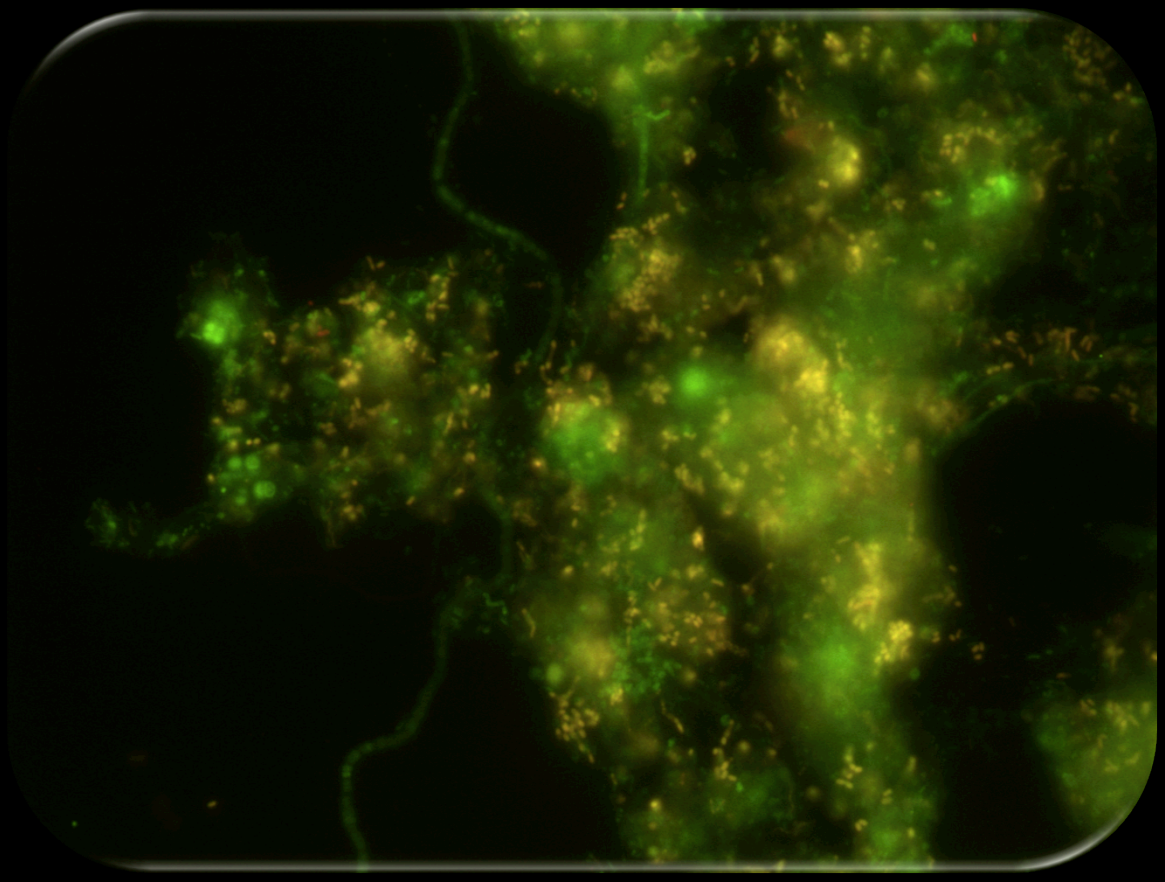
Micropollutant bioremoval in wastewater treatment systems: from microbial population structure to function

Bárbara Fonseca de Almeida



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
February, 2013

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To my family

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The continuous release of micropollutants into receiving waters due to insufficient elimination from wastewater treatment plants (WWTP) raises global concerns regarding their potential risks to the environment and human health. Most treatment plants employ biological systems to degrade organic matter due to economic and environmental advantages but they are not designed to select microbial consortia that possess the necessary enzymes to remove micropollutants. Therefore, it is important to characterise the microbial populations in treatment systems in order to optimise the elimination of these compounds from WWTP. This work characterises the performance, metabolism and identity of the microbial populations involved in the degradation of target micropollutants, namely two pharmaceutical compounds and a heavy metal.

The main factors influencing the performance of activated sludge during the degradation of ibuprofen and ketoprofen are described in this study using mathematical models. The model accurately predicted the degradation of both compounds, which was found to be a function of biomass composition and substrate loading rate. From one of the characterised activated sludge, 12 microorganisms were isolated that were possibly involved in the ibuprofen and ketoprofen biodegradation. One of the isolates was characterised via another model, where ibuprofen degradation performance was dependent on both biomass growth and initial substrate concentration. This isolate with unique

phenotypical and molecular characteristics has been classified as a new species (*Patulibacter medicamentivorans*). Furthermore, the metabolic aspects of ibuprofen degradation by *Patulibacter medicamentivorans* were investigated through quantitative proteomics using a metabolic labelling strategy. The whole genome of this microorganism was sequenced to provide a species-specific protein platform for optimal protein identification. Several proteins were identified that are possibly involved in the degradation of ibuprofen and that will enable further studies aiming at characterising the mechanisms of microbial community development and dynamics in engineered systems. An example of such an application was conducted in this thesis to assess the evolution of mercury reducing microbial communities developed under different operating conditions. The genetic (16S rRNA gene) and functional (*merA* gene) characteristics of the mercury-reducing cultures enriched with different carbon sources (glucose, ethanol or acetate) were investigated. The choice of the carbon source was shown to have profound effects on both the phylogenetic development of microbial cultures and mercury reducing performance.

This thesis represents a step towards a better understanding of the microbial communities involved in the degradation of important micropollutants. This knowledge can be applied in the future for improved design and operation of water and wastewater treatment systems in order to enhance the removal of these compounds.

A emissão constante de micropoluentes em meios aquáticos, devido à eliminação ineficiente destes compostos nas estações de tratamento de água residual (ETAR), tem suscitado preocupações a nível mundial a respeito de riscos adversos potenciais para o meio ambiente e saúde humana.

A maioria das ETARs, utilizam processos biológicos para remover contaminantes orgânicos da água residual por serem processos vantajosos em termos económicos e ambientais. No entanto, estes sistemas não estão projectados para seleccionar consórcios microbianos que possuam as enzimas necessárias para remover micropoluentes, levando à eliminação ineficiente destes compostos. Perante este fato, torna-se importante caracterizar as populações microbianas em sistemas de tratamento, a fim de optimizar a eliminação destes compostos nas ETARs. O trabalho apresentado nesta tese caracteriza o desempenho, o metabolismo e a identidade das populações microbianas envolvidas na degradação de dois compostos farmacêuticos (ibuprofeno e cetoprofeno) e de um metal pesado (mercúrio).

Os principais factores que influenciam o desempenho de lamas activadas durante a degradação do ibuprofeno e do cetoprofeno foram descritos neste estudo através de modelação matemática. O modelo matemático, previu com precisão a degradação de ambos os compostos, em função da composição de biomassa e de concentração inicial de substrato. Doze microrganismos, potencialmente envolvidos na biodegradação do ibuprofeno e cetoprofeno, foram isolados de uma das lamas activadas previamente caracterizada pelo modelo matemático. A degradação de ibuprofeno por um dos isolados foi descrita por outro modelo

matemático, em função do crescimento de biomassa e da concentração inicial de substrato. Este isolado, com características fenotípicas e moleculares exclusivas foi classificado como uma espécie nova, *Patulibacter medicamentivorans*. Adicionalmente, os aspectos metabólicos de degradação do ibuprofeno por *Patulibacter medicamentivorans,* foram investigados por proteómica quantitativa usando uma estratégia de marcação metabólica. O genoma deste microrganismo foi sequenciado e usado como uma plataforma de identificação proteica específica para as proteínas desta espécie. Várias proteínas foram induzidas na presença de ibuprofeno, que provavelmente estarão envolvidas na biodegradação deste composto. A identificação destas proteínas permitirá elucidar os mecanismos de degradação, e seguir a actividade e a dinâmica de desenvolvimento das comunidades microbianas em bioreactores. Um exemplo desta aplicação foi realizada nesta tese para avaliar a evolução das comunidades microbianas reductoras de mercúrio, desenvolvidas sob diferentes condições de operação. As características genéticas (gene rRNA 16S) e funcionais (gene *merA*) de culturas reductoras de mercúrio enriquecidas na presença de diferentes fontes de carbono (glicose, etanol ou acetato) foram investigadas. A escolha da fonte de carbono demonstrou afectar a selecção e a filogenética das culturas microbianas, bem como a sua capacidade de redução do mercúrio.

Esta tese representa um avanço no sentido de uma melhor compreensão das comunidades microbianas envolvidas na degradação de importantes micropoluentes. No futuro, este conhecimento pode ser aplicado na concepção e operação de sistemas de tratamento de águas residuais, com o objectivo de aperfeiçoar a remoção destes compostos e minimizar o seu impacto ambiental.

List of Publications

Almeida, B., Oehmen, A., Marques, R., Brito, D., Carvalho, G., Barreto Crespo, M.T., 2012. Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture. Submitted to J. Hazard. Mater.

Almeida, B., Vaz-Moreira, I., Schumann, P., Nunes, O.C., Carvalho, G., Barreto Crespo, M.T., 2012. *Patulibacter medicamentivorans* sp. nov., isolated from activated sludge of a wastewater treatment plant. Submitted and in revision to the Int. J. Syst. Evol. Microbiol.

Almeida, B., Kjeldal, H., Lolas, I., Knudsen, A.D., Carvalho, G., Nielsen, K.L., Barreto Crespo, M.T., Stensballe, A., Nielsen, J.L., 2012. Quantitative proteomic analysis of ibuprofen-degrading *Patulibacter* sp. strain I11. Biodegradation. doi:10.1007/s10532-012-9610-5.

Carvalho, G., Almeida, B., Fradinho, J., Oehmen, A., Reis, M.A., Crespo, M.T., 2011. Microbial characterisation of mercury reducing mixed cultures enriched with different carbon sources. Microbes Environ., 26, 293-300.

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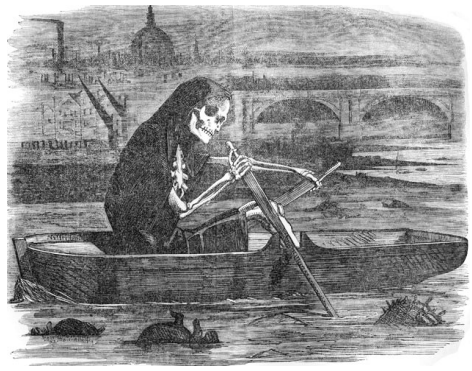
Chapter 1

Introduction

1. Introduction



"The Silent Highway - Man". This cartoon comments on the polluted condition of the Thames river. Death is here associated with pollution and disease. Punch, 10 July 1858.

The industrial progress had a profound positive effect on the social, economic and cultural conditions of our times but it has also been associated with the generation and release into the environment of large amounts of toxic compounds, causing the widespread contamination of land and water.

The presence of toxic compounds in water has raised concerns among drinking-water regulators, governments, water suppliers and the public in general, regarding their potential risks to the environment and to human health. The protection of water resources has become one of the most important environmental challenges of the 21st century.

1.1 Micropollutants in water

The term “micropollutant” refers to an environmental toxic compound at concentrations below the milligram per liter (mg L^{-1}) (Murray et al., 2010). Micropollutants are comprised not only by metals and metalloids such as mercury, cadmium or arsenic, but also by organic contaminants such as pharmaceuticals, biocides and personal care products. Some of which have been classified as priority pollutants due to possible mutagenic, carcinogenic or toxic effects (Kolpin et al., 2002). A list of 33 priority micropollutants has been established by the European Union Water Framework Directive to regulate the levels of these compounds in environmental discharges and in drinking water (European Commission, 2008, Murray et al., 2010). With the advances in analytical techniques, new contaminants are being detected that can have adverse effects to human health or environment. These so called “emerging contaminants” are strong candidates for future regulation (Murray et al., 2010). Furthermore, potential synergistic or antagonistic toxic effects might occur from mixtures of compounds or metabolites (Kolpin et al., 2002).

The routes by which micropollutants enter the environment are diverse and closely related to the final target/function for which they are produced. The major point source is wastewater from industrial, domestic and agricultural activities. A variety of monitoring campaigns showed that micropollutants occur up to the µg L^{-1} level in wastewater treatment plant (WWTP) effluents due to the ineffectiveness of the existing treatment systems (Heberer, 2002; Murray et al., 2010; Reemtsma et al., 2006; Salgado et al., 2010).

A study on 23 European countries detected the presence of organic persistent pollutants in groundwater, such as pharmaceuticals, antibiotics, pesticides,

perfluorinated acids, benzotriazoles, hormones, alkylphenolics, caffeine, diethyltoluamide and triclosan, at concentrations of ng L^{-1} (Loos et al., 2010) suggesting that the when left untreated, these compounds can achieve aquifers used for drinking water supply. While most emerging contaminants are detected at ng L^{-1} concentrations in surface waters, research surveys detected some of them up to the µg L^{-1} range (Kolpin et al., 2002; Loos et al., 2009; Murray et al., 2010; Pal et al., 2010). Toxicological studies often show acute effects at mg L^{-1} concentrations, but sublethal effects have also been reported below the µg L^{-1} level. For example, 133 mg L^{-1} of ibuprofen showed a lethality of 50 % in the population of *Daphnia magna* (Han et al., 2006) but also chronic exposure of ibuprofen concentrations below 100 µg L^{-1}, interfered in the ovulation of Japanese medaka (*Oryzias latipes*) (Flippin et al., 2007).

1.2 Micropollutants in wastewater treatment plants

Conventional biological municipal WWTPs are designed to remove organic carbon, nitrogen and phosphorus loads from domestic and industrial wastewater streams, as well as to reduce the concentration of pathogens. To date, the design of WWTPs does not focus on the removal of micropollutants. The removal of these compounds from wastewater is possible, and has been reported in numerous studies as being affected by the specific properties of the individual compounds (e.g. biodegradability, sorption behaviour, volatility), by the technological and operational characteristics of the treatment system (e.g. temperature, redox potential, sludge and hydraulic retention time), and by the microbial community (e.g. biomass activity and nitrification potential) (Hamid and Eskicioglu, 2012; Helbling et al., 2012; Kummerer, 2009; Onesios et al., 2009). Furthermore, biodegradation was shown to be the predominant mechanism by which pollutants with a low K_{ow} of < 2.5 (partition octanol-water,

which reflect the compound´s hydrophobicity and thus its adsorption potential) are primarily removed in wastewater treatment systems (Carballa et al., 2004; Hamid and Eskicioglu, 2012; Quintana et al., 2005; Salgado et al., 2012).

Pharmaceutical active compounds (PhACs), in the form of the parent compound or active metabolite, are the most studied group of emerging contaminants (Richardson, 2009). Tons of PhACs are annually produced worldwide to be administrated to humans and animals (Santos et al., 2010). In Germany a total of 6000 - 7000 tons of potentially adverse active substances are produced every year (Kummerer, 2009). Generally, their production is intended to have a particular physiological effect in living beings and to resist inactivation before administration. Thus, leading to biocumulative and toxic effects in aquatic and terrestrial ecosystems (Santos et al., 2010). PhACs are internally or externally administrated to humans and animals and excreted primarily via feces and urine. After excretion the parent compound and the originated metabolites enter the sanitary sewer systems, which are directly connected, to the municipal WWTPs (Santos et al., 2010).

Commonly, PhACs feature low biodegradability and high polarity, which affects their removal by conventional wastewater treatments and therefore leads to their continuous discharge into the receiving water bodies (Ternes and Joss, 2006). Wide dissemination at low concentrations in the range of the ng L^{-1} up to the µg L^{-1} in the aquatic environment is evident today (Kummerer et al., 2009). The presence of pharmaceuticals in the River Rhine in Germany was monitored for 10 years (1997-2006) indicating no major decrease on the concentrations. Furthermore, the loading rates exceeded one ton per year at the most downstream site suggesting the inefficient removal of these compounds from WWTPs (Sacher et al., 2008).

