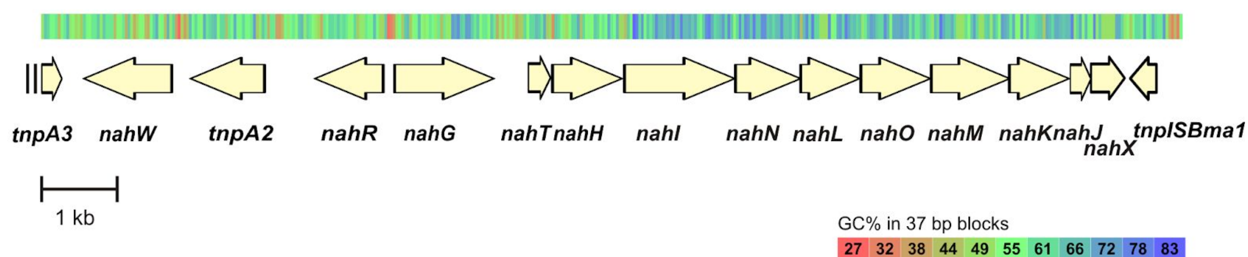

Figure 6. Genetic organization of *sal* operon of *Pseudomonas pseudoalcaligenes* C70. The genes of the *sal* operon - *tnpA*, transposase-like gene: *nahW*, salicylate 1-hydroxylase; *nahR*, regulatory gene; *nahG*, salicylate 1-hydroxylase; *nahT*, chloroplast ferredoxin-like protein coding gene; *nahH*, catechol 2,3-dioxygenase; *nahI*, hydroxymuconic semialdehyde dehydrogenase; *nahN*, hydroxymuconic semialdehyde hydrolase; *nahL*, 2-oxopent-4-enoate hydratase; *nahO*, acetaldehyde dehydrogenase; *nahM*, 2-oxo-4-hydroxypentanoate aldolase; *nahK*, 4-oxalocrotonate decarboxylase; *nahJ*, 4-oxalocrotonate isomerase; *nahX*, hypothetical protein coding gene; *tnpISBma1*, transposase-like gene (Jõesaar *et al*., 2017).

Previous experiments have shown that harbouring of the two phylogenetically different catechol 2,3-dioxygenases, *pheB* and *nahH*, gives to the *P. pseudoalcaligenes* C70 advantage over the strains holding only one copy of gene in the degradation of aromatic compounds (Jõesaar *et al.*, 2017). Strain C70 is effective degrader of phenol and salicylate, especially at higher substrate concentrations, when these compounds are present as a mixture, both catechol *meta* pathways are expressed. Also accumulation of toxic intermediate catechol was avoided in the mixture of salicylate and phenol, because of expression of both catechol 2,3-dioxygenases (Jõesaar *et al.*, 2017). The role of the concentration of the catechol in the growth medium in the degradation rate of aromatic compounds has been recognised also by other workgroups Muňoz *et al.*, 2007). Therefore, strain C70 appears to be a good candidate for bioremediation of contaminated areas.

2. THE AIMS OF THE THESIS

The main aim of the present research was to identify the physiological role of two salicylate 1-hydroxylases in *Pseudomonas pseudoalcaligenes* strain C70. We hypothesize that additional salicylate 1-hydroxylase enables the growth of the strain C70 in the presence of higher salicylate concentrations. Therefore to confirm the hypothesis, the following aims were outlined:

- To determine the growth parameters of the strain C70 and reference strains grown on different salicylate concentrations;
- To determine the maximum concentration of salicylate that the strain C70 and reference strains can tolerate;
- To determine the expression levels of two salicylate 1-hydroxylases, *nahG* and *nahW*, of the strain C70 and reference strains grown on salicylate;
- To construct the mutant strain C70Δ*nahG*, where *nahG* gene is disrupted.

3. EXPERIMENTAL PART

3.1. Materials and Methods

3.1.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. Pure cultures were stored in 30% glycerol at -80 °C (CELMS; available in the Estonian Electronic Microbial database website http://eemb.ut.ee). Batch cultivations of *Pseudomonas* strains were performed in 200 ml Erlenmeyer flasks containing 50 ml minimal medium (M9 salts and trace elements (Adams, 1959; Bauchop and Elsden, 1960) supplemented with sodium salicylate (will be used later in the text as salicylate, concentrations 2-25 mM) or R2A medium (Difco, USA) or R2A medium supplemented with salicylate (3 mM) at 30 °C on a rotary shaker (100 rpm). Growth was followed spectrophotometrically at 580 nm. *Escherichia coli* strain DH5α containing the plasmid pTZ57R/T (Thermo Fisher Scientific, USA) was grown on LB medium with ampicillin (LB-Ap; 15 µg ml^{-1}) or kanamycin (LB-Km; 50 µg ml^{-1}) at 37 °C; *E. coli* CC118λpir and *E. coli* HB101 were grown on LB medium with kanamycin (LB-Km; 50 µg ml^{-1}) at 37 °C .

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
P. pseudoalcaligenes C70	Phe^+ Nah^+ Sal^+	Vedler *et al.*, 2013
P. fluorescens F PC20	Phe^+*p*-$Cre^+$$Nah^+$ Sal^+	Heinaru *et al.*, 2000
P. putida G7	Nah^+ Sal^+	Ghosal *et al.*, 1987
P. putida NCIB 9816-4	Nah^+ Sal^+	Serdar *et al.*, 1985
E. coli		
DH5α	*supE44 ΔlacU169* (*φ80 lacZ ΔM15*) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
HB101	*subE44 subF58 hsdS3* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Boyer and Roulland-Dussoix, 1969
CC118λpir	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (*Am*) *recA1 λpir* phage lysogen	Herrero *et al.*, 1990

Plasmids		
pTZ57R/T	Cloning vector (Ap^r)	MBI Fermentas
pUTmini-Tn*5* Km	Delivery plasmid for mini-Tn*5* Km2 (Ap^r Km^r)	De Lorenzo *et al*., 1990
pGP704 L	Delivery plasmid for homologous recombination (Ap^r)	Pavel *et al*., 1994
pRK2013	Helper plasmid for conjugal transfer of pGP704 L (Km^r)	Figurski and Helinski, 1979
pTZ57R/C70nahG	pTZ57R/T containing the PCR-amplified *nahG* gene of stain C70 from *nah* operon	this study
pTZ57R/C70nahG::km	*nahG* of strain C70 (*nah* operon) in pTZ57R/T is interrupted with Km^r gene from pUTmini-Tn*5* Km2 by replacing *Nhe*I and *Cpo*I generated fragment from *nahG* by Km^r gene	this study
pGP704ΔC70nahG::km	pGP704 L with *Sac*I-*Sal*I fragment of ΔC70nahG::km from pTZ57RΔC70nahG::km in vector plasmid opened with the same restrictases	this study

Ap^r, ampicillin resistant; Km^r, kanamycin resistant

Growth parameters of the strain C70 and reference strains were determined from growth curves obtained on minimal medium supplemented with salicylate (1, 2 or 3 mM) as the sole carbon source at 30 ˚C °C. Bacterial growth was periodically monitored by measuring absorbance at 580 nm using POLARstar Omega Microplate reader (BMG Labtech GmbH). Growth rate of cultures was calculated using the Richards model (Dalgaard and Koutsoumanis, 2001).

