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Study of the genes involved in Naphthenic acids (NAs) degradation by *Rhodococcus* spp.

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Abstract

Naphthenic acids (NAs) are an important group of organic pollutants mainly found in hydrocarbon deposits. Although these compounds are toxic, recalcitrant, and persistent in the environment, we are just learning the diversity of microbial communities involved in NAs-degradation and the mechanisms by which NAs are biodegraded. Studies have shown that naphthenic acids are susceptible to biodegradation, which decreases their concentration and reduces toxicity. Nevertheless, little is still known about their biodegradability. The present PhD Thesis's work is aimed to study the biodegradation of simple model NAs using bacteria strains belonging to the *Rhodococcus* genus. In particular, *Rh.* sp. BCP1 and *Rh. opacus* R7 were able to utilize NAs such as cyclohexane carboxylic acid and cyclopentane carboxylic acid as the sole carbon and energy sources, even at concentrations up to 1000 mg/L. The presence of either substituents or longer carboxylic acid chains attached to the cyclohexane ring negatively affected the growth by pure bacterial cultures. Moreover, BCP1 and R7 cells incubated in the presence of CHCA or CPCA show a general increase of saturated and methyl-substituted fatty acids in their membrane, while the *cis*-mono-unsaturated ones decrease, as compared to glucose-grown cells. The observed lipid molecules modification during the growth in the presence of NAs is suggested as a possible mechanism to decrease the fluidity of the cell membrane to counteract NAs toxicity. In order to further evaluate this toxic effect on cell features, the morphological changes of BCP1 and R7 cells were also assessed through Transmission Electron Microscopy (TEM), revealing interesting ultrastructural changes. The induction of putative genes, and the construction of a random transposon mutagenesis library were also carried out to reveal the mechanisms by which these *Rhodococcus* strains can degrade toxic compounds such as NAs.

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1. Introduction

Soil contamination is a global concern and can be considered a major barrier to sustainable development, since it ruins the balance of the ecosystem, causing economic loss and human health damage [Pimentel et al.; 1995]. These environmental issues derive from inadequate or irresponsible disposal measures, such as improper industrial discharge, mining tailings, waste disposal, and stockpiles [Wuana et al.; 2011]. Even Arctic soil contamination has various causes, such as blowouts and accidental oil leaks from tankers, trucks, pipelines, and storage tanks, in addition to various discharges from industrial sites, and/or military bases [Mohn et al.; 2000, Børresen et al.; 2007, Evdokimova et al.; 2012].

Over the last hundred of years, millions of tons of dangerous pollutants were generated, and a significant part of them consists of hydrophobic organic compounds that will persist in the environment for several decades [Head et al.; 2003].

Nowadays, there is a growing awareness concerning the toxic or even carcinogenic effects of a large fraction of the environmental pollutants [Triska et al.; 2004]. The most common soil contaminants are heavy metals, organics, and radio-nuclides. Increased heavy metal levels in soil have been reported in many industrialized countries and areas. Metals and metalloids, such as chromium, cadmium, mercury, and lead, can threaten the ecosystem and human health through the food chains or direct exposure to the contaminated soil/water [Triska et al.;2004]. Organic pollutants such as volatile chlorinated solvents, polychlorinated biphenyls (PCBs) and petroleum products represent another pervasive concern due to their toxicity, mobility, and abundance. These organic pollutants remain stable in soil, being dangerous to animal and plant species [Arias-Estévez et al.; 2008]. In fact, the low volatility and water solubility of high molecular weight hydrocarbons makes them not only persistent in the environment, but also produce long-term adverse effects on the environment and human health including: prenatal toxicity, skin-related diseases, lung cancer, leukemia, and negative

effects on reproduction [Evans et al.; 2005]. While low-molecular weight aromatics volatize relatively rapidly, the heavier ones such as polycyclic aromatic hydrocarbons with three and more rings, remain in the environment for quite a long time. The biodegradation half-lives in natural habitats have been estimated for several PAHs including anthracene (three-rings, 170 days to 8 years), phenanthrene (three-rings, 2.5 days to 5.7 years), and benzo[α]pyrene (five-rings, 8.2 to 58 years) [Speight et al.; 2011]. Anthropogenic activities also generate radio-nuclides contamination [Elless et al.; 2012], deriving from atmospheric testing of nuclear weapons, the leakage of radioactive waste, and disasters such as those of Chernobyl and Fukushima nuclear-power plants. Apparently the high risk on human health and ecological security, derives from contaminated soils.

Efficient ways to dispose dangerous wastes are physical and chemical techniques that include combustion, photolysis, chemical degradation, and decomposition. Each chemical method can be successfully applied only within a certain range of concentration of organic compounds due to their solubility, toxicity, and persistence and even though these technologies are effective, they are in general too expensive and dangerous due to the use of hazardous chemicals resulting in high amount of wastes [Triska et al.; 2004]. Alternative methods for the decontamination are represented by biodegradation or phytoremediation procedures. Microbial biodegradation is considered the most cost-effective and ecological way to reduce or neutralize environmental contamination [Tandlich et al.; 2011]. In fact, microorganisms play a key role in the fundamental ecological processes such as biogeochemical cycling and organic contaminant degradation. Microorganisms are important degraders of both organic matter and xenobiotics, they represent an unexplored reservoir of genetic diversity and metabolic capability and provide several ecosystem services, most importantly the maintenance of soil and water quality, and products as nutrients to other organisms in the food chain [Megharaj et al.; 2011]. It is demonstrated that there are very few environments

where microbes have not been able to survive, adapt and thrive. Microbes are able to utilize a wide combination of electron donors (pollutants) and electron acceptors (oxidants such as O_2 and, in the case of anoxic respiration NO_3^-, Fe_3^+, $Fe(OH)_3$, SO_4^{2-}) to drive the metabolic pathways required for their growth [Tiedje et al.; 1993]. In addition to these redox reactions, microbes have also developed a series of strategies enabling them to detoxify the environment, such as the so called *cometabolism*, allowing the use of a non-growth substrate (compound that is unable to support the cell growth) in the presence of a growth substrate, as it is observed in the case of chlorinated solvents biodegradation [Horvath et al.; 1972].

Bioremediation strategies are advantageous because they can be implemented *in situ*, which means directly in the contaminated site with no need to remove the contaminated material. Innovative *in situ* technologies allow the biological treatment of contaminated sites through biosurfactant molecules produced by microbes [Pacwa-Plociniczak et al.; 2011]. This provides a simpler, less intrusive, and cheaper method than conventional "pump and treat" systems that often employ hazardous chemicals that create and additional environmental risk, in the case of *ex situ* remediation. Microbes may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. In the latter case, this is referred to as *bioaugmentation*, whereas, if the naturally occurring population is encouraged to proliferate by the addition of exogenous electron donors or electron acceptors, this is called *biostimulation*. The advantage of using microorganisms already living in the contaminated area to be reclaimed, is that they are pre-adapted to a specific environmental condition so to lower the cost of the remediation procedure [Leung et al.; 2004].

2 Naphthenic acids (NAs)

2.1 Origin and distribution of NAs

The world's largest accumulations of "oil sands" reserves occurs in the shallow reservoirs of North and South America [Head et al.; 2003]. One of these deposits, approximately 1200 billions barrels of bitumen, is located in Venezuela and it is slightly bigger than the Athabasca oil sands deposit in Alberta, Canada, with 900 billions of barrels (Fig. 2.1.1) [Head et al.; 2003, Zhou et al.; 2008].

Fig. 2.1.1 Canadian oil sands (National Geographic Photograph by Peter Essick)

Notably, the microbial biodegradation of crude oil leads to a decrease in hydrocarbon and aromatic hydrocarbon content, increasing viscosity, density, acidity, sulfur and metal content. Phenols, acyclic, cyclic, saturated and aromatic carboxylic acids, and heteroatom-bearing carboxlic acids are also produced [Larter et al.; 2008].

Several factors are required for the microbial petroleum biodegradation processes: low temperature of the reservoir (between 20 and 60 °C); presence of electron acceptors such as Fe^{3+}, SO_4^{2-},NO_3^-; nutrients (e.g. nitrate and phosphate); an oil/water contact (the bounding

surface between oil and water in which petroleum is biodegraded by microorganisms) [Head et al.; 2003, Zhou et al.; 2008, Eschard et al.; 2008]. The Athabasca oil sands deposits are featured by having a low temperature (< 10 °C) which favors the biodegradation processes. In contrast, there are oil accumulation reservoirs characterized by a temperature around 80-90 °C, which causes inactivation of most of the hydrocarbon-degrading organisms, e.g. the oil reservoir of Peace River in Canada [Zhou et al.; 2008].

The most common approach for the production of crude oil from oil sands is called: "Clark Hot Water Extraction Process" [Clemente et al.; 2005, Frank et al.; 2009] developed by Dr. Karl Clark in 1920. The oil sand is characterized by loose sand or partially consolidated sandstone, which contains a mixture of inorganic materials such as sand, clay and silt, ranging between 80 and 87%, and water, saturated with a dense and viscous form of petroleum (between 6-16%), technically referred to as bitumen, that must be upgraded before it can be used by refineries to produce gasoline and diesel fuels (Fig. 2.1.2) [Liu et al.; 2005].

Fig. 2.1.2 Example of raw bitumen extracted from Alberta's oil sands (Images courtesy of Syncrude Canada Ltd.)

During the "Clark Hot Water Extraction Process", surface oil sands are mined and crushed to reduce size particles, which are mixed with hot water and caustic soda (NaOH) to allow the heavy bitumen to become less viscous and separate it from the sand. Because of this

treatment, the solubility of oil sands asphaltic acids, mainly aromatics containing oxygen functional group such as phenolic and carboxylic types, is increased due to the alkaline pH (around 8) that promotes their release [Allen et al.; 2008, Rogers et al.; 2002]. By increasing the pH, the superficial and interfacial tensions are reduced resulting in the disintegration of the oil sands ore structure and the recovery of the bitumen [Chalaturnyk et al.; 2002]. The process requires a large amount of fresh water, approximately 2 or 3 m^3 per cubic meter of oil produced, generating 4 m^3 of wastewater of which 80-88% is recycled back to the processing facility [Holowenko et al.; 2002]. The wastewater produced as a result of the bitumen extraction process contains sand particles, clay fines, silts, water, dissolved ions (mainly Na^+ and SO_4^{2-}), heavy metals, unrecoverable bitumen, inorganic and organic compounds. This kind of wastewater is named Oil Sand Tailings Water (OSTW) or Oil Sand Process Water (OSPW), and its composition changes depending on the ore quality, source, extraction processes and age [Crowe et al.; 2001].

OSPWs produced during the extraction processes of crude oil are stored in settling tanks into the environment, known as *tailing ponds* (Fig. 2.1.3) [Del Rio et al.; 2006, MacKinnon et al.; 1989].

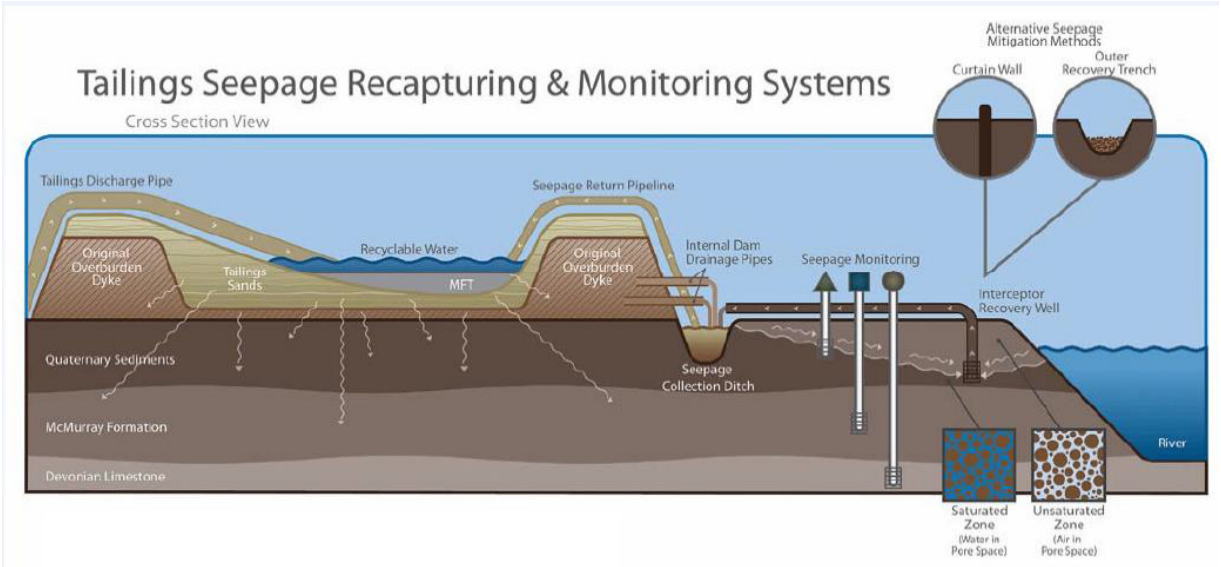

Fig. 2.1.3 Schematic representation of the *tailing pond*

These tailings ponds are open dumps with a high environmental impact and the existing ones (e.g. Athabasca region, Canada) cover an area of around 180 km^2. Once in the pond, the sand quickly sinks to the bottom, and the water from the top three meters is recycled back to the process facility. However, tailings ponds present a number of challenges:

- The bottom layer, a mixture of clay and water called fine tailings, takes a long time to settle down and solidify, e.g. it can take up to 30 years to separate and dry out;
- Due to the extraction process, the remaining water contains chemicals that are toxic to living forms, e.g. fish;
- The risk of wastewater infiltration into the groundwater is significant;
- The small amount of residual oil that floats to the surface of the pond poses a risk for waterfowl.

OSPWs high toxicity is mainly due to the presence of compounds collectively known as **naphthenic acids** (NAs) [Allen et al.; 2008, Madill et al.; 2001, MacKinnon et al.; 1986]. NAs naturally occurring in petroleum and oil sands deposits [Fan et al.; 1991, Seifert et al.; 1969, Dzidic et al.; 1988] are believed to be generated by incomplete aerobic biodegradation of petroleum hydrocarbons. Indeed, earthquakes, erosions or tectonic movements can cause petroleum to be exposed to favorable conditions for the microbial aerobic biodegradation [Behar et al.; 1984, Meredith et al.; 2000, Zhou et al.; 2008]. NAs can also derive from insufficient *catagenesis* that is a physical process that occurs directly in oil deposits under high pressure and temperature conditions, leading to loss of carbonyl groups [Tissot et al.; 1978, Clemente et al.; 2005]. This phenomenon can alter biomolecules through isomerization, aromatization, cyclization and bond cleavage [Sinninghe et al.; 1997]. Finally, NAs can be produced by anaerobic hydrocarbon biodegradation processes in oil sands deposits where aerobic conditions are unlikely. The occurrence of this type of production is demonstrated by

the presence of anaerobic microorganisms and to the discovery of anaerobic hydrocarbon degradation pathways [Head et al.; 2003, Aitken et al.; 2004, Eschard et al.; 2008].

The continuous re-use of recycled water, causes a gradual accumulation of NAs in the tailing ponds at a concentration ranging between 40 and 120 mg/L [Allen et al.; 2008]. In the Athabasca region in Canada and other regions of Russia, Venezuela, Saudi Arabia, Iraq, Romania and West Africa, contamination from NAs were also detected in water resources at a concentration ranging from 0.1 to 0.9 mg/L [Schramm et al.; 2000] and groundwater aquifers (> 55 mg/L) [Conrad et al.; 1998], confirming once again the environmental importance of this issue.

Due to the overall toxicity of NAs in the tailings ponds, remediation procedures aimed at developing efficient treatment of areas holding OSPWs are needed [Quagraine et al.; 2005]. Nowadays, the big challenge for companies, industrial and research communities is to develop reclamation and remediation strategies to treat this wastewater and to release it back into the local environment [Herman et al.; 1994, Young et al.; 2008].

2.2 NAs chemical and physical properties

This class of pollutant compounds was first identified as cyclic carboxylic acids in petroleum or crude oil. Nowadays, the term “naphthenic acids”, refers to all acidic organic compounds found in crude oils including aromatic functionality [Hus et al.; 2000].

NAs are represented by the general formula $C_nH_{2+z}O_2$, where *n* is the number of carbon atoms and *z* indicates the hydrogen deficiency due to ring structure formation [Brient et al.; 1995, Clemente et al.; 2003].

The NAs mixture includes alkyl substituted alicyclic carboxylic acids and a smaller amounts of acyclic aliphatic acids [Brient et al.; 1995, Hus et al.; 2000]. This mixture also includes a low concentration of aromatic olefinic, hydroxy- and dibasic-acids that are described by the general formula $C_nH_{2+z}O_x$, where *x* represents the number of oxygen atoms ranging from 3 to

5 [Barrow et al.; 2009]. These organic acids can be distinguished in acyclic, monocyclic, bicyclic, polycyclic groups, with a number of ring structures up to six or more (Fig. 2.2.1) [Herman et al.; 1993, Brient et al.; 1995].

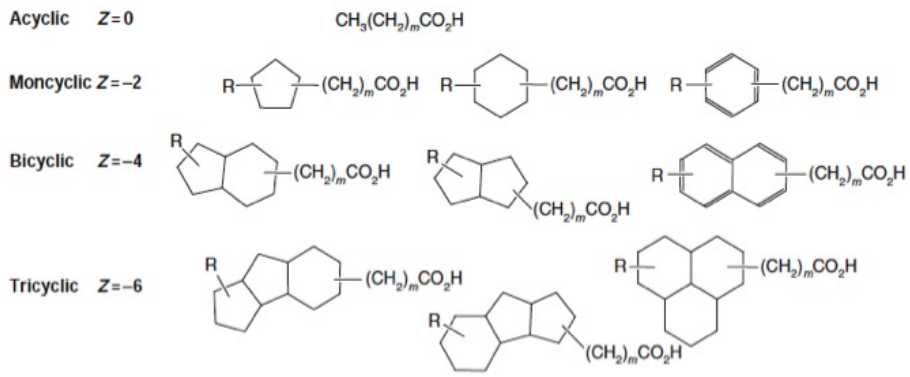

Fig. 2.2.1 Aromatic and non-aromatic NAs structures. R represents an aliphatic group such as methyl; Z indicates hydrogen deficiency due to ring structure formation; m is the number of CH_2 units [Modified from Brient et al.; 1995]

Usually, the carboxylic group is bound to the cyclo-aliphatic ring as a side chain, and it is described by the chemical formula R-$(CH_2)_m$-COOH, where *R* represents the ring structure (single or multi) that generally contains 5 or 6 carbon atoms, while the $(CH_2)_m$ indicates the carbonyl chain. NAs are also characterized by an aliphatic group with variable complex branching [Headley et al.; 2004, Headley et al.; 2007].

Due to their huge heterogeneity, the physical and chemical properties of NAs can significantly vary on the basis of their origin and composition. NAs general chemical composition of carboxylic acids makes them polar, non-volatile (Henry's constant is 8,56 x 10^{-6} atm.m^3/mole) and very stable compounds [Rogers et al.; 2002]. These physicochemical properties are strengthened as their molecular weight increases. NAs dissociation constant (pK_a) is between 10^{-5} and 10^{-6} [Brient et al.; 1995, Headley et al.; 2005a], which is typical of most carboxylic acids such as acetic acid, propionic acid, and palmitic acid. As they contain both a hydrophilic (COOH group) and hydrophobic (non-polar aliphatic) end, NAs are amphipathic compounds that act as surfactants and tend to accumulate in aqueous/non-aqueous interfaces [Armstrong et al.; 2008]. Generally, NAs solubility strongly depends on

the pH, their molecular weight and concentration of inorganic salts in aquatic environments [Quagraine et al.; 2005]. Indeed, at alkaline pH, NAs are generally soluble in water at a concentration up to 50 mg/L. Their solubility decreases with increasing chemical complexity and environmental salinity. They are soluble in organic solvents such as methanol, ethanol, acetone, vegetable and mineral oils. Their boiling points are ranging from 250 °C to 350 °C [Brient et al.; 1995].

NAs can precipitate as metal naphthenate salts, which can cause several problems because of deposit formations that can block pipelines, resulting in higher operating costs or expensive shutdowns. The mechanism through which calcium naphthenates deposition occurs is not fully understood, although it is known that involves a chemical reaction between NAs present in the oil and calcium ions in the water. Calcium naphthenate is the reaction product that is insoluble in both phases (oil and water), which tends to precipitate and accumulate in the oil/water interface [Baugh et al.; 2004, Lutnaes et al.; 2007]. The main fraction of calcium naphthenates deposited in Norwegian, China, West Africa, and UK offshore oilfields, was identified through a multimethod approach. This method consisted of an acidification of the acid mixture in order to collect it from the deposit followed by a fractionation procedure through an ion exchange resin method. The fractions were subsequently injected into a mass spectrometer. In this way, calcium naphthenates deposits were characterized as a family of C80 isoprenoid tetracids referred to as ARN acids with molecular weight between 1227 and 1237 Da [Brient et al.; 1995, Morii et al.; 1998, Clemente et al.; 2005, Smith et al.; 2007, Magnusson et al.; 2008]. Furthermore, NAs can act as oxidizing agents which are highly corrosive. Their corrosion property is due to the temperature of the environment, to the availability of carboxylic groups in their chemical composition to form metal complexes, and to their molecular size and structure [Piehl et al.; 1988, Clemente et al.; 2005]. It is known that the increase of the alkyl side chain length up to three methylene groups is strictly

correlated to the increase of their corrosive effect, while these effects are decreased when more than three methylene groups are present [Turnbull et al.; 1998, Kane et al.; 1999, Slavcheva et al.; 1999, Chen et al.; 2009]. For example, steel alloys that are normally resistant to corrosion, can be damaged by NAs corrosive power leading to safety and reliability problems (Fig. 2.2.2) [Kane et al.; 1999]. Conversely, NAs derivatives such as naphthenate esters can be used as corrosion inhibitors in oil well and petroleum refineries.

Fig. 2.2.2 NA-mediated pipeline corrosion (Image courtesy of Oil Plus Ltd).

2.3 Toxicity of NAs

NAs and naphthenates are considered the main toxic compounds found in OSPWs [Dorn et al.; 1992, Schramm et al.; 2000]. Their concentration can vary between 40 and 120 mg/L, reaching 130 mg/L in fresh oil sands tailings [Allen et al.; 2008]. These pollutant compounds exert their toxic effects towards a large number of organisms including plants, fish, mammals, zooplanktons, phyto-planktons, and amphibians [Wort et al.; 1970, Dokholyan et al.; 1984, Rogers et. al.; 2002, Dorn et al.; 1992, Leung et al.; 2003, Pollet et al.; 2000].

2.3.1 Aquatic environmental toxicity

In aquatic environments, NAs exert their toxic effects on a large number of species of fish even at a low concentration (2.5-5 mg/L) [Dorn et al.; 1992]. NAs toxicity depends on several factors such as water temperature, water hardness, length of exposure, and dissolved oxygen concentration and fish species [Armstrong et al.; 2008, Dokholyan et al.; 1984].

Different studies have shown that OSPWs containing NAs are toxic to a variety of aquatic organisms. Dokholyan and coworkers (1984) studied the acute toxic effects of commercial NAs (used as sodium salts) supplied at a concentration ranging from 10 to 100 mg/L towards *Roach* fish, for ten days. They found that the fish age may play a key role in NAs tolerance, based on the evidence that 50% of two months old *Roach* died when they were exposed to 50 mg/L of commercial NAs, while a concentration of 75 mg/L was found to kill the same percentage of two years old *Roach* [Dokholyan et al.; 1984]. The most evident symptoms were related to changes to the gill and liver [Nero et al.; 2006], decreased in glucose blood level and leukocyte counts, increased muscle glycogen, and incidence deformity [Dokholyan et al.; 1984, Peters et al.; 2007].

2.3.2 Toxicity to Mammals

Information on the toxic effects of NAs on mammals are limited. It is known that commercial NAs can cause death by gastro-intestinal disturbances in rats, the oral LD_{50} evaluated (lethal dose of substance that results in 50% of mortality) was 3-5.2 g/Kg of body weight [Lewis et al.; 2000]. Garcia-Garcia and colleagues (2011) found that commercial NAs had no significant effects on mouse bone marrow derived macrophages (BMDM) viability when these type of cells were exposed to concentrations ranging from 6.25μg/mL to 50μg/mL. *In vitro* exposure of BMDM-derived macrophages to NAs caused down-regulation of the respiratory burst response of BMDM with a pronounced suppression of the reactive

oxygen species production, and down-regulation of genes coding for pro-inflammatory cytokines such as INFγ, IL-1β and CFS-1 [Garcia-Garcia et al.; 2011].

Other NAs toxic effects on mammalian cells are: central nervous system depression, convulsion, hepatoxicity, respiratory arrest [Pennisi et al.; 1977, Lai et al.; 1996, Conrad et al.; 1998, Rogers et al.; 2002].

2.3.3 Toxicity to green plants

NAs exert both stimulatory and inhibitory effects to land plants. Increased yield, dry mass of plants, higher ribonucleic acid proteins, and enzymes involved in nitrogen metabolism were found when *Phaseolus vulgaris* was exposed to commercial NAs [Wort et al.; 1973]. Increased photosynthesis was found when *Typha latifolia* was exposed to wetlands receiving oil sands effluent [Bendell-Young et al.; 2000]. Inhibitory effects regarding leaf growth, stomal conductance, and net photosynthesis were observed when *Populos tremuloides michx* was exposed to a commercial mixture of NAs [Kamaluddin et al.; 2002]. Furthermore, NAs extracted from Athabasca OSPW caused more phytotoxic effects (growth plants reduction) because of their higher bioavailability to wetland plants such as *Typha latifolia*, *Phragmites australis* and *Scirpus acutus* [Armstrong et al.; 2009].

2.3.4 Mechanisms of toxicity in microorganisms

NAs toxic effects on microorganisms are often related to their surfactant characteristics [MacKinnon et al.; 1986, Rogers et al.; 2002, Smith et al.; 2008], while the main factors that generally contribute to the NAs toxicity include pH, chemical structure, molecular size and hydrophobicity [Protic et al.; 1989, Nero et al.; 2006, Frank et al.; 2009]. As surfactants, NAs can easily come in contact with bacterial cell wall and penetrate into biological membranes [Quagraine et al.; 2005]. Because of (i) the lack of functional groups in

NAs that may target a specific receptor, (ii) the solubility of NAs in OSPW as ionic salts, and (iii) the surfactant properties of NAs, the probable mode of acute toxic action for NAs is narcosis [Frank et al.; 2008, 2009, Roberts et al.; 1991]. Narcosis is a phenomenon that correlates the disruption of the cellular cytoplasmatic membrane to the physical introduction of hydrophobic compounds in the lipid bi-layer, leading to the alteration of the membrane properties that include: increase of the fluidity, swelling, thickness, surface tension. These effects cause alterations of the membrane functionality and ultimately cause cell death [Schultz et al.; 1989, Frank et al.; 2008]. Basically, the narcotic effect of hydrophobic compounds is correlated to their size and hydrophobicity [Schultz et al.; 1989, Protic et al.; 1989]. NAs with molecular weight lower than 1000 Da are small and hydrophobic enough to enter into a cell membrane and elicit a greater cell response [Sanderson et al.; 2004, Frank et al.; 2009]. NAs with different molecular structures, in terms of carboxylic groups content and number of rings, have a different degree of hydrophobicity. Hydrophobicity decreases with the increase of the content of carboxylic groups and number of rings [Havre et al.; 2003]. Higher number of carboxylic groups and rings decreases the hydrophobicity of NAs by increasing the electrical charge of the molecule impairing its ability to enter into the cellular membrane. This reduces the toxic effect exerted through the narcotic mode of action [Frank et al.; 2010]. According to this, NAs of the Athabasca's OSPW with a molecular weight around 500 Da were found to exert a more dramatic toxic effect compared to those with higher molecular weight [Seward et al.; 1999].

The toxicity of NAs is also affected by pH in aquatic environments. At pH > 6, NAs are in their ionized form and are less prone to penetrate the biological membranes resulting in lower toxicity. At lower pH, NAs are in their non-ionized form that is soluble in lipid and tends to be facilitated in entering the cytoplasmic membranes [Armstrong et al.; 2009]. NAs toxicity is therefore higher at acidic pH values compared to alkaline ones.

2.3.5 Lipid adaptation mechanisms of bacterial strains

The microbial cytoplasmic membrane represents the main target of pollutant compounds. Different kind of organic pollutants are able to penetrate into the cytoplasmic membrane resulting in the increase of the membrane fluidity. This increase leads to the loss of membrane functionality and to the damage of the bacterial cell. As a consequence, the toxicity of such pollutants can hamper the use of microorganisms for their removal [Heipieper et al.; 1994, Certık et al.; 2002]. Bacterial cells tend to counteract the disruptive effect of organic compounds by readjustment of the membrane fluidity. Most microorganisms have adopted mechanisms aimed to modulate the type of lipid content of the membrane in order to vary the membrane fluidity preventing solvent accumulation [Sikkema et al.; 1994, 1995].