In a Portuguese survey, Salgado (2010) analysed the presence of 65 pharmaceutical and personal care products in 5 WWTPs and confirmed the detection of such compounds up to the 11.5 μg L^{-1} in the influent and 0.9 μg L^{-1} in the effluent. Non-steroidal anti-inflammatory drugs (NSAIDs) were frequently detected and were found to be the most abundant group of PhACs. Non-steroidal drugs are over-the-counter drugs usually administrated to alleviate pain.

Besides the Portuguese study, NSAIDs such as diclofenac, ibuprofen, ketoprofen and naproxen have been detected in influent wastewaters and surface waters of numerous other geographical points (Murray et al., 2010; Loos et al., 2009; Loos et al., 2010; Pal et al., 2010). In particular, ibuprofen and ketoprofen were two of the most recurrent and abundant NSAIDs in WWTPs influents and effluents worldwide (Murray et al., 2010; Onesios et al., 2009; Pal et al., 2010; Salgado et al., 2010; Santos et al., 2010), and therefore they were selected as target compounds in this study.

Several studies have reported the detection of ibuprofen (α-Methyl-4-(2-methylpropyl)-benzeneacetic acid) up to 106 μg L^{-1} and 44 μg L^{-1} in raw sewage and treated effluent wastewater, respectively, and up to 5 μg L^{-1} in surface waters. Similarly, several studies have reported the detection of ketoprofen (3-Benzoyl-α-methyl-benzeneacetic acid) up to 104 μg L^{-1} in raw influents and 0.1 μg L^{-1} in surface waters (Murray et al., 2010; Pal et al., 2010; Salgado et al., 2010; Santos et al., 2010). Low concentrations of ibuprofen (ng L^{-1}) showed to affect the growth and reproduction of aquatic species and to have genotoxic effects for fish. (Flippin et al., 2007; Han et al., 2010; Hayashi et al., 2008; Ragugnetti et al., 2011). So far, the toxic effects of low concentrations of ketoprofen are still unknown. Biodegradation of the two compounds in WWTPs

has been reported by several studies, while contribution of sorption and volatilisation seemed to be negligible (Joss et al., 2006; Salgado et al., 2012; Ternes et al., 2004). Reported removal efficiencies vary greatly, with eliminations of anywhere between 0 % and 100 %, likely due to the different influent concentrations and operating parameters of the different studies (Murray et al., 2010; Pal et al., 2010; Salgado et al., 2012; Santos et al., 2010).

Mercury is the most toxic and frequently detected heavy metal in industrial wastewaters(Wagner-Dobler et al., 2000). Mercury pollution arises from anthropogenic sources such as the chlor-alkali industry, coal burning and gold mining and naturally from volcanoes (Jaysankar et al., 2007). Once discharged into the environment, mercury tends to accumulate in the aquatic media and soils, where it can contaminate drinking water aquifers. Furthermore, it is taken up by aquatic organisms and enters the food chain. Mercury poisoning results in severe chronic disease or death. Therefore, the maximum allowable mercury level in industrial wastewater effluents is 50 µg Hg L^{-1}. The biological reduction of mercury is an efficient process that has been applied in wastewater treatment systems (Chang and Law, 1998; Wagner-Dobler et al., 2000, Wagner-Dobler, 2003).

1.3 Biological removal of micropollutants in wastewater treatment plants

Biological degradation can transform organic molecules to CO_2 and H_2O (mineralisation) or to one or more organic compounds (transformation). In both cases, compounds can serve as primary substrate of carbon and energy source (catabolism) or as secondary substrate coincidentally transformed in the presence of a primary growth substrate (co-metabolism) (Onesios et al., 2009). Measurement of only the parent compound makes it impossible to determine

which type of transformation occurred. Therefore, in this study the term degradation is used in a general way meaning all the possible degradation processes.

Microbial degradation seems to be the predominant mechanism for elimination of polar acidic pharmaceuticals in WWTPs (Quintana et al., 2005; Salgado et al., 2012). Previous lab-scale studies found that activated sludge can partially or completely eliminate such compounds in aerobic and anaerobic conditions. As an example, testes conducted by Quintana et al. (2005) showed that ibuprofen and naproxen were fully mineralised by activated sludge, although these authors only observed degradation in the presence of lactose as additional carbon source. In the same study, activated sludge when incubated with ketoprofen as the sole carbon source produced stable metabolites.

Generally, degradation rates are difficult to predict and can be affected by different variables such as hydraulic retention time, sludge age and environmental conditions, and has been mostly determined experimentally (Daughton and Ternes, 1999).

Modelling and simulating the degradation performance of micropollutants by activated sludge is required to improve process optimisation, design and control of the existing treatment systems or by identifying additional treatment stages.

The activated sludge models (ASMs) are the most commonly implement mechanistic models to describe the biochemical transformations of soluble and particulate compounds in activated sludge. Recently, ASMs models have been extended to predict the degradation of micropollutants in activated sludge

(Plósz et al., 2010; Plósz et al., 2011). Joss et al. (2006) modelled the kinetics of degradation of 35 PhACs by activated sludge using a pseudo first-order equation. The information obtained was used to classify the biodegradability of the 35 studied PhACs according to the obtained degradation constants, classified in three biodegradation levels (Joss et al., 2006). However, reported degradations constants vary greatly among different studies, which were carried out with different environmental conditions, and specific microbial populations.

Other studies have associated the degradation of micropollutants to the presence of another primary substrate used as carbon and energy source (Egli, 2010). For example, the degradation of nitrilotriacetic acid by *Chelatobacter heintzii*, where a higher glucose fraction led to faster nitrilotriacetic acid reduction (Bally and Egli, 1996). Oehmen et al. (2012) also observed this effect using a mixed culture degrading the herbicide propanil, where faster propanil degradation was observed with propionate addition. Nevertheless, competition or inhibition of micropollutant degradation due to the presence of readily growth substrates is also possible (Plósz et al., 2010).

Prior to the degradation of many compounds, a period of adaptation (lag phase) occurs. This period of adaptation has been associated with the acclimatisation to new conditions, enzyme synthesis, increase in the number of degrading microorganisms and inhibition (Alexander, 1999). Chong (2009) proposed a model to describe the degradation of a micropollutant that is dependent on the growth of the micropollutant degrading biomass. This author associated the observed lag-phase to the acclimation of the biomass to the micropollutant, corresponding to an increase in degrading microorganisms.

When subjected to the appropriate operating conditions, microbial communities have the potential to acclimatise by microbial selection or development of catabolic ways to degrade micropollutants, due to their natural genetic flexibility (Rieger et al., 2002). The acclimatisation of microorganisms to a pollutant requires time and the presence of the adequate bacterial enzymes for degradation of that compound (Matsumura, 1989; Spain et al., 1980). González et al. (2006) demonstrated that the acclimatisation of microbial populations improved the degradation rate of diclofenac and selected priority acidic pesticides.

Generally, the adaptations of microbial communities to the target contaminant are observed with an increase of the population that tolerates or degrade the compound. The acquisition of the degradation capacity can be the result of mutations such as nucleotide changes or DNA rearrangements. Genetic information located in mobile genetic elements can be transferred to other community members through horizontal gene transfer. Eventually the community members that are best adapted to resisting or degrading the target compound get selected and their number increases in the microbial community (Top and Springaely, 2003). This microbial genetic flexibility is evident in the mercury reduction mechanism that relies on the *mer* operon, specifically in the *merA* gene, which encodes for the mercuric reductase or MerA enzyme. The *mer* operon can be present in both the chromosomal DNA and mobile genetic elements, such as transposons and plasmids. This suggests that horizontal gene transfer may play an important role in the adaptation of a microbial community to mercury contamination (Osborn et al., 1997).

However, other microorganisms that are not responsible for the degradation might indirectly improve the degradation of micropollutants without actually

degrading it. As an example, the growth of a *Bacillus,* involved in floc formation, enhanced the growth of other two strains that were capable of growing with nonylphenol polyethoxylate surfactants as the sole carbon and energy source (Di Gioia et al., 2004).

Furthermore, in situations where the degrading biomass is present, the necessary genes that encode for the degrading enzymes might require to be induced (Alexander, 1999). Environmental concentrations entering WWTPs might be too low to induce the expression of the appropriate enzymes for degradation of these compounds, but high concentrations can be toxic or inhibit microbial propagation and metabolism (Daughton and Ternes, 1999).

Despite the abundant literature on biodegradation of micropollutants in WWTPs related to occurrence and fate, and the parameters affecting elimination, very little is known about the governing microorganisms. A general interest is growing in research groups worldwide in characterising the microbial communities and their responses to micropollutants, to isolate degrading microorganisms and to identify the genes related to the degradation processes.

1.4 Micropollutant degrading microorganisms

The identification of the microorganisms involved in the degradation of specific contaminants enables the monitoring of their presence in activated sludge, in order to potentially predict the performance of the system as well as to optimise operational parameters that better suit communities of interest.

In the past, the discovery of *Brocadia anammoxidans* as being able to oxidise ammonium to molecular nitrogen under anoxic conditions, made an important

contribution to research on nitrogen elimination in WWTPs (Strous et al., 1999). Nowadays, promising results in studies addressing micropollutant degrading microorganisms have started to appear. For example, Roh et al. (2009) examined the biodegradation potential of triclosan, bisphenol A, and ibuprofen by *Nitrosomonas europaea* and mixed ammonia-oxidizing bacteria in nitrifying activated sludge. Authors observed that microorganisms degraded triclosan and bisphenol A. On the other hand, ibuprofen was more likely degraded by the heterotrophic microorganisms in activated sludge. In a different study, Kraigher et al. (2008) showed a shift in the microbial communities present in activated sludge due to the exposure of 5-500 μg L^{-1} of diclofenac, ibuprofen, ketoprofen and naproxen. Additionally, these authors also observed the growth inhibition of bacteria from the genus *Nitrospira*, correlating it with the decrease in the efficiency of NSAIDs removal in wastewater treatment systems.

When studying micropollutant degrading communities two fundamental questions arise: (1) who are the degrading microorganisms? (2) how do these microorganisms degrade micropollutants?

1.4.1 Assessing the diversity of micropollutant degrading microorganisms

The detailed analysis of the microbial diversity can be addressed using two broad approaches: culture-dependent studies and culture-independent studies.

Culture-dependent methods are based on the cultivability of the microorganisms on selective media. Cultivation is an important tool for the detection of degrading microorganisms, especially when genes of the degraders have not been characterised and thus do not allow the use of nucleic acid

based methods. Isolation and cultivation in an agar medium only allows the characterisation of microorganisms capable of growing in the selected experimental growth conditions. This is even more evident in complex biological systems where only < 1 % of bacterial species are currently known and cultivable. Moreover only 5 – 10 % of the total number assumed to be present in water and wastewater treatment plants can be identified by classical microbiological methods (Kummerer, 2004). Despite that, studying cultured isolates enables the straightforward characterisation of their functions and the evaluation of their biotechnological potential (Cardenas and Tiedje, 2008). Relevant microorganisms have great potential for preparing starter cultures to be used in bioaugmentation purposes. Moreover, cultured isolates can be used in studies concerning the kinetics and process optimisation of micropollutant degradation in biological treatment systems.

The study of microbial communities sometimes results in the identification of new species with specific characteristics regarding the degradation of micropollutants. The identification a new species relies in a polyphasic approach where both phenotypic and genotypic methods are necessary for an appropriate bacterial classification (Uilenberg and Goff, 2006).

To overcome the limitations of traditional microbiological methods a variety of molecular methods independent of cultivation have been developed based on the small subunit of ribosomal ribonucleic acid (16S rRNA gene for prokaryotes and 18S rRNA gene for eukaryotes). The 16S rRNA gene has on average a length of 1,500 nucleotides. This sequence is characterised by its universal distribution, high conservation, as well as differentiation at species level. These characteristics make rRNA genes useful for comparative analysis. Furthermore, full and partial 16S rRNA gene sequencing methods allows the identification of

microorganisms at genus or species level (Cardenas and Tiedje, 2008). The application of 16S rRNA gene based methods in studying the microbial communities dynamics, development and function will be further addressed in the introduction.

1.4.2 Assessing the mechanisms of microbial degradation of micropollutants

Laboratory degradation experiments may be used to test the ability of microorganisms to degrade a target micropollutant by monitoring the decrease of the parent compound concentration or an increase of metabolite concentrations, relative to microbially inhibited controls, under controlled conditions that mimic the real conditions (e.g. temperature, oxygen concentration, pH etc.).

So far, the majority of the studies on the mechanisms of microbial degradation of pollutants primarily relied on the detection and identification of degradation intermediates (Onesios et al., 2009; Quintana et al., 2005; Radjenovic et al., 2008). A complementary approach is to study the enzymatic mechanisms of microbial degradation by identifying key enzymes involved in the process, using proteomic techniques.

Quantitative proteomics has proven to be a valuable tool for analysing the physiological response of microorganisms in the presence of specific compounds or under the influence of different physico-chemical parameters (Kim et al., 2009; Kim et al., 2007). When grown in the presence of the target compound, microorganisms induce the expression of enzymes involved in the degrading metabolic pathway(s). These induced proteins can be identified by quantitative comparisons of the proteomes expressed in presence and absence

of the micropollutant, which give direct measurement of protein expression levels (Kim et al., 2007; Kim et al., 2009). In quantitative proteomics typically cells are primarily lysated and fractionated, and separated in one-dimensional gel electrophoresis. After separation, proteins are enzymatically degraded into peptides and separated by liquid chromatography (LC). Peptides are detected by tandem mass spectrometry (MS/MS) and identified based on MS spectra using bioinformatics tools, therefore resulting on the identification of the proteins (Aebersold and Mann, 2003). Furthermore, the whole genome of model microorganisms can be sequenced to provide a species-specific protein identification platform, which improves the quality of protein identification (Lacerda and Reardon, 2009).

Proteomics has been successfully applied for studying the biodegradation of aromatic hydrocarbons such as benzoate, aniline, and phenol by several microorganisms, e.g. *Acinetobacter* (Giuffrida et al., 2001; Park et al., 2006; Pessione et al., 2003) and *Pseudomonas* strains (Loh and Cao 2008), as well as several mycobacterial (Kim et al., 2004; Liang et al., 2006) and rhodococcal species (Navarro-Llorens et al., 2005; Patrauchan et al., 2005; Tomas-Gallardo et al., 2006).

The identification of key enzymes involved in biodegradation can help discover microbial degradation pathways and suggest candidate biomarkers to monitor micropollutant degradation. Additionally, the identity of the micropollutant degrading microorganisms and the corresponding degrading genes can be used in studies to monitor the degradation potential and microbial communities development under specific environments. This approach is of great use in biotechnological studies aiming the enrichment of degrading microbial communities in engineered systems for WWTP optimisation as well as

bioremediation and bioaugmentation processes (Eyers et al., 2004; Widada et al., 2002).

1.4.3 Characterising micropollutant degrading microbial communities

A fundamental understanding of the microbial community structure and stability, as well as its response to different pollutants in the wastewater, is necessary for efficient and stable WWTP operation. The variations in microbial community structure throughout time can be monitored using molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridisation (FISH). Moreover, under varying experimental conditions it is important not only to know the phylogenetic changes but also to determine the functional changes. Quantitative polymerase chain reaction (qPCR) targeting functional genes that encode enzymes in key metabolic or catabolic pathways enables to assess changes in microbial functions.

1.4.3.1 Denaturing gradient gel electrophoresis

DGGE is the method of choice to demonstrate an effect on microbial communities or differences between microbial communities. It is based on the electrophoretic mobility of denatured DNA-fragments of the same size, with different nucleic acid sequences, in a denaturing gradient polyacrylamide gel containing a linear gradient DNA denaturant made with urea and formamide (Muyzer, 1995). Each gel is composed of DNA band patterns that directly reflect the genetic biodiversity of the sample. The relative intensity of each band and its number roughly corresponds to the abundance of dominant species (Sanz and Kochling, 2007). For determining the phylogenetic identities from DGGE, the DNA bands can be excised, re-amplified and sequenced. DGGE is a useful

technique to monitor the enrichment of microbial cultures. For example, LaPara et al. (2002) used DGGE to characterise the stability of the bacterial community structures treating pharmaceutical wastewater, and concluded that the microbial community was able to adapt to perturbations in the influent wastewater characteristics. More recently, Carvalho et al. (2010) employed 16S rRNA gene-targeted DGGE to investigate the microbial ecology of a propanil-degrading enrichment carried out in a sequencing batch reactor and operated with different feeding strategies.