3.1.2 Phylogenetic analysis of sequences

The deduced amino acid sequences of salicylate 1-hydroxylases of strain C70 were aligned with salicylate-1-hydroxylases of the reference strains from the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using BioEdit version 7.2.5 (Hall, 1999). ClustalW version 2.1 was used for sequence alignments (Thompson *et al*., 1994). The programme TreeViewX (Page, 1996) Version 0.5.0 was applied to visualize the phylogenetic tree to show the difference between proteins of salicylate 1-hydroxylase among a reference strains. Next to the branches is the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

3.1.3 PCR

PCR was applied for the amplification of the gene fragments. The total PCR mixture was 25 µl, including: water, 1x PCR buffer (75 mM Tris-HCl (pH 8.8); 20 mM $(NH_4)_2SO_4$; 0.01% Tween 20), 2.5 mM $MgCl_2$, 200 µM concentrations of each dNTPs (dATP, dGTP, dCTP, dTTP), 0.5 U thermostable Taq DNA polymerase (Thermo Fisher Scientific, USA), primers (10 mM), bacterial cells. The primers used for amplification of genes are listed in Table 2. The reaction was performed in Eppendorf Mastercycler PCR machine. PCR conditions were as follows: denaturation 95 °C for 1 min, annealing of primers (temperature depends on the used primer pair, Table 2) for 45 s, synthesis of DNA 72 °C, time depends of the product size. In order to amplify a sufficient amount of DNA molecules cycles were repeated 32 times. Program ended with final extension step, 72 °C for 10 min.

TABLE 2. PCR primers used for this study

Primer	Nucleotide sequence (5’-3’)	Temperature (°C)	Reference
NahG_F3 NahG_R2 NahG_F7 NahG_R3u NahW_F NahW_R	GGGCTTGAGGACGCCTATTTC GTTGCACACGACAGGCATGAG CTGCAACTGCGCGAAGCCTT GCTTCCCGTACCCAGGGAG CGATGACACTGGCGAACAGC CGTACGGTGGAACGCATCC	61	This study
NahG_algus NahG_lopp	CATGGAGCCTCGCTATGCTG AATGCGGTGGCTGCCTGTGC	59	This study
polAXhoylev polAXhoall	GGGGCAGAACGCCAAGTACG TCTGCGCCAGGCTGTCCAT	61	Sidorenko *et al.*, 2011
KmSac KmOc Luxylemine Luxsisemine	CAGGAGCTCGTTCGATTTATTCAACAAAGCC TCGAGCAAGACGTTTCCC CTTCCTTCTCACTTATCAGCC CAGTCCATTAAGGCTCGGC	57 57 54 54	Hõrak *et al.*, 2004 Saumaa *et al.*, 2006 Saumaa *et al.*, 2006 Saumaa *et al.*, 2006

3.1.4 Gel electrophoresis

Gel electrophoresis was used for analysis of the conducted PCR reactions. PCR probes (5 μl) were mixed with loading color (0.5 μl) and loaded to the agarose gel (0.8%) containing fluorescent dye ethidium bromide (0.5 μg ml^{-1}). A GeneRuler 1kb DNA ladder (Thermo Fischer Scientific, USA) was added to the one line of the gel for determination of the product length. The electrophoresis was performed at 100 V for 17 min in 1xTAE (50 mM Tris-acetate; 1 mM EDTA; pH 8.2) buffer. The resulting gel was visualized under UV light for assessing the presence of bands and their lengths.

3.1.5 Isolation of plasmid DNA

Axygen AxyPrepTM Plasmid Miniprep Kit was used for the isolation of plasmid DNA according to the manufacturer’s protocol.

3.1.6 Preparation of competent cells

120 μl of overnight grown *E. coli* DH5α or *E. coli* CC118λpir cells were transferred to the new 5 ml LB medium. Cells were grown at 37 °C for 2 hours. After that biomass was separated by centrifugation (1 min; 12 000 rpm). Cells were washed twice with 500 μl cold water and twice with 200 μl of 10% glycerol and suspended in 50 μl of 10% glycerol.

3.1.7 Ligation and electroporation

Ligation mixture consisted of 10x ligase buffer, ATP (1 mM), ligase (0.5 U/μl), mQ water, vector DNA, PCR fragment or restricted fragment. Ligation reaction was incubated at room temperature overnight. For precipitation of ligation mixture, 2 μl of a 5 M NaCl solution and 50 μl of a cold 96% ethanol solution were added and left at -20 °C for 20 min. To obtain the precipitate, mixture was centrifuged (12 000 rpm, 15 min) and pellet was washed twice with 150 μl of 70% ethanol. The pellet was dried from ethanol and resuspended in 5 μl of mQ. Then 2.5 μl of DNA (ligation mixture) was added to the competent *E. coli* DH5α or *E. coli* CC118λpir cells (see p.3.1.6) and thereafter pipetted into sterilized electroporation cuvette. Electroporation was carried out with "BioRad" electroporator at 2500 V. Immediately afterwards, LB liquid medium was added to the cells, suspended, poured back to the tube and incubated at 37 °C for 1 hour on a rotary shaker (150 rpm). After incubation, the cell suspension was centrifuged (8 000 rpm, 3 min), about 100 μl of liquid medium was left to suspend the cells. The suspension was plated onto LB-Km (*E. coli* CC118λpir) or LB-Ap (*E. coli* DH5α) selective plate and cells were grown at 37 °C.

3.1.8 Mutagenesis of the salicylate 1-hydroxylase *nahG* gene of *P. pseudoalcaligenes* C70

The description of the bacterial strains and plasmids used for the construction of the *nahG* mutant strain (C70Δ*nahG*) are shown in the Table 1. To disrupt the *nahG* gene in *P. pseudoalcaligenes* C70, the 1.7 kb DNA region containing *nahG* was amplified with the primer pair NahG_algus and NahG_lopp. The amplified PCR product was ligated into the pTZ57R/T vector (Thermo Fisher Scientific, USA) and electroporated into competent *E. coli* DH5α cells (see p.3.1.6-3.1.7). As a result of ligation, pTZ57R/C70nahG construct was produced. To find out the transformant containing the pTZ57R/C70nahG construct, PCR was

performed using primers NahG_algus and NahG_lopp (Table 1). From the positive clone the pTZ57R/C70nahG construct was isolated as described in the paragraph 3.1.5.

In the next step, approximately 1 kb sized DNA fragment of *nahG* region was cutted out from the construct pTZ57R/C70nahG by using the restriction enzymes *Nhe*I and *Cpo*I (Thermo Fisher Scientific, USA). After that the fragment was replaced with the Km resistance gene (Km^r) from the plasmid pUTmini-Tn*5*Km, which was amplified prior by PCR using primer KmSac (Table 2) and then cleaved with *Ecl136*II. Before the ligation of Km^r gene fragment into the restricted construct pTZ57R/C70nahG the ends of the construct must be blunt-ended. For that 1 µl of 2 mM dNTP and 1 U Klenow fragment (Thermo Fisher Scientific, USA) was added to the respective restriction reaction mixture. The mixture was incubated at 37 °C for 30 min and was then heat inactivated at 75 °C for 20 minutes. Restricted Km^r gene fragment was treated with FastAP Phosphatase (Thermo Fisher Scientific, USA) to avoid ligation of the open cloning vector. Therefore 1 U of the FastAP phosphatase was added to the restricted Km^r gene fragment containing solution and sample was incubated at 37 °C for 15 minutes. Enzyme was inactivated by heating at 75 °C for 20 minutes.