A lipid bilayer can exist in either a ordered phase (gel) or a disordered phase (liquid-crystalline). The ordered phase of a biological membrane is characterized by lipid molecules organized in an highly packed and rigid arrangement, while in a disordered phase the lipid molecules are more fluid. This is due to the characteristic phase transition temperature that depends on the degree of saturation and length chain of lipid molecules. The phase transition temperature for long chain saturated fatty acid is very high compared to the unsaturated one. For example, palmitic acid (16:0) and palmitoleic acid (16:1*cis*) have phase transition temperature of 63 °C and 0 °C, respectively. This means that palmitic acid remains in an ordered phase below 63 °C in the lipid bilayer. [Weber et al.; 1996]. Consequently, microorganisms can alter the saturated/unsaturated fatty acids ratio in order to counteract the fluidizing effects of pollutant compounds. This mechanism was described for different gram positive and negative bacteria, such as *Pseudomonas stutzeri* grown in the presence of naphthalene, as well as three different *Rhodococcus* spp. grown in the presence of different aromatic compounds (phenol, 4-chloro-phenol, benzene, and cholo-benzene) [Zoradova et al.; 2011, Tsitko et al.; 1999, Gutierrez et al.; 1999]. Also, microorganisms can alter the content

of methyl-branched fatty acids in order to maintain their membrane rigidity when they grow under adverse conditions. Methyl-branched fatty acids are defined as *iso* and *anteiso*. *iso*-Methyl branched fatty acids have the branch point on the penultimate carbon of the fatty acid, while *anteiso*-methyl-branched fatty acids have the branch point on the ante-penultimate carbon atom (Fig. 2.3.5.1).

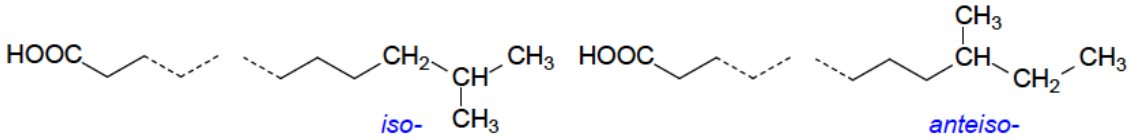

Fig. 2.3.5.1 Example of *iso*- and *anteiso*-methyl-branched fatty acid.

These fatty acids are featured by different phase transition temperatures. In particular, it is higher for *iso*-methyl-branched fatty acid than *anteiso* configuration. This difference causes a remarkable change in the rigidity of the membrane when the *iso/anteiso* ratio is increased in a lipid-bilayer. According to the higher phase transition temperature and the more ordered packing of *iso*- than *anteiso*-methyl-branched fatty acid, microorganisms can alter the ratio *iso*/*antesio*-methyl branched fatty acid ratio in order to make a more rigid cytoplasmic membrane [Kaneda et al.; 1991]. Gram positive bacteria often contain methyl-branched fatty acids [Tsitko et al.; 1999], although they can be found also in Gram negative bacteria [Mrozik et al.; 2005, Zoradova et al.; 2011]. For example, Unell *et al.* (2007) reported that *Arthrobacter chlorophenolicus* was able to increase the *iso/anteiso* ratio in response to the presence of phenols in the growth medium. Methyl-branched fatty acids *de novo* synthesis depends on the energetic status of the cells as well as on *de novo* synthesis of their precursors (leucine, isoleucine, and valine) [Kaneda et al.; 1991]. In relation to this, the mechanism of adaptation cannot be supported by microorganisms that grow under inhibiting conditions.

Another mechanism of adaptation that microorganisms can exploit is the *cis-trans* fatty acid isomerization [Heipieper et al.; 2004]. This mechanism is a short-term response triggered by microorganisms in the presence of hydrophobic chemicals, and it does not require new

synthesis of fatty acids. The biological significance of this mechanism of adaptation is due to the steric structure of these fatty acids. In particular, the acyl chains of fatty acids in *cis* configuration have a non-movable bend of 30° that cause steric hindrance and disturbs the highly ordered fatty acid package. In contrast, the acyl chains of fatty acids in *trans* configuration with a non-movable bend of 6 °C does not hinder the tight fatty acid packing (Fig. 2.5.3.2) [Keweloh et al.; 1996].

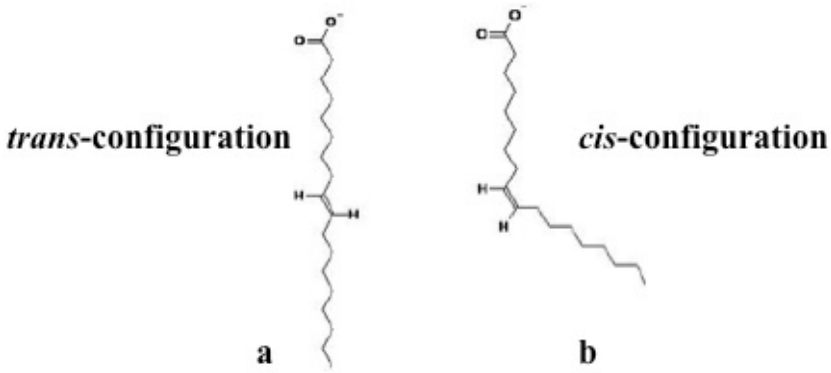

Fig. 2.3.5.2 Elaidic acid in *trans* configuration (a), and oleic acid in *cis* configuration.

This mechanism allows the microorganisms to rapidly modify the lipid membrane when they grow under stress conditions. This, along with the increased saturated and methyl-branched fatty acids alterations, can help bacterial cells to survive during a long time period of adverse conditions.

3. Microbial biodegradation

Microbial biodegradation of NAs in aerobic conditions is the most cost-effective way of reducing their toxicity from wastewaters [Scott et al.; 2008]. However, the diversity of microbial communities involved in NAs degradation, and the mechanisms by which NAs are biodegraded in OSPWs, are poorly described [Holowenko et al.; 2002, Whitby et al.; 2010]. The degradation of NAs via microbial activity results in the production of CO_2 and water. Herman *et al.* (1994) showed that bacterial cultures isolated from oil sands tailings were able to utilize as sole carbon source both a commercial mixture of NAs and a mixture of organic acids derived from oil sands tailings ponds. During 24 days of incubation, the microbial activity converted into CO_2 approximately 50% and 20% of organic carbon of the commercial mixture of NAs and of the organic carbon of the oil sands tailings ponds NAs, respectively [Herman et al.; 1994]. Clemente *et al.* (2004) described the rate of commercial NAs mixture degradation performed by aerobic cultures isolated from oil sands process-affected waters, through high-performance liquid chromatography and gas chromatography-mass spectrometry (GC-MS). Within 10 days of incubation, the amount of NAs dropped from about 100 to <10 mg/L. This was accompanied by the release of about 60% of carbon from NAs as CO_2 and the reduction of toxicity of the culture supernatant. This study also demonstrated that biodegradation can change the composition of the NAs complex mixture and that low molecular weight NAs are degraded faster than high molecular weight ones [Clemente et al.; 2004].

The metabolic pathways putatively involved in the biodegradation of aliphatic and alicyclic carboxylic acids include β-oxidation, combined α- and β-oxidations [Rontani et al.; 1992] and aromatization [Taylor et al.; 1978]. β-oxidation represents the preferred route by which most microorganisms degrade aliphatic and alicyclic carboxylic acids [Quagraine et al.; 2005a,

Taylor et al.; 1978, Trudgill et al.; 1984]. Taylor and co-workers (1978) found that among 33 new microorganisms isolated from mud and soil samples from the Aberystwyth area in Wales (U.K.) 32 microbial strains were able to metabolize cyclohexanecarboxylic acid (CHCA) by β-oxidation of the coenzyme A ester. Cyclohexanecarboxylic acid (CHCA) was also metabolized through the β-oxidation pathway by several microorganisms that include *Acinetobacter anitratum*, a strain named PRLW19 (reclassified as *Alcaligenes fecalis*), and *Pseudomonas putida* [Evans et al.; 1975, Blakley et al.; 1978, Blakley et al.; 1982]. For example, growth medium extracts of PRLW19 strain grown on CHCA were shown to contain 1-ene-CHCA and pimelic acid by GC-MS analysis. The presence of these two compounds in the growth medium and the high oxidative activity of cell suspensions with 1-ene-CHCA and 2-hydroxy-CHCA provided strong evidence for the metabolism of CHCA via β-oxidation pathway (Fig. 3.1). The activity related to the enzymes acyl-CoA synthetase, dehydrogenase, hydrolase and thiolase was found in crude extracts of PRLW19 cells grown on CHCA as sole carbon and energy source [Blakley et al.; 1978]. These two intermediates were also observed in the case of *Rhodopseudomonas palustris*, a purple non-sulfur phototrophic bacterium, during anaerobic degradation of benzoic acid that leads to CHCA formation as intermediate of reaction which is further degraded by a metabolic pathway similar to that found in PRLW19 strain [Pelletier et al.; 2000].

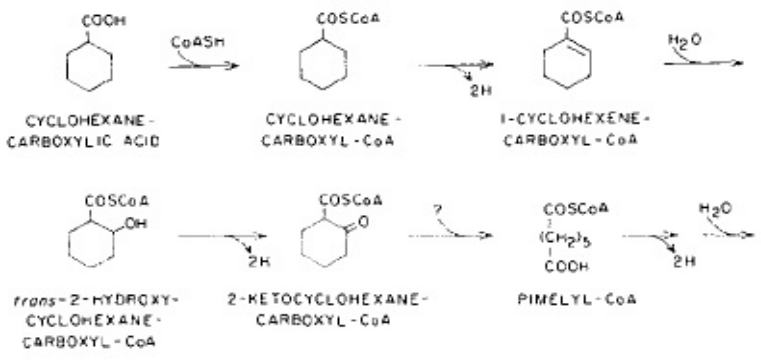

Fig. 3.1 β-oxidation pathway proposed for CHCA degradation by PRLW 19 [modified from Blakley et al.; 1978].

Other studies have provided results that support the aromatization pathway of CHCA [Blakley et al.; 1974, Kaneda et al.; 1974, Taylor et al.; 1978]. In *Arthrobacter* sp. culture, the microbial degradation of CHCA occurred through aromatization of the ring to form *p*-hydroxybenzoic acid before ring cleavage. In particular, cellular extracts of *Arthrobacter* sp. grown on CHCA were able to consume oxygen in the presence of CHCA, 4-hydroxy-CHCA, 4-keto-CHCA, *p*-hydroxybenzoic acid, protocatechuic acid, quinic acid, and shikimic acid. 4-hydroxy-CHCA dehydrogenase and 4-keto-CHCA oxidase were found to be the key enzymes involved in the catabolism of CHCA, by analyzing the activity of the cellular extracts of CHCA-grown *Arthrobacter* sp. cells. The first enzyme requires Mg^{2+} while the second enzyme is very stable and does not require any cofactor for its activity [Blakley et al.; 1974] (Fig. 3.2).

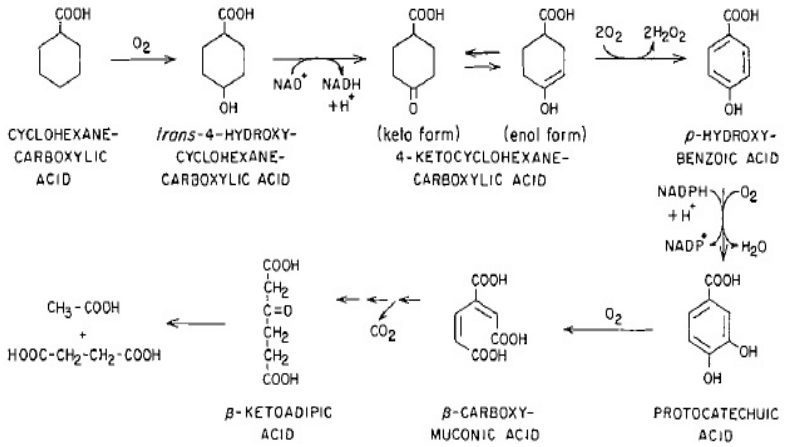

Fig. 3.2 Aromatization pathway proposed for the catabolism of CHCA by *Arthrobacter* sp. [Blakley et al.; 1974].

The CHCA aromatization pathway shown in figure 3.1.3 was previously described in Gram positive bacteria such as *Arthrobacter*, *Corynaebacterium*, and *Alcaligenes* [Blakley et al.; 1974, Kaneda et al.; 1974, Taylor et al.; 1978]. Iwaki and co-workers (2005) described the function of the *pobA* gene coding for the enzyme responsible for the formation of protocatechuic acid from *p*-hydroxy-benzoic acid in the Gram positive *Corynaebacterium cyclohexanicum* [Iwaiki et al.; 2005]. This enzyme was initially described for its involvement

in the aromatic compound breakdown in Gram negative bacteria such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Azotobacter chroococcum* [Entsch et al.; 1988, Quinn et al.; 2001, Bertani et al.; 2001].

Rontani and colleagues (1992) reported that *Alcaligenes* sp. PHY12 when incubated in the presence of cyclohexaneacetic acid (CHAA) shows a combination of α- and β-oxidation to degrade this type NA. In particular, *Alcaligenes* sp. PH12 was able to decarboxylate CHAA to CHCA that was further degraded by β-oxidation [Rontani et al.; 1992]. Ougham *et al.;* (1982) isolated a strain of *Arthrobacter* (named *Arthrobacter* sp. CA1) from a soil contaminated with aviation fuel by enrichment on mineral salts medium containing cyclohexaneacetate (CHA) as unique carbon and energy source. By analyzing the cell extracts obtained from CHA-grown *Arthrobacter* sp. CA1 cells, enzymes responsible for the formation of CHA-CoA were detected. It was proposed that the degradation of CHA proceeded through the formation of a CoA ester followed by initiation of the β-oxidation cycle. Due to the formation of a tertiary alcohol, that is not amenable to dehydrogenation to form a ketogroup, the β-oxidation was blocked. The side chain of CHA was eliminated as acetyl-CoA by the action of a CoA lyase. Consequently, cyclohexanone was produced and it was degraded by a Baeyer-Villiger oxygenase responsible for the insertion of an oxygen into the cyclohexanone ring forming a lactone (6-capro-lactone) (Fig. 3.3).

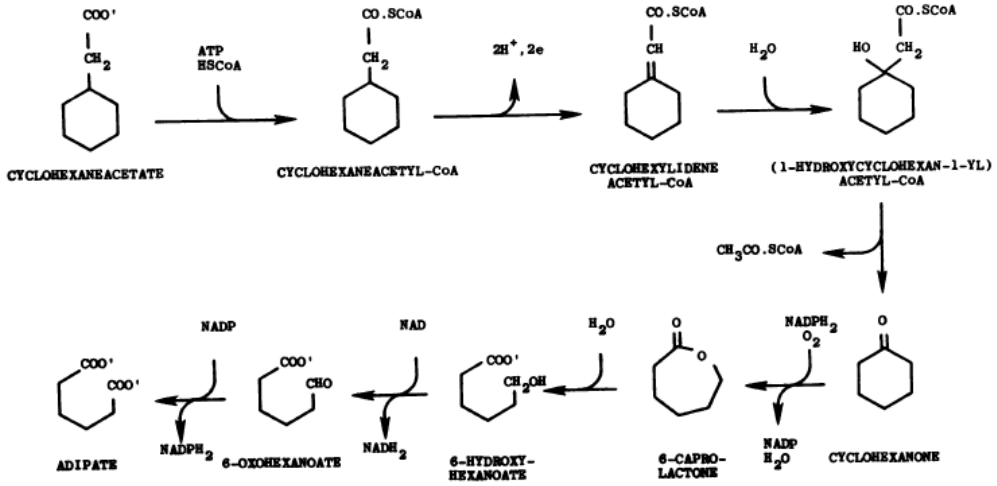

Fig. 3.3 Oxidation pathway of *Arthrobacter* sp. CA1 [Ougham et al.; 1982].

Despite their overall toxicity and recalcitrance in wastewater, little information is available on the degradation of aromatic and highly branched alkanoic carboxylic acids that represent a small portion of NAs mixture contained in oil sands processed water (less than 10 %) [Hsu et al.; 2000]. Recently, Johnson and colleagues (2011) showed that increased alkyl side chain branching is responsible for NAs recalcitrance. In particular, they examined the biodegradation of four aromatic alkanoic acid isomers that differed in the length of the alkyl side chain branch: (4'-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA); (4'-*iso*-butylphenyl)-4-butanoic acid (*iso*-BPBA); (4'-*sec*-butylphenyl)-4-butanoic acid (*sec*-BPBA) and (4'-*tert*-butylphenyl)-4-butanoic acid (*tert*-BPBA) (chemically synthesized) (Fig. 3.1.5 a, b, c, and d) [Smith et al.; 2006]. A hydrocarbon degrading culture provided a pool of microbes putatively capable of BPBA degradation, as demonstrated through GC-MS analysis. By analyzing gas chromatograms after 35 days of incubation, the less branched *n*-BPBA was shown to be readily degraded, while the rate of degradation of the most branched *n*-BPBA was much slower. The major metabolic intermediate of butanoic acid biodegradation was the ethanoic acid (Fig. 3.4 e, f, and g), which, in turn, was further transformed to (4'-carboxybutylphenyl)ethanoic acid, the latter being a diacid formed by addition of a carboxylic acid group to the alkyl side chain. This confirmed that the initial degradation steps of the branched *n*-BPBA involve the removal of two carbons from the carboxyl side chain of the aromatic alkanoic acids, indicative of β-oxidation. Complete mineralization of *n*-BPEA and (4'-carboxybutylphenyl)ethanoic acid occurred in 49 days, while no further degradation of *iso*-, *sec*- or *tert*-BPEA metabolites was detected. Through 454 Pyrosequencing and 16S rRNA anlysis, *Burkholderia*, *Pseudomonas* and *Sphingomonas* spp. were identified as dominant microbial genera and key microorganisms involved in aromatic alkanoic acid transformations [Johnson et al.; 2011].

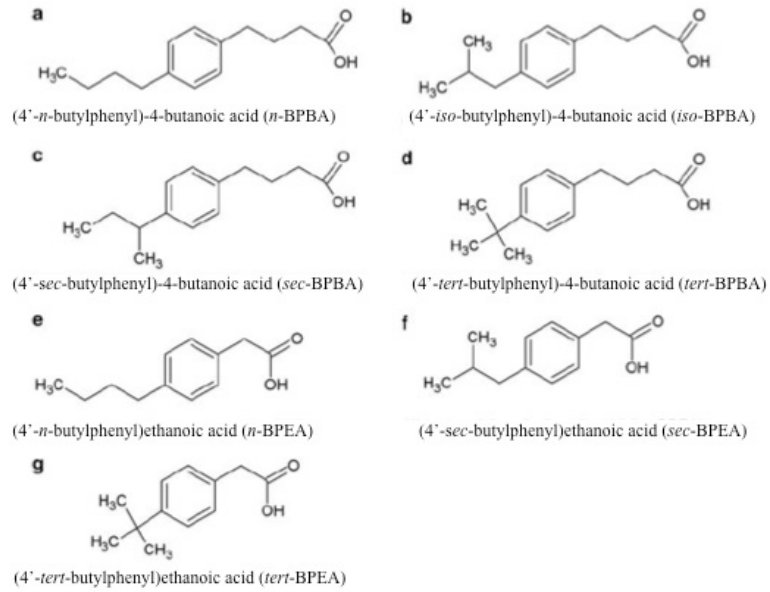

Fig. 3.4 Aromatic alkanoic acids: (4'-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) (a), (4'-*iso*-butylphenyl)-4-butanoic acid (*iso*-BPBA) (b), (4'-*sec*-butylphenyl)-4-butanoic acid (*sec*-BPBA) (c), (4'-*tert*-butylphenyl)-4-butanoic acid (*tert*-BPBA) (d), (4'-n-butylphenyl)ethanoic acid (n-BPEA) (e), (4'-sec-butylphenyl)ethanoic acid (sec-BPEA) (f), and (4'-tert-butylphenyl)ethanoic acid (tert-BPEA) (g) [modified from Johnson et al.; 2011].

Nowadays, some information is available about alkyl phenyl alkanoic acids degradation by pure cultures. Johnson *et al.* (2012) attempted to isolate microbial species that could degrade BPBAs (Fig. 3.1.5 a, b, c, and d). A strain was isolated from hydrocarbon-contaminated sediment from Avonmouth (U.K.) that was used as inoculum of cultures supplied with *n*-BPBA as sole carbon and energy source. The strain isolated was designated as IS2.3 and it was phylogenetically related to *Mycobacterium* genus (16S rRNA 100% to *Mycobacterium aurum*). IS2.3 was able to degrade *n*-BPBA or *t*-BPBA (Fig. 3.1.5 a, d) and to use them as sole carbon and energy sources. During *n*-BPBA and *t*-BPBA degradation by IS2.3, (4'-*n*-butylphenyl)ethanoic acid and (4'-*t*-butylphenyl)ethanoic acid were produced as metabolic intermediates. This suggested that the degradation of BPBAs compounds proceeds via β-oxidation pathway. During *t*-BPBA degradation by the strain IS2.3, the diacids (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid were also formed. These two metabolites were quantified revealing that they were present in low abundance i.e. 4,9% for (4'-carboxy-*t*-butylphenyl)ethanoic acid and 1,5% for (4'-carboxy-*t*-butylphenyl)-4-

butanoic. The detection of these compounds during BPBA degradation could help to explain the presence of diacids, also named as O_4 species in oil sands processed water. These type of compounds are recalcitrant to microbial degradation and tend to concentrate in NAs-contaminated areas. Frank *et al.* (2009) and Headley *et al.* (2011) revealed these toxic O4 species in Athabasca contaminated sites through nuclear magnetic resonance spectroscopy analysis and electro-spray ionization mass spectrometry.

NAs biodegradation is principally affected by their chemical structure. Several studies showed how aerobic microbial activities are able to degrade low molecular weight NAs, while branched, cyclic and high molecular weight toxic compounds tend to accumulate in the contaminated areas as they are more persistent and recalcitrant to biodegradation [Holowenko et al.; 2002, Scott et al.; 2005, Clemente et al.; 2004, Biryukova et al.; 2007]. Smith *et al.* (2008) studied the rate of degradation of *n*-butyl-cyclohexyl-butanoic acid (BCHBA) and the *iso*-, *sec*- and *tert* forms by the bacterial community found in sediments. They demonstrated that the biodegradation efficiency decreases when alkyl side chain branching increases. They found that 97% of *n*-BCHBA with non-branched alkyl side chain was degraded, while 2,5% of *tert*-BCHBA with highly branched alkyl side chain was degraded in 30 days [Smith et al.; 2008]. Han *et al.* (2008) reported similar conclusions on the alkyl side chain branching. They also reported the negative effect of the cyclicity degree of the compound on the biodegradability of NAs.

Differences in biodegradation rates can be observed if we consider different isomers of the same model NAs. Holowenko *et al.* (2002) and Headley *et al.* (2002) studied the biodegradation of NAs from OSPW and/or commercial mixtures, respectively. They showed that *cis*-isomers are less biodegradable than *trans*-isomers. In particular, they investigated the biodegradation rates of *trans*-4-methyl-1-cyclohexaneacetic acid (4-MACH) and *trans*-4-methyl-cyclohexanecarboxylic acid (4-MCCH) and their *cis*-isomers. The faster degradation

of *trans*-isomers compared to the *cis* ones was related to the lower bioavailability of the *cis*-isomers and the higher energy required to break their intra-molecular hydrogen bonds [Holowenko et al.; 2002, Tanapat et al.; 2002].

Other studies showed that methyl substitution can impair or reduce the rate of β-oxidation of both aliphatic- and cyclo-alkane carboxylic acids [Dias et al.; 1971, Hammon et al.; 1972, Beam et al.; 1974]. For example, Herman *et al.* (1994) showed that during 24 days of incubation, only 6-7% of methyl substituted pentyl-cyclohexanecarboxylic acid was mineralized to CO_2, while 50% of CHCA was degraded. Apparently, although these data about model NAs degradation are useful, we are far to fully understand the microbial degradation pathways required to OSPWs detoxification, as they contain thousands of different molecules.

Plants and microorganisms associated to plants have beneficial effects in contaminated soils. Many data show that plants-bacteria association support the degradation of contaminants in the rhizosphere [Davis et al.; 2002, Susarla et al.; 2002; Siciliano et al.; 2001]. The type of microorganisms and fungi that colonize the rhizosphere are influenced by root exudates that alter the chemistry of the soil in the area, and that can be used as selective growth substrates. In turn, microorganisms affect the composition and amount of root exudates by influencing root cell leakage, cell metabolism, and plant nutrition [Yang et al.; 2000]. Considering this, in phytoremediation studies, rhizobacterial populations have been studied for their ability to metabolize NAs and mitigate their toxicity. Biryukova *et al.* (2007) showed for the first time that microorganisms isolated from the rhizosphere in Athabasca contaminated soil can degrade commercially available NAs. After 17 days of growth of these root-associated microbial consortia on NAs, more than 90% of the initial concentration of the toxic compounds was metabolized

Few studies focused on the metabolic abilities of algae regarding NAs biodegradation, as well as the mechanisms by which algae can transform NAs. For example, algal communities have been characterized in the tailings ponds of the Athabasca Oil Sands [Leung et al.; 2001, 2003], while other studies described the ability of the algae to degrade model and tailings associated NAs [Headley et al.; 2008]. Among the 12 algal species that have been characterized by Headley *et al.* (2008), two *Naviculla* strains transformed the model NA *trans*-4-methylycyclohexaneacetic acid (4-MCHAA). One of these two *Naviculla* strains was also able to remove the *cis*-isomer of 4-MCHAA. NAs mixture derived from oil sands tailings water could not be degraded by any of the 12 algal species characterized. In a subsequent study, the green alga *Chlorella pyrenoidosa* was shown to metabolize the CHCA with the production of hydroxylated metabolites [Yoshizako et al.; 1991]. Two green algae (*Dunaliella tertiolecta* and *Chlorella vulgaris*) and a cyanobacterium strain (*Synechococcus leopoliensis*) were investigated by Quesnel *et al.* (2011) for their ability to degrade five model NAs at a concentration of 300 mg/L that exceeds that found in NAs-contaminated oil sands tailing waters (around 120 mg/mL). The NAs tested in this study were the CHCA, the cyclohexaneacetic acid (CHAA), the cyclohexanepropionic acid (CHPA), the cyclohexanebutyric acid (CHBA), along with a more complex NA such as 1,2,3,4-tetrahydro-2-naphthoic acid. As a result, *Dunaliella tertiolecta* was shown to degrade all the NAs under analysis except the 1,2,3,4-tetrahydro-2-naphthoic acid, while *Synechococcus leopoliensis* showed only tolerance towards all the tested NAs. In particular, *Dunaliella tertiolecta* showed the ability to grow on CHBA producing CHAA as metabolic intermediate. By GC-MS analysis of the growth medium derived from algal cultures treated with CHAA as sole carbon and energy source, cyclohexylidene acetic acid was found to be a degradation intermediate, as previously reported for the CHAA-degrading bacterium *Arthrobacter* sp. CA1 [Ougham *et al.;* 1982]. This evidence has suggested that *D. tertiolecta* was capable of CHAA degradation

exploiting a similar metabolic pathway to that described for *Arthrobacter* sp. CA1. *D. tertiolecta* cultures incubated in the presence of CHPA revealed that CHCA was an intermediate of degradation. CHCA was completely degraded via β-oxidation, similarly to that described for PRWl19 strain [Blakley et al.; 1978]. Conversely, algal cultures could not degrade the highly recalcitrant 1,2,3,4-tetrahydro-2-naphthoic acid, despite the fact it has the same molecular weight of CHBA. This suggested that NA persistence is influenced by the ring composition and not by the molecular weight of the molecule [Quesnel et al.; 2011].

In summary, all these data on the degradation of model NAs demonstrate the requirement for more research work in order to clarify the complexity of NAs degradation pathways.

4. The genus *Rhodococcus*

Rhodococci are Gram-positive, non-sporulating, aerobic bacteria. They are classified into the suprageneric actinomycetes group known as mycolate-containing nocardioform, also including the genera *Mycobacterium*, *Nocardia* and *Corynebacterium* [Finnerty et al.; 1992]. *Rhodococcus* genus includes genetically and physiologically diverse bacteria, which are able to colonize various habitats, from the sea level [Bell et al.; 1998] to Alpine soils [Margesin et al.; 2003], and from the deep sea [Heald et al.; 2001] to coastal sediments [Langdahl et al.; 1996] and Arctic and/or Antarctic environments [Whyte et al.; 2002, de Carvalho et al.; 2005]. Some *Rhodococcus* strains are also pathogens; for instance, *R. fascians* causes the formation of leaf gall in many plants while *Rh. equi* is an equine pathogen with the ability to infect other domestic animals [Gurtler et al.; 2004].

Rhodococcus genus members have many peculiar properties that can be summarized as following:

- a peculiar cell wall, containing long aliphatic chains of mycolic acids, which facilitates the uptake of hydrophobic substrates into the cells. The presence of diverse organic compounds

in the growth medium may induce changes in the fatty acids composition of the membrane lipids and can alter the fluidity of the cell envelope. Notably, the ability to modulate the fatty acids composition of the cell envelope is important for the resistance of *Rhodococcus* cells to many toxic compounds [de Carvalho et al.; 2005b].