1.4.3.2 Fluorescence *in situ* hybridisation

FISH is a technique that allows the qualitative and quantitative investigation of the composition of bacterial communities through the use of rRNA-targeting fluorescent probes. These probes consist of a short sequence of 16-20 nucleotides conjugated to a fluorescent dye on the 5' end that enable the detection/identification of microorganisms (Sanz and Kochling, 2007). A variety of molecular probes targeting rRNA genes have been reported at various taxonomic levels (Amann et al., 1995). In FISH, cells are fixed and permeabilised to enable the penetration of the probes inside the cell and to protect the RNA from degradation. Hybridisation of the probe to the target rRNA gene is carried out in stringent conditions adjusted by varying either the formamide concentration or the hybridisation temperature. Labelled cells can be visualised by fluorescence microscopy (Moter and Gobel, 2000). Multiple group-specific oligonucleotide probes targeting different microbial taxa can be used in a FISH experiment for simultaneous phylogenetic classification and quantification of active microbial populations. FISH is a valuable tool for the identification and quantification of microbial populations. This technique has been extensively used for the identification of the abundant microorganisms present in key

processes in wastewater treatment plants (Amann et al., 2001; Wagner and Loy, 2002). For example in the detection and quantification of *Candidatus* Accumulibacter phosphatis, the polyphosphate-accumulating organism, in full-scale systems (Crocetti et al., 2000).

Recent studies demonstrated the use of FISH to characterise the variations in microbial communities in the presence of micropollutants. Haseborg and Frimmel (2007) employed FISH to evaluate the impact of clofibric acid and ibuprofen on the nitrifying bacterial population in fixed bed biofilm reactors. In a different study, Serrano et al. (2011) used FISH to follow the evolution of the microbial populations present in a membrane bioreactor operating in a sequential mode for the simultaneous removal of nutrients and pharmaceutical compounds from synthetic urban wastewater.

However, FISH is an exclusively taxonomic technique that does not reveal the function or metabolic features of the microorganisms, although in some cases these characteristics can be deduce from comparison with other phylogenetically-related bacteria (Sanz and Kochling, 2007), or investigated through combination of FISH with e.g. microautoradiography (MAR).

1.4.3.3 Quantitative polymerase chain reaction

Quantitative PCR (qPCR) has been used in microbial ecology studies to measure the abundance and expression of taxonomic and functional gene markers related to the removal of micropollutants (Bustin et al., 2005; Smith and Osborn, 2009). Quantitative-PCR works in the same way of an end-point PCR, i.e. multiple amplification cycles in which the DNA is initially denatured and annealed with specific oligonucleotide primers, followed by extension of a

complementary strand from each annealed primer, resulting in an exponential increase in amplicon numbers. In qPCR, oligonucleotide-intercalating fluorescent dyes such as SYBR Green or fluorescent probes (*Taq*Man) are used to detect the increase in amplicon numbers. Software records in 'real-time' the increase of amplicons concentration during the early exponential phase of amplification, enabling the quantification of gene numbers when these are proportional to the starting template concentration (Bustin et al., 2005; Smith and Osborn, 2009).

As qPCR is a sensitive and specific method to detect changes in the abundance of target functional genes, it can be applied to investigate the effects of experimental parameters on the numbers of the target gene and subsequently on the process that these genes encode for. Several functional genes encoding key reactions in biodegradation pathways of environmental pollutants, such as herbicides, aromatic hydrocarbons, halogenated and chlorinated compounds, have been targeted by qPCR analysis (Smith and Osborn, 2009).

Aims and layout

Wastewater treatment plants (WWTPs) are one of the main routes for the introduction of micropollutants into the environment but can also represent a major treatment opportunity for preventing significant environmental exposure.

Most conventional treatment plants use biological systems to degrade organic matter due to their economical and environmental advantages. However, knowledge on the microbiology and biodegradation pathways involved in micropollutants removal in WWTPs is still reduced.

A variety of biochemical and molecular methods can be applied to reveal the microbial community structure and dynamics over time in response to the presence of specific micropollutants. These approaches allow linking micropollutants degradation performance with the microbial community. This knowledge facilitates the optimisation of the process operation conditions that enable the acclimatisation of microbial populations for micropollutants removal in WWTPs.

The main objective of the work presented in this thesis was to characterise the microbial populations involved in the removal of micropollutants in water and wastewater treatment systems, focusing essentially in understanding and predicting the main factors influencing the microbial degradation of important emerging pollutants, in identifying and characterising key microorganisms involved in the degradation process, as well as the metabolic aspects of the degradation. Finally, the potential application of the knowledge of such characterisation is explored in monitoring the acclimatisation of microbial communities for the degradation of a target micropollutant, where microbial community structure and dynamics were linked with degradation performance.

The ultimate goal of this thesis is to provide a step forward towards understanding and improving the microbial degradation of micropollutants in WWTP.

This thesis is divided into six chapters.

- Chapter one consists of a general introduction on the challenges of micropollutants in water and wastewater treatment systems. Emphasis

is given to the biological degradation of micropollutants, particularly on the importance of addressing the microbial population dynamics, structure and function during the degradation process.

- In Chapter two, the biodegradation of two NSAIDs (ibuprofen and ketoprofen) by activated sludge and a pure culture was described using mathematical models. Mathematical models can help to understand and predict the main factors influencing the biodegradation of micropollutants and are essential for process optimisation, design and control of wastewater treatment systems. The effects of substrate loading rate and mixtures of compounds on the microbial degradation performance of ibuprofen and ketoprofen were characterised using three different activated sludges. Since the micropollutant degrading biomass constitutes only a small fraction of activated sludge, more specific investigation of the performance and growth of these organisms required the use of pure cultures. Several ibuprofen and ketoprofen degrading microorganisms were obtained from isolation and cultivation in an agar medium. One isolate was further characterised in terms of ibuprofen degradation kinetics using an additional mathematical model.

- Chapter three presents the unique phenotypic and genotypic characteristics of the ibuprofen degrading isolate, previously kinetically characterised for ibuprofen degradation, which enabled its classification as a new species, *Patulibacter medicamentivorans*.

- In Chapter four the metabolism of ibuprofen degradation by *Patulibacter medicamentivorans* was investigated. This Chapter

describes the proteomic analysis of *Patulibacter medicamentivorans,* with and without exposure to ibuprofen, carried out by *in vivo* metabolic labelling. Furthermore, the whole genome of this microorganism was sequenced and employed to provide a species-specific protein identification platform, for improved quality of protein identification.

- The identification of key enzymes involved in biodegradation can suggest candidate biomarkers to monitor micropollutant degradation. The knowledge of the genes encoding for micropollutant degrading enzymes, as well as the identity of degrading microorganisms, can be applied in studies to monitor the degradation potential and microbial communities development in engineered systems. Chapter five explores the potential application of such knowledge. The taxonomic (16S rRNA gene) and functional (*merA* gene) characteristics of mercury-reducing cultures enriched with different carbon sources (glucose, ethanol or acetate) were investigated aiming at understanding the long term effect of the carbon substrate (that can be supplemented to the system) on the microbial structure and function. The dynamics of the microbial cultures during the enrichment period were monitored using complementary molecular techniques such as DGGE and FISH. Additionally, functional changes were determined through the abundance of the *merA* gene using qPCR, which were then related with mercury removal efficiency.

- To finalise, Chapter six, encloses a general discussion of all the conclusions from the other chapters and presents perspective knowledge gaps to be addressed in future work.

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Chapter 2

Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture

Submitted for publication to the Journal of Hazardous Materials:

Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture.

Bárbara Almeida, Adrian Oehmen, Ricardo Marques, Dulce Brito, Gilda Carvalho, Maria Teresa Barreto Crespo.

Almeida B. was involved in all the experimental work presented in this chapter, except for the NSAIDs quantification that was made by IBET Analytical Services, Oeiras, Portugal and the sequencing of the 16s rRNA gene that was performed by Biopremier, Lisbon, Portugal. Mathematical modelling was performed together with Adrian Oehmen. Ricardo Marques and Dulce Brito helped performing the biodegradation assays.

Abstract

A model describing ibuprofen and ketoprofen biodegradation by activated sludge from three different wastewater treatment plants (WWTPs) was developed in this study. This model successfully described the biodegradation profiles observed at two different initial concentrations of each compound, where a lag-phase was observed prior to the biodegradation of each compound. Twelve ibuprofen and ketoprofen degrading isolates were identified in this study from the WWTP sludge showing the best removal performance. One of these isolates was characterised via another model, where biodegradation was dependent on biomass growth rate as well as the ibuprofen concentration. The fact that different models were needed to describe the biodegradation by activated sludge and a pure culture suggests that the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) depends on the microbial community, thus pharmaceutical biodegradation models may require adaptation depending upon the system. This study provides an advance towards modelling pharmaceutical biodegradation in WWTPs.

Introduction

The presence of pharmaceutically active compounds (PhACs) in wastewater treatment plants (WWTPs) as well as in natural matrices (soil, sediments and water) has been an issue of concern in recent years. PhACs are known to possess potentially harmful effects to the environment and public health (mutagenic, carcinogenic, endocrine disruptor) (Heberer, 2002; Kolpin et al., 2002). PhACs have been detected in concentrations up to μg L^{-1} in influents and effluents of WWTP worldwide and in surface waters (Heberer, 2002; Kolpin et al., 2002; Murray et al., 2010; Pal et al., 2010; Salgado et al., 2010). The elimination of such compounds from wastewater treatment is possible, and has been reported in numerous studies, through the action of different removal mechanisms such as sorption, biodegradation and abiotic degradation (e.g. Salgado et al., 2012). Biodegradation was shown to be the predominant mechanism by which polar acidic pharmaceuticals are primarily removed in wastewater treatment systems (Quintana et al., 2005; Salgado et al., 2012).

Ibuprofen and ketoprofen are two common non-steroidal anti-inflammatory drugs (NSAIDs) that are among the most abundantly detected (in the ng L^{-1} to μg L^{-1} range) in WWTP influents and effluents, as shown by various surveys carried out all over the world (Marco-Urrea et al., 2010; Onesios et al., 2009; Salgado et al., 2010; Zwiener et al., 2002).

Ibuprofen was detected up to 106 μg L^{-1} and 44 μg L^{-1} in raw sewage and treated effluent wastewater, respectively, and up to 5 μg L^{-1} in surface waters (Murray et al., 2010; Pal et al., 2010; Salgado et al., 2010; Santos et al., 2010). At concentrations of ng L^{-1} ibuprofen was shown to affect the growth and reproduction of aquatic species and having genotoxic effects for fish (Han et al.,

2010; Hayashi et al., 2008; Pomati et al., 2004; Ragugnetti et al., 2011). The range of detection of ketoprofen in wastewater and freshwaters is lower as compared to ibuprofen, with detections of up to 104 µg L^{-1} in raw influents and 0.1 µg L^{-1} in surface waters (Murray et al., 2010; Pal et al., 2010; Salgado et al., 2011; Santos et al., 2010). The toxic effects that such concentrations might pose to the aquatic environment are still unknown.

Biodegradation of the two compounds in WWTPs have been reported by several studies, which differ in removal efficiencies due to the varying influent concentrations and operating factors, with eliminations of anywhere between 0 % to 100 % (Daughton and Ternes, 1999; Murray et al., 2010; Pal et al., 2010; Salgado et al., 2012; Santos et al., 2010). Biodegradation models are still in their infancy, and are needed to evaluate, understand and predict the main factors influencing the biodegradation of PhACs in WWTPs. Developing an appropriate mathematical model to describe NSAID biodegradation by activated sludge was indeed an objective of this study. Further, previous studies have not examined the biodegradation of a mixture of NSAIDs (or other PhACs), and the synergistic effects that the presence of one compound can have on another. It is known that mixtures of compounds can affect microbial degradation due to inhibition by either competition or toxicity (Reardon et al., 2002), and therefore this was evaluated in this work.

Some PhAC biodegradation models are derived from first-order kinetic expressions, as proposed by Joss et al. (2006). In general, the most commonly used activated sludge models (ASMs) employ Monod-based kinetic expressions (Hauduc et al., 2012). Previous studies incorporating PhAC degradation into ASMs have usually employed one of these two approaches (Collado et al., 2012; Lust et al., 2012; Plósz et al., 2010). Chong (2009), have also proposed a model

to describe the degradation of a xenobiotic, which is dependent on the growth of xenobiotic-degrading biomass. Chong (2009) proposed that degradation was a process dependent upon the acclimation of biomass, affecting the growth of the degrading fraction. Such a hypothesis is very difficult to test with activated sludge, which contains a wide diversity of organisms, where the PhAC degraders likely represent only a minor fraction (Chong, 2009; Majewsky et al., 2011). We investigated this hypothesis by using a pure culture to study the growth dependency on NSAID removal. For this purpose, we obtained a number of isolates from a WWTP and simultaneously identified new species not previously associated with NSAID biodegradation.

Few studies have been conducted to understand the degradation of ibuprofen and ketoprofen by microorganisms present in activated sludge. To date only two microorganisms were isolated from activated sludge as being able to utilise ibuprofen, a *Nocardia* sp. (Chen and Rosazza, 1994) and *Sphingomonas* Ibu-2 (Murdoch and Hay, 2005). However, no kinetic studies were performed with these strains in order to understand their ibuprofen removal performance. So far, no microorganism has been isolated from activated sludge that is associated to ketoprofen degradation, but the capability of white-rot fungi to degrade ketoprofen has been demonstrated (Marco-Urrea et al., 2010).

Therefore, the first aim of this work was to characterise and model the kinetics of ibuprofen and ketoprofen biodegradation by activated sludge from three different WWTP, varying the initial concentrations of each compound alone or in a mixture. The second aim of this work was to isolate NSAID-degrading microorganisms and characterise their degradation efficiency. Ibuprofen was chosen as a model NSAID to perform kinetic characterisation from one of the

pure cultures, which was subsequently described through a mathematical model.

Material and Methods

Activated sludge sampling

Activated sludge and influent samples were collected from the recirculation tank and from the primary clarifier, respectively, of three municipal WWTPs – Beirolas WWTP (SIMTEJO, Lisbon), Fernão Ferro WWTP (SIMARSUL, Seixal) and Setúbal WWTP (Águas do Sado, Setúbal). All of the plants treat mainly municipal wastewater, where industrial discharges incorporate <20 % of the flow. The samples were transported to the laboratory in a refrigerated isothermal container and used immediately. The activated sludge was settled and the supernatant was discarded. Influent was filtered through a glass microfiber filter GF/D (Whatman, Dassel, Germany) to remove suspended particles and adjusted to pH 7. Activated sludge samples were adjusted to an average of 3 g L^{-1} (volatile suspended solids - VSS) with wastewater influent.

Degradation of ibuprofen and ketoprofen by activated sludge

Biodegradation experiments were carried out in 500 mL Erlenmeyer flasks placed in a rotary shaker at 27 ºC and at 110 rpm. Activated sludge was spiked with ibuprofen and ketoprofen to test the following conditions: i) using ibuprofen or ketoprofen as the sole carbon and energy source, ii) feeding a mixture of the two NSAIDs to test substrate competition, iii) using two initial concentrations - 1000 µg L^{-1} or 500 µg L^{-1} for ibuprofen and 250 µg L^{-1} or 100 µg L^{-1} for ketoprofen – to test the difference in degradation kinetics. Samples were

taken at regular intervals during 36 h to measure the degradation of ibuprofen and ketoprofen as described below. Autoclaved biomass and uninoculated ibuprofen-containing media (abiotic test) were used as controls in this study.