Restricted blunt-ended vector pTZ57R/C70nahG and Km^r gene fragment were precipitated and ligated as previously described (see p.3.1.7). Received pTZ57RΔC70nahG::km construct was electroporated into the *E. coli* DH5α cells (see p.3.1.7) and cells were plated on the selective LB-Km plates (see p.3.1.1). The presence of the pTZ57RΔC70nahG::km construct was determined by PCR using NahG_algus and KmOc primers (Table 2; see p.3.1.3) and afterwards the plasmid was isolated from the positive clone (see p.3.1.5). In the last step the sequence of the ΔC70nahG::km was excised with *Sac*I-*Sal*I from the obtained construct pTZ57RΔC70nahG::km and ligated into the suicide vector plasmid pGP704L (opened with *Sac*I-*Sal*I) to get the construct pGP704ΔC70nahG::km. This construct was electroporated into the *E. coli* CC118λpir cells (see p.3.1.6-3.1.7). The transformants were inoculated on the selective medium LB-Km (see p.3.1.1) and presence of the constructs pGP704ΔC70nahG::km was checked by PCR (primers NahG_algus and KmOc, Luxylemine and Luxsisemine).

3.1.8.1 Conjugation

Conjugation was applied for obtaining the mutant strain of *P. pseudoalcaligenes* C70 where gene *nahG* is substituted with Km resistance gene and only one salicylate 1-hydroxylase (encoded by *nahW*) is intact. Therefore, the following three strains were used: donor strain *E. coli* CC118λpir containing construct pGP704ΔC70nahG::km, helper strain *E. coli* HB101 and recipient strain *P. pseudoalcaligenes* C70. Cells of donor and helper strains were grown overnight in LB medium containing Km (50 μg ml^{-1}) at 37 °C and strain C70 in LB medium at 30 °C. In the following day 100 μl of pregrown cells of each strain were reinoculated into a new LB media (5 ml). After certain time point (1.5, 2 and 2.5 hours) 100 μl of each strain was pipetted onto LB plate as a blotch and grown overnight at 30 °C. Overnight grown cells from the one plate were suspended in 1 ml of 1xM9 solution, centrifuged (8,000 rpm, 3 min) and liquid was taken away. Cell pellet was carefully mixed with 700 μl of 1xM9 solution and 100 μl of suspension was plated in parallel on minimal medium containing Km (50 μg ml^{-1}) and Na-pyruvate (0.2%) or naphthalene. Colonies of the transconjugants were reinoculated in parallel on minimal medium supplemented with penicillin (1 mg ml^{-1}) and Na-pyruvate (0.2%) or naphthalene to exclude the vector. For the confirmation of the selection of C70Δ*nahG*, the transconjugants were tested with PCR using primer pairs NahG_F7 and NahG_R3u; Luxylemine and Luxsisemine (Table 2).

3.1.9 qRT-PCR

The quantitative reverse transcription-PCR (qRT-PCR) method was used for the evaluation of the expression levels of *nahG* and/or *nahW* genes of the strains C70, PC20, NCIB 9186-4 and G7 grown on R2A and R2A supplemented with 3 mM salicylate. Total RNA was isolated with the NucleoSpin RNA II kit (Macherey-Nagel, USA) from the samples taken from the middle of the logarithmic growth phase and from the beginning of the stationary phase. The RNA samples were treated with DNase I (Thermo Fisher Scientific, USA) to avoid the contamination with DNA. The concentration and purity of the isolated RNA was measured using a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, USA). According to the protocol of the SuperScript III Platinum SYBR green one-step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, USA) the qRT-PCR was performed using Rotor-Gene Q system (QIAGEN). A total volume of reaction was 10 μl and

it contained 10 ng of the total RNA. The *nahG* gene of the strain C70 was amplified with primers NahG_F7 and NahG_R3u while primers NahG_F3 and NahG_R2 were used in the case of the reference strains. The *nahW* gene of C70 was amplified with primer pair NahW_F and NahW_R. For all the strains *polA* was used as a reference gene and it was amplified with polAXhoylev and polAXhoall primers (Table 2). The PCR was performed using the following program: 50 °C for 3 min and 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 30 s, extension at 72 °C for 20 s and 40 °C for 1 min. At the end of the run, melting curve analyses were performed by increasing the temperature from 72 °C to 95 °C (0.35 °C per 3 s) with continuous fluorescence recording. The Rotor-GeneQ software version 2.02 (QIAGEN) was used to analyse the raw data. The LinRegPCR software version 2013.0 (Ruijter *et al*., 2009) was used for the calculation of the mRNA amounts. Data from three separate qRT-PCR experiments performed on three independently extracted RNAs were averaged and normalized against *polA* levels. Analysis of data was performed by supervisor.

3.2. RESULTS AND DISCUSSION

3.2.1 Phylogenetic analysis of salicylate 1-hydroxylases

Pseudomonas pseudoalcaligenes C70 possesses in the genome two chromosomal salicylate 1-hydroxylases encoded by the genes *nahG* and *nahW*. First of them is a part of the *sal* operon as in the classical *nah* lower operon of *P. putida* G7, but second one is between two transposons and situates upstream of the *sal* operon (Fig. 6). The deduced amino acid (aa) sequences of NahW is shorter than the sequence of NahG, 389 aa and 437 aa, respectively. Comparison of the sequences with reference sequences from the GenBank database is shown in the Figure 7. Alignment with ClustalX2 revealed the similarity between NahW of the strain C70 and NahW of the *P. stutzeri* AN10 and NahG of *Sphingomonas* AJ1, both in length and sequence identity, 100% and 56%, respectively. The two salicylate 1-hydroxylases of the strain C70 have low similarity, identity of sequences is only 26% (Fig. 8). NahG of C70 is more similar with other well-characterized NahGs of reference strains, for example *P. putida* G7, *P. putida* NCIB 9816-4, *P. stutzeri* AN10 etc., sharing 81-99% identity (Fig. 8). At the same time lowest identity, 56%, was determined between the NahGs of the C70 and *P. putida* S-1 (Yamamoto *et al.*, 1965). Pair-wise comparison of sequences of the salicylate 1-hydroxylases of C70 with those determined in the genomes of the strains *P. stutzeri* AN10 (NahG and NahW; Bosch *et al.*, 2000) and *Pseudomonas* sp. ND6 (NahG and NahU; Zhao *et al.*, 2005) revealed higher homology with the first strain, identities 99.8-100% (Fig. 8). Jõesaar *et al.*, (2017) showed also that *sal* operon of the C70 has almost identical structure with *sal* operon of the AN10. The sequences of other two salicylate 1-hydroxylases possessing strain *Pseudomonas* sp. ND6 were more distinct, although NahU was also shorter than NahG as it was in the case of NahW and NahG of the strains C70 and AN10 (Fig. 7).