- the capacity to produce surfactants improving the metabolism of hydrophobic substrates. Surfactants, such as trehalose-containing glycolipids, promote the adhesion of cells to hydrophobic phases in a two-phase system, decreasing the interfacial tension between phases and hydrophobic compounds [Finnerty et al.; 1992, de Carvalho et al.; 2005b];

- the ability to persist under stress conditions such as starvation [Martínková et al.; 2009] or after desiccation [LeBlanc et al.; 2008];

- the capacity to degrade pollutants though in the presence of more easily degradable carbon sources [Bell et al.;1998, Martínková et al.; 2009];

- the high frequency of recombination described in some *Rhodococcus* strains contributes to the plasticity of their genomes improving the ability to acquire new genes (by horizontal gene transfer) and consequently, new enzymatic activities [Larkin et al.; 2005].

- the large genomes of *Rhodococcus* strains, such as *Rhodococcus jostii* RHA1 with a genome around 9.7 Mb in size, provide a redundancy of catabolic pathways [McLeod et al.; 2006]. Moreover, *Rhodococci* also contain mega-plasmids carrying a large number of catabolic genes [McLeod et al.; 2006]. The wide range of *Rhodococcus* metabolic activities includes the degradation of alkanes and aromatic hydrocarbons, biotransformation of steroids and a significant set of xenobiotic compounds [Martínková et al.; 2009], antibiotics along with amino acids production [Wakisaka et al.; 1980, Yamada et al.; 1973], lignin degradation [Andreoni et al.; 1991], chemo-litho-autotrophic growth in the presence of hydrogen and carbon dioxide [Reh et al.; 1981], and production of biosurfactants [Bicca et al.; 1999, Philp et al.; 2002].

In summary, since *Rhodococcus* strains are characterized by numerous of enzymatic activities, unique and peculiar cell wall structure and suitable biotechnological properties, they are ideal organisms to be employed for bioconversion and biodegradation of many organic compounds in environmental remediation and in the pharmaceutical and chemical industries [Bell et al.; 1998, Larkin et al.; 2005, Van der Geize et al.; 2004]. Unfortunately, most of the genetic systems and regulatory mechanisms of genes and proteins required for these degradation/biosynthetic pathways in *Rhodococcus*, are still far from being understood. Indeed, the progress in *Rhodococcus* genetics and biochemistry is limited by the following features, namely:

- the high rhodococcal genetic diversity creates obstacles for finding molecular tools applicable to all *Rhodococcus* spp. [Shao et al.; 1995, Powell et al.; 1998].
- the recalcitrance of *Rhodococcus* strains cell walls to mechanical and chemical treatments hampers both the nucleic acids extraction and the introduction of exogenous DNA [Larkin et al.; 2005];
- the pleomorphism whereby many strains grow as short rods, cocci or branched multinucleated filaments [Williams et al.; 1976, Powell et al.; 1998] leads to problems in the segregation of mutants [Larkin et al.; 2005];
- the genomic instability may create problems with illegitimate integration upon electroporation of *Rhodococcus* cells with exogenous DNA [Larkin et al.; 2005];
- the high GC content genome creates problem in PCR amplification and DNA-DNA/ or RNA-DNA hybridization techniques;
- the presence of effective endogenous restriction systems that recognize unmethylated sites in exogenous DNA can cause the cleavage and the subsequent degradation of newly introduced

DNA with the consequence of low 'fertility' of some *Rhodococcus* strains in intergenic matings and/or transformation [Denome et al.; 1993, Schäfer et al.; 1990];

- the peculiar genetic codon usage by which the start codon of gene translation is often a GTG triplet instead of the typical ATG triplet and by which stop and start codons of consecutive genes clustered in operons often overlaps.

These genetic aspects can hamper the heterologous expression of rhodococcal proteins in *E. coli* or *Pseudomonas* strains that are the typical host for protein functional experiments because of the broad knowledge about their protein expression mechanisms and the significant amount of expression vectors that exist for these genera.

Due to the above reported limitations, and despite the importance of many potentially valuable *Rhodococcus* strains, the genetic analysis of this genus has hindered for a long time by the lack of efficient molecular tools [Sallam et al.; 2006]. During the last decade, several efforts have been towards the development of genetics strategies for the manipulation of *Rhodococcus* members, namely: the construction of *E. coli-Rhodococcus* shuttle vectors [Matsui et al.; 2007, Hirasawa et al.; 2001, Nakashima et al.; 2004], the development of transposon systems to create random transposon libraries [Sallam et al.; 2006, Fernandes et al.; 2001, Mangan et al.; 2001] and unmarked mutagenesis deletion systems with *Sac*B as counter selection to generate mutants as a result of two consecutive DNA single crossover events [Jager et al.; 1995, Denis-Larose et al.; 1998, Van der Geize et al.; 2001]. Additionally, the following approaches have been recently applied in *Rhodococcus* strains: transcriptomic and proteomic techniques [Goncalves et al.; 2006, Hara et al.; 2007, Patrauchan et al.; 2005], analyses of regulator-operator interactions [Eulberg et al.; 1998, Nga et al.; 2004], studies of transcription using reporters [Nga et al.; 2004, Veselý et al.; 2007, Takeda et al.; 2004, Knoppová et al.; 2007], development of systems for the overexpression of genes involved in

key catabolic pathways and enzymes [Veselý et al.; 2003, Lessard et al.; 2004, Nakashima et al.; 2004, Na et al.; 2005].

The genomes of several *Rhodococcus* strains such as *Rhodococcus jostii* RHA1 [McLeod et al.; 2006], *Rhodococcus opacus* B-4 [Takarada unpublished] and *Rhodococcus erythropolis* PR4 [Sekine et al.; 2006] have recently become available on public databases. The genome sequences revealed very large and complex genomes, partly owing to the presence of (multiple) large (linear) plasmids along with catabolic complexity and diversity.

In conclusion, further efforts to improve *Rhodococcus* genomic knowledge and to develop new and more efficient tools for genetic engineering of this genus are needed. Breakthroughs in *Rhodococcus* genetics and molecular biology will support attempts to construct *Rhodococcus* strains with suitable properties for environmental and biotechnological applications [Van der Geize et al.; 2004].

4.1. NAs degradation by *Rhodococcus* spp.

Thanks to their metabolic capability and versatility, *Rhodococcus* genus, along with other closely related high-GC actinomycetes (*Mycobacterium*, *Corynebacterium*, *Gordona*, *and Nocardia*), are widely recognized as ideal candidates for the biodegradation of pollutant compounds found in petroleum deposits [Yu et al.; 2006, Quek et al.; 2006].

Presently, only one experimental work is available on the *Rhodococcus* spp. ability to grow on NAs [Demeter et al.; 2014]. These authors isolated two *Rhodococcus* spp. from a microbial community derived from Athabasca OSPW, *Rh*. sp. MTF and *Rh*. sp. OSPW strains, which were shown to belong to *Rh*. *erythropolis* and *Rh*. *fascians* species, respectively. In addition to MTF and OSPW strains, other isolates were obtained belonging to *Cyanobacteria*, *Pseudomonas*, *Xanthobacter*, and *Ancylobacter* genera. The microbial

community was able to grow on a mixture of CHCA and CHAA, while none of the isolates could degrade these NAs. [Demeter et al.; 2014].

A few reports describe the ability of *Rhodococcus* spp. to degrade alicyclic aliphatic hydrocarbons that can be considered the chemical backbones of most of the NAs structures. Lee *et al.* (2007) characterized a microbial consortium able to utilize cyclo-hexane as sole carbon and energy source. Microorganisms isolated from the microbial consortium were tested for their ability to degrade cyclo-hexane. The 16S rDNA of the most efficient cyclo-hexane-degrading strain shared 97% of similarity with the 16S rDNA of the nitrile-metabolizing bacterium *Rhodococcus* USA ANO 12 [Brandao et al.; 2002]. This new strain, named as *Rh*. sp EC1, was characterized by a versatile metabolism, being able to utilize structurally diverse compounds, including high carbon number diesel and lubricant oils [Lee et al.; 2007].

Rh. sp. EC1 cyclo-hexane degradation occurred via aromatization pathway. In particular, 2-cyclohexen-1-one, gamma-butyrolactone and phenol were identified as intermediates by GC-MS analysis of the growth medium of *Rh*. sp. EC1 culture incubated in presence of cyclo-hexane. It has been suggested that the cyclo-hexane can be degraded via aromatization pathway. Since that gamma-butyrolactone is considered as an intermediate of tetrahydrofuran (THF), and that THF was not directly detected as a metabolite of cyclo-hexane, the degradation pathway of THF was investigated to confirm the aromazation of cyclo-hexane as main removal mechanism of aromatic hydrocarbons by *Rh*. sp. EC1. The results showed that *Rh*. sp. EC1 was able to degrade THF producing 2,3-hydrofuran and lactone as intermediates. These evidences support the hypothesis that *Rh*. sp. EC1 could degrade cyclo-hexane via aromatization pathway (Fig. 4.1.1) [Yi et al.; 2011].

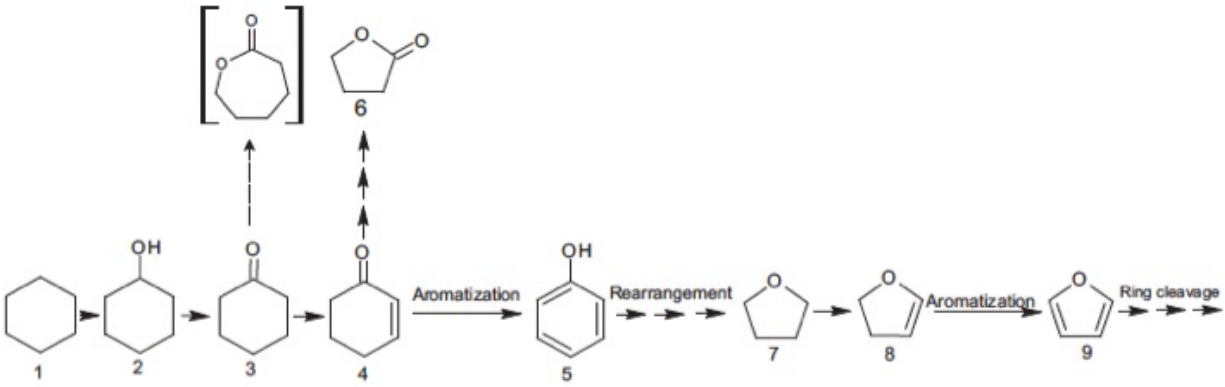

Fig. 4.1.1 Proposed cyclohexane degradation pathways by *Rhodococcus* sp. EC1; (1) cyclohexane (2) cyclohexanol (3) cyclohexanone (4) 2-cyclohexen-1-one (5) phenol (6) gamma-butyrolactone (7) tetrahydrofuran (8) 2, 3-hydrofuran (9) furan [Yi et al.; 2011].

Another mechanism described for the ring cleavage of alicyclic compounds is achieved through lactone formation. For example, *Rh.* NDKK48 was capable of cyclo-hexane and methyl-cyclo-hexane degradation when it was cultivated in the presence of hexadecane as co-carbon source. In particular, this *Rhodococcus* strain cleaved the ring of methyl-cyclo-hexane producing the following intermediates: 4-methylcyclohexanone, 4-methyl-2-oxepanone, and 3-methyladipic acid. The formation of 4-methyl-2-oxepanone indicated that the ring cleavage was performed by Baeyer-Villiger oxidation [Koma et al.; 2005]. Interestingly, a similar pathway was described regarding the degradation of cyclododecane by *Rhodococcus ruber* CD4 which was isolated from a mixed culture enriched with cyclododecane as unique carbon and energy source [Schumacher et al.; 1999]. On the basis of the metabolic intermediates detected, a putative pathway for the degradation of cyclododecane degradation by *Rh*. *ruber* CD4 was proposed. The substrate was initially hydroxylated to cyclododecanol and then dehydrogenated to the keto-cyclododecanone. This alicyclic ketone was then subjected to a Baeyer-Villiger oxidation, resulting in the formation of the lactone oxacyclo-tridecan-2-one, which was converted into 1-12-dodecandioic acid by two subsequent dehydrogenation steps (Fig. 4.1.2) [Schumacher et al.; 1999].

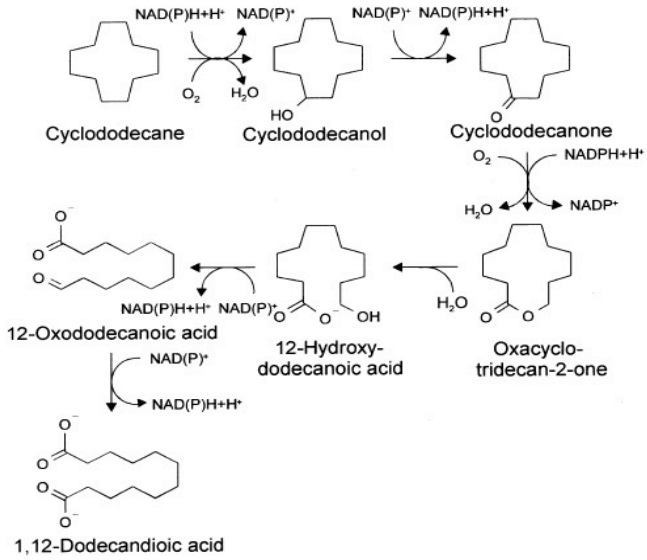

Fig. 4.1.2 *Rhodococcus* ruber CD4 proposed pathway for the degradation of cyclododecane [Schumacher et al.; 1999].

Baeyer-Villiger oxygenases are enzymes responsible for the homonym reaction that forms an ester from a keton or a lactone from a cyclic ketone. Kostichka and colleagues (2001) showed data about the characterization of a gene cluster involved in cyclododecanone degradation in *Rh. ruber* strain SC1. This *Rhodococcus* strain was isolated from a mixed culture derived from an industrial wastewater bioreactor by selecting for microbes that can utilize cyclododecanone as the sole carbon source. The characterization of *Rh. ruber* SC1 gene cluster revealed genes coding for cyclododecanone monooxygenase, an esterase, an alcohol dehydrogenase, and an aldehyde dehydrogenase. The four genes were cloned in suitable vectors for *E. coli*, and the enzyme activities were evaluated analyzing the intermediates produced during biotransformation experiments with cyclododecanol or cyclododecanone as substrates. The first two genes encode for the enzymes catalyzing the first two steps of the cyclododecanone oxidation. The last two genes encode for two different dehydrogenases responsible for the formation of docecanedioic acid from the oxidation of 12-hydroxylauric acid. On the basis of the intermediates produced during these experiments, a postulated pathway for *Rh. ruber* SC1 was proposed (Fig. 4.1.3) [Kostichka et al.; 2001].

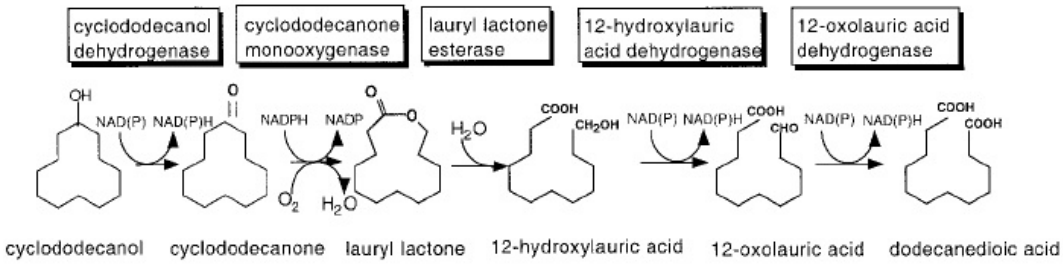

Fig. 4.1.3 Postulated pathway for the degradation of cyclododecanol performed by *Rh. ruber* SC1. The boxes above the reactions indicate the enzymes required for each step of dergadation [Kostichka et al.; 2001].

Few information are presently available about the cyclopropane biodegradation. In this respect, in 1995 a strain of *Rhodococcus rhodochrous* able to grow on cyclopropanecarboxylate was isolated [Nishihara et al.; 1995], and the putative degradation pathway was described [Toraya et al.; 2004]. Cell extracts obtained from *Rh. rhodochrous* cells grown on cyclopropanecarboxylate were tested in order to assess their ability to catalyze the oxidation of cyclopropanecarboxylate with NAD^+. The results showed that the reduction of NAD^+ to NADH was coupled with oxidation of cyclopropanecarboxylate, and the reduction was dependent on ATP, CoA and cyclopropanecarboxylate. By TLC analysis of the growth medium of *Rh. rhodochrous* grown on cyclopropanecarboxylate acid, intermediates such as cyclopropanecarboxyl-CoA, and 3-hydroxybutyryl-CoA, were identified. Moreover, putative CoA thioesters (3-hydroxybutyryl-CoA, and crotonyl-CoA) were examined in absence of ATP and CoA, in order to assess if they were substrates for the oxidation system in the cell extracts. The results showed that both putative intermediates were oxidized with the reduction of NAD^+ to NADH. These findings suggested that *Rh*. *rhodochrous* was able to degrade cyclopropanecarboxylic acid by β-oxidation sequences, involving CoA thioester derivatives, before ring opening (Fig. 4.1.4) [Toraya et al.; 2004].

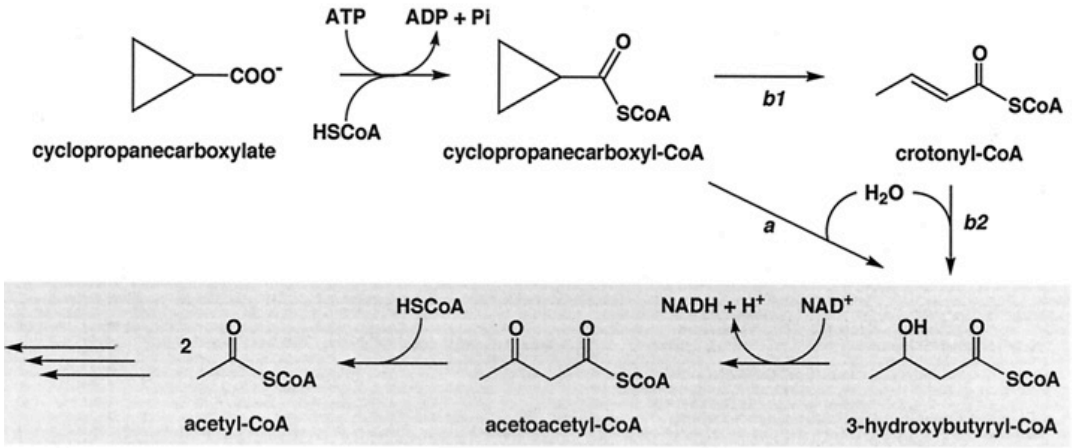

Fig.4.1.4 Postulated pathway for the degradation of cyclopropane carboxylate by *Rh. rhodochrous* [Toraya et al.; 2004].

In conclusion, *Rhodococcus* species thanks to their wide metabolic properties and growth versatility, are able to degrade several alicyclic compounds, which have different structural complexity, in addition to other pollutants such as *n*-alkanes and aromatic compounds present in fuel oils [Cappelletti et al.; 2011, Auffret et al.; 2009, Kim et al.; 2011].

4.3. *Rhodococcus* sp. BCP1

Rhodococcus sp. strain BCP1 (DSM 44980) was isolated from an aerobic *n*-butane-grown consortium [Frascari et al.; 2005]. It is able to cometabolically degrade chloroform, vinyl chloride, and trichloroethylene [Frascari et al.; 2006, Cappelletti et al.; 2012]. BCP1 is also able to catabolize a wide range of aliphatic, alicyclic, and carboxylated alkanes. Due to its metabolic plasticity, it represents a strain of considerable environmental and industrial interest [Cappelletti et al.; 2011].

In the past, BCP1 resting cells pre-grown on *n*-butane were exposed to different concentrations of short-chain halogenated hydrocarbons in order to define the kinetic parameters relative to the degradation processes (Frascari et al., 2006). The results showed that BCP1 cells were able to degrade chloroform concentrations up to 119 mM (14.2 mg/L).

Notably, BCP1 strain showed the highest ability to tolerate chlorofom toxicity among the bacterial strains described in the literature [Cappelletti et al.; 2012]. BCP1 was also able to transform vinyl chloride and trichloroethylene while did not show any degradation ability towards 1,2-trans-dichloroethylene. Cappelletti and colleagues (2011) also showed that BCP1 was able to grow on medium- and long-chain alkanes ranging in length from C_{12} to C_{30}. The *Rh*. sp. BCP1 *AlkB* gene cluster was described and the activity of the *alkB* promoter was assayed by cloning the promoter region together with a promoterless *lacZ* gene into a suitable vector. This construct was then introduced in *Rh*. sp. BCP1 cell by electroporation. The data obtained revealed that among the substrates tested (C_6-C_{22}), those in the C_{12} to C_{16} range were the most efficient inducers of the *alkB* promoter. In addition, the *alkB* promoter was not negatively affected when alternative carbon sources (LB, glucose, or succinate) were present together with the *n*-alkane inducer (*n*-hexane or *n*-dodecane) [Cappelletti et al.; 2012].

On the basis of the 16s rRNA sequence, *Rh.* sp. BCP1 appears to be closely related to *Rhodococcus aetherivorans* and *Rhodococcus ruber* (Frascari et al., 2006). Recently, a phylogenetic study was done to assess the presence of a dominant *alkB* gene among *Rhodococcus* species. On the basis of the *Rhodococcus alkB* gene phylogenetic analysis, the results showed that *Rh.* sp. BCP1 belongs to the *aetherovorans* species [Táncsics et al.; 2015].

Rh. sp. BCP1 genome was recently sequenced by 454 Sequencing Technology (Roche GS FLX Titanium). The genome is around 6.2 Mb in size with a G+C content of 70.4%. Two scaffolds constituting a total of ~0.2 Mb are likely to be plasmids, as they carry genetic signatures typical of *Rhodococcus* plasmids. NOTE: (the whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. AVAE00000000) [Cappelletti et al.; 2013].

4.4. Rhodococcus opacus sp. R7

Rhodococcus opacus R7 was isolated from a soil sample contaminated by polycyclic aromatic hydrocarbons, in Italy, by enrichment culture techniques using naphthalene as sole carbon and energy source. This *Rhodococcus* strain showed the ability to degrade not only naphthalene but also *o*-xylene that is the xylene isomer most recalcitrant to microbial degradation [Di Gennaro et al.; 2001].

Two genomic regions involved in naphthalene (*nar* gene cluster) and salicylate (*gen* gene cluster) degradation were identified in *Rhodococcus opacus* R7 (Di Gennaro et al.; 2011). The gene organization of these two regions was described and RT-PCR experiments were performed in order to define the range of substrates (naphthalene and salycilate) inducing the expression of the gene clusters. RT-PCR data indicated that both naphthalene and salicylate induced the transcription of the genes involved in naphthalene degradation. The genes involved in the salicylate metabolism were induced only by salicylate. Due to this, it was evident that the salicylate could be degraded independently from naphthalene [Di Gennaro et al.; 2011].

Rh. opacus R7 which is known to grow and degrade xenobiotic compounds such as naphthalene and *o*-xylene, can also grow on *n*-alkanes ranging from C_{10} to C_{36}, while no growth has been observed when *n*-alkanes in the range C_6-C_8 were added to the minimal medium. Notably, the *alk* gene cluster responsible for *n*-alkane degradation in *Rhodococcus opacus* R7 was identified and characterized [Zampolli et al.; 2014]. The R7 *alkB* gene was cloned into the *E. coli*/*Rhodococcus* shuttle vector and transferred in *Rhodococcus erythropolis* AP in order to evaluate the alkane degradation in comparison to the wild type strain. Resting cells of *Rh*. *erythropolis* AP incubated in the presence of C_{12} revealed that the percentage of biodegradation in 6 h was near 80% in the recombinant strain and 37% in the

wild type strain. These data suggested the involvement of the *alkB* gene regarding the *n*-alkane degradation in R7 strain.

Recently, *Rhodococcus opacus* R7 genome was sequenced by 454 Sequencing Technology (Roche GS FLX Titanium). The total genome size is around 10.1 Mb with a G+C content of 66%. Five scaffolds, constituting a total of ~1.65 Mb, are likely to be plasmids, as they carry genetic signatures typical of *Rhodococcus* plasmids; NOTE: the whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession numbers CP008947, CP008948, CP008949, CP008950, CP008951, and CP008952) [Di Gennaro et al.; 2014].

Aims

Naphthenic acids (NAs) belong to an important and heterogeneous group of organic contaminants, highly toxic and recalcitrant to degradation. Moreover, concerns about NAs are associated to their wide distribution and significant impact from the environmental and economic point of view. Nevertheless, little is known about their biodegradability. Moreover, considering the lack of information on the mechanisms through which these pollutant compounds can be degraded, NAs contamination issue is an important and actual challenge. It is clear that the development of efficient bioremediation strategies aimed to reduce both the concentration and toxicity of NAs in the environment is necessary. To this end, it is crucial to extend the knowledge on microorganisms capable of degrading NAs as well as the molecular mechanisms that underlie these processes.

Rh. sp. BCP1 and *Rh. opacus* R7, belonging to the *Rhodococcus* genus, have been described in the past for their capacity in the degradation of different pollutant compounds [Frascari et al.; 2006, Cappelletti et al.; 2011, Di Gennaro et al.; 2001, Zampolli et al.; 2014]. In order to get deep into the peculiar metabolic and genetic properties of BCP1 and R7 strains, the present PhD Thesis's work investigated on the ability of these *microorganisms* to grow and degrade NAs such as cyclohexanecarboxylic acid (CHCA), cyclopentanecarboxylic acid (CPCA), methyl-cyclohexanecarboxylic acid (m-CHCA), and cyclohexaneacetic acid (CHAA).

Fatty Acid Methyl Esters (FAMEs) analysis was performed to define the membrane lipid composition in BCP1 and R7 cells growing on NAs compared to succinate. These modifications are supposed to occur as mechanism of cell adaptation to counteract the toxicity derived from NAs. In order to further evaluate this toxic effect on cell features, the

morphological changes of BCP1 and R7 cells were also assessed through Transmission Electron Microscopy (TEM).

The induction of putative genes, and the construction of a random transposon mutagenesis library were also carried out to reveal the mechanisms by which these *Rhodococcus* strains can degrade toxic compounds such as NAs.

5. General Materials and Methods

5.1. Bacterial strains, media and growth conditions

Table 1: Bacterial strains, plasmids for general applications.

Bacterial strains	**Relevant genotype or characteristics**	**Reference**
Escherichia coli DH5α	supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	[Hanahan, et al.; 1983]
Rhodococcus sp. BCP1	Ability to grow on n-alkanes and co-metabolize low chlorinated solvent	[Frascari et al.; 2005]
Rhodococcus opacus R7	Ability to grow on aromatic hydrocarbons and utilizes naphthalene and o-xylene	[Di Gennaro et al.; 2001]
Plasmids	**Relevant genotype or characteristics**	**Reference**
pSC-A	Strataclone® cloning vector ampr/kmr	Agilent technologies
pNC9503	Shuttle vector E.coli/Rhodococcus thior/kmr	[Kalscheuer et al.; 1999]
pTNR-TA	Non replicant trasposon vector ampr/thior, IstA, IstB, Pnit promoter, IS border (left & right), duplicated target DNA, pGEM3ZM ori region	[Sallam et al.;2007]

Table 2: Antibiotic stock solutions.

Antibiotic stock solution	**Concentration**	
	E. coli	*Rhodococcus* sp.
Ampicillin, 50 mg/ml, water solution	50 µg/mL	-
Kanamycin, 50 mg/ml, water solution	25 µg/mL	-
Thiostrepton, 25 mg/ml, DMSO solution	-	5 or 10 µg/mL
Isoniazid, 50 mg/ml, water solution	-	6 µg/mL

Antibiotic stock solutions were prepared as reported in Table 2. The solutions were stored at -20 °C in 1 ml aliquots until use.

Liquid cultures of all strains were grown in agitation at 150 rpm at the optimum temperature (i.e. *E. coli* at 37 °C while *Rhodococcus* sp. BCP1 and *Rhodococcus opacus* R7 at 30°C for).

The composition of the media used in this study are reported in Table 2

Table 3: Composition of the media.

Media	Composition	Concentration
Luria Bertani (LB) pH 7-7.2	Tryptone	10 gr/L
	NaCl	10 gr/L
	Yeast Extract	5 gr/L
	H_2O	to volume
Mineral Salts Medium (MSM) pH 7 (Schlegel 1961)	Na_2HPO_4 x 12 H_2O	9 gr/L
	KH_2PO_4	1,5 gr/L
	NH_4Cl	1 gr/L
	$MgSO_4$ x 7 H_2O	0,2 gr/L
	Fe(III) NH_4-Citrate	1,2 mg/L
	$CaCl_2$ x 2 H_2O	20 mg/L
	Trace elements SL 6	
	$ZnSO_4$ x 7 H_2O	10 mg/L
	$MnCl_2$ x 4 H_2O	3 mg/L
	H_3BO_3	30 mg/L
	$CoCl_2$ x 6 H_2O	20 mg/L
	$CuCl_2$ x 2 H_2O	1 mg/L
	$NiCl_2$ x 6 H_2O	2 mg/L
	Na_2MoO_4 x 2 H_2O	3 mg/L
	H_2O	to volume
Electrocompetent medium for *Rhodococcus sp.* BCP1 pH7	Tryptone	10 gr/L
	NaCl	10 gr/L
	Yeast Extract	5 gr/L
	Glycine	3,5% w/v
	Sucrose	1,8% w/v
	Isoniazid	6 μg/mL
	H_2O	to volume
Electrocompetent medium for *Rhodococcus opacus* R7 pH7	Tryptone	10 gr/L
	NaCl	10 gr/L
	Yeast Extract	5 gr/L
	Glycine	1,5% w/v
	Sucrose	1,5% w/v
	H_2O	to volume

The stock solutions ($MgSO_4$ x 7 H_2O, Fe(III) NH_4-Citrate, $CaCl_2$ x 2 H_2O and Trace elements SL 6 at the concentration of 1000X) were prepared and separately autoclaved.