Putative NSAID degrading microorganism isolation, identification and biodegradation characterisation

Putative NSAID degrading microorganisms were isolated from activated sludge collected from the Beirolas WWTP (Lisbon, Portugal). Briefly, activated sludge (3 g L^{-1}) was incubated in minimal medium M9 (Oehmen et al., 2009) supplemented with 500 µg L^{-1} of ibuprofen or 250 µg L^{-1} of ketoprofen at 28 ºC for 15 days. A new addition of ibuprofen or ketoprofen was made once the parent compound had been fully removed from the medium, as determined by ultra performance liquid chromatography. Ibuprofen and ketoprofen degradation capacity of the microbial community was evaluated during the period of incubation. In order to identify possible NSAID degrading microorganisms, samples were serially diluted in sterile saline solution (NaCl 0.85 %, w/v), spread on agar plates containing minimal medium supplemented with 250 µg L^{-1} of ibuprofen or ketoprofen and 15 g L^{-1} agar and incubated at 28 ºC for 3-4 days. Individual colonies were selected, re-inoculated onto the same medium supplemented with 1 g L^{-1} of yeast extract (Oxoid, Hampshire, England) and tryptone (Oxoid, Hampshire, England) (50 % of each) for isolation into pure cultures. Pure cultures were stored in Tryptone Soya Agar (Oxoid) at 4 ºC.

The biodegradation capacity of the isolated microorganisms was evaluated by cultivating them in 250 mL Erlenmeyer flasks containing 100 mL of minimal medium supplemented with 1 g L^{-1} of yeast extract and tryptone (50 % of each) and 100 µg L^{-1} of the corresponding NSAID. Cultures were incubated on a horizontal shaker at 110 rpm, 28 ºC during 100 hours. Isolates able to degrade

the NSAIDs were identified by Biopremier (Lisbon, Portugal) through sequencing the 16S rRNA gene, using the primers PA/907r (Muyzer et al., 1995).

The effect of initial ibuprofen concentration on the degradation performance of one isolate (I11) was also assessed. The isolate was grown in Tryptone Soya Broth (TSB) medium (Oxoid) up to the mid-logarithmic growth phase (assessed through optical density measurements at 600nm), after which cells were pelleted by centrifugation at 6000 g for 5 min at 4 ºC and washed twice with M9 medium. 0.2 g L^{-1} of this biomass was used as inoculum for the degradation tests. Experiments were conducted in 250 mL flasks containing 100 mL of M9 medium. Different initial concentrations (250 µg L^{-1} and 1000 µg L^{-1}) of ibuprofen were tested with or without the presence of yeast extract and tryptone (initial concentration of 3 g L^{-1}, 50 % of each). Flasks were incubated on a horizontal shaker at 110 rpm, 28 ºC for approximately 100 hours.

The bacterial isolate I11 was also tested at different initial concentrations of yeast extract and tryptone (initial concentrations of 0.5 g L^{-1}, 1 g L^{-1}, 2 g L^{-1}, 3 g L^{-1} and 5 g L^{-1}, containing 50 % of each substrate) using an initial ibuprofen concentration of 250 µg L^{-1}. A pre-culture of I11 was grown in TSB medium without ibuprofen up to the mid-logarithmic growth phase. Cells were pelleted by centrifugation at 6000 g for 5 min at 4 ºC and washed twice with M9 medium. Biomass, at a concentration of 0.2 g L^{-1}, was used to inoculate 250 mL Erlenmeyer flasks with 100 mL of M9 medium containing the ibuprofen, yeast extract and tryptone. Autoclaved cells and uninoculated ibuprofen-containing media (abiotic test) were used as controls in this study.

Analytical methods

All reagents, acetonitrile (Fisher Scientific, Fair Lawn, USA), 85 % phosphoric acid (Sigma Aldrich, Germany) and methanol (Fisher Scientific, Fair Lawn, USA), were of HPLC gradient grade, with a purity of ≥ 99%, according to the supplier. Prior to use, solutions were filtered through 0.2 µm SPARTAN filters (Whatman, Dassel, Germany). The laboratory water used in the study was provided by the Milli-Q water system (Millipore, CA, USA). Ibuprofen and ketoprofen were obtained from Sigma-Aldrich (Germany). Total suspended solids (TSS) and VSS were determined according to Standard Methods (APHA et al., 1995).

Samples were taken during the biodegradation assays, centrifuged at 6000 g for 5 min and the supernatant filtered through a 0.2 µm SPARTAN (Whatman, Dassel, Germany) filter prior to ibuprofen analysis. The ibuprofen and ketoprofen quantification was determined using ultra performance liquid chromatography (UPLC) as previously described (Almeida et al., 2012). Ibuprofen was detected at 220 nm and ketoprofen at 257 nm.

Results and discussion

Ibuprofen and ketoprofen biodegradation by activated sludge

Ibuprofen and ketoprofen biodegradation was studied using activated sludge from three different WWTPs, using different concentrations of each compound, alone or in a mixture, in order to study possible substrate competition effects and kinetic differences. Figures 2.1 and 2.2 show that each sludge was able to remove both of the tested compounds.

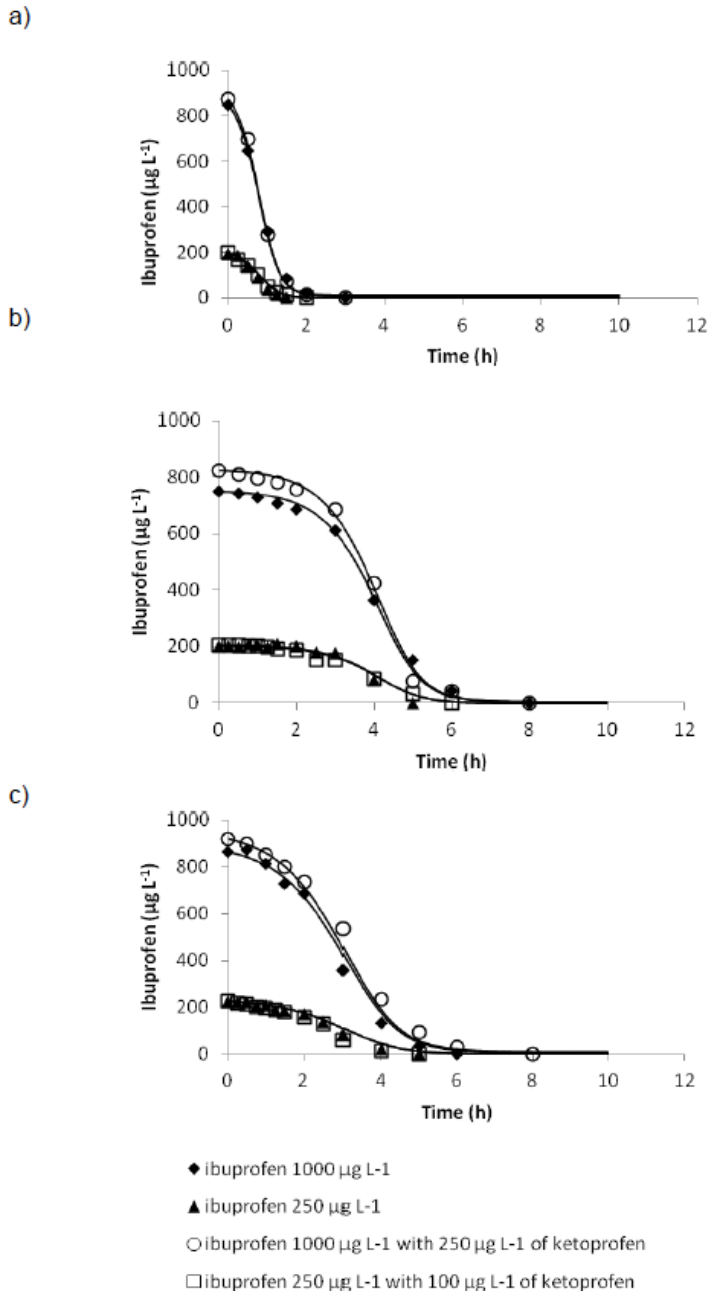

Figure 2.1. Experimental data (symbols) and kinetic model fitting (lines) for the degradation of ibuprofen by the three activated sludges: a) Beirolas WWTP; b) Setúbal WWTP and c) Fernão Ferro WWTP.

Correlation between experimental values and model predictions: a) R^2 = 0.99, b) R^2 = 0.99, c) R^2 = 0.99.

a)

b)

c)

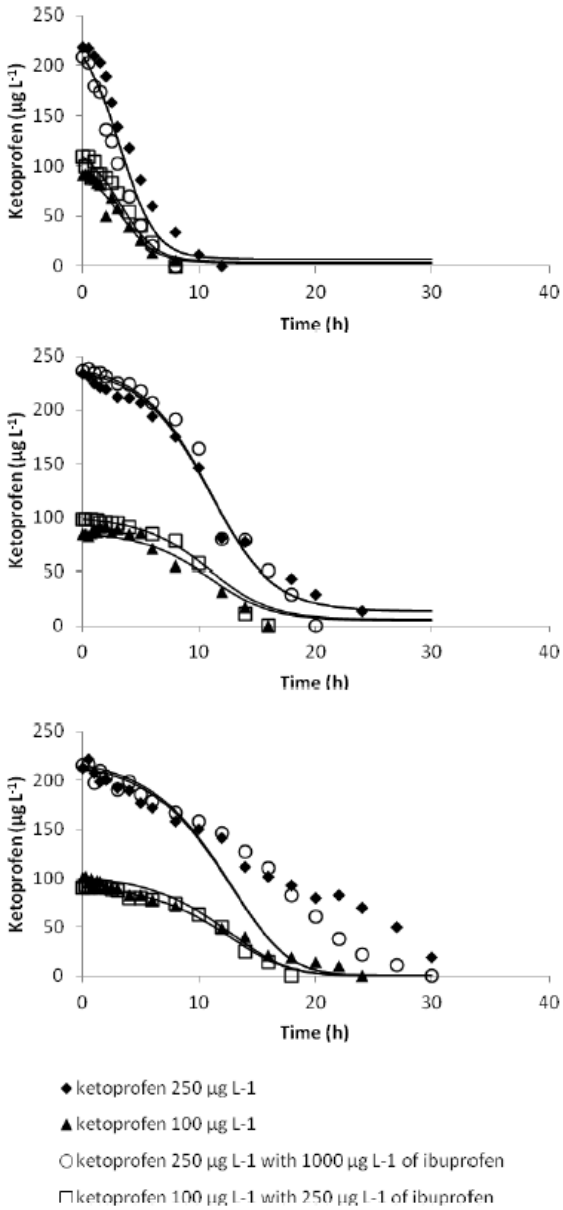

Figure 2.2. Experimental data (symbols) and kinetic model (lines) fitting for the degradation of ketoprofen for the three activated sludges: a) Beirolas WWTP; b) Setúbal WWTP and c) Fernão Ferro WWTP.

Correlation between experimental values and model predictions: a) R^2 = 0.95, b) R^2 = 0.99, c) R^2 = 0.85.

In these tests, it was observed that biomass growth was negligible, since the biomass concentration remained constant at 3.0 ± 0.1 g L^{-1}. Furthermore, there was no ibuprofen or ketoprofen removal observed in the autoclaved sludge and abiotic controls, confirming that the removal was due to biological activity. Previous studies have shown that ibuprofen and ketoprofen are commonly removed through biodegradation, while sorption and volatilisation seem to be negligible (Joss et al., 2006; Ternes et al., 2004; Salgado et al., 2012), which is in good agreement with our results.

Furthermore, Figure 2.1 and Figure 2.2 show that all tested activated sludge communities removed both NSAIDs, where neither stimulation nor inhibition was observed in cases where ibuprofen and ketoprofen were fed simultaneously as compared to the cases where each NSAID was fed individually. A lag phase was observed prior to the commencement of NSAID biodegradation in all tests, which was also observed during the ibuprofen and ketoprofen biodegradation tests of Quintana et al. (2005). Moreover, for each sludge, the total degradation time for each NSAID was similar at the two different concentration levels tested. Indeed, in Figure 2.1a, ibuprofen was removed in approximately the same period of time (2h) when either 1000 µg L^{-1} or 250 µg L^{-1} was added. Similar trends were observed for the other two WWTPs, although the degradation time was 6h. These results contrast with those previously obtained by Collado et al. (2012), where a lag phase was not observed for ibuprofen degradation, and the degradation rate of ibuprofen was found to be higher at lower ibuprofen concentrations when using the same initial biomass concentration. While it is unclear why our results contrast with those of Collado et al. (2012), the differences in degradation performance

observed between these two studies highlight the importance of testing the PhAC degradation profile for each specific activated sludge.

The biodegradation of ketoprofen by the three activated sludges followed a similar trend as compared to ibuprofen, although the kinetics were considerably slower and the lag phase considerably longer. The fact that ibuprofen was more easily biodegraded than ketoprofen is likely due to differences in their chemical structures. Furthermore, Quintana et al. (2005) also observed that ibuprofen biodegradation was faster than ketoprofen, which is in good agreement with our results.

Modelling NSAID biodegradation by activated sludge

Previous studies focussed on modelling the biodegradation of PhACs from activated sludge have generally found that either first-order or Monod kinetic expressions describe well the experimental data (Collado et al., 2012; Joss et al., 2006, Lust et al., 2012; Plósz et al., 2010). However, this was not the case in the present study. Chong (2009) proposed a model to describe xenobiotic degradation that employed a modified logistical function, with a sigmoidal shape. This showed much better agreement with the data obtained from the three wastewater treatment plant sludges tested in this study, and was thus chosen as the basis for model development.

Chong (2009) proposed a model based on the acclimation of a biomass fraction corresponding to the xenobiotic degraders. Since this degrader fraction of activated sludge is difficult to measure in practice, we propose a modified model where the sigmoidal function is applied directly to describe the xenobiotic (NSAID) substrate degradation rate in equation 2.1, instead of

describing the degrading biomass fraction as proposed in Chong (2009). Furthermore, the NSAID degradation was described by a modified-first order function with respect to the NSAID substrate (S), as opposed to a modified-Monod equation. This change minimises the number of parameters to estimate in the model equation. Finally, the NSAID degradation is also described as a function of the total biomass concentration (X), as shown in equation 2.1:

$$\frac{dS}{dt} = S \cdot X \frac{ab}{(1 + e^{-b(t-c)})} (1 - \frac{1}{1 + e^{-b(t-c)}})$$

where a, b and c are constants. These constants were estimated using non-linear regression fitting to the experimental data using Aquasim (Reichert, 1994). The simultaneous parameter estimation for the three constants utilised the experimental data of three tests at both initial concentrations (i.e. 1000 µg L^{-1} and 250 µg L^{-1} for ibuprofen and 250 µg L^{-1} and 100 µg L^{-1} for ketoprofen) alone or in combination with the other NSAID. The fourth test was utilised for model validation. Using the estimated constants, the rate constant (k_1) of NSAID degradation can be calculated according to equation 2.2:

$$k_1 = ab$$

while the length of the lag phase (L) can be calculated according to equation 2.3 (Chong, 2009):

$$L = c - \frac{2}{b}$$

As shown in Figures 2.1(a-c) and 2.2(a-c), the model predictions were in good agreement with the experimental data describing ibuprofen and ketoprofen by all three activated sludges. The estimated values of the parameters are shown in Table 2.1, and were used to describe both initial concentration levels simultaneously for each compound at each plant, both when they were fed alone and in combination with one another.

Table 2.1. Parameter values used for model simulation of the degradation of ibuprofen and ketoprofen by activated sludge.

Parameter	Beirolas WWTP	Setúbal WWTP	Fernão Ferro WWTP
a ibuprofen (L g^{-1})	1.46 ± 0.18	1.76 ± 0.45	1.61 ± 0.45
a ketoprofen (L g^{-1})	1.21 ± 0.40	0.96 ± 0.13	3.12*
b ibuprofen (h^{-1})	2.95 ± 0.22	1.27 ± 0.09	1.04 ± 0.11
b ketoprofen (h^{-1})	0.52 ± 0.12	0.32 ± 0.03	0.25*
c ibuprofen (h)	1.34 ± 0.08	5.46 ± 0.33	4.67 ± 0.48
c ketoprofen (h)	5.88 ± 1.18	14.7 ± 0.91	21.81*
k_1 ibuprofen (L g^{-1} h^{-1})	4.31 ± 0.89	2.23 ± 0.77	1.67 ± 0.69
k_1 ketoprofen (L g^{-1} h^{-1})	0.63 ± 0.40	0.31 ± 0.07	-
L ibuprofen (h)	0.66 ± 0.13	3.89 ± 0.45	2.75 ± 0.71
L ketoprofen (h)	2.03 ± 2.03	8.45 ± 1.56	-

*The parameter estimator in Aquasim was unable to converge, therefore the standard deviations for these experiments could not be estimated.