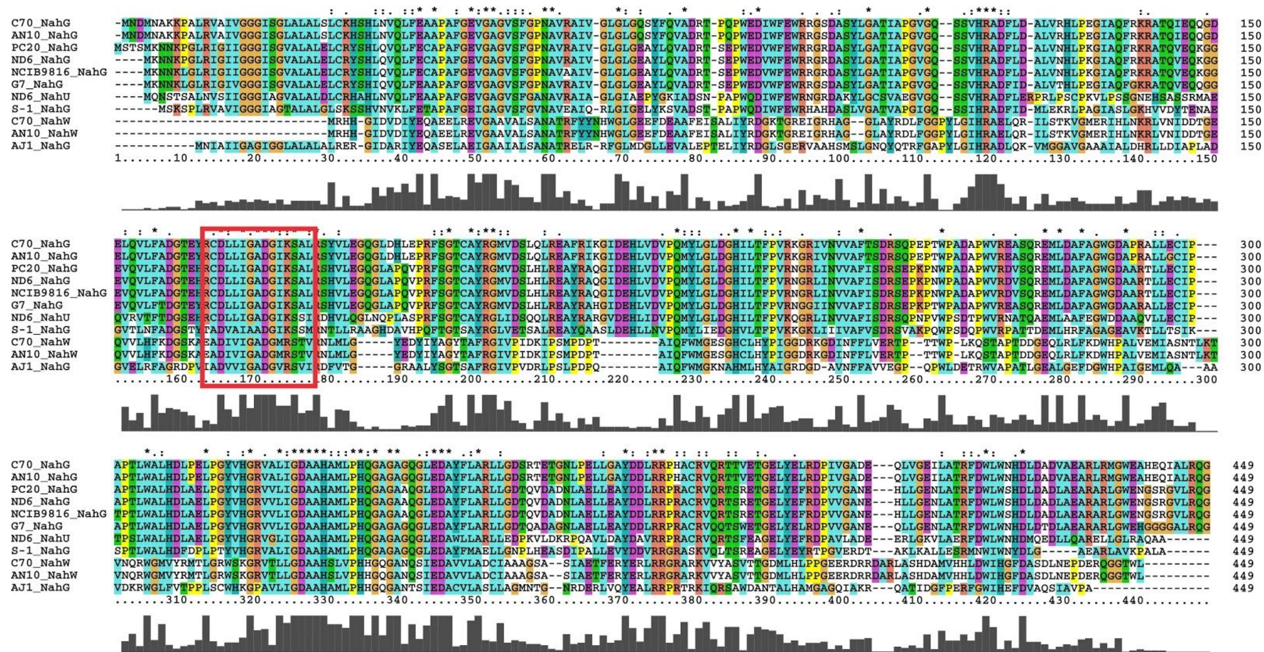

Figure 7. Comparison of deduced amino acid sequences of salicylate 1-hydroxylase of *P. pseudoalcalines* C70 (ANC68233, ANC68230), *P. stutzeri* AN10 (AAD02146, AAD02157), *P. fluorescens* PC20 (AAY21678), *Pseudomonas* sp. ND6 (NP943149, NP943122), *P. putida* NCIB 9816-4 (X83926), *P. putida* G7 (M60055), *P. putida* S-1 (AB010714) and *Sphingomonas* sp. AJ1 (AB000564). Red box indicates conserved region containing lysine (K) residue essential for binding of NADH.

Red box in the Figure 7 indicates conserved region containing lysine (K) residue (174, numbering based on the sequence of PC20) essential for the binding of the NADH. Suzuki *et al*., (2000) showed that Lys163 (corresponds to Lys174 in the Fig. 7) has an important role in the binding of NADH at the active site through an ionic bond rather than playing a role in catalysis as was proposed by Bosh *et al*., (1999). All NahGs of the pseudomonads presented in the Fig. 7 possess in this position lysine, but NahWs of the strains C70 and AN10 and also NahG of the *Sphingomonas* sp. AJ1 possess at this position arginine (R). Suzuki with co-workers (2000) replaced Lys163 with Arg163, like it is in case of NahW, and concluded that enzyme kinetics did not altered compared to the wild type.

The alignment of the sequences of deduced amino acids of the salicylate 1-hydroxylases (Fig. 7) revealed that NahWs of the strains C70 and AN10 do not have the well-conserved N-terminal FAD-binding site (GxGxxG) which is present in all previously described NahGs (Bosch *et al*., 1999; Zhao *et al*., 2005). This element comprises the secondary structure elements β1 and α1, forming a domain, which interact with the ADP

moiety of the FAD cofactor (Costa *et al.*, 2019). The putative second FAD-binding site is very conserved in all salicylate hydroxylases and is presented also in both NahG and NahW sequence (Bosch *et al.*, 1999).

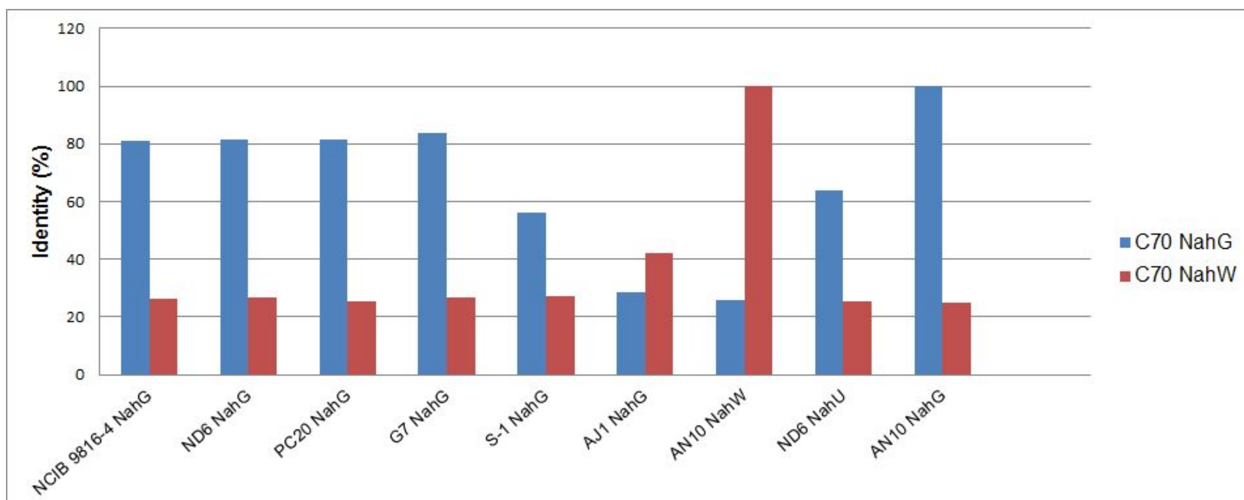

Figure 8. Identity % of deduced amino acid sequences of salicylate 1-hydroxylase of *P. pseudoalcalines* C70 (ANC68233, ANC68230), *P. stutzeri* AN10 (AAD02146, AAD02157), *P. fluorescens* PC20 (AAY21678), *Pseudomonas* sp. ND6 (NP943149, NP943122), *P. putida* NCIB 9816-4 (X83926), *P. putida* G7 (M60055), *P. putida* S-1 (AB010714) and *Sphingomonas* sp. AJ1 (AB000564).

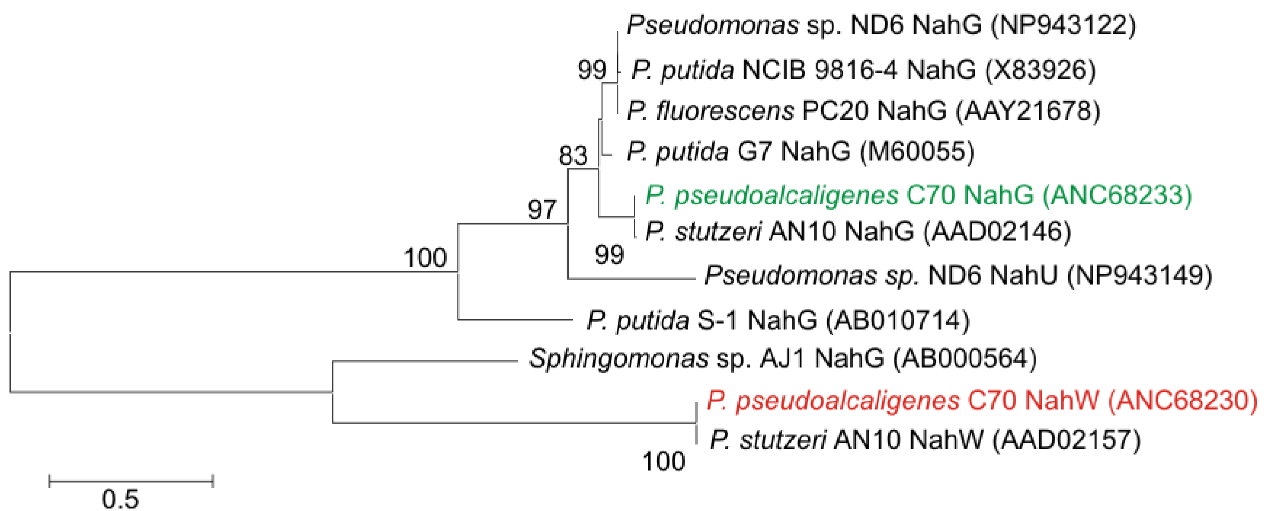

Figure 9. A phylogenetic tree based on deduced amino acid sequence of salicylate 1-hydroxylase of *P. pseudoalcaligenes* C70 and reference strains available from the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Phylogenetic tree (Fig. 9) was constructed from the sequences of salicylate 1-hydroxylases of the strain C70 and reference strains from the GenBank database to study the phylogenetic relationship between sequences. Analysis of the phylogenetic tree revealed that sequences of the NahW of the strains C70 and AN10 form a separate group on phylogenetic tree, being more similar to the NahG of the *Sphingomonas* sp AJ1, which can degrade naphthalene and phenanthrene (Komukai-Nakamura *et al*., 1996). The NahG of the strain C70 clusters to the same group with the salicylate 1-hydroxylase of the well-characterized strain G7 (You *et al*., 1991).