Mineral salt medium (MSM) was prepared adding Na_2HPO_4 x 12 H_2O, KH_2PO_4 and NH_4Cl, after adjusting the pH, it was autoclaved at 121ºC for 20 minutes. Subsequently, it was mixed with volumes of stock solutions of $MgSO_4$ x 7 H_2O, Fe(III) NH_4-Citrate, $CaCl_2$ x 2 H_2O and Trace elements SL6 in order to have a final concentration of 1X.

For growth on solid media, agar was added at the final concentration of 15 g/L.

5.2. Extraction of genomic DNA from Rhodococcus spp.

The genomic DNA was isolated from BCP1 cells using the protocol here described. A 100 mL culture grown on LB media to an OD_{600}=1.0 was centrifuged at 7000 rpm at 4°C for 10 minutes and washed twice with 1 mL of TE buffer (20 mM Tris, 10 mM EDTA pH 8.0). The cell pellet was resuspended in 500 µL of TE (20 mM Tris, 10 mM EDTA pH 8.0) containing lysozyme at the concentration of 30 mg/mL. Before being incubated 1 hour at 37ºC, mutanolysin (final concentration of 100U/mL) and proteinase K (final concentration of 1mg/mL) were also added to the solution. At the end of the incubation, 150 µL of a 10% SDS solution were added and the suspension was mixed and incubated in a water bath at 65°C for 5 min.

The cell suspension was centrifuged at 13000 rpm for 5 min at 4 °C, and the aqueous phase was collected. One volume of a phenol-chloroform-isoamyl alcohol 25:24:1 v/v mixture was added and the sample was vortexed for 30 seconds. The water phase containing the genomic DNA was separated from the organic phase and cell debris by centrifugation at 13000 rpm at 4 °C for 5 minutes. The extraction was repeated twice and phenol traces were removed by adding 1 volume of a 24:1 v/v mixture of chloroform-isoamyl alcohol. The aqueous phase

was recovered after centrifugation at 13000 rpm at 4ºC for 5 minutes and 0.1 volumes of 3 M sodium acetate (pH 4.8) and 0.7 volumes of isopropanol were added. Extracts were incubated for 10 min at room temperature and then they were centrifuged for 30 min at 4ºC at 13000 rpm. The DNA pellet was washed with ice-cold EtOH 70% and finally it was dried out. The resuspended genomic DNA was stored at -20ºC and the aliquot to use in each experiment was previously treated with RNase at 37ºC for 30 minutes.

5.3. RNAs isolation procedure

The protocol for the RNA isolation was based on the "Protocol for the isolation of total RNA from *E. coli* for microarray" with several modifications. Moreover all steps were performed swiftly to minimise RNA degradation and RNase free or DEPC treated solutions, RNase free microfuge tubes and RNase free filter pipette tips were used.
(http://derisilab.ucsf.edu/data/microarray/pdfs/Total_RNA_from_Ecoli.pdf)
Each frozen pellet was thawed on ice and washed twice in TE buffer (20 mM Tris, 10 mM EDTA pH 8.0). After this step, the cell pellet was resuspended in 500 µL of TE buffer containing:

- Lysozyme at the final concentration of 30 mg/ml;
- Proteinase K at the final concentration of 30 mg/ml;
- Rnase inhibitor (RNasin Plus RNase Inhibitor – Promega) at the final concentration of 1U/50µl;

The cells thus resuspended were incubated for 30 minutes at 30 °C.
After this incubation incubation step, 200µl of an SDS 10% solution was added to the sample and mixed by vigorous inversion and incubated in a water bath for 5 minutes at 65 °C. Subsequently, 200µl of RLT buffer (QIAGEN) supplied of β-Mercaptoethanol according to the manufacturer's instructions, was added to the sample and mixed by vortex and inversion

for 10 minutes at room temperature.

The cell suspension was centrifuged at 13,300 rpm for 4 minutes at 4 °C, and the aqueous phase collected in a new tube. 3M NaOAc pH 5.2 solution (1/10 of the volume) and acidic phenol (1 volume) were added to the sample.

Sample was mixed for inversion and incubated in a water bath for 6 minutes at 65 °C, inverting tube every 40 seconds. After this treatment, the sample was centrifuged for 10 minutes at 13,300 rpm at 4 °C, and the aqueous phase was collected in a new tube and was added an equal volume of chloroform. After vigorous inversion, the sample was centrifuged at 13,300 rpm for 5 minutes at 4 °C. Chloroform treatment was repeated twice to remove as much as possible phenol traces. After phenol-chloroform extraction, 3M NaOAc pH 5.2 solution (1/10 of the volume) and 2 volumes of cold 100% EtOH were added to the aqueous phase containing total RNAs. The sample was incubated at -80ºC over night and the day after it was centrifuged at 13,300 rpm for 30 minutes at 4ºC. The pellet was then washed with 1 mL of 80% cold ethanol solution and it was dried at 37 °C for 15-20 minutes.

The total RNAs were treated twice with 4U of Q1 RNase-Free DNase (Promega) for 45 minutes at 37°C. RNAs DNA-free were cleaned by phenol-chloroform purification before being used for further experiments.

5.4. DNA manipulations and genetic techniques

All restriction digests, ligations, cloning and DNA electrophoresis, were performed using standard techniques [Sambrook et al.; 1989]. Taq polymerase, restriction endonucleases and T4 DNA ligase were used as specified by the vendors (Roche or Promega, Milan, Italy). The Strataclone cloning vector (Agilent technologies) was routinely used and recombinant plasmids were introduced into StrataClone SoloPack Competent Cells by transformation thermal shock mediated.

To detect the presence of insert DNA, X-Gal was added to agar media at a final concentration of 40 µg/ml. X-Gal stock solutions were prepared at a final concentration of 40 mg/ml in N-N-dimethylformamide and stored as 1 ml aliquots at - 20 °C protected from light.

Kits for plasmid mini- and midi-preps, PCR purification and DNA gel extraction were obtained from QIAGEN (Milan, Italy) and used according to the manufacturer's instructions.

5.5. Electroporation of Rhodococcus species.

DAY 1

A 10-mL culture of *Rh* sp. BCP1 or *Rh opacus* R7 is inoculated in LB.

DAY 2

500 µl of the overnight culture of *Rh* sp. BCP1 or *Rh opacus* R7 is transferred to 50 mL of electrocompetent media described in table 3 for both *Rhodococcus* species

DAY 3

At an OD_{600} approximately of 0,6, 3 µg/mL of ampicillin is added to the BCP1 culture, and incubated for 1.5 hours at 30 °C with shaking (150 rpm), while R7 culture is ready for further processing.

Subsequently, the cells are collected by centrifugation for 10 minutes at 6,000 rpm at 4ºC.

The cells are washed twice with 25 mL ice-cold EPB1 (20 mM Hepes pH 7.2, 5% glycerol) and once with 10 mL ice-cold EPB2 (5mM Hepes pH7.2, 15% glycerol). The cells are harvested by spin at 6,000 rpm for 10 minutes at 4ºC and after discarding the supernatant, they were suspended in 1-2 mL EPB2. 200 µL of cells were mixed with 200 µL of EPB2 buffer in cold sterile tubes on ice and immediately used for the electroporation.

1 µg of foreign DNA was added to the cells and transferred in 0.2 cm-cuvettes (Biorad). The electroporation was performed with Eporator (EPPENDORF) as follows: 2.5 kV, 25 µF, and 400 Ω. Pulsed cells were immediately supplied with 1 mL of LB and transferred into new 2

mL tube. The cells were regenerated for 5-6 hours with shaking at 150 rpm at 30ºC, before they were plated onto LB agar plates containing the appropriate antibiotic. Resistant transformants were available after 5 days of incubation at 30°C.

6. Metabolic and physiological aspects of *Rhodococcus* single species growing on NAs

The microbial NAs degradation by *Rhodococcus* species is poorly understood. Demeter *et al.* (2014) have recently provided evidence on NAs degradation by a microbial consortium, in which members of the genus *Rhodococcus* were part of this microbial community. In this respect it is interesting to note that due to the hydrophobic nature of NAs, bacterial cells are likely to modify their lipid membrane composition, as a mechanism of adaptation, to counteract these hydrophobic pollutants as shown in the past for other hydrophobic molecules [Tsitko et al.; 1999, Gutierrez et al.; 1999]. Based on this prediction, *Rhodococcus* strains (*Rh.* BCP1, and *Rh. opacus* R7) were initially examined to assess their capacity to grow on model NAs [cyclohexanecarboxylic acid (CHCA), methyl-cyclohexanecarboxylic acid (mCHCA), cyclohexaneacetic acid (CHAA), and cyclopentanecarboxylic acid (CPCA)] that are characterized by a different structural complexity. In addition, Fatty Acid Methyl Esters (FAMEs) analysis was performed to evaluate possible changes in the composition of the membrane fatty acids of the *Rhodococcus* species under investigation. Finally, Transmission Electron Microscopy (TEM) analysis was also carried out to verify the presence of morphological changes in *Rhodococcus* cells grown on NAs.

6.1. Materials and Methods

6.1.1. Preparation of Rhodococcus strains inocula

Rhodococcus strains (*Rh.* BCP1, and *Rh. opacus* R7) were cultivated in Mineral Salt Medium (MSM, see chapter 5, table 2) in the presence of different NAs, such as: cyclohexanecarboxylic acid (CHCA), or cyclopentanecarboxylic acid (CPCA), or methyl-cyclohexanecarboxylic acid (mCHCA), or cyclohexaneacetic acids (CHAA). Bacteria stocks were thawed on ice, streaked out on LB agar plates and grown for two days at 30 °C. The biomass was collected with a sterile loop and accurately suspended in 2 mL of saline solution. Cell suspension was centrifuged at 13,300 rpm for 3 min and washed three times in a saline solution. *Rhodococcus* cells were inoculated in 500 mL flasks containing 100 mL of MSM at an initial OD_{600} of 0,05. CHCA, CPCA, and mCHCA at the concentration of 200, 500 or 1000 mg/L, while CHAA 100 or 200 mg/L were added as sole carbon and energy source. The flasks were incubated at 30 °C and stirred (150 rpm) for four weeks.

Growth curves were performed as **C**olony **F**orming **U**nits (CFU) collecting aliquots of the culture every 24h (from 0 to 672 hs). Aliquots of the supernatants were also collected, in order to evaluate the rate of degradation of the carbon sources through GC-MS analysis.

6.1.2. NAs stock solution preparation

The working solution of each NA was obtained by diluting the stock solutions in 0.1 N NaOH. In particular, 10 N NaOH solution (150 µl) was added to the exact amount of the NA stock solution in order to solubilize it. The final volume (15 mL) of the working solution was reached by adding MSM. Working solutions concentrated 30 mg/L or 10 mg/L of each NAs were routinely prepared.

6.1.3. Extraction and derivatization of model NAs

Supernatant (500 µl for each sample) obtained from *Rhodococcus* spp. NAs-grown cells was collected at different times (every 24 hs until to 672 hs). The samples were stored in appropriate vials (Agilent Technologies CL Vial screw 2mL clr WrtOn). To extract NAs from the supernatant, two volumes of dichloromethane were added to the sample and sealed with a Teflon cap to avoid evaporation during the sonication process performed at 4 °C for 1 h. After sonication, the organic phase was collected in a new vial and it was supplied with an appropriate volume of N,O-Bis (trimethylsilyl)-trifluoroacetamide (20 µl each 25 mg/L of NAs) for the derivatization process. By incubating samples in a water bath at 60 °C for 10 minutes, NAs derivatization process was carried out.

6.1.4. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The biodegradation of NAs was evaluated by analyzing their residual concentrations in the supernants through gas chromatography-mass spectrometry, utilizing a column Supelco SPB-5 (30 m x 0,25 mm, d_f 0,25 µm) and He as gas-carrier. Each analysis was carried out through the injection, with a syringe gas-tight, of 2 µl of derivatized sample. The initial temperature of the injector was 70 °C, it was held for 3 min, followed by an increase of 5 °C/min to a final temperature of 280 °C. NAs were identified on the basis of the atomic mass unit and retention time compared to standard peaks at a known concentration. The NAs calibration curve was constructed by analyzing standard NAs ranging from 50 up to 1000 mg/L. The NAs residual concentration was calculated on the basis of the NAs calibration curve (Fig. 6.1.1).

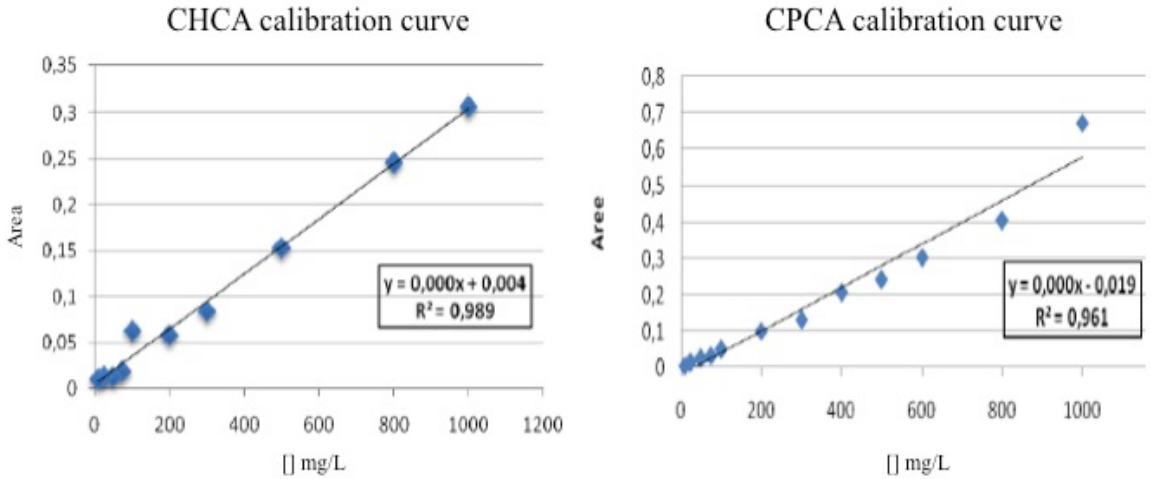

Fig. 6.1.1 Calibration curves for CHCA (left graph), and CPCA (right graph).

For data processing the following softwares were used:

- "The Uscrumbler" was used to normalize peak areas obtained as output through gas chromatography;

- "Origin" was used to select the area under the peak of interest, which was then integrated and subsequently interpolated to the calibration curve;

- "Microsoft Office Excel 2011" was used for the graphics rapresentation of the data obtained.

6.1.5. Fatty acid methyl esters (FAMEs) analysis

Rh. sp. BCP1 and *Rh. opacus* R7 were cultured in MSM supplemented with 500 mg/L of CHCA or CPCA, and Glucose 1 g/L for 72 hs, 96 hs, and 72 hs respectively, at 30 °C in an orbital stirrer (150 rpm). These incubation times correspond to the late exponential growth phase. Bacterial cells were collected from the media by centrifugation at 13,000 rpm for 5 min and washed twice with water. The total lipids were isolated by Folch's method [Folch et al.; 1957].

6.1.6. Preparation of samples for TEM analysis

The strain BCP1 was incubated in the presence of CHCA or CPCA at the concentration of 200 mg/L for 96 hs to reach the optimal cell density, while the strain R7 was incubated for 96 hs in CHCA and 168 hs in CPCA. BCP1 and R7 were incubated on Glucose 1% w/v for 12 hs and 72 hs, respectively, as positive control experiment. The different inoculum were prepared with a delay of 24 hs from each other in order to collect and process the biomass produced at the same time for TEM analysis.

Samples were pre-fixed in a solution of 0.1 M cacodylate buffer (pH = 7.3) containing 2.5% (v/v) glutaraldehyde (buffer A), 0.075% (w/v) of ruthenium red and lysine acetate 0.075 M, for 30 min at 4 °C. After washing with the buffer A (3 times, 10 min each at 4 °C), samples were fixed with buffer A for 2 hs at 4 °C. The washing step was repeated as described above. Then, the samples were post-fixed with a 2% solution (v/v) osmium tetroxide in buffer A for two hours at 4 ° C, washed in the same buffer (3 times, 15 min each at 4 °C), and finally dehydrated in a series of ethanol solutions of increasing concentration. Through the use of the critical point method, using CO_2, the drying was carried out in a Balzers CPD 020 Union. The samples were then infiltrated for three days with decreasing ratios of ethanol-LR white resin (SPI Supplies, West Chester, PA). After this procedure, the samples were embedded in LR White resin and cut with a Reichert Ultracut ultramicrotome (Leica Microsystems Srl, Milan, Italy) using a diamond blade. The thin sections obtained were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX electron microscope (JEOL). The micrographs were acquired by the charge-coupled device camera Olympus SIS VELETA with iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

6.2. *Rhodococcus* spp.: Growth and degradation of model NAs

6.2.1. Cyclohexanecarboxylic acid (CHCA) and Cyclopentanecarboxylic acid (CHCA)

Rh. sp. BCP1 and *Rh*. *opacus* R7 capacity to grow and degrade NAs were evaluated by of CHCA at variable concentration (200, 500, and 1000 mg/L) as the sole source of carbon and energy as compared to the mineral salts medium (Fig. 6.2.1.1, a, b, c, d, e, and f).

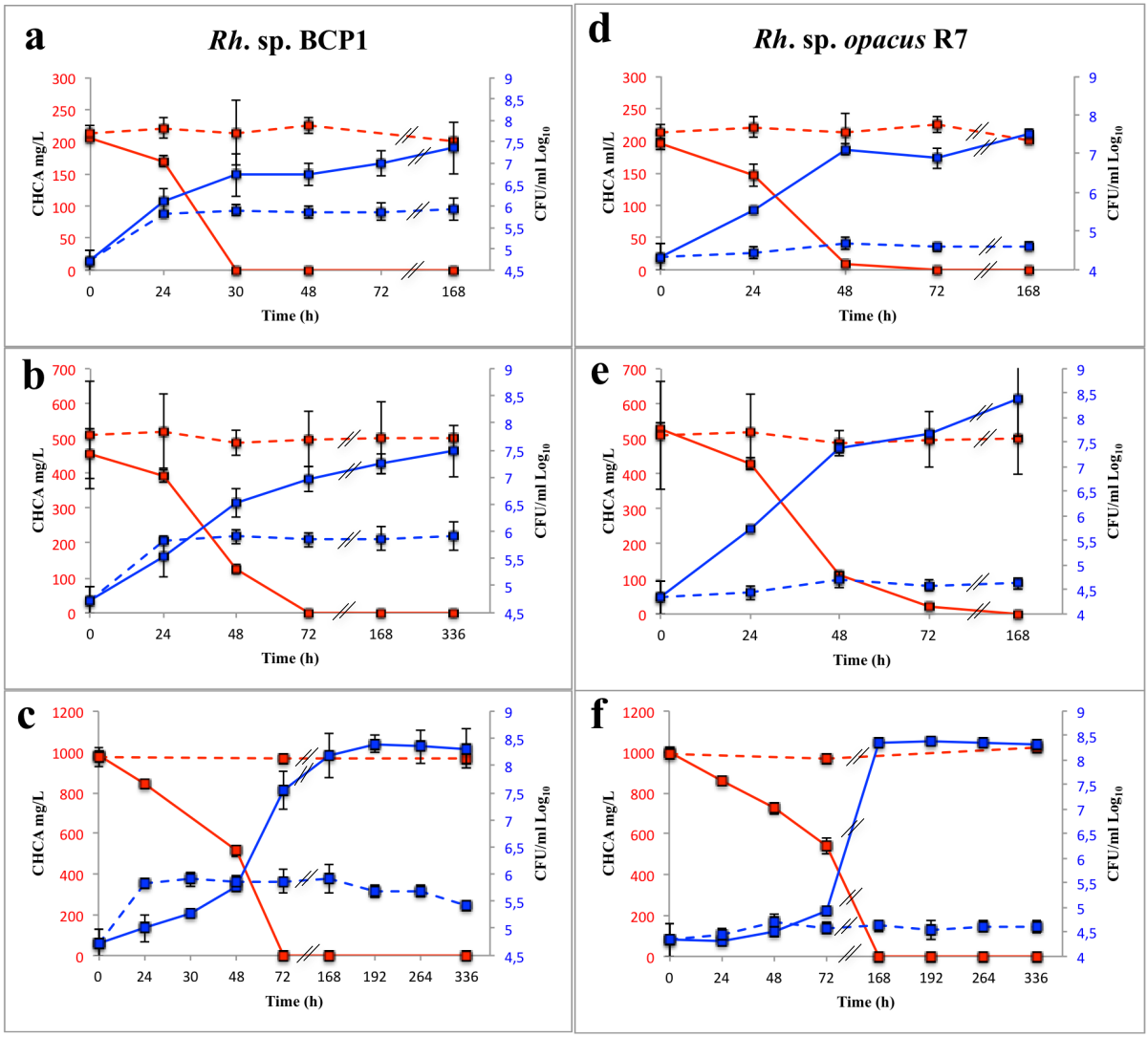

Fig. 6.2.1.1 Growth (continuous blue line) and degradation (continuous red line) curves of *Rh*. sp. BCP1 (a, b and c) and *Rh*. *opacus* R7 (d, e, f) cultures in the presence of CHCA 200 mg/L (a and d), 500 mg/L (b and e) and 1000 mg/L (c and f). Red dashed lines represent the sterile control to asses abiotic substrate degradation; blue dashed lines represent the growth of the *Rhodococcus* strains in MSM without carbon source (MSM).

BCP1 showed a robust growth reaching around $2{,}06x10^8$ CFU/mL when it was grown in the presence of CHCA at the concentration of 1000 mg/L for 168 hs (Fig. 6.2.1.1, c). The highest biomass development ($1{,}2x10^7$ CFU/mL) at 48 hs of growth was seen when cells were incubated with 200 mg/L of CHCA (Fig. 6.2.1.1, a). During a period of 14 days, analysis of the growth medium by GC-MS showed that BCP1 strain was able to degrade 200 mg/L of CHCA within 30 hs (Fig. 6.2.1.1, a), while 72 hs were necessary when CHCA was added at 500 or 1000 mg/L (Fig. 6.2.1.1, b, and c). An initial decrease biomass content ($1{,}88x10^5$ CFU/mL after 48 hs of incubation), and a delayed plateau were observed when BCP1 was incubated with the highest concentration of CHCA. These data suggested that the cytotoxic effect caused by CHCA to the microbial population is dose-dependent. It occurs when bacterial cells are exposed to 1000 mg/L of CHCA causing the decrease in the number of CFU/mL during the initial 30-48 hs.

Strain R7 shows that both growth and degradation rates are comparable to those seen with BCP1 when it was incubated in the presence of 200 or 500 mg/L of CHCA (Fig. 6.2.1, d, and e). R7 cells incubated in the presence of CHCA at 1000 mg/L showed a lag phase of 72 hs, probably due to the CHCA toxicity, and a rapid growth followed by a stationary phase after 168 hs (Fig. 6.2.1.1, f). In particular, no microbial growth was related to the initial CHCA depletion. To this aspect, the phenomenon of CHCA absorption into the cell wall may explain the decrease of the CHCA concentration without any evident cell growth. NAs adsorption into a microbial cell wall was previously reported by Herman *et al.* (1993) studying several carboxylated cycloalkanes produced by the extraction of bitumen from the Athabasca oil sands.

Growth and degradation rates were also evaluated (Fig. 6.2.1.2).

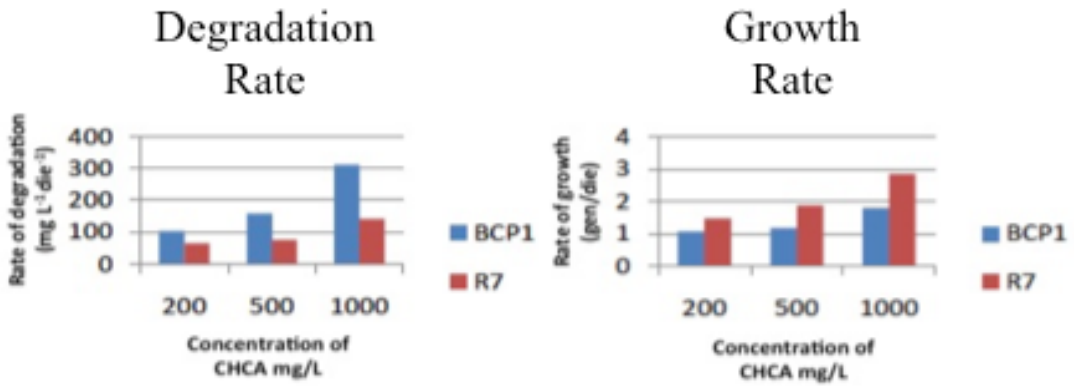

Fig. 6.2.1.2 Rate of degradation and growth* of *Rh*. sp. BCP1 and *Rh*. *opacus* R7 cells grown on CHCA 200, 500 and 1000 mg/L.

***rate of growth: v= n/t where n= [LogCFU/mL$_{end}$ - Log CFU/mL$_{in}$]/Log 2 and *t* indicates growth time.**

BCP1 showed a CHCA degradation rate two times faster than R7, while its growth rate was not significantly dependent on NAs concentration.

R7 showed rates of degradation and growth that were strongly dependent on CHCA concentration. In particular, R7 growth rate was two times higher when it was incubated in the presence of 1000 mg/L than 200 mg/L of CHCA. This observation suggested that the plateau observed at 200 mg/L was due to the exhaustion of the growth substrate.

Rh. sp. BCP1 and *Rh. opacus* R7 were also investigated regarding their ability to grow and degrade CPCA (Fig. 6.2.1.3, a, b, c, d, e, and f).

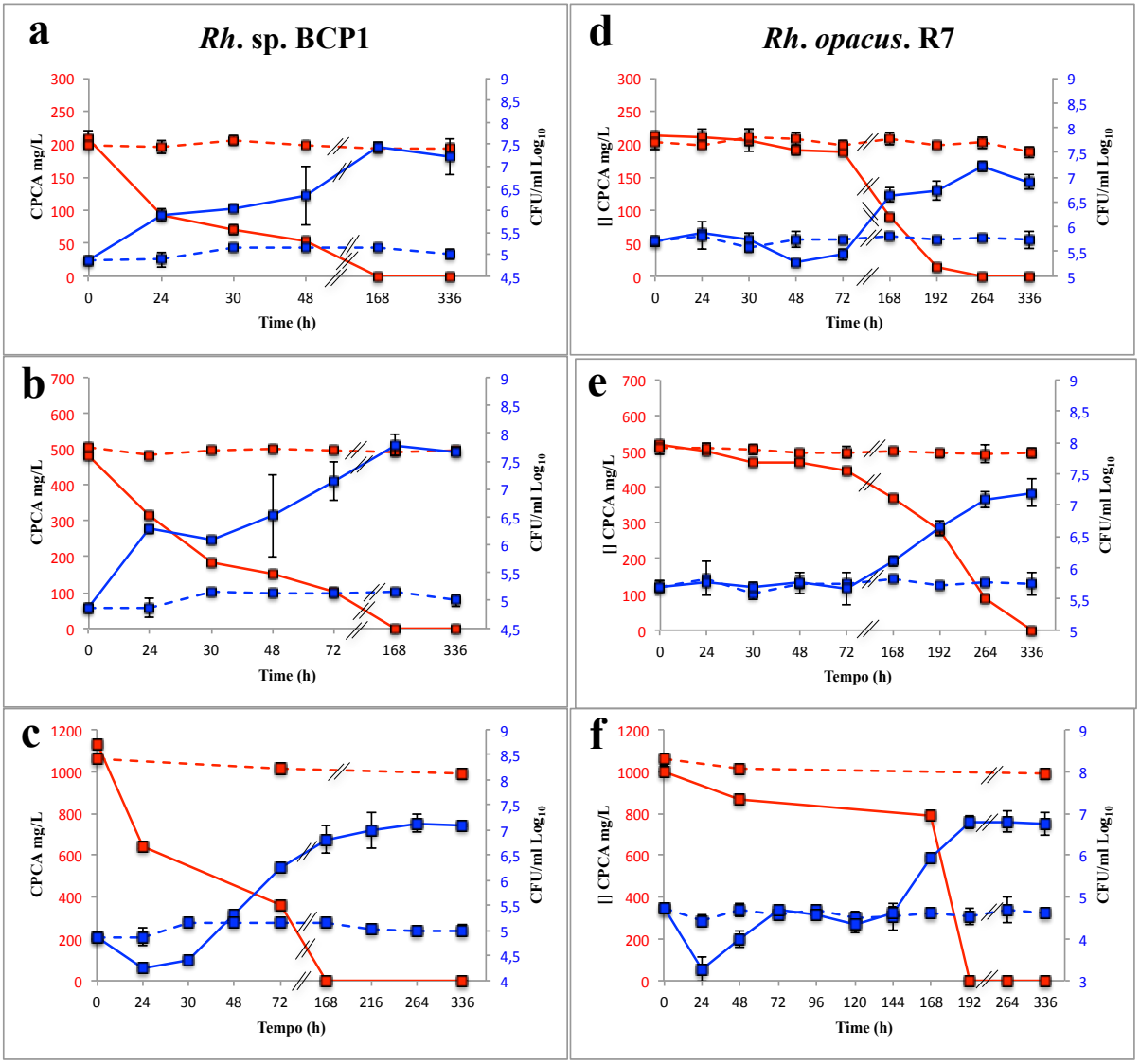

Fig. 6.2.1.3 Growth (continuous blue line) and degradation (continuous red line) curves of *Rh*. sp. BCP1 (a, b and c) and *Rh*. *opacus* R7 (d, e, and f) cultures in the presence of CPCA 200 mg/L (a and d), 500 mg/L (b and e) and 1000 mg/L (c and f). Red dashed lines represent the sterile control to assess abiotic substrate degradation; blue dashed lines represent the growth of *Rhodococcus* strains in MSM without carbon source (MSM).