As shown in Figures 2.1 and 2.2, the model predicted well the experimental data, with the only exceptions being the tests where ketoprofen was fed to Fernão Ferro sludge at 250 µg L^{-1}. Indeed, these tests (Figure 2.2c) were the only tests that showed a different behaviour, as the sigmoidal trend of NSAID biodegradation was less pronounced at this initial ketoprofen concentration. While the reason for this difference is unclear, one possibility is that this concentration of ketoprofen was inhibitory for this particular sludge, thereby affecting its behaviour.

Table 2.1 also shows the k_1 and L values for each test performed with the 3 sludges. For all WWTP sludges, the k_1 was significantly higher and the L values were significantly lower for ibuprofen degradation as compared to ketoprofen degradation. It is clear that the Beirolas sludge displayed significant advantages as compared to the other 2 WWTPs. The lag phase was significantly shorter with respect to both ibuprofen and ketoprofen biodegradation, and the ibuprofen degradation rate constant was also higher in this sludge. The Beirolas

sludge required far less operational time to degrade both ibuprofen and ketoprofen (Figure 2.1 and 2.2), mainly due to the fact that the lag phase was generally >4 times shorter with this sludge as compared to the other 2 plants for both NSAIDs.

The different kinetic properties observed could be related to the different microbial communities in each treatment plant. However, it was not clear from the aforementioned experiments if the lag phase found for NSAID biodegradation was due to the acclimation of degrading microorganisms and coupled with their growth, as proposed by Chong (2009), or if it was rather due to the induction and expression of the necessary NSAID-degrading enzymes by the same microbial community. For this reason, further experiments were carried out to isolate ibuprofen and ketoprofen degraders, where one of the isolates was characterised with respect to its NSAID degradation kinetics. Unlike the activated sludge experiments, where the concentration of NSAID degraders could not be measured, the growth of ibuprofen degraders could readily be monitored throughout the pure culture experiments.

Isolation of putative ibuprofen and ketoprofen degrading organisms

Since Beirolas sludge was shown to have the best NSAID degradation kinetics in the previous experiments, this sludge was used to obtain NSAID degrading isolates through plating in NSAID-containing agar. Sixty-five microorganisms were isolated, where twelve isolates were able to degrade either ibuprofen or ketoprofen. The identification and NSAID removal efficiency of each isolate are presented in Table 2.2. It was the first time that each of these microorganisms were reported as being capable of degrading either ibuprofen or ketoprofen. While the isolates identified in this study were obtained from WWTP sludge, the role and significance of these bacteria in WWTPs is still unknown. Further

study is needed in order to characterise and identify the microbial populations responsible for PhAC biodegradation in WWTPs.

Table 2.2. Phylogenetic affiliation of isolates and their ability to degrade ibuprofen or ketoprofen, observed 100 h after the addition of ibuprofen or ketoprofen.

Isolate	% Similarity	Type strain	Accession number	Ketoprofen %removal	Ibuprofen %removal
I2	100	*Gordonia amicalis*	EU266486.1	-	26
I4	99	*Acinetobacter bouvetii*	JF681285	-	12.8
I5	99	*Paracoccus aminophilus*	NR_042715.1	-	16.2
I11	97	*Patulibacter americanus*	NR_042369	-	35
K1	100	*Methylobacterium populi*	NR_029082.1	12	-
K3	99	*Gordonia hydrophobica*	NR_026254.1	22	-
K11	100	*Tsukamurella spumae*	NR_042800.1	20.6	-
K12	99	*Paracoccus aminovorans*	NR_025857.1	14.1	-
K13	99	*Rhodococcus qingshengii*	NR_043535.1	13.6	-
KA1	100	*Gordonia terrae*	NR_037022.1	15.3	-
KA2	99	*Rhodococcus zopfii*	NR_041775.1	14.9	-
KA6	99	*Bosea thiooxidans*	NR_041994.1	13.4	-

The biodegradation of ibuprofen by a pure culture

The bacterial isolate I11 showed the highest NSAID removal capacity as compared to the other isolates (Table 2.2), and was thus chosen for further characterisation.

The kinetics of degradation of ibuprofen by I11 was assessed in this study through a series of batch tests using either different initial ibuprofen concentrations or different levels of additional carbon sources (yeast extract and tryptone). Varying the carbon source addition was necessary to influence the corresponding level of biomass growth by the isolate (Figure 2.3), allowing

the investigation of the link between biomass growth rate and ibuprofen degradation rate.

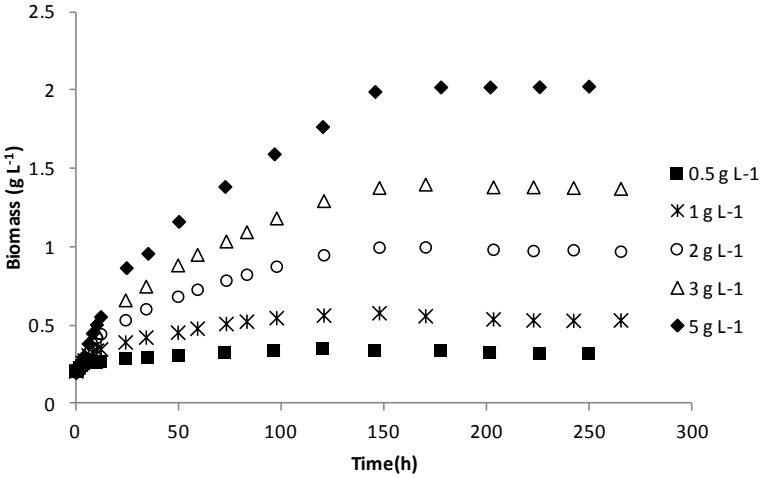

Figure 2.3. I11 growth profile (g L^{-1} TSS) in the presence of 250 µg L^{-1} ibuprofen at different concentrations of yeast extract and tryptone.

Unlike the experiments with activated sludge, the degradation of ibuprofen began immediately after its addition to the medium without a lag phase (Figure 2.4). There was no biodegradation activity in the autoclaved and abiotic control tests, confirming that the degradation activity was biologically mediated.

I11 was not able to use ibuprofen as the sole carbon source, although degradation was observed in the presence of other carbon sources, concomitantly with cell growth. In previous studies, two bacterial strains were described as being able to utilise ibuprofen, a *Norcardia* sp. (Rosazza and Chen, 1994) and *Sphingomonas* Ibu-2 (Murdoch and Hay, 2005). Only *Sphingomonas* Ibu-2 was able to degrade ibuprofen as the sole carbon source.

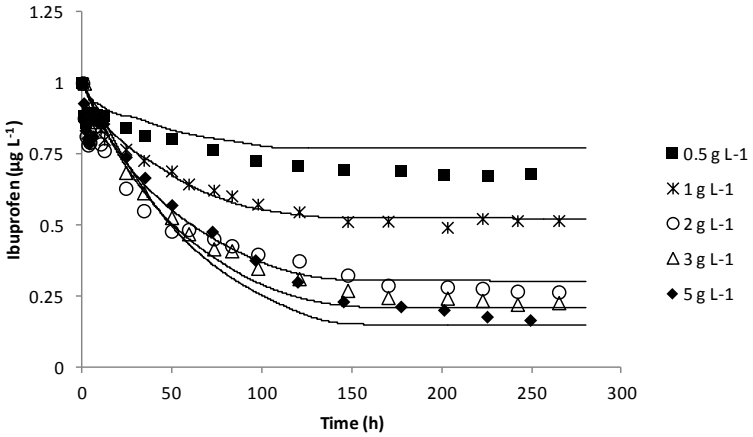

Figure 2.4. Predicted values (lines) and experimental values (symbols) for the degradation of ibuprofen by the isolate I11 at different initial concentrations of tryptone and yeast extract.
R^2 = 0.95 between experimental values and model predictions.

Additionally, the effect of initial ibuprofen concentration on the degradation performance, as sole carbon source or in the presence of other carbon sources was investigated. After 323 h of incubation, I11 was able to reduce 28 % and 50 % of 1000 µg L^{-1} and 250 µg L^{-1} of ibuprofen, respectively (Figure 2.5). At the highest initial concentration of ibuprofen, the maximum degradation rate was 1.97 µg L^{-1} h^{-1}, which is substantially higher than the 0.88 µg L^{-1} h^{-1} observed at 250 µg L^{-1}. This shows that the ibuprofen degradation rate was dependent on the ibuprofen initial concentration, similarly to what was observed in the activated sludge experiments.

Moreover, it was shown that the degradation rate of ibuprofen increased with an increase in the initial concentration of yeast extract and tryptone (Table 2.3), which corresponded to an increase in biomass growth rate (Figure 2.3).

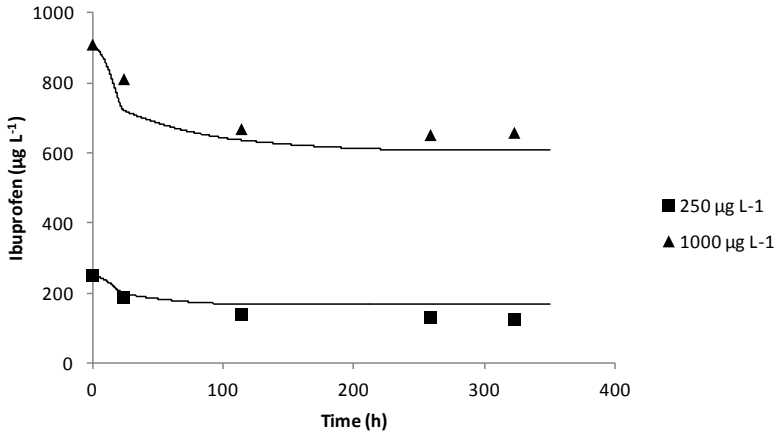

Figure 2.5. Predicted values (lines) and experimental values (symbols) for ibuprofen degradation by the isolate I11 at two initial concentrations (250 µg L^{-1} and 1000 µg L^{-1}). R^2 = 0.97 between experimental values and model predictions.

Table 2.3. Ibuprofen and biomass maximum degradation rates at different initial concentrations of yeast extract and tryptone.

Experiment	0.5 g L^{-1}	1 g L^{-1}	2 g L^{-1}	3 g L^{-1}	5 g L^{-1}
Maximum degradation rate (µg L^{-1} h^{-1})	0.36	0.53	0.98	1.25	1.24
Maximum biomass growth rate (g L^{-1} h^{-1})	0.001	0.002	0.004	0.006	0.010

The simultaneous utilisation of supplemental carbon sources with a compound that is less energetic (e.g. ibuprofen) may sustain cell growth and maintenance, facilitating biodegradation of that compound. A similar effect was observed during the degradation of nitrilotriacetic acid (NTA) by the bacterial strain *Chelatobacter heintzii*, where a higher glucose fraction led to faster NTA reduction (Bally and Egli, 1996). Oehmen et al. (2012) also observed this effect

using a mixed culture degrading the xenobiotic herbicide propanil, where faster propanil degradation was observed with propionate addition.

From Table 2.3, it can be observed that while the maximum cell growth rate continued to increase with increasing initial carbon source concentration, the maximum ibuprofen degradation rate in each test approached a plateau at 1.25 µg L^{-1} h^{-1}, obtained using an initial ibuprofen concentration of 250 µg L^{-1}. Nevertheless, a higher maximum degradation rate was obtained for a concentration of 1000 µg L^{-1} of ibuprofen. This shows that the ibuprofen degradation rate by I11 is simultaneously dependant on the growth rate as well as the ibuprofen concentration.

Modelling the biodegradation of ibuprofen by a pure culture

Since the biodegradation of ibuprofen by a pure culture showed different behaviour as compared to activated sludge, the equation describing NSAID biodegradation was modified, as shown below in Equation 2.4:

$$\frac{dS}{dt} = k_2 \frac{S}{S_o} \frac{dX}{dt} (e^{-z\frac{dX}{dt}})$$

where k_2 represents the ibuprofen degradation rate constant, S_o is the initial concentration of ibuprofen present in the media, dX/dt represents the cell growth rate, and z is an inhibition constant. The constants 'k_2' and 'z' were estimated using non-linear regression fitting to the experimental data using Aquasim (Reichert, 1994). The simultaneous parameter estimation for both constants utilised the experimental data of the five tests at the different initial carbon substrate concentrations, where the estimates are shown in Table 2.4.

Table 2.4. Parameter values used for model simulation of the degradation of ibuprofen by the isolate I11.

Parameter	k_2 (µg g^{-1} h^{-1})	z
Value	2.76 ± 0.17	65.7 ± 6.41

Since the degradation of ibuprofen was found to only occur during the biomass growth phase, equation 2.4 is dependent on the rate of biomass growth. Furthermore, since the ibuprofen degradation rate was found to approach a maximum at high biomass growth rates, it was necessary to add an inhibition function that regulated this effect. This was achieved through the exponential term in equation 2.4.

The model predictions and the experimental data for ibuprofen degradation by I11 are shown in Figure 2.4. The model was able to describe well the trends observed in each of the 5 tests. Furthermore, the model was applied to describe the two tests fed with different ibuprofen concentrations (250 µg L^{-1} and 1000 µg L^{-1}) without changing any of the model parameters, in order to validate the model. The results are shown in Figure 2.5, whereby the model also describes well the experimental data.

It is clear from the results of this study that it was not possible to develop a universal model to describe NSAID biodegradation by both activated sludge as well as pure cultures, despite the fact that the same compounds were used under similar environmental conditions. Furthermore, the differences observed between xenobiotic biodegradation profiles reported in literature (Chong, 2009; Collado et al., 2012; Joss et al., 2006; Lust et al., 2012; Plósz et al., 2010; Quintana et al., 2005) support the hypothesis that the applicability of PhAC biodegradation models depends on the composition of the biomass as well as

the loading rate of these compounds. When applied towards different WWTPs, biodegradation models may require adaptation to describe different systems, where model validation will be of high importance in these situations. Further model development is recommended for the future in order to successfully optimise PhAC biodegradation from WWTPs. This study advances our ability to describe PhAC biodegradation and provides useful modelling options that should be considered during future applications.

Conclusions

Iburofen and ketoprofen biodegradation kinetics were investigated using activated sludge from three different WWTPs. A similar profile was observed with each sludge, where a lag phase was found to occur prior to NSAID biodegradation, and when the two NSAIDs were simultaneously supplied, their removal was independent from the other. This activated sludge performance was described using a model where the degradation rate was a function of the NSAID concentration. Twelve ibuprofen/ketoprofen degrading isolates were identified from one of the WWTP. The ibuprofen biodegradation kinetics of one of these isolates (I11) was shown to be dependent on the biomass growth rate as well as the NSAID concentration, which was incorporated in a different model. Overall, this work provides a step forward towards describing PhAC removal in WWTPs. The behaviour of NSAID removal may be subject to variation between WWTPs, thus PhAC biodegradation models may require adaptation to different systems.

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Chapter 3

Patulibacter medicamentivorans sp. nov., isolated from activated sludge of a wastewater treatment plant

Submitted and in revision for publication to the International Journal of Systematic and Evolutionary Microbiology:

Patulibacter medicamentivorans sp. nov., isolated from activated sludge of a wastewater treatment plant

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Almeida B. was involved in all the experimental work presented in this chapter, except for the analysis of the isoprenoid quinones, cellular fatty acids and cell diamino acids that were performed by Peter Schumann, at the Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; and the Biolog assays that were performed by the IBET Analytical Services Unit, Oeiras, Portugal. The analysis of the polar lipids was performed in collaboration between the author, Ivone Vaz-Moreira and Olga C. Nunes at FEUP, Porto, Portugal.