3.2.2 Growth on salicylate

Growth experiments were performed in microtiter plates containing 0.1 ml of minimal medium supplemented with salicylate (1-3 mM) to determine growth parameters of the *P. pseudoalcaligenes* C70 and reference strains (Fig. 10 and 11). Growth was measured spectrophotometrically at 580 nm after every 7 minutes. The inspection of the representative growth curves revealed that strains have different growth parameters. Strain C70 and reference strains *P. putida* G7, *P. fluorescens* PC20 and *P. putida* NCIB 9816-4, have closely related *nahG* and possess for the cleavage of the catechol catechol 2,3-dioxygenase (C23O), but *sal* operon of reference strains locates in plasmid. But in the strain NCIB 9816-4 the insertion of IS element between *nahG* and *nahT* produces a stem-loop structure, preventing the expression of the downstream genes including the C23O (Dennis and Zylstra, 2004). Accumulating catechol induces catechol 1,2-dioxygenase, but the specific activity of enzyme is low (Williams *et al*., 1975). According to this, complete naphthalene degradation in the strain NCIB 9816-4 is carried out via catechol *ortho* pathway, but not via catechol *meta* pathway (Dennis and Zylstra, 2004), although the catechol *meta*-cleavage enzymes were constitutively expressed at low levels (Serdar and Gibson, 1989).

The growth curves of the studied strain C70 revealed that the lag-phase length did not change significantly on used salicylate concentrations (Fig. 10). Similar effect was observed in the case of the strains G7 and PC20, although on 3 mM substrate concentration PC20 had longer lag-phase than strains C70 and G7. Lanfranconi with co-workers (2009) showed that lag-phase length of the strain AN10, possessing also two salicylate 1-hydroxylases as strain C70, increased when higher salicylate concentrations were used, especially above 6 mM.

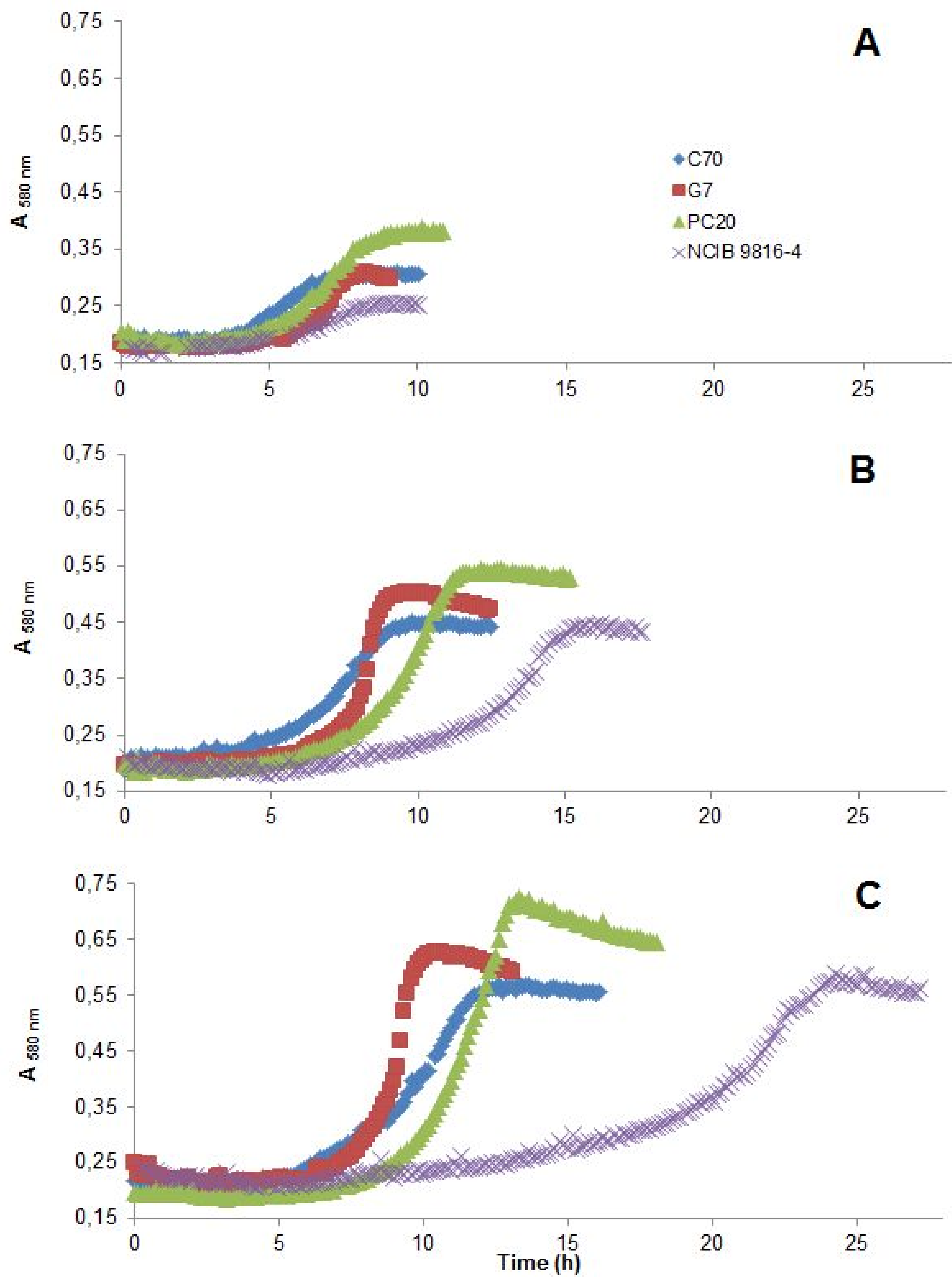

Figure 10. Representative growth curves of the strain C70 and reference strains in minimal medium supplemented with 1 mM (A), 2 mM (B) or 3 mM (C) salicylate.