The results summarized in Fig. 6.2.1.3 indicate that after 168 hs of incubation, BCP1 was able to completely degrade CPCA supplied to the growth medium at the concentration of 200 or 500 mg/L. The 60% of CPCA was depleted during the initial 30 hs of growth, with no evident lag phase. BCP1 also displayed a significant growth featured by an increase of the cell number of two order of magnitude (from $6{,}75 \times 10^4$ to $1{,}1 \times 10^7$ CFU/mL) during the first 48 hs (Fig. 6.2.1.3, a and b).

Conversely, BCP1 showed a lag phase of 48 hs when it was incubated in the presence of CPCA at the concentration of 1000 mg/L. An increased biomass content was observed after 48 hs of growth (from $1{,}95x10^5$ to $1{,}82x10^7$ CFU/mL), and a plateau was reached within 216 hs (i.e. stationary phase of growth) along with the complete depletion of CPCA (Fig. 6.2.1.3, c). These data suggested an efficient adaptation, internalization and degradation of the substrate by BCP1.

R7 growth and degradation rates were lower than those of BCP1. R7 showed an initial lag phase of about 72 hs for each concentration of CPCA, followed by exponential growth. The stationary phase was reached between 192 and 336 hs. In particular, GC-MS data revealed that only the 30% of the initial substrate amount was depleted during 168 hs when R7 was incubated in the presence of 200 or 500 mg/L of CPCA (Fig. 6.2.1.3, d and e). Also, the 21% depletion was observed in R7 cultures supplied with 1000 mg/L of CPCA in 168 hours, and the biomass content was decreased of one order of magnitude (from $1{,}73x10^4$ to $2{,}98x10^3$ CFU/mL after 24 hours of growth) (Fig. 6.2.1.3, f). This decrement was not observed in R7 cultures supplied with CPCA $\leq$ 500 mg/L (from $7{,}02x10^4$ to $9{,}23x10^4$ CFU/mL after 24 hs of growth) so to suggested a dose-dependent CPCA cytotoxic effect in R7 cells.

R7 showed a rate of growth and degradation which were dose-dependent; in particular, these two parameters increased in parallel with the concentration of CPCA supplied to the growth medium. Also BCP1, showed a dose-dependent degradation rate, but its growth rate was constant and not dependent on the concentration of CPCA (Fig. 6.2.1.4).

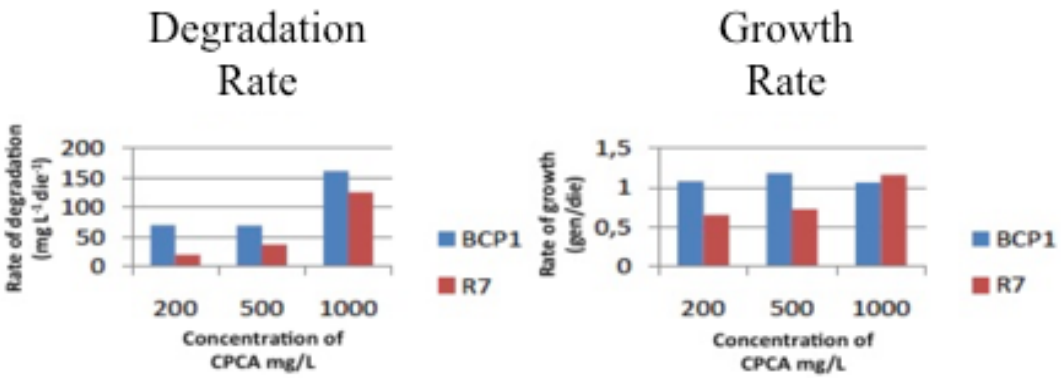

Fig. 6.2.1.4 Rate of degradation and growth of *Rh*. sp. BCP1 and *Rh*. *opacus* R7 cells grown on CPCA 200, 500 and 1000 mg/L.

It should be underlined that (CHCA and CPCA) differ for one carbon atom only. CPCA shows a more cytotoxic effect than CHCA, based on the lag phase displayed by R7 at each concentration added to the growth medium. BCP1 did not show any lag phase except for the highest concentration of CHCA and CPCA. In summary, since higher rates of degradation were observed with BCP1 cultures compared to R7 under each growth condition, we can conclude that *Rh*. sp BCP1 is more efficient than *Rh. opacus* R7 to degrade NAs such as CHCA and CPCA.

6.2.2. Cyclohexaneacetic acid (CHAA) and methyl-cyclohexanecarboxylic acid (mCHCA)

BCP1 and R7 were also tested for their ability to grow on CHAA and mCHCA that are NAs with a higher structural complexity than CHCA and CPCA. Both *Rhodococcus* strains were unable to grow on MSM supplied with 100 or 200 mg/L of CHAA as sole carbon and energy source (Fig. 6.2.2.1).

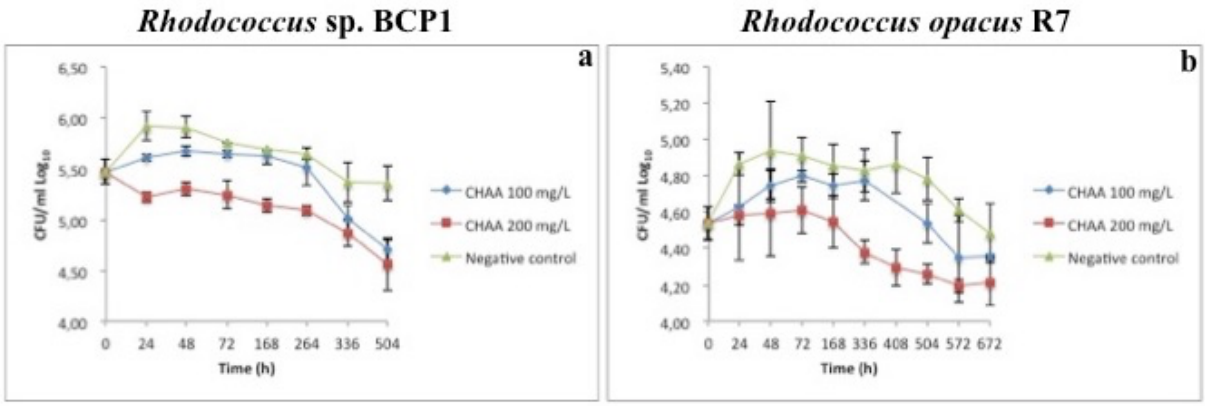

Fig. 6.2.2.1 *Rh.* sp. BCP1 (a) and *Rh. opacus* R7 (b) grown in the presence of 100 mg/L (Blue curve) and 200 mg/L (Red curve) of CHAA. In negative controls (Green curve) no carbon source was added to the growth medium.

Among the metabolic pathways described in the literature for the degradation of such alicyclic compounds, β-oxidation is the most probable one [Rho et al.; 1975, Blakley et al.; 1978, Blakley et al.; 1982]. Due to the CHAA alkyl-side chain characterized by an even number of carbon atoms, the β-carbon (position of the carbon atom respect to the carboxylic group) is in tertiary position. Consequently, during the CHAA β-oxidation a tertiary alcohol is produced, which is less suitable to microbial oxidation via the β-oxidation pathway. CHAA degradation has previously been reported by Rontani *et al.* (1992) in *Alcaligenes* sp. PH12. This strain was capable of CHAA degradation combining α- and β-oxidation. The absence of growth could be explained by considering the metabolic inability displayed by BCP1 and R7 for CHAA degradation.

When BCP1 and R7 are incubated in the presence of mCHCA, only BCP1 has the capacity to grow. In particular, at each concentration tested of mCHCA (200, 500, and 1000 mg/L), BCP1 cells showed a growth lag phase of approximately two weeks (Fig. 6.2.2.2, a).

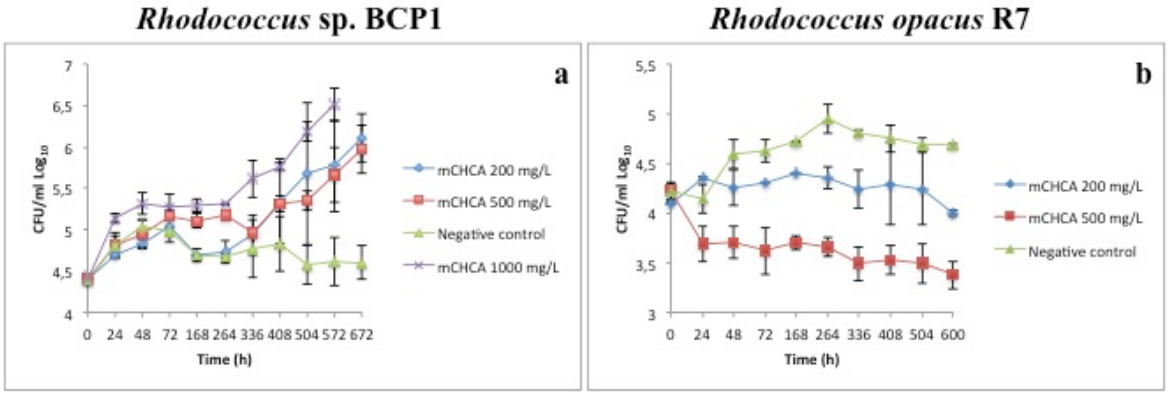

Fig. 6.2.2.2 *Rh.* sp. BCP1 (a) and *Rh. opacus* R7 (b) grown in the presence of 200 mg/L (Blue curve), 500 mg/L mCHCA (Red curve). The (a) growth curves also include the growth of BCP1 on 1000 mg/L mCHCA (Purple curve). In negative controls (Green curve) no carbon source was added to the growth medium.

The above reported data on BCP1 and R7 mCHCA-grown cells suggested that one methyl group was sufficient to increase the overall toxicity and recalcitrance of this NA compared to CHCA. Also, BCP1 growth rate was lower than that observed when it was incubated in the presence of CHCA (Fig.7.2.7).

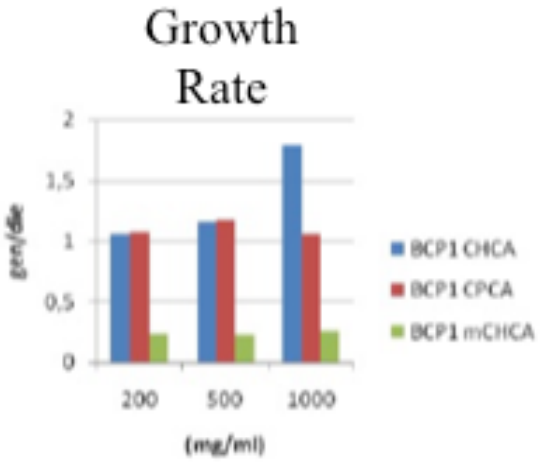

Fig. 6.2.2.3 Comparison of the BCP1 growth rates on various NAs: CHCA (Blue), CPCA (Red), and mCHCA (Green).

In particular, the BCP1 growth rate, which was constant and not dependent on the concentration of mCHCA, resulted to be five times less than that of CHCA-grown cells. Moreover, a decrease of the initial mortality of mCHCA-grown cells was observed at the highest concentration. This aspect can be explained as following: BCP1 might partially

oxidize mCHCA producing a non-toxic intermediate that accumulates within the cells because of its recalcitrance to degradation. During the degradation of this intermediate by BCP1, a microbial sub-population would be selected undergoing to the physiological/biochemical changes possibly manifested by the lag phase observed. Of course this hypothesis must be experimentally verified.

6.3. Fatty Acid Methyl Esters (FAMEs) analysis

Since hydrophobic compounds tend to fluidize the cytoplasmic membrane through their accumulation between the acyl-chain of fatty acids, most microorganisms have adopted a series of mechanisms to modulate the type of lipid molecules (e.g. saturated fatty acids, and methyl-substituted fatty acids) of the membrane [Segura et al.; 1999]. The membrane fatty acids composition of BCP1 and R7 grown in the presence of CHCA or CPCA was compared to that of glucose-grown cells, in order to identify possible changes of the lipid molecules which may allow tolerance to these NAs. Both *Rhodococcus* strains were incubated in the presence of glucose (1%) and CHCA (200 mg/L) for 48 hs and 72 hs, respectively. BCP1 and R7 were also cultured in the presence of CPCA (200mg/L) for 96 hs and 168 hs, respectively. These periods of incubation corresponded to the late exponential growth phase.

Among all the fatty acids analyzed, BCP1 showed a significant increase of methyl-substituted fatty acids such as methyl-heptadecanoate (Me17:0), methyl-octadecanoate (Me18:0), and methyl-hexadecanoate (Me16:0) content for both NAs tested (Fig. 6.3.1 and listed in table 4).

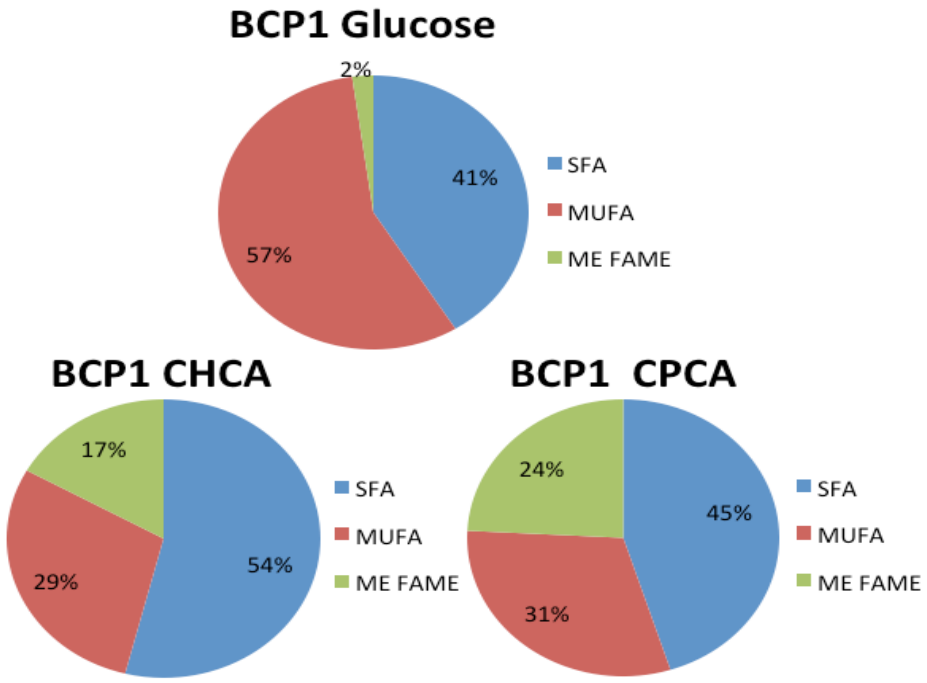

Fig. 6.3.1 The charts show the relative percentages of *Rh*. sp. BCP1 lipid molecules clustered as methyl-saturated fatty acids (ME FAME), saturated fatty acids (SFA), and mono-unsaturated fatty acids (MUFA).

The relative percentages of methyl-fatty acids were 17% (CHCA), 24% (CPCA), and 2% (glucose). Taking into account the saturated fatty acids, only, such as stearic acid (18:0) and palmitic acid (16:0) (listed in table 4), a similar trend to that of methyl-substituted ones was displayed by BCP1, although palmitic acid slightly decreased when the cells were incubated in the presence of CPCA. The overall relative percentages of saturated fatty acids were 54% (CHCA), 45% (CPCA), and 41% (glucose). A significant decrease was also observed regarding the *cis*-mono-unsaturated fatty acids content. Indeed, these lipid molecules represented the 29% (CHCA), and the 31% (CPCA) of the total lipids, while the 57% of *cis*-mono-unsaturated fatty acids characterized the biological membrane of BCP1 glucose-grown cells. Among *trans*-mono-unsaturated fatty acids, only eldainic acid (18:1 9 T) (0,44%) was detected in BCP1 CPCA-grown cells (listed in table 4).

Table 4. The relative percentages of each fatty acid under analysis in BCP1 cells grown on glucose (1%), CHCA and CPCA (200 mg/L). Nd, means not detected.

FAME	BCP1 Glu rel. %	BCP1 CHCA rel. %	BCP1 CPCA rel. %
14:0	1,009	0,967	1,225
15:0	0,656	0,703	0,72
16:0	35,463	42,567	30,002
16:1 6 T	nd	nd	nd
16:1 9 T	nd	nd	nd
16:1 6 C	1,244	2,585	1,193
16:1 9 C	12,531	1,75	2,2
Me 16:0	0,914	1,47	4,348
Me 17:0	0,298	1,715	5,85
18:0	4,155	10,097	13,298
18:1 9T	nd	nd	0,44
18:1 11T	nd	nd	nd
18:1 9 C	42,249	23,184	25,619
18:1 11 c	0,924	1,884	1,798
Me 18:0	1	13,792	14,061

Regarding R7, the relative percentages of methyl-substituted fatty acids were 12% (CHCA), 15% (CPCA), and 5% (Glucose) (6.3.2).

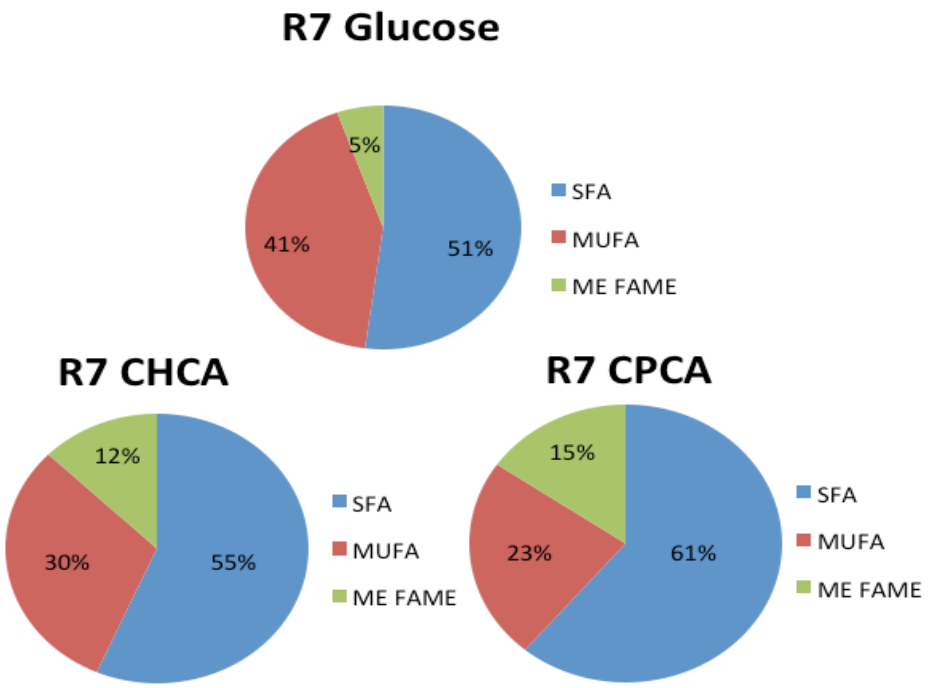

Fig. 6.3.2 The charts show the relative percentages of *Rh. opacus* R7 lipid molecules clustered as methyl-saturated fatty acids (ME FAME), saturated fatty acids (SFA), and mono-unsaturated fatty acids (MUFA).

Except for methyl-hexadecanoate (Me16:0) that slightly decreases in R7 CHCA- and CPCA grown-cells compared to the control, the amount of methyl-heptadecanoate (Me17:0) and methyl-octadecanoate (Me18:0) (listed in table 5) were increased, even if their content was lower than that observed in BCP1. Similarly to BCP1, R7 showed an increased content of saturated fatty acid, and their relative percentages were 55% (CHCA), 61% (CPCA) and 51% (glucose), with a general decrease regarding the content of *cis*-mono-unsaturated ones (Fig. 6.3.2 and table 5).

Table 5. The relative percentages of each fatty acid under analysis in R7 cells grown on glucose (1%), CHCA and CPCA (200 mg/L). Nd, means not detected.

FAME	R7 Glu rel. %	R7 CHCA rel. %	R7 CPCA rel. %
14:0	2,454	2,217	1,405
15:0	2,342	1,4	1,096
16:0	39,9	40,945	48,263
16:1 6 T	nd	nd	nd
16:1 9 T	nd	nd	nd
16:1 6 C	3,384	3,946	1,159
16:1 9 C	7,273	1,879	2,023
Me 16:0	2,082	1,69	1,314
Me 17:0	0,324	1,516	2,774
18:0	6,435	10,896	10,332
18:1 9T	nd	nd	Nd
18:1 11T	nd	nd	Nd
18:1 9 C	30,14	22,591	18,679
18:1 11 c	0,852	1,79	1,623
Me 18:0	2,931	9,27	11,283

The biological significance of these lipid modifications in the membrane is correlated to the increase of the membrane rigidity in order to better tolerate hydrophobic hazardous compounds. Basically, such compounds tend to accumulate between the acyl-chain of the microbial membrane phospholipids increasing the membrane fluidity. This effect results in the loss of the membrane functionality that can cause ultimately the cell death. Bacterial cells can also modulate the membrane rigidity by increasing the degree of lipid saturation, the number of methyl-substituted fatty acids, as well as decreasing the content of unsaturated

fatty acids. The data about BCP1 and R7 CHCA- and CPCA-grown cells showed that these *Rhodococcus* species can exploit different mechanisms of adaptation in order to counteract the fluidizing effect of hydrophobic pollutants such as NAs.

6.4. Transmission Electron Microscopy (TEM) analysis

Transmission electron microscopy of BCP1 and R7 cells grown on CHCA and CPCA (200 mg/L) were performed in order to investigate the physiological effects derived from these types of NAs. Glucose (1%) grown cells were used as a positive control. These experiments were conducted with cells harvested in their late exponential phase.

Micrographs of both *Rhodococcus* strains grown on glucose exhibited electron-transparent inclusion bodies, which were different in shape and size (Fig. 6.4.1). The inclusion bodies have been shown in the past to contain triacylglycerols and other neutral lipids [Alvarez et. al 2002].

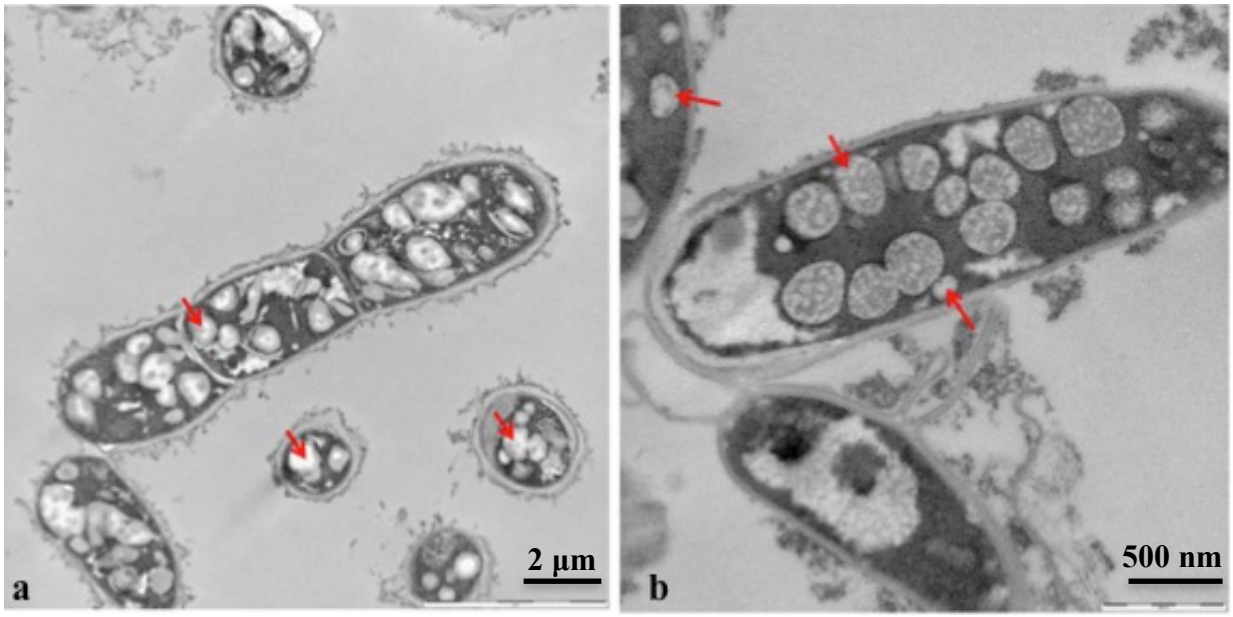

Fig. 6.4.1 TEM micrographs of *Rh*. sp. BCP1 (a) and *Rh. opacus* R7 (b) cells grown on Glucose 1% w/v for 12 and 72 hours, respectively. Inclusion bodies are indicated by red arrows. The scale bars correspond to 2 µm (a) and 500 nm (b).

In contrast to control cells, BCP1 and R7 CHCA- or CPCA-grown cells were featured by electron-dense inclusion bodies, visible as dark granules (6.4.2). In some cases, white cavities

were observed instead of the dark granules, this phenomenon possibly due to the loss of these inclusion bodies during the preparation of the samples.

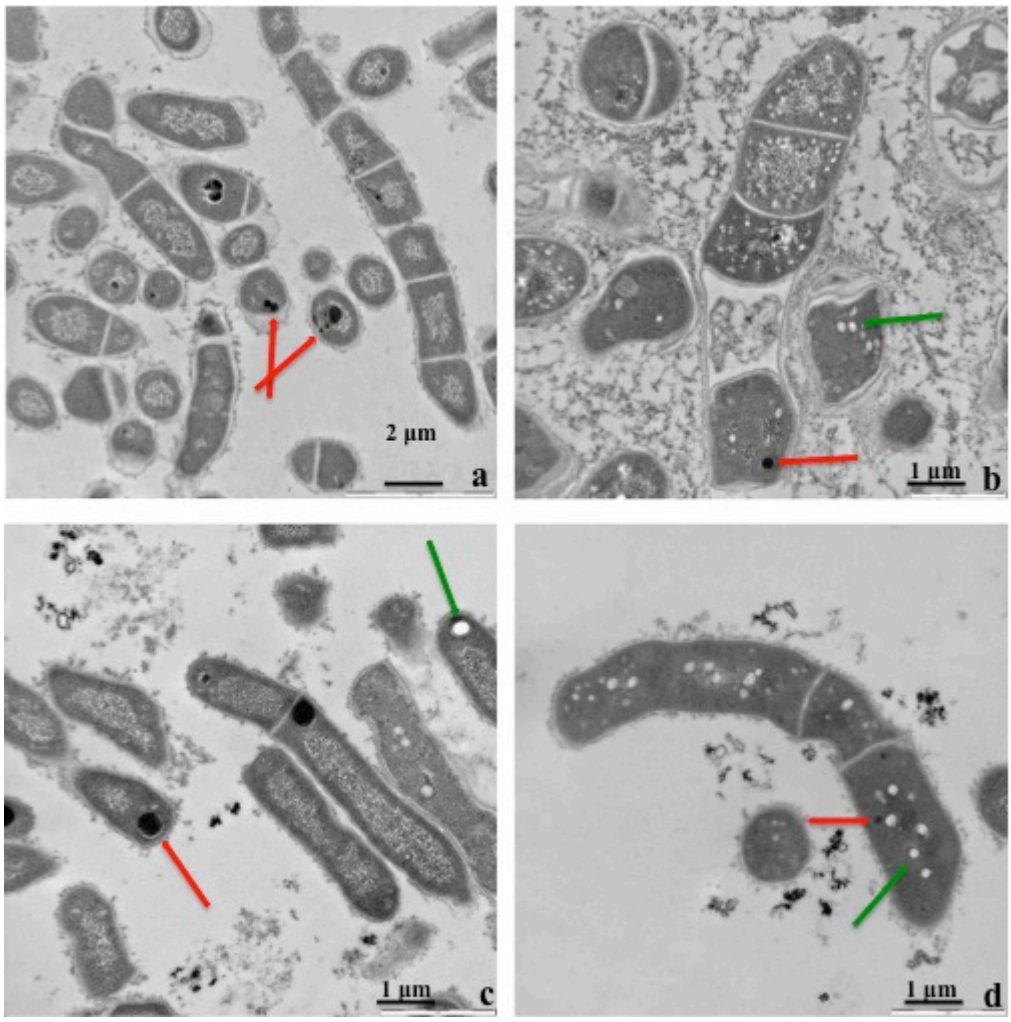

Fig. 6.4.2 TEM micrographs of *Rh*. sp. BCP1 (a, and b) and *Rh*. *opacus* R7 (c, and d) cells grown on CHCA (a, and c) or CPCA (b, and d). Dark granules are indicated by red arrows. White cavities are indicated by green arrows. The scale bars correspond to 2 µm (a), and 1 µm (b, c, and d).