Abstract

A Gram-positive, aerobic, non-motile, non-endospore-forming rod with ibuprofen degrading capacity, designated I11^T, was isolated from activated sludge from a wastewater treatment plant. The major respiratory quinone was demethylmenaquinone DMK-7, $C_{18:1}$ CIS 9 was the predominant fatty acid, phosphatidylglycerol was the predominant polar lipid, the cell wall contained *meso*-diaminopimelic acid as the diagnostic diamino acid and the G+C content of the genomic DNA was 74.1 mol%. On the basis of 16S rRNA gene sequence analysis, the closest phylogenetic neighbours of strain I11^T were *Patulibacter ginsengiterrae* CECT 7603^T (96.8 %), *Patulibacter minatonensis* DSM 18081^T (96.6 %), and *Patulibacter americanus* DSM 16676^T (96.6 %). The phenotypic characterisation supports the inclusion of strain I11^T within the genus *Patulibacter.* However, distinctive features and the 16S rRNA gene sequence analysis suggest the proposal of a new species. Therefore, the name *Patulibacter medicamentivorans* sp. nov. is proposed, and the type strain is I11^T (=DSM 25962^T =CECT 8141^T).

Introduction

Biodegradation of pharmaceutically active compounds (PhACs), such as ibuprofen, has been an increasing concern in recent years due to their detection in natural matrices such as soil, sediments and water and to their potential risk to the environment and health of living beings (Santos et al., 2010). The presence of ibuprofen, a non-steroidal anti-inflammatory drug, and other PhACs in the environment is mostly due to their inefficient removal from wastewater treatment plants (WWTP). Microbial degradation plays an important role in wastewater treatment processes and several studies are being conducted in order to understand and improve microbial degradation of PhACs in WWTP (Ying et al., 2009; Onesios et al., 2009; Salgado et al., 2012).

The strain described in this study, designated I11^T, was isolated from activated sludge of Beirolas WWTP (Lisbon, Portugal). Although sharing some phenotypic and phylogenetic characteristics with members of the genus *Patulibacter*, strain I11^T could not be affiliated to any of its described species. The genus *Patulibacter* was first proposed by Takahashi et al. (2006) with *Patulibacter minatonensis* and further emended by Reddy and Garcia-Pichel (2009) to include *Patulibacter americanus*. Recently, *Patulibacter ginsengiterrae* was isolated from a ginseng field (Kim et al., 2012a) and characterised as a new species belonging to the genus *Patulibacter*. Based on a polyphasic taxonomic study, isolate I11^T is proposed in this study as type strain of a new species of this genus.

Materials and Methods

Activated sludge (3 g L^{-1}) collected from Beirolas WWTP (Lisbon, Portugal) was inoculated in minimal medium (Oehmen et al., 2009) supplemented with 500 µg L^{-1} of ibuprofen as the only carbon source and grown at 28 °C on a rotary shaker at 110 r.p.m. for approximately 15 days. Within this period of time, a new ibuprofen addition was carried out whenever all the parent compound had been removed from the medium, as determined by high performance liquid chromatography (Almeida et al., 2012). After 15 days the enrichment culture was serially diluted by a series of ten-fold dilutions in sterile saline solution (0.85 % NaCl, w/v) and streaked on agar plates containing minimal medium supplemented with 250 µg L^{-1} of ibuprofen and 15 g L^{-1} agar. After 1 week of incubation at 28 °C, all the colonies with distinct morphotypes were isolated and purified on the same medium. Among the isolated organisms, strain I11^T showed higher ibuprofen degradation capacity. Strain I11^T was stored in culture medium supplemented with 20 % glycerol at -80 °C. For the experiments reported in this study, strain I11^T was subcultured in Tryptic Soya Broth (TSB, Oxoid) or on Tryptic Soya Agar (TSA, Oxoid), at 28 °C for 3 days, unless stated otherwise. Type strains *Patulibacter minatonensis* DSM 18081^T, *Patulibacter americanus* DSM 16676^T and *Patulibacter ginsengiterrae* CECT 7603^T were used for comparison purposes under identical growth conditions.

The colony and cell morphology, Gram-staining, cytochrome *c* oxidase and catalase tests, production of endospores and motility were analysed based on the methodologies of Murray et al. (1994) and Smibert and Krieg (1994). The temperature (4, 10, 15, 20, 25, 27, 30, 32, 37, 39, 42, 45 and 48 ºC), salinity (1.5, 2.5, 3.5, 5.5, 7.5 and 10 % (w/v) of NaCl) and pH ranges (5-9 with increments of 0.5 pH units) for growth were determined by measuring the

turbidity (600 nm) of cultures grown in 50 mL falcon tubes containing 5 mL TSB, and incubated at 32 ºC. The pH of the TSB medium was adjusted using MES (pH 5.0-6.0), MOPS (pH 6.0-8.0) and CAPS (pH 8.0-9.0) and further sterilised using a 0.2 µm Whatman filter.

Antibiotic susceptibility was determined by the Kirby-Bauer disk diffusion method (Bauer et al., 1966), using antibiotic disks from Oxoid containing the following (µg): ampicillin (10), amikacin (30), aztreonam (30), bacitracin (10 U), ceftriaxone (30), ciprofloxacin (5), ceftazidime (30), chloramphenicol (30), erythromycin (15), gentamicin (10), novobiocin (30), polymyxin B (300 U), tetracycline (30) and vancomycin (30). Additional physiological and biochemical characteristics were determined using API 20NE and API ZYM test strips (bioMérieux), and GP2 microplate from the Biolog identification system, according to the manufacturer's instructions.

The polar lipids were analysed as described previously (Vaz-Moreira et al., 2007) using the methods of Manaia et al. (2004). Isoprenoid quinones were extracted and purified as described by Collins et al. (1977) and analysed by HPLC and electron-impact mass spectrometry (320 Singlequad equipped with a direct insertion probe, Varian) as described by Yi et al. (2007). Cellular fatty acids were extracted from cells cultivated on TSA at 28 °C. Strain I11^T and the three reference strains grew similarly well and provided sufficient cells of comparable physiological age from the third streak quadrant of the plates after cultivation under the given conditions for 72 hours. Fatty acid methyl esters were obtained following the method of Stead et al. (1992) and were analysed by gas chromatography using the Microbial Identification System (MIDI, Sherlock Version 6.1; database, ACTIN6; gas chromatograph, model 6890N, Agilent Technologies). The identity of hydroxy fatty acids was confirmed by

GC/MS (320 Singlequad equipped with a gas chromatograph CP-3800 and a column VF-5ms [30m x 0.25 mm I.D., film thickness 0.25 µm], Varian). The cell wall diamino acid of strain I11^T was determined from whole-cell hydrolysates as described by Hasegawa and Takizawa (1983).

Both, 16S rRNA gene sequence and the G+C content value were obtained from the whole genome sequencing of I11^T strain (Almeida et al., 2012). The identification of phylogenetic neighbours was initially carried out by the BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programs against the database of type strains with validly published prokaryotic names (Kim et al., 2012b). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012b). Phylogenetic analysis was performed using MEGA version 5 (Tamura et al., 2011). Sequence similarity was estimated using the model of Jukes and Cantor (1969) and values were used to construct the phylogenetic tree by the neighbour-joining method. Tree stability was assessed by comparison with other trees constructed with the maximum parsimony and maximum-likelihood methods. Bootstrap analysis was performed employing 1000 re-samplings. A total of 1296 nucleotide positions in each 16S rRNA gene were included in the analysis. Non-homologous and ambiguous nucleotide positions were excluded from the calculations.

Results and Discussion

After 3 days on TSA at 28 °C, strain I11^T formed irregular, semi-translucent creamy white colonies with undulate margins, and 1-2 mm in diameter. Cells were catalase positive, oxidase-negative, Gram-stain-positive, non-motile and

non-spore forming rods. Growth was not observed below 20 °C or above 39 °C, with an optimum at around 32 °C. Good growth occurred at pH 6.0-7.5. Growth with more than 1.5 % salinity was not observed. Strain I11^T degraded ibuprofen. Other phenotypic characteristics were summarized in Table 3.1 and in the species description.

The complete 16S rRNA gene sequence (1541nt) obtained from the whole genome sequencing of strain I11^T and determined using RNAmmer (Almeida et al., 2012) shared sequence similarities of 96.8 % with *P. ginsengiterrae* CECT 7603^T, 96.6 % with *P. minatonensis* DSM 18081^T and *P. americanus* DSM 16676^T, and 93 % or less, with other *Rubrobacteriales* members. The neighbour-joining tree placed I11^T in a cluster within the genus *Patulibacter* (Figure 3.1) but with a distinct position suggesting that the novel strain deserves a separate species status. Since it has been observed that strains belonging to the same species share commonly > 97 % 16S rRNA gene sequence similarity (Stackebrandt and Goebel, 1994; Tindall et al., 2010) and the sequence similarity between strain I11^T and the other type strains did not exceed this value, DNA-DNA hybridisation was not conducted for species delineation in this study.

The inclusion of strain I11^T within the genus *Patulibacter* is supported by some genotypic and chemotaxonomic features. Indeed, the genomic DNA G+C content of I11^T strain was 74.1 mol%, which fell within the range of 72-75 mol% observed for the other species of this genus (Kim et al., 2012a). The peptidoglycan diamino acid of strain I11^T was *meso*-diaminopimelic acid and the predominant cellular fatty acid was $C_{18:1}$ CIS 9 (46.4 %, Table 3.2), similar to other *Patulibacter* members (Takahashi et al., 2006; Reddy and Garcia-Pichel, 2009; Kim et al., 2012a).

Table 3.1. Distinctive characteristics of the strain I11^T and of the other type strains of *Patulibacter* species.

Strains: *P. ginsengiterrae* CECT 7603^T, *P. americanus* DSM 16676^T and *P. minatonensis* DSM 18081^T. Data are from this study unless otherwise indicated. -, Negative; +, positive; v, variable and ND, no data available.

Characteristic	**I11^T**	***P. ginsengiterrae* CECT 7603^T**	***P. americanus* DSM 16676^T**	***P. minatonensis* DSM 18081^T**
Colony pigmentation	White	White	Pink	Pale yellow
Motility	-	+	+	+
pH range for growth**	5.5-8.0	5.5-9.0[a]	5.0-9.0[b]	6.0-8.0[c]
T range for growth (ºC)**	20-39	5-37[a]	10-30[b]	16-28[c]
3% NaCl	-	+	-	-
Oxidase	-	+	-	-
Nitrate reduction	+	-	-	-
Tween 40	+	+	+	-
Tween 80	+	+	+	-
Carbon utilisation				
L-Alanine	+	-*	-	-
D-Arabitol	+	+*	-	-
L-Arabinose	-	+	+*	+
Citrate	-	-*	-	-
D-Fructose	+	-	-	+
Gentiobiose	+	-	-	-
Glycogen	-	-*	-	-
Ibuprofen	+	-	-	-
Inositol	-	-*	-	-*
D-mannitol	-	+	-	-*
D-mannose	-	-*	+*	+
D-melibiose	-	+	-*	-
D-trehalose	-	-*	-	-
Enzymes				
Alkaline phosphatase	+	-	+	-*
Cystine arylamidase	+	+	-	-
β-glucosidase	+	-	-	-
Urease	-	-*	-	-
Susceptibility				
Bacitracin	+	-	+	+
Ciprofloxacin	+	-	-	+

Characteristic	I11^T	*P. ginsengiterrae* CECT 7603^T	*P. americanus* DSM 16676^T	*P. minatonensis* DSM 18081^T
Chloramphenicol	+	+*	+	-*
Tetracyclin	-	-	+	+
Isoprenoid quinone	DMK-7	DMK-7	MK-7(H_2)	DMK-7
DNA G+C content (mol%)**	74.1	74.6[a]	72.0[b]	72.0[c]

*Differs from the original description.

**Data were taken from: a, Kim et al. (2012a); b, Reddy and Garcia-Pichel (2009); c, Takahashi et al. (2006).

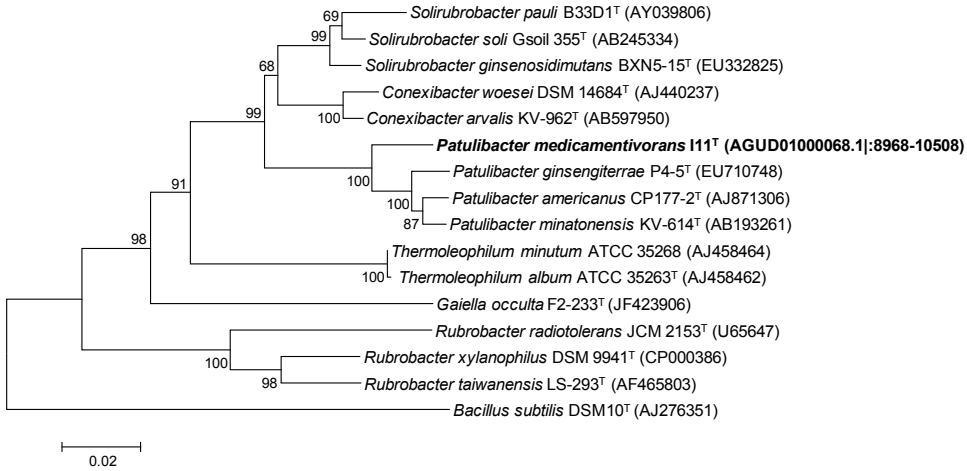

Figure 3.1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences (1296 bp), showing the relationships of strain I11^T with members of the genus *Patulibacter* and other closest genera.

All the branches were also recovered by the maximum-likelihood and maximum parsimony methods. Bar, 2 substitutions per 100 nt.

The respiratory quinone extracted from strain I11^T eluted by a retention time shorter by 5 % than this of menaquinone MK-7 from *Bacillus subtilis* subsp. *subtilis* DSM10^T and was identified by mass spectrometry as demethylmenaquinone DMK-7 (base peak 211 m/z, molecular ion 636 m/z). DMK-7 represented 92.2 % of the sum of all integrated peaks. The area of other five additional minor peaks, which could not be attributed to any menaquinone standards used [MK-5, MK-6, MK-7, MK-8, MK-7(H_2), MK-8(H_2) or MK-9(H_2)], varied between 0.5 and 2.6 %. The polar lipid profile of I11^T consisted of the major phosphatidylglycerol (PG), four unknown phospholipids (PL1-PL4) and small amounts of diphosphatidylglycerol (DPG) (Supplementary Figure 7.1). A similar polar lipid profile was described for *Patulibacter gisengiterrae* CECT 7603^T (Kim et al., 2012a).

The new species status of strain I11^T was supported by other physiological, biochemical and chemotaxonomic differences (Tables 3.1 and 3.2). The presence of demethylmenaquinone DMK-7 as the main respiratory quinone, and the presence of higher proportion of $C_{17:0}$ 10-methyl in the fatty acids profile of strain I11^T are among these distinctive features. Based on these differences strain I11^T is suggested to represent a novel species within the genus *Patulibacter*, for which the name *Patulibacter medicamentivorans* sp. nov. is proposed, and the type strain is I11^T (=DSM 25962^T =CECT 8141^T).

Table 3.2. Cellular fatty acid compositions of strain I11^T (1) and its closest phylogenetic neighbours *P. ginsengiterrae* CECT 7603^T (2), *P. americanus* DSM 16676^T (3) and *P. minatonensis* DSM 18081^T (4).

All strains were cultivated on TSA at 28 °C for 72 hours and tested in parallel in the present study.