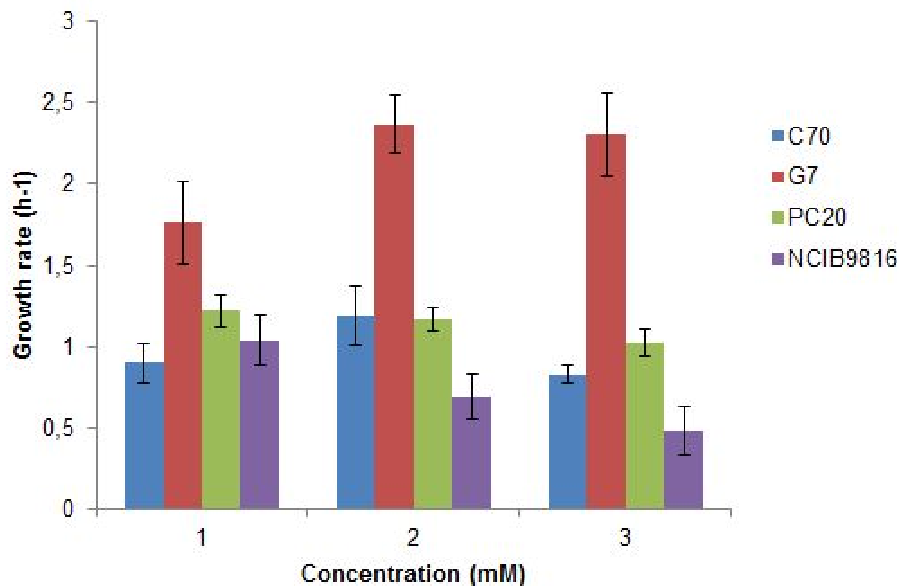

Figure 11. Growth rate of the strain C70 and reference strains in minimal medium supplemented with different concentrations of salicylate. Error bars indicate the standard deviation.

The length of the lag-phase increased faster in the case of the strain NCIB 9816-4 (Fig. 10) when the concentration of substrate in the growth medium was higher. Previous studies have shown that strain NCIB 9816-4 is quite sensitive to naphthalene and salicylate due to the imbalanced metabolism and the accumulation of catechol (Park *et al*., 2004). In the performed experiments this strain had also the lowest growth rate values that decreased with increasing the salicylate concentration (Fig. 11). At the same time the reference strain G7 had the highest growth rate values among the strains on all growth conditions and values did not decrease while concentration of substrate was enhanced. These data are in accordance with the study of Colbert *et al*. (1993) that showed the ability of the strain G7 tolerate high concentrations of salicylate, whereas the optimum concentration for the growth was 10 mM. The growth rate of the strains C70 and PC20 on salicylate were almost at the same level, although slight decrease was observed on highest substrate concentration in the case of the strain C70 (Fig. 11). Jõesaar *et al*. (2017) showed that 3 mM of salicylate inhibited the growth of the strain C70.

The highest growth yield on salicylate were obtained for the strain PC20 while strains C70 and NCIB 9816-4 had the lowest ones (Fig. 10).

The conducted experiments showed that strain C70 and reference strains had different growth parameters on salicylate (1-3 mM) that lead us to next research question: how high concentrations of the salicylate do these strains tolerate?

3.2.3 Tolerance to higher concentrations of salicylate

Several studies have shown that salicylate can be toxic for the bacteria (Pomposiello *et al.,* 2001; Lee *et al.,* 2005; Riordan *et al.,* 2007). For example, growth of *E. coli* was inhibited when 5 mM salicylate was added to the LB medium (Pomposiello *et al*., 2001), while the growth of the *Burkholderia cepacia* in phenanthrene and salicylate containing growth medium was diminished when the concentration of salicylate was higher than 1.25 mM (Lee *et. al*., 2005). The growth of the gram-positive *Staphylococcus aureus* exposed to 2 mM salicylate in LB medium was slightly reduced (Riordan *et al*., 2007).

The tolerance of the strain C70 and reference strains to higher concentrations of the salicylate was determined in batch cultivation experiments in 200 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with sodium salicylate (concentrations 2-25 mM) (Fig 12). After two days of incubations, the growth of the strain C70 was observed in growth medium containing up to 25 mM salicylate, although the formation of clumps of cells was determined when concentrations of salicylate were higher than 17 mM (Fig. 12A). Jõesaar *et al*. (2017) showed that at limited oxygen concentrations (manometric respiratory system OxiTop) the consumption of the oxygen by the cells of the C70 diminished already in minimal medium supplemented with 3 mM salicylate. Madsen *et al*. (1996) indicated that reduced oxygen levels contribute to sensitivity of the cells to the naphthalene and also to salicylate. Similarly to the strain C70 the wild-type strain *P. stutzeri* AN10, which contains additional salicylate 1-hydroxylase *nahW* gene, is able to grow in the medium containing 24 mM salicylate (Lanfranconi *et al*., 2009). While for the mutant strain of AN10, where *nahW* was eliminated, toxic effect of salicylate was observed already at 3 mM concentration and strain did not grow at salicylate concentrations higher than 9 mM. They concluded that additional salicylate 1-hydroxylase improved tolerance of the strain AN10 to salicylate (Lanfranconi *et al*., 2009).

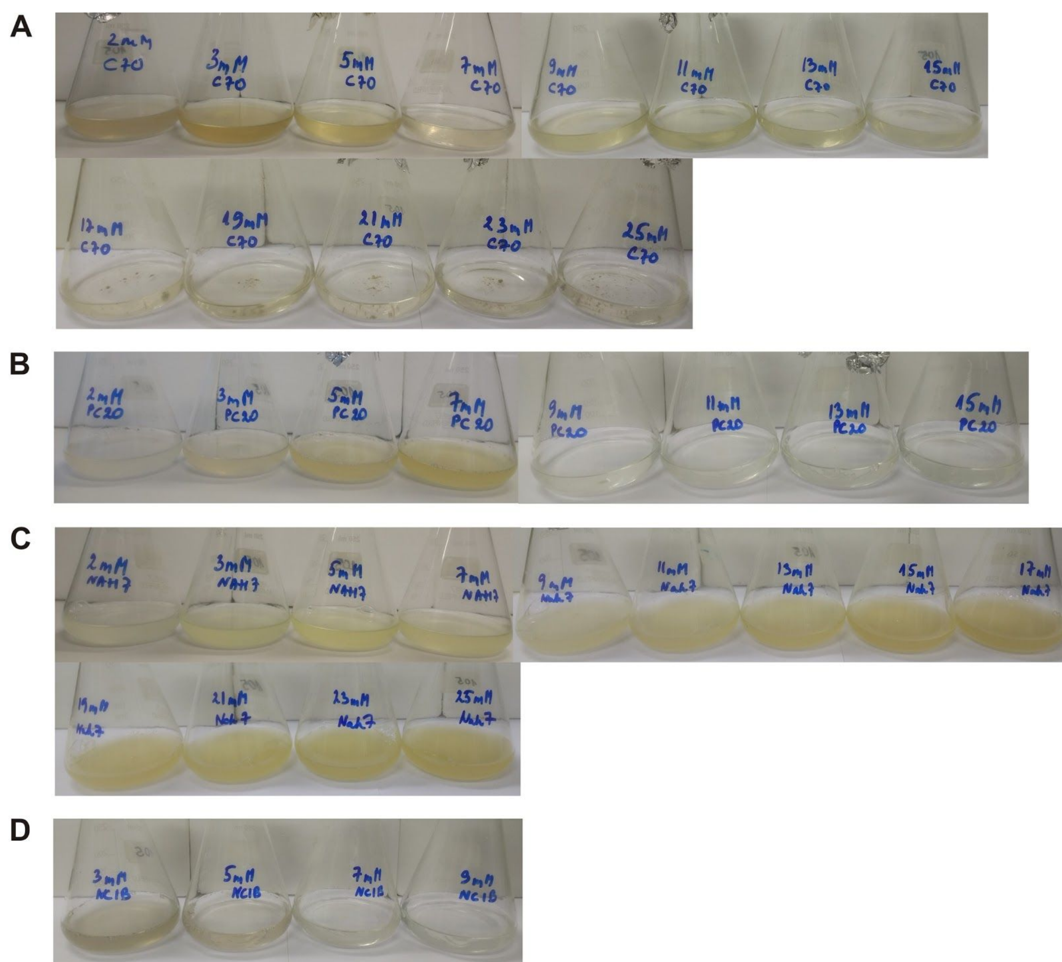

Figure 12. Photos of the flasks containing minimal medium supplemented with salicylate (from 2 mM up to 25 mM) and inoculated with C70 (A), PC20 (B), G7 (C) or NCIB 9816-4 (D) after two days incubation at 30 °C.