These inclusion bodies, that are observed in several Gram positive and negative bacteria strains, including *Rhodococcus* strains and other actinomycetes are believed to derive from hydrophobic compounds [Scott et al.; 1976, Singer et al.; 1984, Alvarez et al.; 1996]. Analogously, Veeranagouda *et al.* (2009) observed similar inclusion bodies in *Rh.* sp. KL96 grown in the presence of alkyl-phenol such as *p*-cresol, as well as Whyte *et al.* 1999 in *Rh*. sp. Q15 cells grown in the presence of diesel fuels. Alvarez & Steinbuchel (2002) reported that bacteria strains belonging to actinomycetes, particularly *Streptomyces* and *Rhodococcus*,

accumulate lipid inclusion bodies under stress conditions (e.g. toxicity derived by pollutant compounds or starvation). During prolonged starvation, microorganisms can use these stored substances as a sole carbon and energy [Alvarez et al.; 2004]. Interestingly, TEM analysis also shows that BCP1 and R7 grown on CHCA or CPCA are present numerous non-orthodox shaped cells (6.4.3).

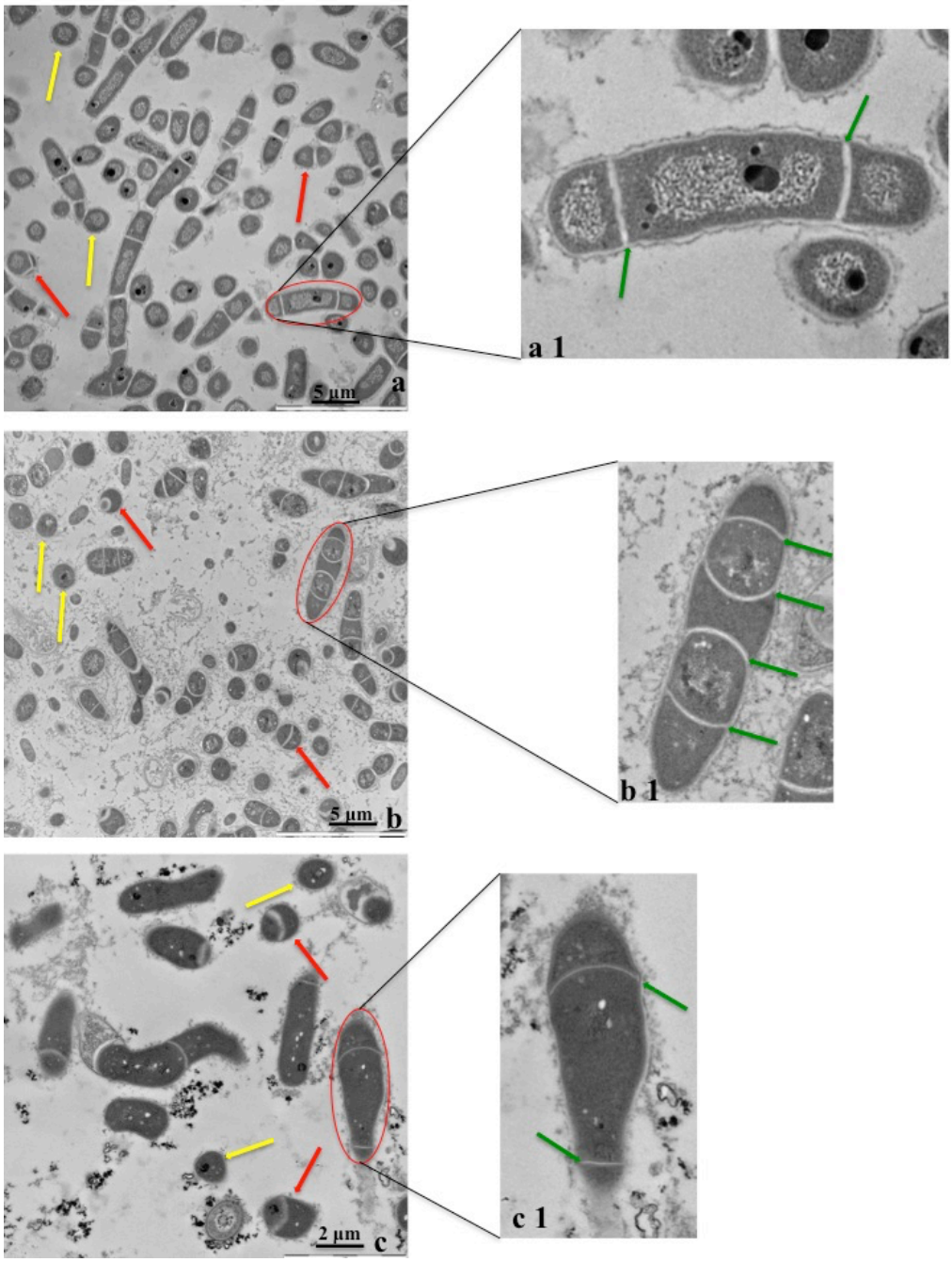

Fig. 6.4.3 TEM micrographs of *Rh*. sp. BCP1 grown on CHCA (a, and enlargement a 1), and CPCA (b, and enlargement b 1); *Rh. opacus* R7 grown on CPCA (c, and enlargement c 1). Short rod cells are indicated by yellow (coccus shape) and red (coccus shape arrangement in pairs) arrows. Septa of cell division are indicated by green arrows. The scale bars correspond to 5 µm (a, and b), and 2 µm (c).

In particular, bacterial cells that displayed the shape of a long-rod were featured by the presence of several septa of cell division (6.4.3, a1, b1, and c1). This finding suggests that BCP1 and R7 incubated in the presence of CHCA or CPCA did not complete their cellular division. BCP1 and R7 short-rod cells could be distinguished in two forms: (i) coccus cells, probably due to the transversal section of large-rod cells (Fig. 6.4.3 a, b, and c indicated by yellow arrow); (ii) short rod cells, arranged in pairs, probably indicating a further division step that resulted in the formation of coccus cells (Fig. 6.4.3 a, b, and c indicated by red arrows).

Capsular material, similar to EPS (Extracellular Polymeric Substance), was observed around the cells of both *Rhodococcus* strains. BCP1 showed an organized capsule when it was incubated in the presence of glucose, CHCA or CPCA (Fig. 6.4.4, b, and c).

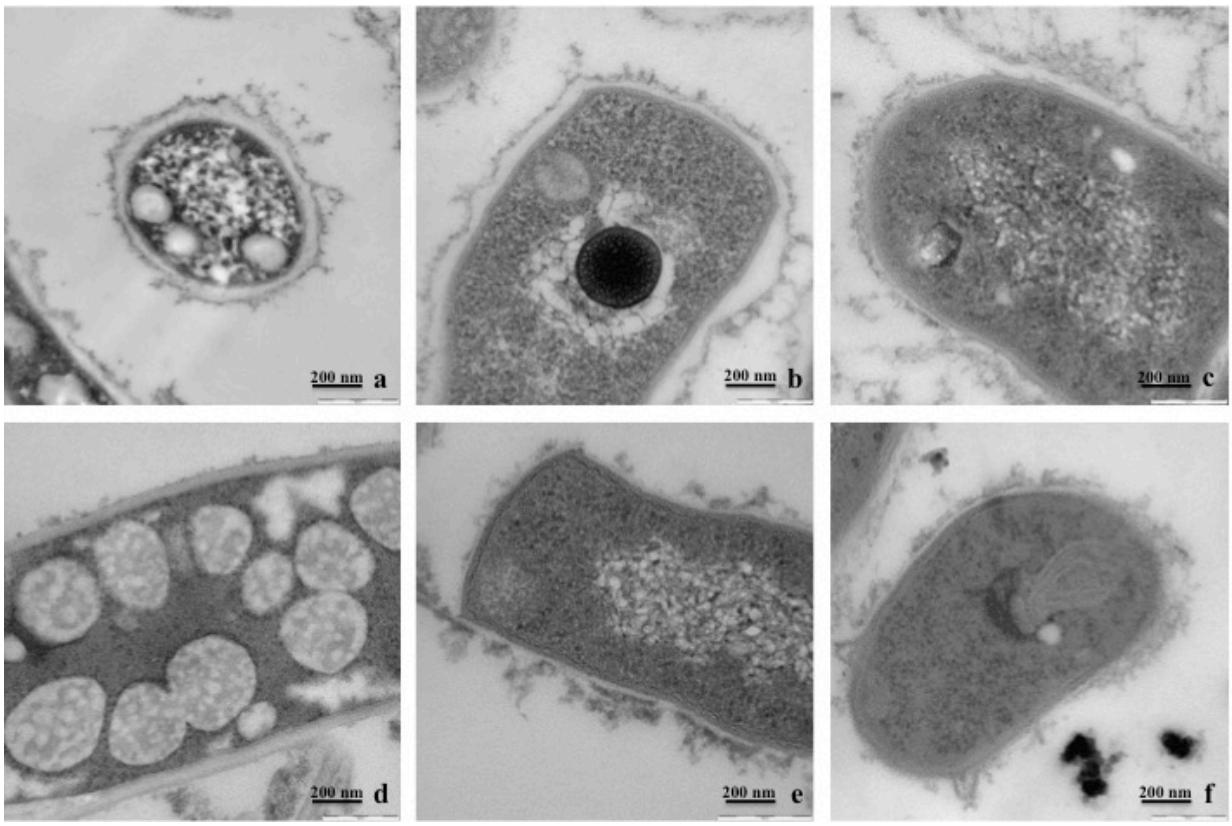

Fig. 6.4.4 TEM micrographs: particular of the EPS material surround the cells for *Rh*. sp. BCP1 and *Rh*. *opacus* R7 grown on glucose (a, and d), CHCA (b, and e) or CPCA (c, and f). The scale bars correspond to 200 nm.

In contrast, R7 exhibits a capsule featured by extracellular material highly disorganized, less packed; in particular, this disordered arrangement was more evident when R7 was grown in the presence of CPCA (Fig. 6.4.4, e, and f). EPS was also found dispersed around the cells without any particular association to the cells. Similarly, Whyte and coworkers (1999) found cells of *Rhodococcus* sp. Q15 clumped by EPS when grown in the presence of glucose-acetate and diesel fuels.

6.5. Discussion

In this PhD thesis's work section we have examined the capacity of *Rh*. sp. BCP1 and *Rh. opacus* R7 to degrade a few NAs, along with the impact of these toxic compounds on bacterial cells growth and structure. Notably, the only work presently available in the literature which describes the NAs degradation by *Rhodococcus* species within a microbial consortium is that of Demeter *et al.* (2014). These authors evaluated the metabolic capability of a microbial community derived from Athabasca OSPW to degrade a mixture of two model NAs (CHCA-CHAA). This microbial consortium was characterized by *Rhodococcus*, *Cyanobacteria*, *Pseudomonas*, *Xanthobacter*, and *Ancylobacter* species. As almost no information is available on the ability of *Rhodococcus* spp. to degrade model NAs, the first aim of the present experimental work was to assess the metabolic potential of BCP1 and R7 strains, regarding NAs such as CHCA, CPCA, mCHCA, and CHAA. Growth and degradation data showed that both *Rhodococcus* species show great degradation abilities. In particular, these strains were able to degrade, below the detectable levels, CHCA and CPCA up to 1000 mg/L, a concentration of NAs far above than those found in the tailing ponds (40-120 mg/L) [Allen et al.; 2008].

BCP1 showed a faster rate of degradation than R7 for each amount of substrate (CHCA and CPCA) tested. Further, BCP1 exhibited a pronounced lag phase only in the case of the highest

CHCA or CPCA concentration. R7 showed a delayed degradation as compared to BCP1 and a larger lag phase when it was incubated with the highest concentration of CHCA and for each concentration of CPCA. These data, taken together, suggest that R7 is more prone to NAs toxicity than BCP1. Unfortunately, no metabolic intermediates were detected by GC-MS analysis during CHCA or CPCA catabolism with no significant improvement of our present knowledge on the metabolic pathways already described in the literature [Trudgill et al.; 1984, Rontani et al.; 1992, Johnson et al.; 2012].

Both strains were unable to grow on CHAA although one of the most probable way to be used would be the β-oxidation pathway [Evans et al.; 1975, Blakley et al.; 1978, Blakley et al.; 1982]. A possible explanation for the absence of growth displayed by BCP1 and R7 might be related to the molecular structure of CHAA. This compound is characterized by an alkyl side chain with an even number of carbon atoms. The CHAA recalcitrance is due to the position of the β carbon (that is tertiary) within the ring. Consequently, the formation of a tertiary alcohol during β-oxidation cycle would impair the further dehydrogenation step because of its steric hindrance [Quesnel et al.; 2011, Kannel et al.; 2012].

As far as concern methyl-substituted NAs, only BCP1 was able to grow when it was incubated in the presence of the mono-methyl-sustituted mCHCA. In particular, BCP1 showed a lag phase of 21 days when it was incubated in the presence of 1000 mg/L of mCHCA. After this significant lag phase, the number of CFU/mL was increased by two order of magnitude (from $2{,}1 \times 10^4$ to $1{,}83 \times 10^6$ CFU/mL). The data reported in the literature on the degradation of mono- or multi-substituted NAs have showed that their rate of degradation was slower than in the case of the unsubstituted NAs [Johnson et al.; 2011, 2012]. Moreover, the presence of a methyl substitution can hamper or even impair the degradation of aliphatic- and cyclo-alkane carboxylic acids [Herman et al.; 1994]. The growth data reported about BCP1

and R7 mCHCA-grown cells suggested that mono-substituted NAs are more recalcitrant and toxic as compared to CHCA.

In general, many hydrophobic compounds can penetrate into a membrane causing swelling and increase of fluidity, with the loss of membrane functionality and damage of the bacterial cell [Jurkiewicz et al.; 2012]. In this respect, most of the microorganisms can exploit mechanisms aimed to modulate the type of lipid content of their membrane, for example, increasing the content of saturated and methyl-saturated fatty acids in order to maintain the membrane rigidity [Segura et al.; 1999].

FAMEs analys was performed in order to evaluate changes in the lipid membrane content in BCP1 and R7 cells incubated in the presence of CHCA or CPCA as compared to glucose grown cells. Results revealed a general increase of saturated and methyl-substituted fatty acids, while the *cis*-mono-unsaturated ones decreased. These evidences are in line with previous data by Tsitko *et al.* (1999) in three different *Rhodococcus opacus* strains (GM-14, GM-29, and 1CP) grown on aromatic compounds such as benzene, phenol, 4-chlorophenol, chlorobenzene, or toluene. The strongest modification was shown by *Rh*. *opacus* 1CP that increased its content of methyl-substituted fatty acids from 3.1% to 24% or 34% when it was incubated in the presence of fructose, phenol, and 4-chlorophenol, respectively. This adaptation has also been described in *Rh*. sp. 33 which shows an increase in the percentage of saturated fatty acids when it was incubated in the presence of mannitol (57%), benzene (59%), or mannitol along with benzene (63%). It has been proposed that methyl-branched and saturated fatty acids require *de novo* synthesis that depends on the energetic status of bacterial cells [Kaneda et al.; 1991]. This aspect could explain the significant lag phase displayed by BCP1 and R7, when they were grown under stress conditions caused by the highest concentration of NAs tested. Among the *trans*-fatty acid analyzed, only BCP1 displayed a slight increase of the eldainic acid content (18:1 9 T) when it was incubated in the presence of

CPCA for 96 hs. This evidence suggests that BCP1 is capable of *cis-trans* isomerization, which is considered a short-term response triggered by the presence of hydrophobic chemicals not linked to the synthesis of new fatty acids [Heipieper et al.; 2003]. These data suggested that both BCP1 and R7 cells can modulate the lipid type content of the membrane to overcome an adverse toxic condition.

Transmission Electron Microscopy (TEM) analysis has shown interestingly ultra-structural changes, including electron-transparent inclusions, irregular cellular division, production of extracellular material that was supposed to be EPS (Extracellular Polymeric Substance). Inclusions bodies in the form of dark granules were observed for both BCP1 and R7 strains, when they were incubated in the presence of CHCA, and CPCA. Inclusions bodies are a common feature in bacteria grown on hydrocarbons, and similar structures have been observed in several Gram-negative and Gram-positive bacteria, including *Rhodococcus* strains [Alvarez et al.; 2002, 2004]. These inclusion bodies are believed to be hydrophobic storage compounds, [Scott et al.; 1976, Singer et al.; 1984, Alvarez et al.; 1996], and tend they to accumulate within the cells during stress conditions (toxicity due to pollutant compounds or starvation) [Alvarez et al.; 2004]. BCP1 and R7 NAs-grown cells were characterized by several cells having a long-rod shape with irregular septa of division, suggesting incomplete cell division, along with cells in the form of short-rods. These structural anomalies have previously been reported in *Rh.* sp. KL96 grown in the presence of *p*-cresol [Veeranagouda et al.; 2009]. Similarly to a previous report by Whyte *et al.;* (1999) in *Rh*. sp. Q15, the presence of capsular material was also found in BCP1 and R7 cells grown on NAs. The main difference between the two *Rhodococcus* strains is linked to the organization of the capsule, which is efficiently structured in BCP1 as compared to R7 cells. This aspect suggests a better adaptability of BCP1 than R7 cells when are incubated in the presence of

NAs. All these ultra-structural changes might suggest a further mechanism of adaptation that bacterial cells exploit to counteract experimental stress conditions.

In conclusion, the *Rhodococcus* strains BCP1 and R7 have been shown to utilize model NAs as sole carbon and energy source, with the exception of CHAA. BCP1 and R7 were also able to exploit mechanisms of adaptation involving the membrane fatty acids composition and fluidity. Morphological changes such as electron-transparent inclusion bodies, irregular cellular division, production of extracellular material that was supposed to be EPS, were also present as evidence of the cell response to the toxicity of NAs.

7. Genetic analysis of model NAs (CHCA and CPCA) degradation by *Rhodococcus* sp. BCP1 and *Rhodococcus opacus* R7

The available literature shows that the most probable metabolic pathways involved in NAs degradation include β-oxidation, a combination of α- and β-oxidation, and aromatization [Blakley et al.; 1982, Rontani et al.; 1992, Iwaki et al.; 2005].

Here the results of our studies on the putative "biodegradative" genes involved in the degradation of model NAs such as CHCA and CPCA in *Rh*. sp. BCP1 and *Rh. opacus* R7 cells is reported. . In particular, the presence of genes homologous to those already present in the literature and possibly involved in NAs biodegradation pathways was investigated in *Rh*. sp. BCP1 genome. Further, the expression of these genes was studied in BCP1 cells grown in the presence of NAs through semi-quantitative RT-PCR and quantitative Real-Time RT-PCR (RT-qPCR). In BCP1 cells grown in the presence of CHCA, the induction of the genes encoding for enzymes involved in the degradation of this substrate via β-oxidation pathway, was demonstrated. Moreover, a random mutagenesis library was constructed for *Rh. opacus* R7. A non-replicant transposon vector (pTNR-TA) containing a *Rh. erythropolis* transposable element (IS*1415*) was used to transform R7. Among the transposon-mediated mutants obtained, 11 recombinant R7 clones were defective in the growth on CPCA.

7.1. Materials and Methods

7.1.1. Semiquantitative RT-PCR

BCP1 cell growth in the presence of CHCA, CPCA (both used at 500 mg/L), or succinate (60 mM) as sole carbon and energy source is described in chapter 6 § 6.1. The biomass was

harvested and total RNAs were isolated as described in the chapter 5 (RNA isolation procedure). RNAs isolated from BCP1 succinate-grown cells were used as a reference sample in the negative control. The total RNAs were treated twice with 4U of Q1 RNase-Free DNase (Promega) for 45 min at 37°C. RNAs DNA-free were cleaned by phenol-chloroform purification before being used for further experiments.

RNAs (500ng) were retro-transcribed with 0.25 μg of random hexamers (Invitrogen) in 10-μL reaction mixtures. After denaturation for 3 min at 94°C and annealing for 5 min at 37°C, 1 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and 50 U of the RNase inhibitor RNasin (Promega) were added and reverse transcription was performed at 42°C for 1 h. When necessary, specific primers (forward and reverse, listed in table 6) were used instead of random hexamers.

PCR reaction was performed at 20, 25, or 30 cycles in a final volume of 25 μL using Taq DNA Polymerase (recombinant) (Thermo scientific). Thermocycling conditions were as follows: 95 °C for 3 min (initial denaturation step), 95 °C for 30 sec (denaturation), 60 or 63 °C for 30 sec (annealing temperature on the basis of the T_m of the primer), 72 °C for 90 sec (extension), and 72 °C for 10 min (final extension). 4 μL of cDNA diluted 1:5 (around 40ng) was used as template for each PCR reaction.

Colony PCR was performed as following: *E. coli* and/or *Rhodococcus* clones were picked up from agar plate and suspended in 20 μL of 10mM Tris 1 mM EDTA pH 8 solution (this solution contained also SDS 10%, and 1% v/v Triton X-100 in the case of *Rhodococcus* clones). The samples were boiled at 95 °C for 5 min and then cooled down on ice for 2 min. After this treatment, the samples were centrifuged for 3 min at the max speed and 4 °C. 1 μL of the sample was used as template for PCR reaction.

PCR products were evaluated by gel-electrophoresis, and GeneRuler 1Kb DNA Ladder (Thermo scientific) was used as marker of molecular weight.

7.1.2. RT-qPCR

To assess the transcriptional level of the gene that encodes for the glutaryl-CoA dehydrogenase (KDE15142.1) induced by growth of the strain BCP1 on CHCA or CPCA, RT-qPCR experiments were performed. The effect of alternative carbon sources on the expression of this gene was also evaluated by adding succinate (60 mM), and glucose (1% v/v). The reverse-transcribed samples were amplified using the CFX96 Real-Time System (Bio-Rad). Each sample, at a final volume of 20 µL, contained 40 ng of cDNA (4 µL), 250 nM of each primer (5 µL of a mix 1 µg forward and reverse, listed in table 6), and 10 µL of SsoAdvanced universal SYBR Green supermix (Biorad), and 1 µL of milliQ water. Thermocycling conditions were as follows: 30 sec at 95 °C, followed by 30 cycles of 5 sec at 95 °C, and 5 sec at 60 ºC. The expression level of the housekeeping gene 16S rDNA was used as reference gene to normalize the tested gene in *Rh.* sp. strain BCP1. The relative fold change in mRNA quantity was calculated for the gene of interest in each sample using the ΔΔCt method followed by Student's one-sample t test to evaluate whether the gene targets were differentially regulated. Data are expressed as mean value ± standard deviation derived from at least three independent experiments. Differences were considered significant at $P < 0.05$. For each RNA preparation, at least three independent Real-Time PCR experiments were conducted.

Table 6: Primers list.

Semiquantitative-PCR Primer	Forward	Reverse
Cyclohexanone monooxygenase KDE14614.1	gtgacctcgatggtgtggaa	gcacgtcgatctcgtactcg
Cyclohexanone monooxygenase KDE14662.1	gcagccggtacttcatctcc	aagtcgacgtggtcctcgat
Cyclohexanone monooxygenase KDE14308.1	gggcgagatcttccactcc	tgcaggttgcggttgtattc
Cyclohexanone monooxygenase KDE12215.1	ctgccgtacttccagcagtg	cgcagcatcgtccagtagtc
Cyclohexanone monooxygenase KDE12242.1	tcgaggtcgacgagagtgaa	atgaacggcatgaagacacg
Cyclohexanone monooxygenase KDE11803.1	gcaagatgctcggggactac	tcgaagaacgggaaggtgat
Cyclohexanone monooxygenase KDE10995.1	ccggccctgtactcgtactc	ggagctggctctcgatcat
Cyclohexanone monooxygenase KDE11012.1	cctggttctggaaccgctat	cccttgtccgtgaactcctc
Cyclohexanone monooxygenase KDE11542.1	gacgtggacagccacgacta	gcggttgaaggtctcgtacc
Cyclohexanone monooxygenase KDE11570.1	ctgcagcagtggaactggac	gatgttggtgaacgggctct
Cyclohexanone monooxygenase KDE10307.1	ggactggagccgaatctttg	gcatgtcgaggatgtggaag
Cyclohexanone monooxygenase KDE12207.1	gcggcaagatgttccactc	gggacgggtcacgtagttct
2-hydroxycyclohexanecarboxyl-CoA dehydrogenase KDE15144.1	atgagcaacatcgcactggt	gaacgggatggccttgat
2-hydroxycyclohexanecarboxyl-CoA dehydrogenase KDE12227.1	cttcgacctgaacggaaagg	ctcggagcacagccacag
Long chain fatty acid-CoA ligase KDE15143.1	cgacaccgaactcaccgata	aacttggccctctcctggtc
Enoyl-CoAhydratase KDE15139.1	gtcaccacacgcgaagacc	gacgaatccttccagcaagc
Naphthoate synthase KDE15145.1	gaccgtgttcgaggacatca	gaggccctcgtcggactt
Butyryl-Coa dehydrogenase KDE15141.1	gaggtcgtccgtgacttcgt	cacgaactgggtgttctgga
Glutaryl-CoA dehydrogenase KDE15142.1	gttccgaggagcagaagcag	acggggtactcgagggagat
pobA KDE11135.1	agcacgaggtcaacaaggac	gacatccaccacgagaagtg
16rDNA	agagtttgatcmtggctcag	tacggytaccttgttacgactt
RT-qPCR Primer	**Forward**	**Reverse**
16SrDNA	attagtggcgaacgggtgag	cccgaggtcctatccggtat
Glutaryl-Coa dehydrogenase KDE15142.1	cttcggcaagctcaacaaca	gatgacggggtactcgagg

7.1.3. Transposon mutagenesis library

To identify defective growth mutants of *Rh. opacus* R7 in the prence of CHCA or CPCA as only carobon and energy source, a transposon mutagenesis library was carried out. The transposon vector used in this study was a non-replicating transposon plasmid named pTNR-TA by Sallam et al (2006). This vector is featured by the *E. coli* origin of replication (*ori*), the coding sequences *istA* and *istB* responsible for the transposition activity from IS*1415* elements derived from *Rh. erythropolis*, two inverted repeated sequences (IR1 upstream of *istA*, and IR2 downstream of *istB*, nit promoter (P_{nit}), ampicillin and thiostrepton resistant genes. (Fig. 7.1.3.1) [Sallam et al.; 2007].

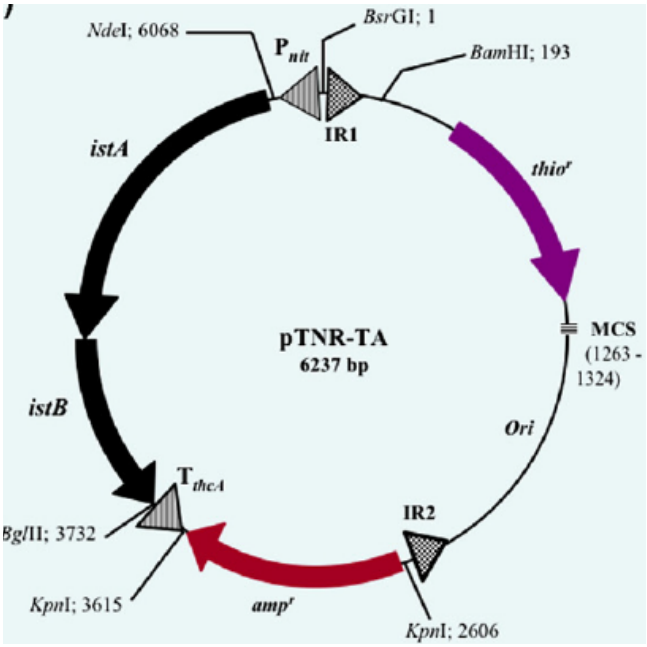

Fig. 7.1.3.1 Non-replicanting transposon plasmid (pTNR-TA) [Sallam et al.; 2007].

R7 cells were grown on electrocompetent media (chapter 5, table 3), harvested at their initial exponential growth phase, and pocessed in order to perform electroporation experiments as described in chapter 5 (Electroporation of *Rhodococcus* spp.). R7 transformant cells were spread onto LB agar plates supplied with the thiostrepton (final concentration of 5 µg/mL) as selectable marker. After this step, the R7 colonies were streaked out on LB agar plates with thiostrepton to leave out false-positives. R7 colonies resistant to thiostrepton were streaked out onto three different types of MSM agar medium supplied with glucose (0,5% v/v) to avoid auxotrophic mutants, or CHCA (100 mg/L, or CPCA (100 mg/L) (Fig. 7.1.3.2). The R7 clones that were able to grow on glucose and were defective for the growth on CHCA or CPCA were further analyzed.