Fatty acid	1	2	3	4
Saturated straight-chain				
$C_{9:0}$	0.1			
$C_{10:0}$	2.4			0.1
$C_{12:0}$	1.1			
$C_{14:0}$	0.5		0.2	
$C_{15:0}$			0.3	
$C_{16:0}$	0.8	3.1	4.7	2.7
$C_{17:0}$			0.5	
$C_{18:0}$	1.2	3.1	2.7	1.7
$C_{19:0}$			0.6	0.6
$C_{20:0}$			0.5	
Unsaturated straight-chain				
$C_{16:1}$ B				0.4
$C_{16:1}$ CIS 9	2.0	0.5	3.4	0.9
$C_{17:1}$ CIS 9	0.6	0.6	3.1	
$C_{18:1}$ CIS 9	46.4	48.8	56.2	54.5
$C_{20:1}$ CIS 11	0.6			
$C_{18:3}$ CIS 6,12,14			3.2	
Saturated branched-chain				
iso-$C_{10:0}$	1.5			
iso-$C_{12:0}$	1.7			
iso-$C_{14:0}$	3.4	1.5		0.3
iso-$C_{16:0}$	2.9	0.8		1.7
iso-$C_{18:0}$	4.9	0.8		1.5
anteiso-$C_{11:0}$	0.4			
anteiso-$C_{13:0}$	0.4			
anteiso-$C_{15:0}$	3.2	8.8	11.5	5.6
anteiso-$C_{17:0}$	2.5	3.7	2.0	9.4
anteiso-$C_{19:0}$	0.7	0.7	1.6	1.8
$C_{17:0}$ 10-methyl	9.3	0.8	1.0	1.9
Unsaturated branched-chain				
iso-$C_{16:1}$ F	0.2			
iso-$C_{18:1}$ H	0.2			
iso-$C_{19:1}$ I				1.4
Hydroxy branched-chain				
iso-$C_{14:0}$ 2OH*	5.1	1.1		0.6

Fatty acid	1	2	3	4
anteiso-$C_{15:0}$ 2OH	6.8	9.4	3.9	13.7
Summed Features				
Sum In Feature 7 ($C_{18:1}$ CIS11/t9/t6 / $C_{18:1}$ TRANS 6/t9/c11)	1.1	0.9	2.9	1.1
Sum In Feature 9 (un 18.846/18.858 / $C_{19:0}$ CYCLO C9-10/un)		15.3		

Description of *Patulibacter medicamentivorans* sp. nov.

Patulibacter medicamentivorans (me.di.ca.men.ti.vo'rans N.L. n. *medicamentus* pharmaceutical drug; L. part. adj. vorans *devouring*; N.L. adj. *medicamentivorans* eater of pharmaceutical drug).

Cells are aerobic, Gram-stain-positive, catalase positive, oxidase negative, non-motile, non-sporulating rods (0.3-0.4x1.0-1.1 µm). Colonies on TSA are creamy white, semi-translucent, irregular with undulate margins. Optimum temperature and pH for growth are 32 °C and 6.0-7.5, respectively. Growth is not observed in media containing more than 1.5 % (w/v) NaCl. The Biolog substrates dextrin, mannan, Tween 40, Tween 80, D-arabitol, arbutin, D-cellobiose, D-fructose, gentiobiose, α-D-glucose, maltotriose, D-melezitose, 3-methyl-D-glucose, β-methyl-D-glucoside, palatinose, L-rhamnose, D-ribose, salicin, D-tagatose, turanose, D-xylose, acetic acid, β-hydroxybutyric acid, α-ketovaleric acid, D-lactic acid methyl ester, succinic acid mono-methyl ester, propionic acid, pyruvic acid, L-alanine, glycyl-L-glutamic acid, D-fructose-6-phosphate and D-glucose-6-phosphate are utilised. It is able to reduce nitrate and has enzyme activity for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine and cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. The Biolog substrates α-

cyclodextrin, β-cyclodextrin, glycogen, inulin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygdalin, L-arabinose, L-fucose, D-galactose, D-galacturonic acid, D-gluconic acid, m-inositol, α-D-lactose, lactulose, D-mannitol, D-mannose, D-melibiose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, α-methyl-D-mannoside, D-psicose, D-raffinose, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-trehalose, xylitol, α-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, lactamide, D-malic acid, pyruvic acid methyl ester, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2′-deoxy adenosine, inosine, thymidine, uridine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate, α-D-glucose-1-phosphate and D-L-α-glycerol phosphate are not utilized. The API 20 NE substrates L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate, and phenylacetic acid are not utilised. Indole production is not observed. Fermentation of D-glucose does not occur, and activities of valine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, urease and gelatinase are not detected. It is susceptible to ampicillin (10 μg), amikacin (30 μg), bacitracin (10 U), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), novobiocin (30 μg), polymyxin B (300 U) and vancomycin (30 μg), and resistant to aztreonam (30 μg) and tetracycline (30 μg). The cell wall peptidoglycan contains *meso*- diaminopimelic acid. Demethylmenaquinone DMK-7 is the predominant respiratory quinone. Major cellular fatty acid is $C_{18:1}$CIS 9. The DNA G+C content is 74.1 mol%. The polar

lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and four unknown phospholipids (PL1-PL4).

The type strain I11^T (=DSM 25962^T =CECT 8141^T) was isolated from activated sludge enriched for ibuprofen degradation.

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Chapter 4

Quantitative proteomic analysis of ibuprofen-degrading *Patulibacter* sp. strain I11

This chapter contains data published in:

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Almeida B. and Henrik Kjeldal equally contributed to the work presented in this chapter. The author was involved in all the experimental work presented in this chapter, except for: 1) the identification and quantification of the proteome that was performed by Henrik Kjeldal and Allan Stensballe at AAU, Aalborg, Denmark; 2) ibuprofen degradation analysis that was performed by IBET Analytical Services, Oeiras, Portugal; 3) qPCR analysis that was performed by Ihab Lolas at AAU, Aalborg, Denmark. The whole genome sequencing of I11 was performed in collaboration with Kaare Nielsen at AAU, Aalborg, Denmark. The whole genome assembly and annotation was performed together with Ihab Lolas at AAU, Aalborg, Denmark.

Abstract

Ibuprofen is the third most consumed pharmaceutical drug in the world. Several isolates have been shown to degrade ibuprofen, but very little is known about the biochemistry of this process. This study investigates the degradation of ibuprofen by *Patulibacter* sp. strain I11 by quantitative proteomics using a metabolic labelling strategy. The whole genome of *Patulibacter* sp. strain I11 was sequenced to provide a species-specific protein platform for optimal protein identification. The bacterial proteomes of actively ibuprofen-degrading cells and cells grown in the absence of ibuprofen was identified and quantified by gel based shotgun-proteomics. In total 251 unique proteins were quantitated using this approach. Biological process and pathway analysis indicated a number of proteins that were up-regulated in response to active degradation of ibuprofen, some of them are known to be involved in the degradation of aromatic compounds. Data analysis revealed that several of these proteins are likely involved in ibuprofen degradation by *Patulibacter* sp. strain I11.

Introduction

The emergence of pharmaceutically active compounds (PhACs) in natural matrices such as soil, sediments and water has been an issue of increasing concern in recent years. Several studies have been carried out to elucidate the magnitude of the risks that PhACs may impose to the environment and health of living beings (Daughton and Ternes 1999; Pal et al., 2010; Murray et al., 2010; Santos et al., 2010). Many PhACs are potentially harmful to the environment and the public health, and several PhACs are considered to have mutagenic, carcinogenic and endocrine disruptor effects (Daughton and Ternes 1999; Pal et al., 2010; Kummerer 2009; Santos et al., 2010). Other compounds appear harmless when considered individually, but a potential risk of unknown additive or synergistic effects in the presence of mixtures of compounds may exist. Presence of PhACs in the environment suggests their inefficient removal in wastewater treatment plants (WWTPs). PhACs can typically be detected in the ng L^{-1} to µg L^{-1} range in influents and have highly varying removal degrees in wastewater treatment (Heberer 2002; Kolpin et al., 2002; Onesios et al., 2009; Daughton and Ternes 1999; Salgado et al., 2010; 2011; Santos et al., 2010).

Elimination of organic contaminants and some micropollutants from wastewater treatment is due to the action of different removal mechanisms, such as sorption, biodegradation and abiotic degradation (Salgado et al., 2012). Removal of polar acidic pharmaceuticals seems to be predominantly removed by microbial degradation in wastewater treatment systems (Salgado et al., 2012; Quintana et al., 2005).

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) detected in the ng to µg per litre range in wastewater, as shown by numerous surveys that have

been carried out all over the world (Daughton and Ternes 1999; Kolpin et al., 2002; Onesios et al., 2009; Salgado et al., 2010; 2011; 2012; Ying et al., 2009). Ibuprofen is globally the third most produced pharmaceutical compound and a highly diffuse consumption makes it one of the most frequently detected compounds in wastewater (Buser et al., 1999). Typical values of ibuprofen detection range from 22 μg L^{-1} to 84 μg L^{-1} in raw sewage and treated effluent water, respectively, while up to 5 μg L^{-1} has been detected in surface waters (Santos et al., 2010; Pal et al., 2010).

Only few studies have focused on the biochemistry of the biodegradation of PhACs. Studies on the biodegradation of ibuprofen in activated sludge have suggested the formation of hydroxy-ibuprofen, carboxy-ibuprofen, and carboxy-hydratropic acid (carboxy-HA) as degradation products (Quintana et al., 2005; Buser et al., 1999; Zwiener et al., 2002; Zwiener and Frimmel 2003; Winkler et al., 2001). Genetic and metabolite analysis of ibuprofen degradation by *Sphingomonas* Ibu-2, revealed several key metabolites possibly associated with ibuprofen degradation, such as ibuprofen CoA; 1,2-cis-diol-2-hydroibuprofen CoA and 4-isobutylcatechol – and a cluster of five putative genes (*ipfABDEF*) was used to propose a degradation pathway for ibuprofen by this isolate (Murdoch and Hay 2005; Kagle et al., 2009). This approach has provided insights that are potentially of great significance for the understanding of ibuprofen microbial degradation. However, it remains to be elucidated if phylogenetically different microorganisms as well as the strain *Sphingomonas* Ibu-2 do in fact degrade ibuprofen through this proposed pathway or by some other unknown mechanism.

Quantitative proteomics has proven to be a valuable tool for analysing the physiological response of microorganisms in the presence of specific

compounds or under the influence of different physico-chemical parameters (Kim et al., 2007; 2009). Proteomics has also been succesfully applied for studying the biodegradation of aromatic hydrocarbons such as benzoate, aniline, and phenol by several microorganisms, e.g. *Acinetobacter* (Giuffrida et al., 2001; Park et al., 2006; Pessione et al., 2003) and *Pseudomonas* strains (Loh and Cao 2008), as well as several mycobacterial (Kim et al., 2004; Liang et al., 2006) and rhodococcal species (Navarro-Llorens et al., 2005; Patrauchan et al., 2005; Tomas-Gallardo et al., 2006). Many microorganisms capable of degrading micropollutants induce the expression of enzymes involved in the metabolic pathway(s) related to the degradation itself, when grown in the presence of the target compound. These induced proteins can be identified by quantitative comparisons of the proteomes expressed in presence and absence of the micropollutant, which give direct measurement of protein expression levels (Kim et al., 2007; 2009). Furthermore, proteomics is a powerful tool for providing functional information on prokaryotic gene expression at the protein level essentially allowing for bridging the gap between genome and phenotype.

The main objective of this study was to investigate the degradation pathway of ibuprofen by *Patulibacter sp*. strain I11, a bacterial strain isolated from activated sludge, using quantitative proteomics combined with genetic information of the microorganism. Global changes in the proteome of *Patulibacter* sp. strain I11 when grown in the presence and absence of ibuprofen, were characterised by stable isotope metabolic labelling in combination with 1-D gel based shotgun-proteomics. To the best of our knowledge, this study is the first proteomic analysis on an ibuprofen-degrading microorganism, providing novel information on the potential proteins related to the degradation of ibuprofen, and represents a step towards a better

understanding the conditions that promote the degradation of this NSAID in WWTPs.

Material and Methods

Bacterial isolation and characterization

The bacterial strain I11, used in this study, was isolated from activated sludge collected from Beirolas WWTP (Lisbon, Portugal). Activated sludge (3 g L^{-1}) was inoculated in M9 minimal medium (Oehmen et al., 2009) supplemented with 500 µg L^{-1} of ibuprofen and grown at 28 ºC for 15 days. Ibuprofen degradation capacity of the enriched microbial community was evaluated during the period of incubation. Ibuprofen was followed by high performance liquid chromatography and regularly added to avoid depletion. I11 was subsequently isolated from this microbial enrichment by a series of ten-fold dilutions in 0.85 % (w/v) NaCl streaked on agar plates containing minimal medium supplemented with 250 µg L^{-1} of ibuprofen and 15 g L^{-1} agar. The plates were incubated at 28 ºC for 3-4 days and individual colonies were selected, re-inoculated onto the same medium supplemented with 1 g L^{-1} of yeast extract (Oxoid, Hampshire, England) and tryptone (Oxoid, Hampshire, England) (50% of each) for isolation into pure cultures. Cells were subjected to ibuprofen biodegradation in OD2-medium (Silantes, München, Germany) and M9 medium supplemented with 1 g L^{-1} yeast extract and tryptone in batch experiments at different initial ibuprofen concentrations (1,000 µg L^{-1}, 250 µg L^{-1} and 50 µg L^{-1}). All incubations were aerobic and run at 28 ºC on a rotary table (110 rpm), and the ibuprofen degradation and OD_{600} were measured at regular intervals.

Bacterial cultivation conditions and protein sample preparation for proteomic analysis

A differential proteomic approach was carried out by metabolic labelling of the proteome of strain I11 grown in the presence or absence of ibuprofen as outlined in Figure 4.1. Two independent experiments were conducted by metabolically labelling biological replicates using a forward and reverse labelling strategy (Figure 4.2).

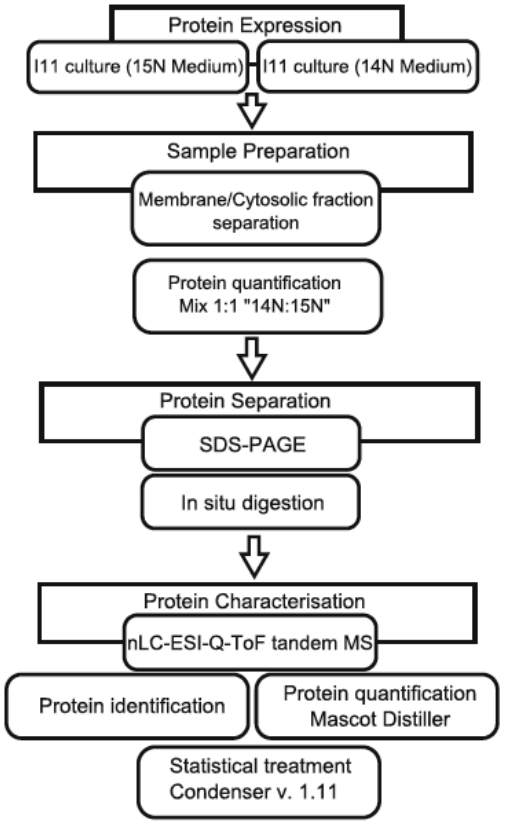

Figure 4.1. Analytical scheme for the relative quantification of proteins derived from the proteome of the isolate *Patulibacter* sp. I11 grown in the presence and absence of ibuprofen.

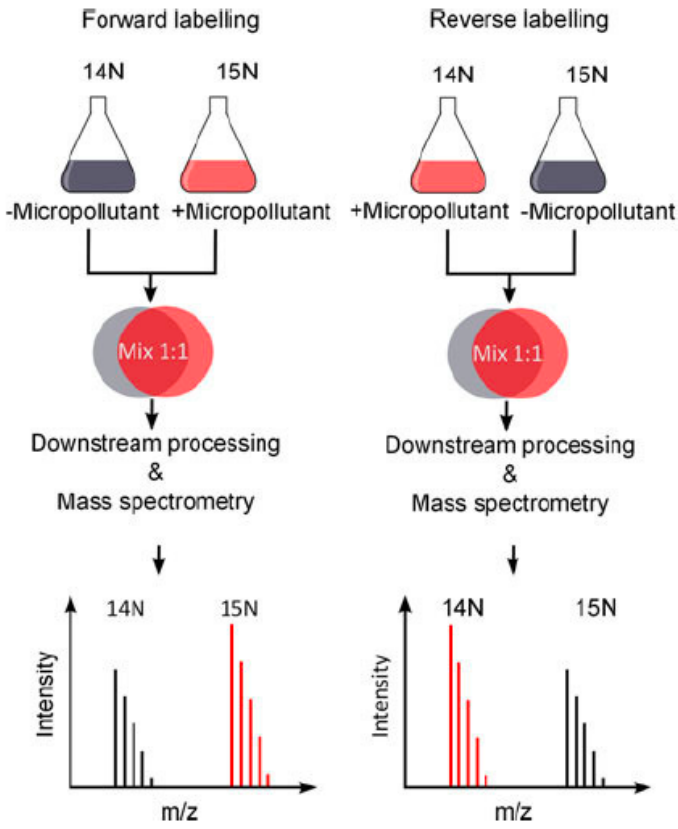

Figure 4.2. A schematic of the forward and reverse metabolic labelling of the bacterial isolate *Patulibacter* sp. I11 grown in the presence and absence of ibuprofen. Following labelling, proteins were extracted processed and subjected to tandem mass spectrometry.