Although strain C70 was able to tolerate higher concentration of salicylate, the most efficient strain in these experiments was strain G7 (Fig. 12C). The growth of the strain was fast and without the formation of clumps in all used salicylate concentrations. Colbert *et al.* (1993) determined that the optimum concentration of salicylate for the growth of the strain G7 was 10 mM.

The other reference strains, PC20 and NCIB 9816-4 were able to grow on minimal medium supplemented with up to 7 mM and 5 mM SAL, respectively (Fig. 12B and D). The sensitivity of the NCIB 9816-4 to higher concentrations of substrate has been proposed to be caused by the presence of IS*Pre1*-like element in the *sal* operon of the pDTG1 plasmid disrupting the lower naphthalene operon and expression of the C23O (Park *et al.*, 2002;

Dennis and Zylstra, 2004). However, it is known that during the growth on salicylate catechol *ortho* pathway is induced instead of the C23O (Dennis and Zylstra, 2004).

After three days of incubation the growth medium of the strain NCIB 9816-4 containing 5 mM salicylate turned black (Fig. 13). These dark pigments - quinonic intermediates - are formed in solution after the oxidation of catechol and are toxic even to bacteria able to degrade catechol (Park *et al*., 2004; Jimenez *et al*., 2014). Several researchers have pointed out that accumulation of H_2O_2 during the degradation of naphthalene contributes to the oxidation and polymerization of the catechol-like intermediates (Park *et al*., 2004; Hassett *et al*., 2000). Formed compounds affect the activity of the catechol 2,3-dioxygenase that results in the accumulation of catechol and finally in the cell death (Park *et al*., 2004; Hassett *et al*., 2000). Also the growth medium containing 7 mM salicylate inoculated with the cells of the strain C70 turned light brown after three days (Fig. 13). These observations are in the agreement with the results of Jõesaar *et al*. (2017), who showed that catechol accumulates in the growth medium when strain C70 is degrading salicylate.

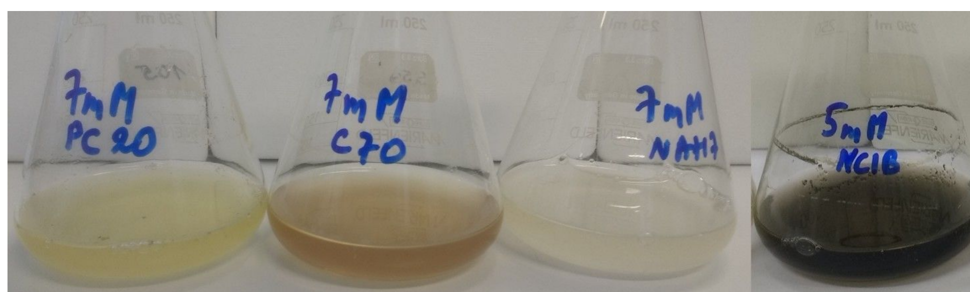

Figure 13. Photo of the flasks containing minimal medium supplemented with 7 mM salicylate inoculated with strain PC20, C70, G7 or NCIB 9816-4 after three days incubation at 30 °C. Indication of accumulation of intermediates of the salicylate degradation pathway.

The conversion of catechol into 2-hydroxymuconic semialdehyde in catechol *meta* pathway results in appearance of yellow colour (Schweizer, 1993). During experiment, we observed the formation of yellow colour with all strains, because all strain have C23O activity. At higher concentrations of salicylate the growth medium of the strains G7 and PC20 turned intensive yellow colour that remained until the end of the experiment (5 days). While the growth medium of the strain NCIB 9816-4 turned during the longer incubation (3 days) brown or even black (Fig. 13).

To conclude this experiment, two salicylate 1-hydroxylase possessing strain C70 can tolerate up to 25 mM of salicylate in the growth medium, but high concentrations (> 17 mM) are stressful for the cells. The most tolerant to the high salicylate concentrations was reference strain G7. To clarify the role of the two salicylate 1-hydroxylases of C70, induction experiments with salicylate were carried out and the expressions levels of respective genes were determined by using the qRT-PCR method.

3.2.4 Expression levels of the salicylate 1-hydroxylases

qRT-PCR method was used for elucidation of the expression levels of the salicylate 1-hydroxylases in the presence or the absence of the salicylate in the growth medium. These experiments will give us information about the role of two salicylate 1-hydroxylases (*nahG* and *nahW*) in the strain C70. To better understand the physiology of strain C70 we used also three reference strains, namely *P. fluorescens* PC20, *P. putida* G7 and *P. putida* NCIB 9816-4, possessing plasmidial *nah* upper and lower operons, but they do not have a second salicylate 1-hydroxylase (*nahW*) as C70 has.

The cells were grown on following media: R2A and R2A supplemented with salicylate (3 mM), whereas the samples were taken from the mid-logarithmic (I) and the early stationary (II) growth phase. The obtained relative mRNA expression levels of the *nahG* and *nahW* of the strain C70 and the reference strains are presented in the Figure 14.

The results of qRT-PCR experiments revealed that *nahG* of the strain C70 grown in R2A medium was not expressed, while *nahW* had low expression level. Supplementation of the growth medium with the salicylate resulted in higher expression levels of *nahG* and *nahW*, especially in the cells taken from the mid-logarithmic growth phase and in the case of the *nahW* (Fig. 14). Bosch with co-workers (1999) showed that in the strain AN10 specific activity of the NahW was a bit higher than NahG in medium containing salicylate. Both *nahW* and *nahG* of the strain C70 were expressed at the similar level in the samples taken from the early stationary growth phase as it has been also described in the strain AN10. Further, Bosh *et al*. (1999) showed that *nahW* and *nahG* of the strain AN10 are expressed at high level when 3-chlorosalicylate and methylsalicylates (different positions of the substituents) are used as inductors, respectively. Do the salicylate 1-hydroxylases of the strain C70 have different substrate specificity needs to be studied.

Expressions of *nahG* of reference strains G7, PC20 and NCIB 9816-4 at different growth conditions are distinct (Fig. 14). First, very low expression levels were obtained for the strain G7 that may be explained with the use of the too low concentration of the salicylate (3 mM) in the experiments. The previous experiments have shown that the strain G7 can tolerate higher concentrations of naphthalene (Ahn *et al*., 1998). Similarly to the strain C70, the expression level of *nahG* of the G7 was low in the R2A medium. Second, the highest expression level of *nahG* was determined in the case of the strain NCIB 9816-4. Park *et al*. (2004) found that the strain NCIB 9816-4 is very sensitive to higher concentrations of naphthalene and salicylate due to the accumulation of intermediates into the growth medium. It has been also shown that intermediates can inhibit activity of the enzymes, for example, catechol inhibits catechol 2,3-dioxygenase activity (Muñoz *et al*., 2007; Jõesaar *et al*., 2017).