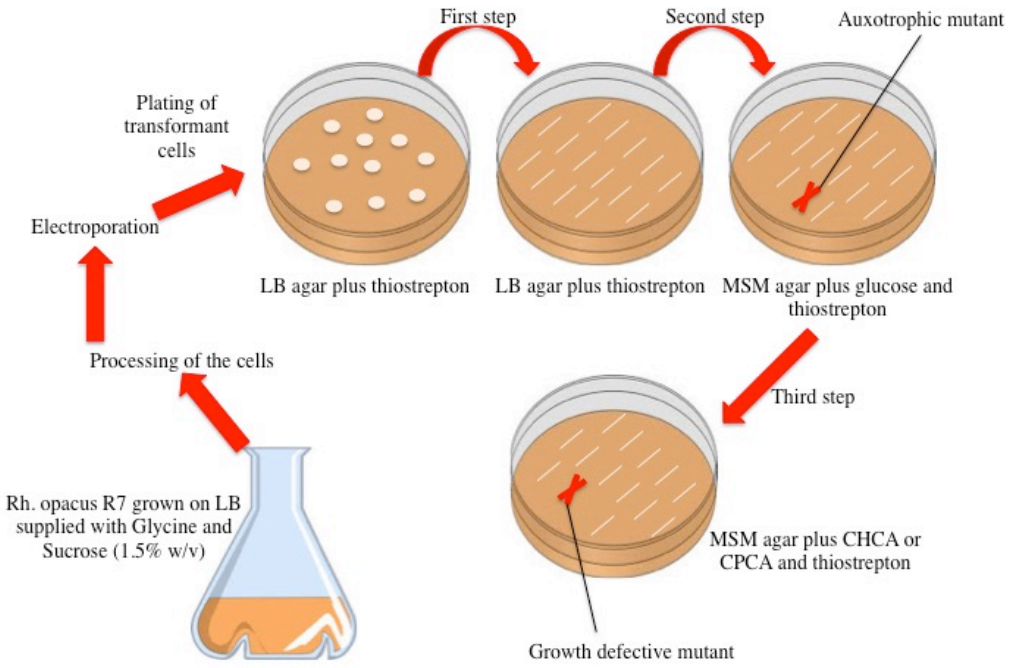

Fig. 7.1.3.2 Visual represantation of the strategy used to perform the random transposon mutagenesis library for *Rh. opacus* R7.

7.1.4. Two-step gene walking method

The two-step gene walking method was used to quickly map the locus where the transposon was inserted in the genome of R7. The PCR reaction was performed with only one primer named *walking-thio 1* (forward) and/or *walking-thio 2* (reverse) that are listed in table 7. These primers are both complementary to the known sequence of the thiostrepton gene. The thermocycling condition were as following: (walking-step 30 cycles) 98 °C for 3 min (initial denaturation), 98 °C for 30 sec (denaturation), 65 °C ± 5°C for 30 sec (T-Gradient annealing) and 72 °C for 3 min (extension); (ii) (aspecific step 1 cycle) 98 °C 30 sec (denaturation), 40 °C 30 sec (annealing), and 72 °C for 3 min (extension); (iii) Walking-step was repeated again for 30 cycles. The first 30 cycles of PCR (walking step), single strand DNA (ssDNA) product is produced due to the presence of the forward and/or reverse primer, only. The second step is characterized by one PCR cycle with non-stringent annealing temperature, that allows the unspecific bind of the primer in different sites of ssDNA produced during the first step as

reverse primer. During this step, dsDNA of different length was produced because of the random incorporation of the sequence primer at each 5 prime end of ssDNA produced. The last 30 cycles of PCR are performed with stringent annealing temperature that specifically amplifies dsDNA. This PCR product can be directly sequenced using a nested primer (Fig. 7.1.4.1).

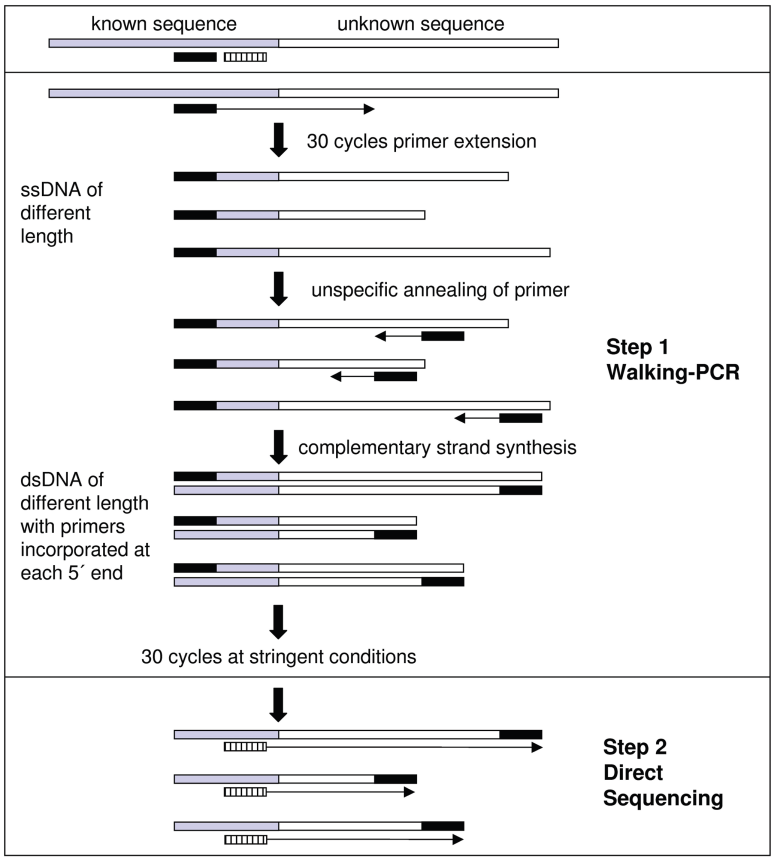

Fig. 7.1.4.1 Two-step walking primer scheme [Pilhofer et al.; 2007].

Table 7: Primers list.

Two-step gene walking primer	Forward	Reverse
Walking thio 1	ggaaaaggactgctgtcgctgcc	-
Sequencing thio 1	gccgtagacctcgatgaactccac	-
Walking thio 2	-	aaggcggatggcgacatttcc
Senquencing thio 2	-	tccgtgaaggaactcggggac
Thiostrepton gene	ggatccgccagagagcgacgac	cgccttcgaggagtgcccg

7.2. Genetic analysis about NAs degradation by *Rh*. sp. BCP1

In order to identify "biodegradative" genes involved in NAs degradation, total RNAs were isolated from BCP1 grown in the presence of CHCA, CPCA or succinate as sole carbon and energy source. The quality of RNA preparation, as well as genomic DNA contamination, were evaluated by analyzing RNAs preparation on agarose gel (Fig. 7.2.1).

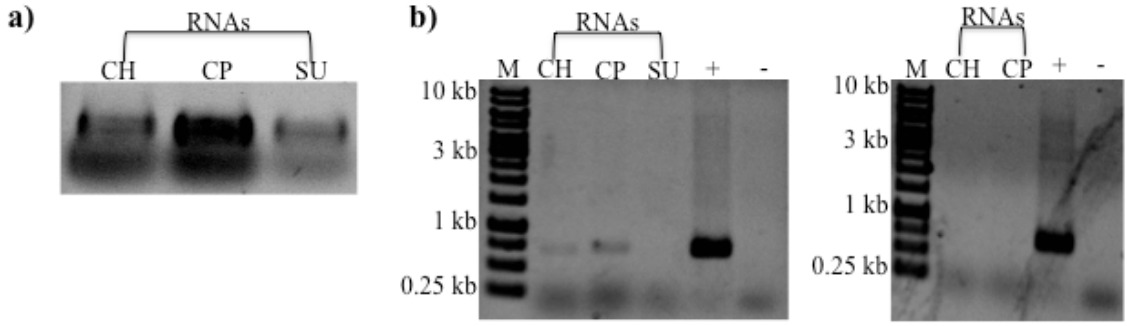

Fig. 7.2.1 RNA samples isolated from *Rh*. sp. BCP1 (a) grown on CHCA (CH), CPCA (CP), or succinate (SU); RNAs DNA-free (b); "+" indicates positive control; "-" indicates negative control; "M" 1Kb molecular weight marker.

DNA-free RNA samples were evaluated by PCR reaction using RNA as template, 2-hydroxycyclohexane dehydrogenase primers (listed in table 6); BCP1 genomic DNA was used as positive control. DNA-free RNA isolated from BCP1 succinate-grown cells was obtained after two step of digestion with DNase RNase-free (Fig. 7.2.1, b, left agarose gel). Three steps of DNase digestion were usually needed to obtain RNA-free DNA preparation from BCP1 CHCA- or CPCA-grown cells (Fig. 7.2.1, b, right agarose gel).

As alicyclic aliphatic hydrocarbons are the chemical backbone of most of the NAs, our study was focused on the putative genes of the BCP1 genome, potentially coding for enzymes involved in degradative pathways including reactions such as aromatization, oxidation through lactone formation, and β-oxidation steps [Iwaki et. al. 2005, Yi et. al. 2011, Toraya et. al. 2004]. Among the putative genes described in the literature and involved in alicyclic compounds degradation, *pobA* gene (aromatization) and genes coding for cyclohexanone

monooxygenases (Baeyer-Villiger oxidation) have been extensively studied [Iwaki et al.; 2005, Yi et al.; 2011]. In particular, the *pobA* gene codes for a 4-hydroxybenzoate 3-hydroxylase responsible for the formation of protocatechuate from 4-hydroxybenzoic acid during CHCA aromatization pathway by *Corynebacterium cyclohexanicum* [Iwaki et al.; 2005]. Cyclohexanone monooxygenases are reported to be responsible for the formation of a lactone (6-capro-lactone) during CHCA Baeyer-Villiger oxidation in *Arthrobacter* sp. CA1 [Ougham et al.; 1982].

In the BCP1 genome sequences [Cappelletti et al.; 2013], one *pobA* (KDE11135.1) gene homologue and 12 different genes coding for cyclohexanone monooxygenase (KDE14614.1, KDE14662.1, KDE14308.1, KDE12215.1, KDE12242.1, KDE11803.1, KDE10995.1, KDE11012.1, KDE11570.1, KDE11542.1, KDE12207.1) were found and their expression was analyzed by semi-quantitative RT-PCR (Fig.7.2.2).

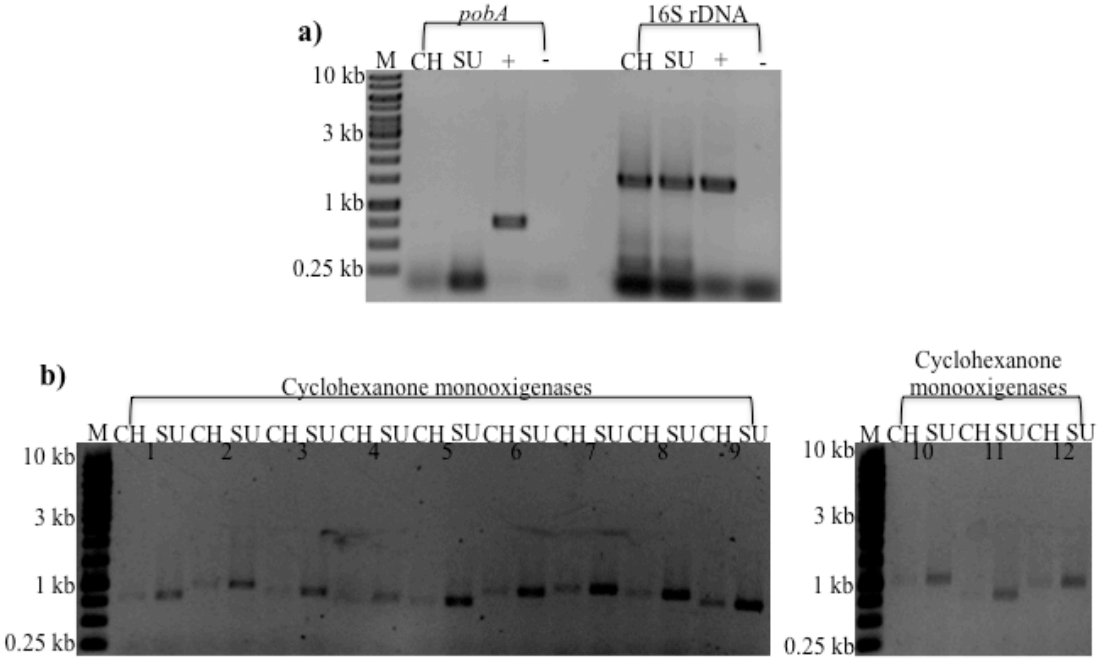

Fig. 7.2.2 Semi-quantitative RT-PCR analysis using total RNAs isolated from BCP1 grown on CHCA (CH) or succinate (su). *pobA* gene (a); 12 different genes coding for cyclohexanone monooxygenase (b, from 1 to 12); "+" indicates positive control; "-" indicates negative controls; "M" 1Kb molecular weight marker.

It was seen that *pobA* and 12 cyclohexanone monooxygenase genes were not expressed when BCP1 was grown in the presence of CHCA as the only source of carbon and energy. These data suggested the fact that CHCA degradation by BCP1 does not undergo neither aromatization or Baeyer-Villiger monooxygenase-mediate oxidation.

β-oxidation represents the preferred route by which most microorgnaisms degrade aliphatic and alicyclic carboxylic acids [Quagraine et. al. 2005a, Taylor et. al. 1978, Trudgill et. al. 1984]. Blakley *et al.* (1978) found that CHCA can be converted to CHCA-CoA through β-oxidation cycle in a strain named PRLW19. CHCA was an intermediate of degradation during anaerobic degradation of benzoic acid by *Rhodopseudomonas palustris* [Pelletier et. al. 2000]. During *Rh. palustris* CHCA degradation by β-oxidation, 2-hydroxycyclohexanecarboxyl-CoA was produced and converted into 2-ketocyclohexane-carboxyl-CoA by a dehydrogenase enzyme. On the basis of the amino acid identity percentage as compared with the 2-ketocyclohexane-carboxyl-CoA dehydrogenase of *Rh. palustris*, several genes were found in the BCP1 genome. The two best hits were analyzed (Fig. 7.2.3).

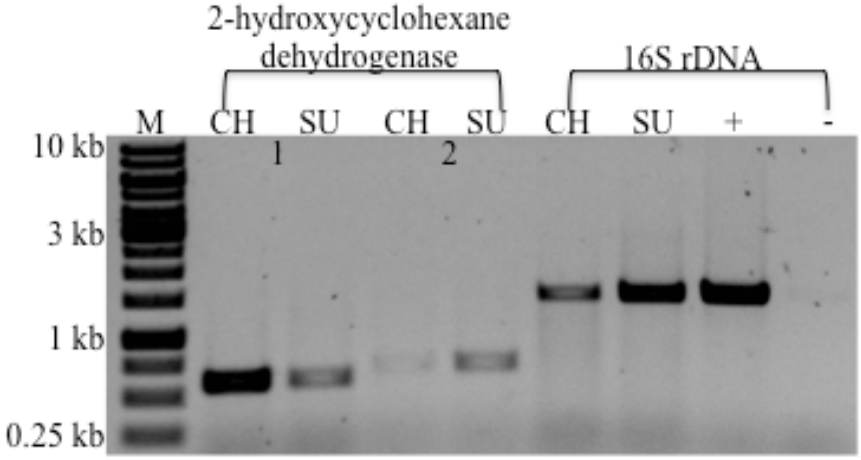

Fig. 7.2.3 Semi-quantitative RT-PCR analysis using total RNAs isolated from BCP1 grown on CHCA (CH) or succinate (su). 2-hydroxycyclohexanecarboxyl-CoA; "+" indicates positive controls; "-" indicates negative control; "M" 1Kb molecular weight marker.

The *orf* 1 (KDE15144.1) and the *orf* 2 (KDE12227.1) encode two enzymes that share 38% and 53% of identity (amino acid sequence) with the corresponding enzyme of *Rh. palustris*,

respectively. Only the *orf* 1 was expressed by BCP1 CHCA grown-cells. By analyzing the genomic region surrounding the *orf* 1, all the genes corresponding to those described in *Rh. palustris* for CHCA degradation were found. This gene cluster was named BCP1 *chca* gene cluster. The gene cluster organization, the amino acid percentage of identity, and the semi-quantitative RT-PCR results are reported in Fig. 7.2.4.

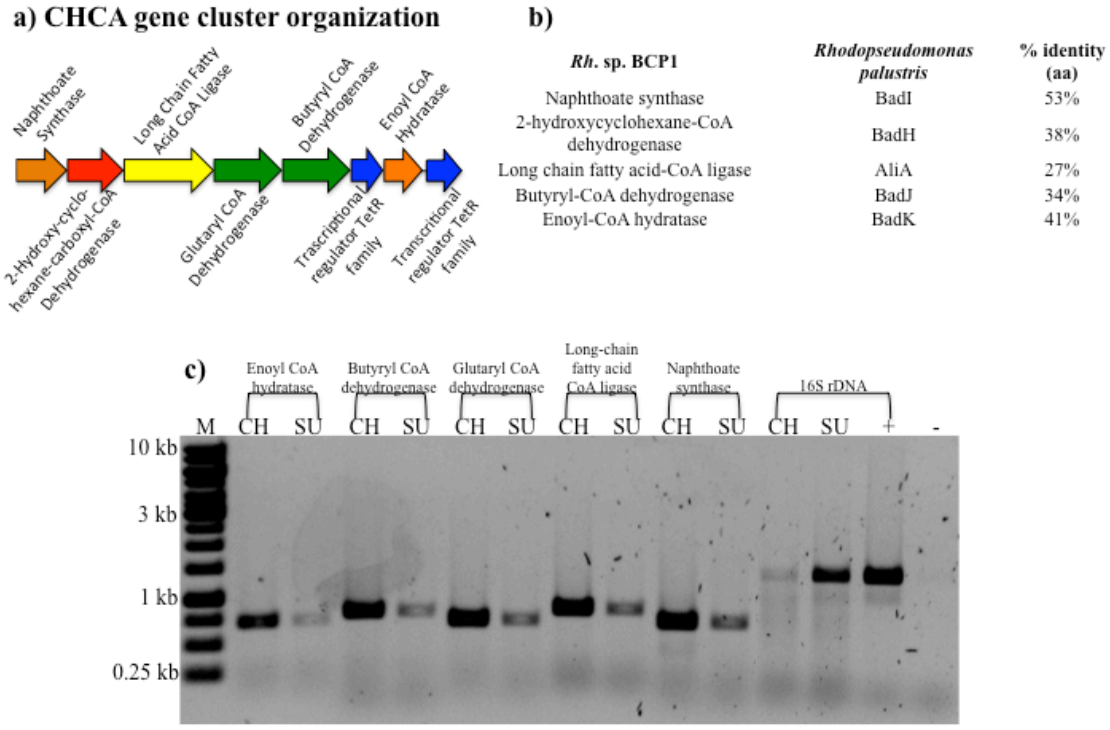

Fig. 7.2.4 (a) CHCA gene cluster organization in BCP1; (b) correspondence between BCP1 and *Rhodopseudomonas palustris* gene cluster and amino acid percentages of identity; and (c) semi-quantitative RT-PCR analysis using total RNAs isolated from BCP1 grown on CHCA (CH) or succinate (su); 2-hydroxycyclohexanecarboxyl-CoA; "+" indicates positive controls; "-" indicates negative control; "M" 1Kb molecular weight marker.

In addition to the genes, homologous to those found in *Rh. palustris*, the BCP1 *chca* gene cluster includes an additional dehydrogenase (annotated as glutaryl-CoA dehydrogenase KDE15142.1) and two *orfs* coding for transcriptional regulators belonging to TetR family (KDE15140.1, and KDE15138.1).

RT-PCR experiments using RNAs isolated form BCP1 CHCA- or CPCA- grown cells were carried out to assess whether the *chca* gene cluster is transcribed as mono- or polycistronic unit (Fig.7.2.5).

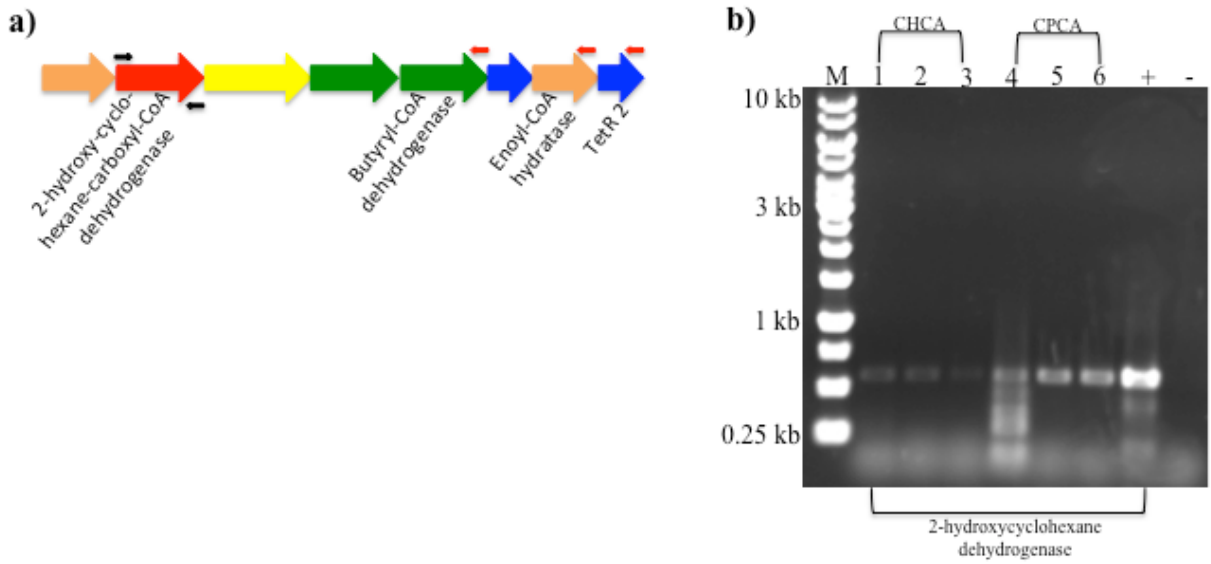

Fig. 7.2.5 Fig.7.2.6 Schematic representation of the experimental procedure; red and black arrows indicate the specific reverse primers used to perform RNAs retro-transcription and the primers used to amplify the target gene (shown in a). RNAs were isolated from BCP1 CHCA- or CPCA-grown cells (b). The retro-transcription was carried out using reverse primer complementary to the mRNA coding for TetR2 (lanes 1, and 4), enoyl-CoA hydratase (lanes 2, and 5), and butyryl-CoA dehydrogenase (lanes 3, and 6); Gene coding for 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase was used as target; "+" indicates positive control (BCP1 gDNA); "-" indicates negative control; "M" 1Kb molecular weight marker.

The retro-transcription step of RNA samples was carried out using specific reverse primer for TetR2 (KDE15138.1), enoyl-CoA hydratase (KDE15139.1), and butyryl-CoA dehydrogenase (KDE15141.1). An RT-PCR product relative to the gene coding for 2-hydroxy-cyclohexanecarboxyl-CoA (KDE15144.1) dehydrogenase was obtained for each condition of reverse-transcription tested. These data suggested that the *chca* gene cluster was expressed as monocistronic unit.

Quantitative Real-Time RT-PCR (RT-qPCR) experiments were carried out using RNAs isolated from BCP1 grown in the presence of CHCA, CPCA, succinate, and/or glucose as the only source of carbon and energy. As the *chca* gene cluster was transcribed as a monocistronic unit, our studies were focused on the analysis of the expression of one gene of the cluster that could represent the transcription of the entire operon. The gene coding for glutaryl-CoA dehydrogenase (KDE15142.1) (target gene) was chosen to evaluate the relative fold change of the *chca* gene cluster in the presence of NAs compared to succinate-grown

cells. The influence of alternative carbon sources (glucose or succinate) on the *chca* operon expression induced by CHCA or CPCA was also evaluated (Fig. 7.2.6, a, and b).

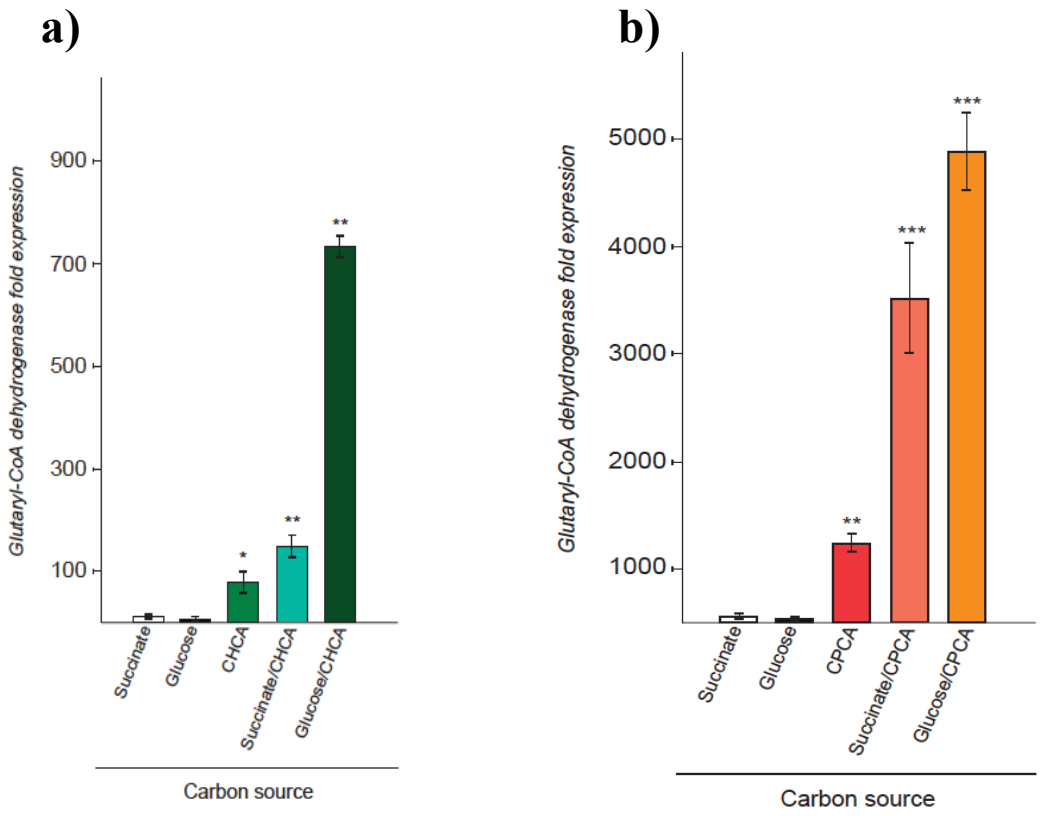

Fig.7.2.6 RT-qPCR. Glutaryl-CoA dehydrogenase fold expression in BCP1 grown on: succinate, and glucose (a, b); CHCA, glucose-CHCA, or succinate-CHCA (a); CPCA, glucose-CPCA, or succinate-CPCA (c). Data are expressed as mean ± SD of at least three different experiments. "*" denotes values significantly different (P<0.05); ""(P<0.005); "***"(P<0.0005) .**

The data showed that the mRNA level of the target gene was around 70- and 1200-fold higher when BCP1 was grown on CHCA or CPCA, respectively, versus succinate as the sole carbon source. Conversely, the transcriptional level of glutaryl-CoA dehydrogenase (KDE15142.1) induced by the growth on glucose was at the basal level (0.6-fold). A strong increase of the target gene expression was obtained when BCP1 was grown in the presence of CHCA or CPCA along with glucose or succinate as co-carbon source. The mRNA level were seen around 700- (CHCA-glucose), 4900- (CPCA-glucose), 149- (CHCA-succinate), and 3400-fold (CPCA-succinate) as compared to succinate. These results suggest that the enzymes encoded by the *chca* gene cluster are involved in CHCA and CPCA degradation. The gene

expression level varied depending on the substrate inducer, being CPCA a better inducer than CHCA. The presence of an alternative carbon source (glucose or succinate) was shown to improve the expression of the *chca* gene cluster.

7.3. pTNR (TA)-mediated transposon mutagenesis of *Rh. opacus* R7

The non-replicant transposon vector pTNR-TA containing IS*1415* elements derived from *Rh. erythropolis* was used to generate transposon-induced mutants of *Rh. opacus* R7 [Sallam et al.; 2007]. Initially, the *Rhodococcus* electroporation procedure was optimized by using the *E. coli*/*Rhodococcus* shuttle vector pNC9503 [Kalscheuer et al.; 1999], that conferred thiostrepton resistance to R7 transformant cells. The electroporation procedure described by Kalscheuer and colleagues (1999) was modified and adapted as following (i) cultivation of R7 cells in LB medium containing 1.5% (w/v) glycine and 1.5% (w/v) sucrose; (ii) 1 μg of foreign DNA/μL added to the cell suspension for the electroporation; (iii) a field strength of 2.5 kV/cm applied during the electroporation. R7 transformants, were spread out onto LB agar plates supplied with different concentrations of thiostrepton (Fig. 7.3.1).

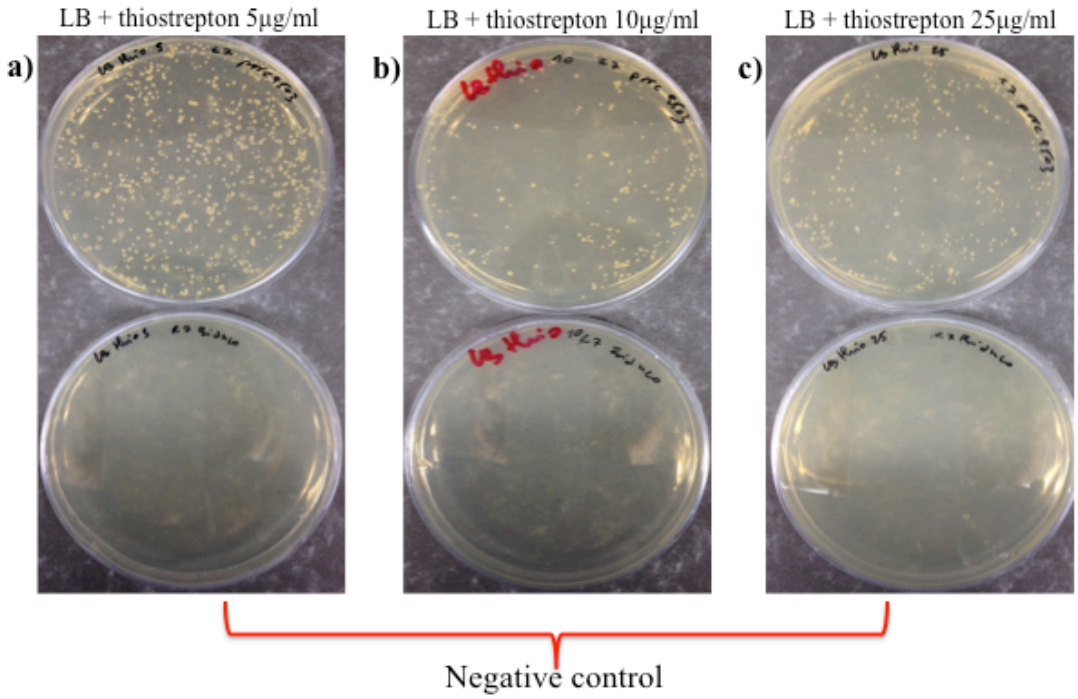

Fig. 7.3.1 *Rh. opacus* R7 cells transformed with pNC9503 *E. coli*/*Rhodococcus* shuttle vector plated onto LB agar plates with 5 µg/mL (a), 10 µg/mL (b), and 25 µg/mL (c) of thiostrepton as selectable marker. In negative controls, sterile water was substituted for foreign DNA.

Results show that R7 transformants were able to grow at each concentration of thiostrepton tested. In order to assess whether these transformants cells received the shuttle vector used, sixty random colonies were picked up and streaked out on the same plates (Fig. 7.3.2).

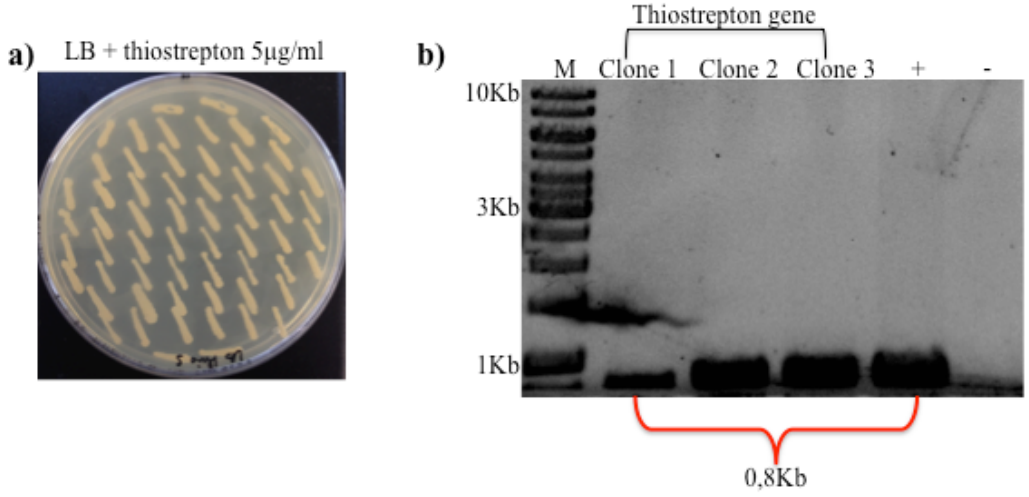

Fig. 7.3.2 R7 transformants streaked out onto LB agar plate containing 5 µg/mL of thiostrepton (a); colony PCR screening for 3 clones randomly chosen (b); "+" indicates positive controls (thiostrepton-gene carried by pNC9503); "-" indicates negative controls; "M" 1 Kb molecular weight marker.

The results showed that all the clones were able to grow in approximately 20 hs at 30 °C, suggesting that pNC9503 was received and maintained by R7 cells. The efficiency of transformation of R7 cells was around 2,4 x 10^5 CFU/mL when R7 was selected with thiostrepton used at 5 μg/mL. Among the R7 clones obtained, three clones were chosen to perform a colony PCR using the thiostrepton-gene primer (Fig. 7.2.3, b). The positive results obtained with PCR, confirmed that pNC9503 was inside R7 cells. Since the concentration of 5 μg/mL thiostrepton gave the best result in terms of transformation efficiency, this antibiotic concentration was used to generate a transposon-induced mutant library. pTNR-TA was the transposon vector used in this present work and it is characterized by the following elements: (i) IS*1415* elements derived from *Rh. erythropolis*; (ii) the selectable marker gene (*thio*^{r}); (iii) *istA* and *istB* genes coding for enzymes responsible for the transposition of inverted repeated sequences (IR1 and IR2) flanking the selectable marker. Since pTNR-TA vector does not contain an origin of replication recognized in *Rhodococcus*, R7 transposon-mutants are resistant to thiostrepton only following the transposition of the selectable marker (*thio*^{r}) into the genome (Fig. 7.3.3, a).

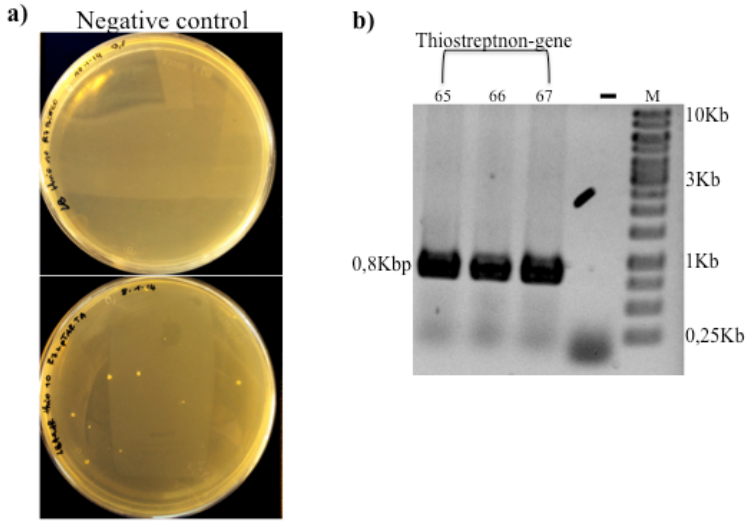

Fig. 7.3.3 *Rh. opacus* R7 cells transformed with pTNR-TA transposon vector plated onto LB agar plates with 5 μg/mL of thiostrepton (a); colony PCR screening for three clones randomly chosen using primers to amplify thiostrepton resitance gene (b); "-" indicates negative control; "M" 1Kb molecular weight marker.

Due to the low efficiency of recombination, the results showed a drastic decrease of the number of transformants obtained as compared to the experiments in which the shuttle vector was used. Among those obtained, three clones were chosen and evaluated by colony PCR enhancing the gene responsible for the thiostrepton resistance (Fig. 7.3.3, b). As a result, a PCR product of the expected size was obtained, confirming the transposition of the selectable marker into R7 genome. During the mutant library construction, to avoid auxotrophic mutants, all the clones obtained were streaked out onto MSM agar plates supplied with glucose (0,5% v/v). R7 defective-growth mutants were evaluated by analyzing their phenotype (as: growth/no growth) on MSM agar plates supplied with CHCA, or CPCA (100 mg/L) (Fig. 7.3.4). Thiostrepton was always added to maintain the selection for the presence of thiostrepton resistant gene.

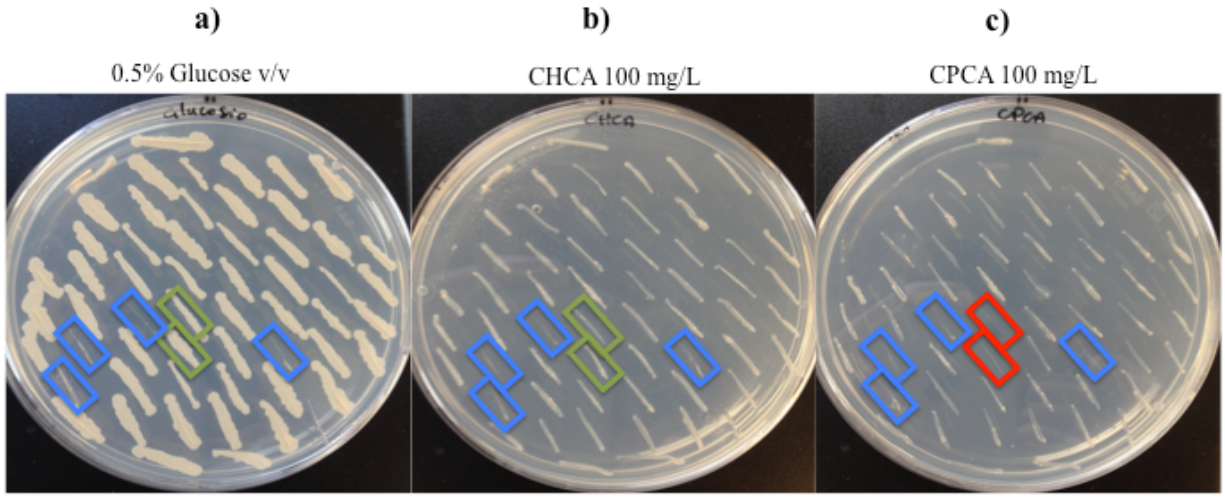

Fig. 7.3.4 R7 transposon-mediated mutants streaked out on MSM agar plates supplied with glucose (0,5% v/v) (a), CHCA or CPCA (100 mg/L) (b, and c). The auxotrophic mutants are indicated by blue rectangles, while CPCA defective-growth and glucose or CHCA non defective-growth mutants are indicated by red and green rectangles, respectively.

Up to date, among 70,000 clones obtained, 4,980 were stable transposon-mutants. The efficiency of recombination was assessed to be around 7%. Eleven mutants were CPCA defective-growth mutants, while they maintained the growth phenotype regarding CHCA. The insertion locus of the transposon was mapped by two-step gene walking method (§ 7.1).

The identification of the genes disrupted in R7 is listed in table 8.

Table 8. R7 mutants identified by the two-step gene walking primer method.

Mutants names	Phenotype			Identity of disrupted genomic regions	Genomic Localization
	Glucose	CHCA	CPCA		
1-24	Y	Y	N	Enoyl-acyl-carrier protein reductase	plasmid pDG2
1-26	Y	Y	N	Large subunit naph/bph dioxygenase	plasmid pDG3
1-35	Y	Y	N	Nitrilotriacetate monooxygenase	plasmid pDG3
3-27	Y	Y	N	Long-chain-fatt-acid-CoA ligase	plasmid pDG1
10-14	Y	Y	N		
22-26	Y	Y	N		
23-12	Y	Y	N		
29-9	Y	Y	N		
29-20	Y	Y	N		
39-47	Y	Y	N		
58-55	Y	Y	N		

The transposon-mutants were shown to be disrupted in *orfs* present only in R7 plasmids (pDG1, pDG2, pDG3). According to Sallam and co-workers (2007), pTNR-TA is able to transpose in genomic regions with a high GC content. Related to this, R7 plasmids are featured by hot-spot regions in which the transposition might easily occurs. The genetic loci of the transposon-mutants are shown in figure 7.3.5.

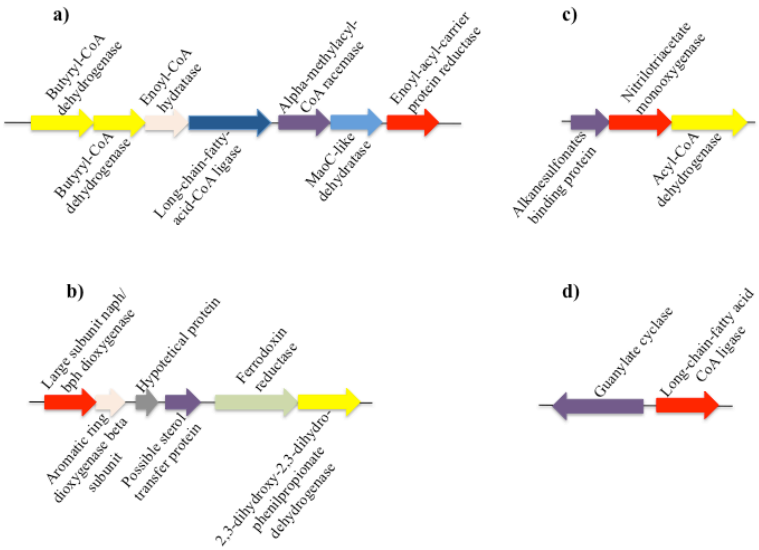

Fig. 7.3.5 Genetic loci of R7 transposon-mutants. The *orf* interrupted are marked by a red arrow: Enoyl-acyl-carrier protein reductase (a); Large subunit naphthalene/biphenyl dioxygenase (b); Nitrilotri-acetate monooxygenase (c); Long-chain-fatty-acids-CoA ligase (d).

The transposon-mutant 1-24 was characterized by the interruption of the *orf* coding for an enoyl-acyl-carrier protein reductase (AII10922.1), which is clustered along with *orf* (AII10921.1, AII10920.1, AII10919.1, AII10918.1, AII10917.1) responsible for the metabolism of fatty acids (Fig. 7.3.5, a). The transposon-mutant 1-26 presents the disruption of an *orf* annotated as a large subunit naphthalene/biphenyl dioxygenase (AII11493.1). As reported by Parales *et al.* 2000, naphthalene dioxygenase mediates the formation of *cis*-1,2-dihydronaphthalene-1,2-diol from naphthalene with the oxidation of NADH to NAD^+. In the same genetic locus, *orfs* coding for the dioxygenase β-subunit (AII11494.1) hypotetical protein (AII11495.1), a possible sterol transfer protein (AII11496.1), a ferrodoxin reductase (AII11497.1), and 2-3-dihydroxy-2-3-dihydrophenilproprionate dehydrogenase (AII11498.1) were found (Fig. 7.3.5, b). The transposon-mutant 1-35 is featured by the interruption of an *orf* coding for a putative nitrilotri-acetate monoxygenase (AII03608.1). This enzyme was described to oxidize nitrilotri-acetate utilizing reduced flavin mononucleotide (FMNH2) and oxygen (O_2) to iminodiacetate and glyoxylate [Uetz et al; 1992]. In addition, this protein contains an alkanesulfonate monooxygenase domain responsible for the desulfonation reaction of a wide range of alkanesulfonates. It is also classified as sulfate starvation-induced protein 6. This *orf* is grouped with genes coding for an alkane sulfonate binding protein (AII03609.1) and a dehydrogenase (AII03607.1) probably involved in alkanesulfonate assimilation (Fig. 7.3.5, c). Eight transposon-mutants were characterized by the interruption of the *orf* coding for a long-chain-fatty-acid CoA ligase (AII10677.1). This enzyme is responsible for the activation of fatty acids in the presence of ATP and CoA, as fatty acid-CoA, and it is clustered with an open reading frame that encodes for a guanylate-cyclase (AII10676.1) (Fig. 7.3.5, d).

7.4. Discussion

In this thesis work, a genetic study was performed aiming to identify the potential "biodegradative" genes possibly involved in NAs (CHCA, and CPCA) degradation by *Rh*. sp. BCP1 and *Rh. opacus* R7 cells.

Analysis of putative biodegradative enzymes in cells of the BCP1 strain was based on the detection of genes that are homologous to those described in the literature as involved in NAs biodegradation. In this respect, CHCA has been described as a metabolic intermediate of benzoic acid degradation by *Rhodopseudomonas palustris* under anaerobic condition [Pelletier et al.; 2000]. The amino acid sequences of the enzymes involved in this metabolism were utilized for a sequence similarity search in BCP1 genome. Interestingly, a few homologous genes were found in the BCP1 genome and their expression was analysed in NAs-growing cells (Fig. 7.4.1). On the basis of the amino acid identity percentage, two different genes encoding for 2-hydroxy-cyclohexanecarboxyl-CoA dehydrogenase (KDE15144.1, KDE12227.1) were found in the BCP1 genome. Further, in CHCA- and CPCA-grown cells, the RT-qPCR showed a high expression level of the gene KDE15142.1, that shares the 38% of amino acid identity with the homologue described in *Rh. palustris*. As a matter of fact, most of the genes described in *Rh. palustris* were found in BCP1 cells. Since these genes (KDE15145.1, KDE15144.1, KDE15143.1, KDE15142.1, KDE15141.1, KDE15140.1, KDE15139.1, and KDE15138.1) are clusterd and transcribed as a monocistronic unit it might be suggested that the enzymes encoded by the genes included in the *chca* operon are likely to be involved in CHCA or CPCA degradation. The putative reactions catalized by these enzymes are represented in Fig. 7.4.1.

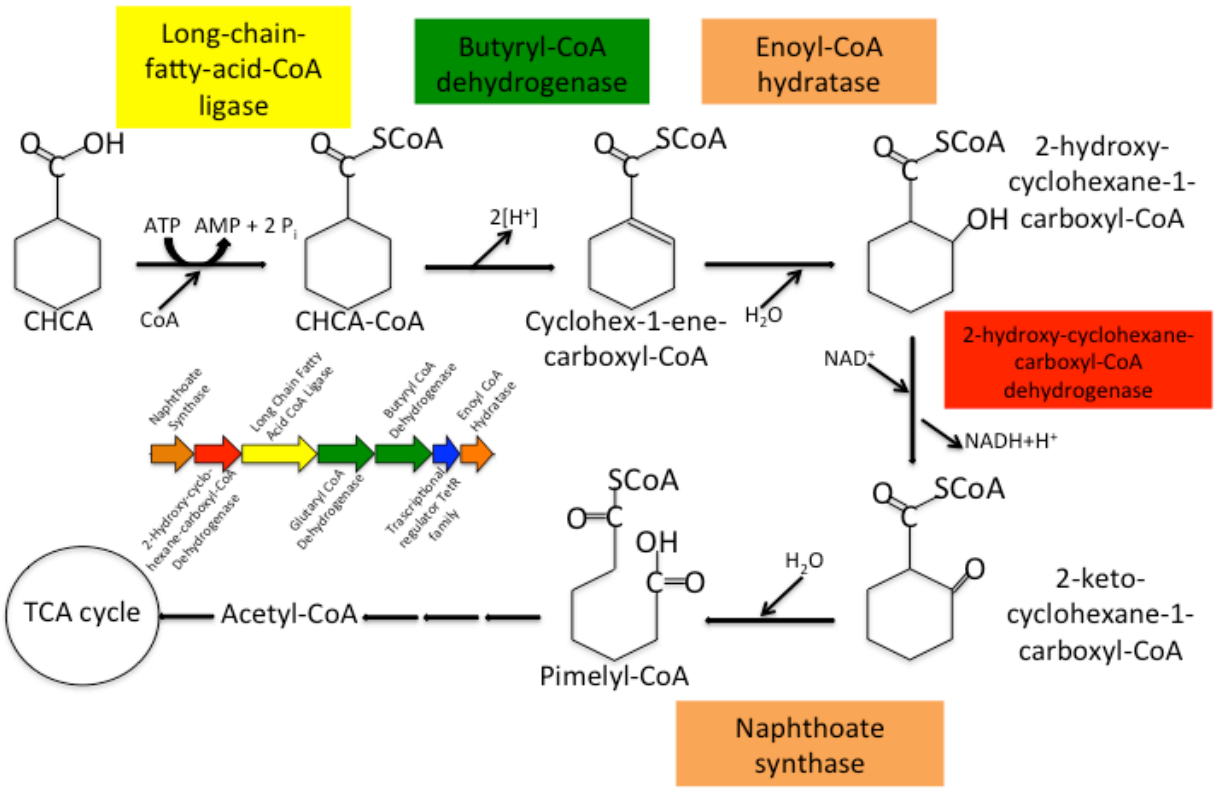

Fig. 7.4.1 Putative metabolic pathway proposed for *Rh*. sp. BCP1 CHCA-grown cells.

Based on of the type of reactions catalysed by the enzymes coded by the *chca* operon, the following CHCA transformation steps can be hypothized. After the cytosolic internalization, the CHCA might be converted into its CoA thioester through the activity of the long-chain-fatty-acid CoA-ligase (KDE15143.1). The thioester can therefore enter into the β-oxidation cycle. The first step of this cycle would be mediated by a dehydrogenase (butyryl-CoA dehydrogenase KDE15141.1 in *chca* operon of BCP1) that converts CHCA-CoA to the corresponding aldehyde (cyclohexan-1-ene-carboxyl-CoA). The second step could be due to an hydratase (enoyl-CoA hydratase KDE15139.1) activity that produces a secondary alcohol (2-hydroxycyclohexane-carboxyl-CoA). During the third step, another dehydrogenase (2-hydroxy-cyclohexane-carboxyl-CoA dehydrogenase KDE15144.1) might form the keto-CHCA-CoA from the secondary alcohol. The fourth step could be mediated by the hydratase (naphthoate synthase KDE15145.1) activity responsible for the ring opening that produces pimelic acid. Rho and Evans (1974) detected pimelic acid studing the metabolism of CHCA

labelled with C14 in *Acinetobacter anitratum*. A similar pathway was also proposed by Torya *et al.* (2004) studing the cyclopropane degradation by *Rhodococcus rhodocrous,* which was able to degrade cyclopropane through the β-oxidation pathway. Other studies showed that CHCA ring can get into the aromatization pathway forming protocatechuate as intermediate [Kaneda et al.; 1974, Iwaki et al.; 2005]. Iwaki and co-workers (2005) studied the *pobA* gene coding for the enzyme responsible for the formation of protocatechuate from 4-hydroxybenzoic acid in *Corynebacterium cyclohexanicum* CHCA-grown cells. Notably, in the BCP1 genome the homologous *pobA* gene was found, but RT-PCR experiments using RNAs isolated from BCP1 CHCA-grown cells revealed that this gene was not expressed.

The evaluation of the involvement of cyclohexanone monooxygenases in CHCA degradation by BCP1 was based on two differet studies, namely: i) Ougham *et al.* (1982) reported that CHCA can be oxidized to capro-lactone by a Baeyer-Villiger monooxygenase; ii) Yi *et al.* (2011) detected the lactone formation (gamma-butyrolactone) during CHCA degradation by *Rhodococcus* sp. EC1. In the BCP1 genome, twelve different cyclohexanone monooxygenases were found and their expression was analysed through RT-PCR experiments. The results showed that none of them were expressed by BCP1 under CHCA growth condition. These data, taken together, suggest therefore that the β-oxidation is the preferential metabolic pathway exploited by BCP1 for CHCA degradation.

For many actinomycets characterized by a variety of metabolic properties, genetic studies are often limited due to the lack of molecular tools and efficient electroporation methods. In this thesis work, a random mutagenesis library of *Rh. opacus* R7 was constructed through the utilization of a transposon-based vector system named pTNR-TA. This plasmid was designed and constructed by Sallam *et al.* (2007) not only to generate transposon-mediated mutants, but also to insert in *Rhodocccus* species genes coding for recombinant protein. For example, *prcA* and *prcB* that encodes for α- or β-subunits of *Streptomyces coelicolor* proteasome were

successfully expressed in different *Rhodococcus* species (*Rh. fascians*, *Rh. erythropolis*, *Rh. rhodochrous*). Here we have successfully used this transposon-mediated system to create mutants of *Rhodococcus opacus* R7 unable to grow on NAs. In particular, 11 transposon-mutants defective in the growth on CPCA were identified. The sequence data of the genomic regions that were interrumpted by the transposon in these 11 mutants defined the genes that are essential for the growth on this NA. The transposon-mutants 1-24 was characterized by the disruption of the *orf* coding for an enoyl-acyl-carrier protein reductase (AII10922.1) that catalyse the reduction of 2-trans-octenoyl-CoA. Vilcheze *et al.* (2000) reported that a mutant of *Mycobacterium smegmatis* lacking the gene coding for the enoyl-acyl carrier protein of the fatty acid synthase was affected in micolyc acid bsiosynthesis, along with an increase in its tretracosanoic acid ($C_{24:0}$) content plus a decrease in hexadecanoic acid ($C_{16:0}$), similarly to *M. smegmatis* cells grown in the presence of isoniazide (antitubercolosis grug). In particular, these Authors demonstrated that the inactivation of *InhA* is per se sufficient to induce the lysis of *M. smegmatis*. According to this, the absence of growth on CPCA by R7 strain lacking the enoyl-acyl-carrier protein reductase (AII10922.1) might be explained by the prevention of a correct fatty acids metabolism. This hypothesis is also supported by the FAMEs data analysis reported in § 6.3 which shows significant membrane fatty acid modifications in R7 cells growing on CPCA. Notably, in R7, the AII10922.1 is clustered together with *orfs* coding for additional enzymes involved in the metabolism of fatty acids: AII10921.1, AII10920.1, AII10919.1, AII10918.1, AII10917.1.

The disruption of the *orf* coding for a long-chain-fatty-acid-CoA ligase (AII10677.1) also impaired the R7 growth on CPCA. Fatty acids are used as sources of carbon and energy by prokaryotes, and their catabolism requires activation into the corresponding fatty acid-CoA forms before they can be converted into metabolites that can enter the central metabolism. Moreover, these compounds are the most important building blocks of the cellular material. In

this respect, fatty acids occur mainly in bacterial cell membranes as the acyl constituents of phospholipids [Kaneda et al.; 1991]. For example, mycolic acids are described as the main and specific long-chain fatty acids of the cell envelope in important human pathogens (e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Corynebacterium diphtheriae*). Their biosynthesis is essential for the growth of these bacteria strains, and also as a target for developing new antituberculous drugs [Portevin et al.; 2005]. Portevin *et al.* (2005) showed that *Corynebacterium diphtheriae* mutant in the gene coding for Acyl-AMP Ligase FadD32 was affected in micolyc acids synthesis. This gene is also essential for the survival of *Mycobacterium smegmatis.* These evidences can therefore explain the absence of growth on CPCA displayed by R7 mutants in the *orf* coding for the long-chain-fatty-acid-CoA ligase (AII10677.1).

The transposon-mutants 1-26 and 1-35 were characterized by the interruption of *orfs* coding for the components of two different oxygenase systems i.e. the large subunit naphthalene/biphenyl dioxygenase (AII11493.1) and the nitrilotriacetate monooxygenase (AII03608.1), respectively. The first enzyme has been described to be involved in naphthalene or biphenil oxidation [Parales et al.; 2000], while nitrilotriacetate monooxygenase oxidizes the nitrilotriacetate to iminodiacetate and glyoxylate utilizing reduced flavin mononucleotide (FMNH2) and molecular oxygen (O_2) [Uetz et al.; 1992]. The gene coding for the nitrilotiacetate monooxygenase (AII03608.1) is clustered along with *orfs* putatively involved in alkanesulfonate assimilation. These enzymes are reported to be synthetized during sulfate starvation in *E. coli* EC1250 cells, and the deletion mutant in the operon containing *orfs* coding for enzymes involved in the alkanesulfonate uptake and desulfonation showed a diauxic growth as compared to the wild type strain [Eichhorn et al.; 1999]. The *orf* coding for the large subunit naphthalene/biphenyl dioxygenase (AII11493.1) and disrupted in R7 mutant 1-26 is clustered with the putative dioxygenase β-subunit

(AII11494.1), and *orfs* coding for the enzymes that might be putatively involved in naphthalene and/or biphenyl oxidation. Notably, the mutations of naphthalene/biphenyl dioxygenase (AII11493.1) and nitrilotriacetate monooxygenase (AII03608.1) impaired the growth of R7 on CPCA but not on CHCA. This might be due to the presence of additional metabolic pathways involved in CHCA degradation by R7 or to the presence of an alternative catabolism of CHCA compared to CPCA in this strain.

In conclusion, this genetic analysis was aimed to reveal the mechanisms by which these *Rhodococcus* strains can degrade NAs toxic molecules. These data suggested that different pathways can be exploited by the two *Rhodococcus* strains investigated. Although we can conclude that BCP1 is capable of CHCA- or CPCA β-oxidation the construction of a deletion mutant in the CHCA gene cluster is necessary to assess whether or not the proposed pathway is the only one used by BCP1. Regarding R7, our data tend to show that CHCA- and CPCA-degradation pathways are different in this strain. Further, the absence of growth of R7 transposon-mutants in the presence of CPCA is likely to result from an impairement in the fatty acids metabolism or in the oxidation mechanism exploited by this strain. Apparently, more transposon-mutants are required to obtain a reliable picture of the degradative potential of *Rh*. strain R7 as far as concern toxic NAs.

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