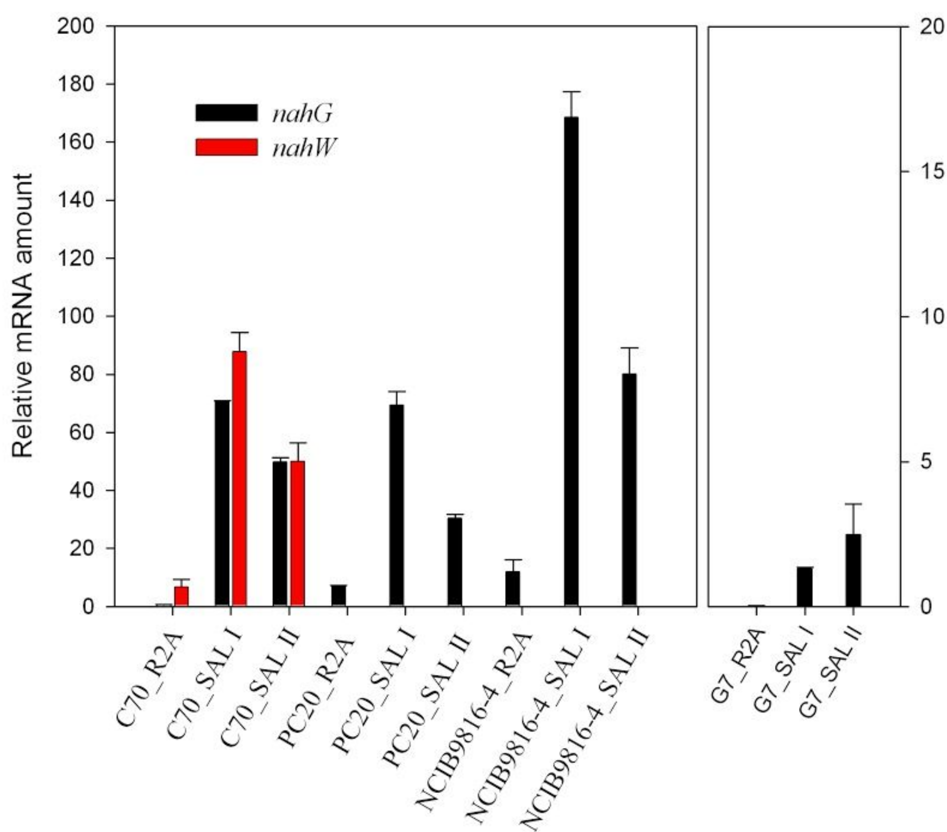

Figure 14. Expressions levels of *nahG* and *nahW* in strain C70 and reference strains. The cells were grown on R2A medium, R2A and salicylate (3mM) medium. The samples were taken in mid-log (I) and in the start of stationary (II) growth phase. The relative expression ratios (y-axis) were calculated as relative values of the reference gene *polA* expression level of the respective strains.

We propose that in R2A medium supplemented with 3 mM salicylate the expression of the salicylate 1-hydroxylase of the NCIB 9816-4 is not inhibited as growth medium did not darken and the expression level of *nahG* was high (Fig. 14). The third reference strain, PC20, had similar *nahG* expression levels as the strain C70 in the logarithmic growth phase. The *nahG* of the strains PC20 and NCIB 9816-4, and *nahW* of the strain C70 were constitutively expressed in R2A medium grown cells (Fig. 14). Specific activities of the salicylate 1-hydroxylases of the reference strains were determined also in the work of the Liivak (2007). Consequently to our study, they used in induction experiments 5 mM salicylate and casamino acid medium instead of the R2A medium. At these conditions strains NCIB 9816-4 and PC20 had highest and lowest value, respectively while the specific activity of salicylate 1-hydroxylase of the strain G7 was between those values. The differences in expression levels obtained in two studies may indicate that reference strains have distinct tolerance to higher salicylate concentrations that was actually seen also in current work (Fig. 12).

To conclude, the performed experiments showed that both salicylate 1-hydroxylases, *nahW* and *nahG,* of the strain C70 are active and expressed at the similar level while cells are introduced to salicylate. To find out the physiological role of these two redundant salicylate 1-hydroxylases, the next step was construct the mutant strain of C70 where one salicylate 1-hydroxylase is disrupted.

3.2.5 Mutagenesis of *nahG* gene encoding the salicylate 1-hydroxylase

One of the aims of the current work was to construct the mutant strain C70Δ*nahG* in which the *nahG* gene encoding salicylate 1-hydroxylase is disrupted in order to determine the physiological role of NahG in the strain C70. For that the donor strain *E. coli* CC118λpir containing the construct pGP704ΔC70nahG::km was transferred by conjugation to recipient wild-type strain C70 with the help of helper strain *E. coli* HB101 (see p. 3.1.8, 3.1.8.1). The transconjugants were then plated on kanamycin containing minimal medium with naphthalene or 0.2% pyruvate to select cells of the strain C70 where functional *nahG* gene has been replaced with kanamycin resistance gene as a result of homologous recombination. Only the cells in which the original *nahG* gene has been replaced with the interrupted *nahG* gene were able to form colonies on the kanamycin selective medium. Parallel inoculations were done also on the selective medium with penicillin to exclude the cells carrying construct pGP704ΔC70nahG::km. The final conformation of the presence of the disrupted *nahG* gene

was done by PCR analysis (see p.3.1.8). Unfortunately, due to the limited time, we have not been able to isolate the mutant strain C70Δ*nahG* after the triple conjugation experiment. Further work is required to establish this.

Summary

Pseudomonas pseudoalcaligenes strain C70 harbours two redundant salicylate 1-hydroxylase genes in the chromosome, *nahG* and *nahW,* for the degradation of salicylate. We found only the reports about the two strains that own also two salicylate 1-hydroxylases in their genome, these strains are *P. stutzeri* AN10 and *Pseudomonas* sp. ND6 (Bosch *et al.*, 1999; Lanfranconi *et al*., 2009).

To describe and find out the role of two salicylate 1-hydroxylases in the strain *P. pseudoalcaligenes* C70, we first analysed the deduced amino acid sequences of NahW and NahG and constructed the phylogenetic tree. We also determined growth parameters of strain C70 and reference strains *P. fluorescens* PC20, *P. putida* G7 and *P. putida* NCIB 9816-4 and ascertained the tolerance to salicylate. Finally, the last aim was to construct the mutant strain of C70, where the *nahG* is disrupted.

From results of our work we can conclude:

1. The comparison of the deduced amino acid sequence of redundant genes, *nahW* and *nahG* of C70 showed that they are almost identical with those of the *P. stutzeri* strain AN10. Additional salicylate hydroxylases (NahW) of C70 and AN10 formed separate group on phylogenetic tree and they were phylogenetically different from classical NahGs.
2. The lag-phase length of C70 did not change significantly on used salicylate concentrations (1-3 mM). Strain C70 had very similar growth rate values as PC20 in the presence of salicylate; the highest growth rate values were revealed strain G7, and the lowest one NCIB 9816-4.
3. The results of qRT-PCR experiments revealed that in R2A medium grown strain C70 cells *nahG* was not expressed, while *nahW* had low expression level. Both *nahW* and *nahG* of the strain C70 were expressed at the similar level in the early stationary growth phase, but in the mid-logarithmic growth phase the *nahW* expression level was higher than *nahG*.

4. In the salicylate tolerance experiment, strain C70 grows in the minimal medium containing up to 25 mM salicylate, but high concentrations (> 17 mM) were stressful for the cells, probably due to accumulation of catechol.
5. Construction of *nahG* disruption mutant was not finished due to time limit, but is going to be continued.

To summarize, the studied strain *P. pseudoalcaligenes* C70 owning two salicylate 1-hydroxylases can tolerate quite high concentrations of salicylate and both *nahW* and *nahG* are expressed while cell are exposed to salicylate. Our hypothesis that the presence of two salicylate 1-hydroxylases is advantageous to the host to deal with toxic compounds at higher concentrations was partly confirmed. To give an accurate answer about the physiological role of additional *nahW*, the construction and analysis of *nahG* or *nahW* disruption mutants must be finished.

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