Pre-cultures of the isolate I11 were aerobically cultured in Erlenmeyer flasks at 28 ºC, 110 rpm with unlabelled or ^{15}N-labelled OD2-medium (Silantes, München, Germany). The forward and reverse labelling strategy served the purpose of evaluating potential influence of the ^{14}N- and ^{15}N-medium on protein expression levels. 10 mL of pre-culture, grown until mid-logarithmic growth phase (OD_{600}~0.6), was used as inoculum. The forward/reverse labelling experiment was carried out aerobically in 100 mL Erlenmeyer flasks in a total volume of 25 mL of unlabelled and ^{15}N-labelled OD2-medium, respectively, with

or without 65 µg L^{-1} of ibuprofen. Cultures were harvested at the mid-logarithmic growth phase (OD_{600}~0.6). Cells were pelleted by centrifugation at 6,000 xg for 10 min at 4 ºC. The supernatant was discarded and the pellet was resuspended in phosphate buffered saline (PBS) supplemented with protease inhibitors (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets; 1 Tablet in 50 mL solution; Boehringer Mannheim, Mannheim, Germany). Cells were subsequently disrupted by sonication (Sonopuls HD2200, Bandelin Electronic, Berlin, Germany) running 10 cycles at 20 W for 1 min, maintaining a 1 min break in-between cycles and keeping samples on ethanol/ice bath. Cell debris was removed by centrifugation at 6,000 xg for 10 min at 4 ºC. The supernatant was collected and centrifuged at 30,000 xg for 60 min at 4 ºC. The supernatant and the pellet (resuspended in 150 µL MilliQ water) constituted the cytosolic and the membrane fraction, respectively. Protein content was determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL), and the ^{14}N- and ^{15}N-labelled samples were mixed in a 1:1 ratio in accordance with the forward/reverse labelling strategy (Figure 4.2). Samples were subsequently resuspended in SDS-sample buffer containing 40 mM dithiothreitol and denatured at 95 ºC (5 min), and separated on a 12 % (w/v) SDS-PAGE gel (Bio-Rad, USA). The gel bands were visualized with Coomassie Brilliant Blue staining. Each lane was excised into 16 pieces, and then subjected to in-gel reduction, alkylation, and tryptic digestion as previously described (Shevchenko et al., 2006). Samples were resuspended in 5% (w/v) formic acid prior to analysis by tandem mass spectrometry.

Identification and quantification of proteins by NanoFlow-LC-ESI Q-ToF tandem MS

Automated LC-ESI MS/MS was performed using a hybrid Q-ToF Mass Spectrometer (Bruker microTOFQ, Bremen, Germany) and a nano-HPLC system (Agilent1200; Santa Clara CA, United States) mounted with a single column setup. A reversed phase nanocolumn (15 cm, 75 µm id) was packed in-house with ReproSil-Pur C18-AQ 3.5 µm resin (Dr. Maisch GmbH, Ammer-Buch-Entringen, Germany) using a high-pressure vessel. The sample was loaded with a flow rate of 350 nL min^{-1} and subsequently eluted at 200 nL min^{-1} using a 35 min gradient of 5-40 % (v/v) acetonitrile in 0.6 % (v/v) acetic acid and 0.005 % (v/v) heptafluorobutyric acid. The mass spectrometer was operated in data dependent acquisition mode to automatically switch between MS and MS/MS acquisition selecting the three most abundant precursor ions. The mass spectrometer was calibrated externally using Tunemix (Agilent G1969-85010) prior to sample acquisition resulting in a sub 5 ppm mass accuracy (R 13.000).

Analysis of proteome data using bioinformatics tools

The MS/MS data was deconvoluted, deisotoped, and exported in a Mascot generic format (mgf-files) prior to database searching. The data were analysed using the MS/MS ion search by the Mascot 2.3 search engine (Matrix Science, UK) against in-house protein database (4,408 sequences; 1,430,678 residues) based on full genome sequencing (isolate I11 whole genome, see below). Protein modifications were fixed for carbamidomethylation of Cys and variable oxidation of Met. The trypsin-derived peptide and peptide fragment mass accuracy were set to 0.05 Da. Results from the automatic database search were evaluated in accordance with the MCP Guidelines (MIAPE) (Taylor et al., 2008).

Identified proteins were further quantified by Matrixscience Distiller toolbox with the following settings for Matrixscience Distiller 2.3 (Ratio name B/U; Fraction 0.7; Correlation 0.9; Peptide_SD 0.1; Protein score 50; Peptide score 15; Max ppm 50; Significance level 0.05; Boundary factor 4; Significance level confidence interval 0.95). Following statistical analysis proteins were Gene Ontology (GO) categorized using STRAP (Bhatia et al., 2009).

RNA isolation and real-time PCR analysis

I11 strain was grown under similar conditions to those described earlier for the proteomic labelling. Bacterial cells were harvested at two time points, before spiking the culture with 65 µg L^{-1} of ibuprofen (at OD_{600}~0.6) and 1h after. 5 mL culture from each time point were collected and cells were harvested by centrifugation at 6,000 xg for 10 min and immediately frozen by dipping in liquid nitrogen and stored at -80°C. Total RNA was extracted from frozen pellets using the Ribopure Bacterial kit (Ambion) according to the manufacturer's instructions. The RNA was then treated with DNase I (Fermentas) to remove trace amounts of genomic DNA. The RNA quality was checked by running 1 % agarose gel and the concentration was quantified using NanoDrop spectrophotometer (NanoDrop Technologies, USA). The incubations were done in duplicate.

Complementary DNA (cDNA) was synthesized by reverse transcription of equal amounts of total RNA (0.5 µg) from each time point (in total 4) using the AffinityScript qPCR cDNA Synthesis kit (Stratagene) following the kit protocol. Triplicate 25 µL SYBR-based real-time PCR reactions were run containing: 12.9 µL Brilliant III SYBR Green REAL-TIME PCR Master mix (Stratagene), 0.8 µg $µL^{-1}$ BSA (New England Biolabs), 900 nM of both forward and reverse primer, 24 nM

ROX reference dye (Stratagene), and 5 µL of undiluted cDNA for target genes, or 5 µL of 1:100 dilution for the reference gene. Reactions were run on Mx3005P (Stratagene) 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 20 s at 60 °C, 20 s at 72 °C, and finally melting profile analysis. Fluorescence was measured after each annealing step. The specificity of the PCR reactions performed for each run was confirmed by the melting curve analysis and gel electrophoresis. For data analysis, the fold change in expression was calculated for each target gene between the two time points using the CT method (Liu and Saint, 2002) as described in detail in (Schmittgen and Livak, 2008). For the normalization of the results, the expression of the 16S rRNA gene was used as an endogenous reference (Talaat et al., 2002; Zhang et al., 2000). The gene-specific primers used in this study were designed using the GeneFisher (Bielefeld University, Bielefeld, Germany) and synthesized by Invitrogen Life Technologies and are listed in supplementary Table 7.1.

Ibuprofen degradation

Samples were taken during the metabolic labelling, centrifuged at 6,000 xg for 5 min and the supernatant filtered through a 0.2 µm SPARTAN (Whatman, Dassel, Germany) filter prior to ibuprofen analysis.

The ibuprofen quantification was determined by an ultra-high-speed liquid chromatography system (ACQUITY UPLC System, Waters Chromatography, Milford, MA, USA) equipped with a photodiode array detector and Empower2 data acquisition software (Waters Chromatography). Samples were separated using a reversed phase C18 column (Hibar Purospher STAR RP-18, 3 µM, Merck). Ibuprofen was eluted in isocratic mode, using 50% (v/v) acetonitrile and 50% (v/v) of Milli-Q water acidified with 10 mM phosphoric acid, as

eluents, for 3.5 min, at 35 ºC with a constant flow rate of 0.30 mL min^{-1}. Ibuprofen was detected at 220 nm and samples were injected twice in a volume of 20 µL. The retention time of the compound (2.9 min) was compared with a standard for identification, and the peak area was used for quantification.

All reagents, acetonitrile (Fisher Scientific, Fair Lawn, USA), phosphoric acid 85% (Sigma Aldrich, Germany) and methanol (Fisher Scientific, Fair Lawn, USA), were of HPLC gradient grade, with a purity of ≥ 99%, according to the supplier. Prior to use, solutions were filtered through Whatman filters (Dassel, Germany) – SPARTAN™ (0.2 µm)). The water used in the study was produced by the Milli-Q water system (Millipore, CA, USA). Ibuprofen was obtained from Sigma-Aldrich (Germany).

Bacterial whole-genome sequencing

Library preparation for Illumina sequencing

DNA was purified from the bacterial isolate I11 cultivated on LB-medium using the UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). A library for paired-end sequencing (PE) was prepared from 5 µg of PicoGreen (Invitrogen, UK) quantified DNA using Paired-End Sample Prep Kit (Illumina, CA, USA), according to manufacturer´s instructions. Briefly, DNA was fragmented by nebulization to generate fragments < 800 bp. The genomic fragments were blunt ended and phosphorylated and a single 'A' nucleotide was added to the 3' ends of the fragments. Adapters were ligated to the A-tailed fragments according to the manufacturer's instructions. Size fractionation and purification of ligation products were performed using a 1 % (w/v) agarose gel. Gel slices

containing DNA in the 500 bp range were cut, purified and the library enrichment was achieved by using 12 PCR cycles with primers PE1.0 and PE2.0 supplied by the kit manufacturers. Finally, the PE library was purified using QIAquick PCR Purification Kit (Qiagen, Germany) and validated by running 10 % of the product on an agarose gel. The stock was kept at -20 ºC until used.

Sequencing and *de novo* genome assembly

The flow cell was prepared according to the manufacturer's instructions using a Paired-End Cluster Generation Kit (Illumina, CA, USA). PE sequencing was performed on an Illumina GAII (Illumina, CA, USA). Five picomolar of the final library was used to achieve approximately 200,000 clusters per tile producing paired-end reads of a 2 x 72 bp in length. Base-calling was performed with the GAPipeline 1.5 using default parameters, which include purity filtering (chastity 0.6). All PE reads were trimmed for length and quality using the CLC Genomics Workbench v.4.5.1 (CLCBio, Denmark) with a quality score limit of 0.01. All reads resulting in less than 35 bp in length were discarded. The de novo assembly of the PE reads was performed using both the CLC Genomics Workbench and ABySS v. 1.2.7 (Simpson et al., 2009) (k = 47 and n = 10). Default parameters were used for assembly in CLC except that the size range (minimum and maximum) between the PE reads was adjusted to 100-600 bp. The resulting contigs from CLC and ABySS were merged into one set of contigs using Minimus from the AMOS software package (Sommer et al., 2007). Finally, the annotation of the genome was determined by uploading the contigs into RAST (Rapid Annotations using Subsystem Technology) server (Aziz et al., 2008) and structural rRNAs and tRNAs were determined using RNAmmer and tRNAscan-SE (Lagesen et al., 2007).

Results

In this study a bacterial strain (I11) isolated from activated sludge capable of degrading ibuprofen was subjected to quantitative proteomics, characterizing the relative changes in the proteome associated with growing this strain in the presence/absence of ibuprofen.

Strain I11 did not grow on ibuprofen as a sole source of carbon and energy (data not shown). However, simultaneous growth and degradation of ibuprofen was observed on M9 medium supplemented with yeast extract and tryptone at different initial ibuprofen concentrations and in OD2-medium (Figure 4.3).

The effect of the initial concentration of ibuprofen on the degradation by I11 revealed that after 300 h the concentration of the parent compound was reduced 28 % and 50 % when grown in M9 media with 1000 and 250 µg L^{-1}, respectively. A reduction of 62 % and 92 % after 90 h was observed on M9 medium and OD2-medium, respectively, for the initial ibuprofen concentration of 50 µg L^{-1}.

Metabolic labelling of this isolate with ^{15}N, using a forward/reverse-labelling strategy, was applied yielding biological duplicates (see Figure 7.2 in the supplemental material). Ibuprofen degradation always followed a linear degradation throughout the first 30 h. To avoid *Patulibacter* sp. I11 entering stationary growth phase, all incubations were carried out until the mid-logarithmic growth phase (OD_{600} ~0.6).

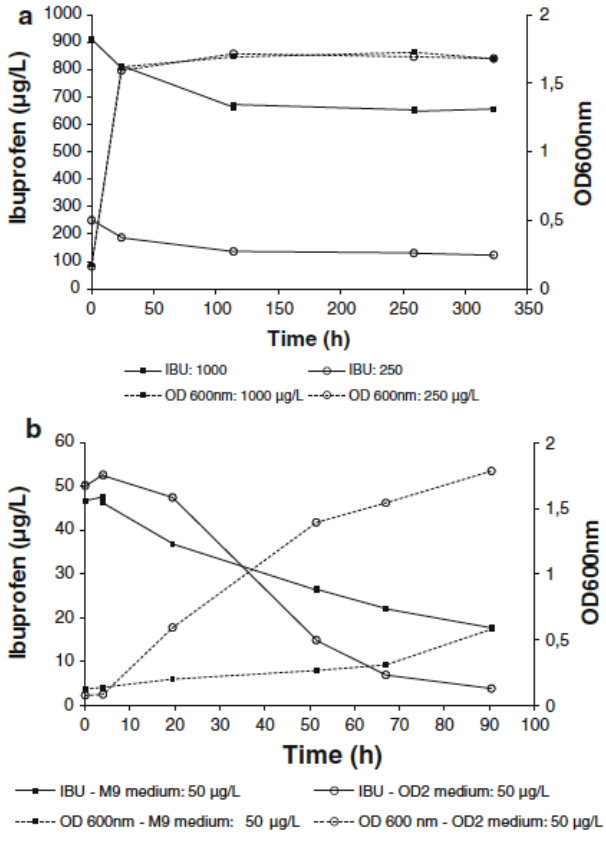

Figure 4.3. Degradation of ibuprofen and growth performance of *Patulibacter* sp. I11 in: a M9 medium supplemented with yeast extract and tryptone and 1,000 or 250 µg L^{-1} of ibuprofen; b M9 medium supplemented with yeast extract and tryptone and 50 µg L^{-1} of ibuprofen, and OD2-medium supplemented with 50 µg L^{-1} ibuprofen.

Whole genome sequencing

To increase the efficiency of the MS-based protein identification, which is known to decrease as amino acid divergence between measured peptides and database entries increases (VerBerkmoes et al., 2009), whole-genome sequencing of I11 was carried out. The assembly resulted in a total of 358 contigs with total 173,723,517 reads; 2,000 fold coverage; mean read length 64

bp and an N50 contig length of 24.7 kb. The uncompleted draft genome included 5,093,141 bases (5.1 Mb) and comprised 4,408 predicted coding sequences (CDSs), with a G+C content of 74 %. Among the 4,408 predicted protein coding sequences, 1,387 (31.4 %) were of unknown function. The retrieved assembled genome was annotated by RAST (Aziz et al., 2008) using sequence-based comparison against genome database of *Conexibacter woesie* (CP001854), closest phylogenetic sequenced genome available with the highest protein similarity with I11. A comparative alignment with 16S rRNA gene sequences showed that this strain had a 96 % similarity with *Patulibacter minatonensis* (AB193261). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AGUD00000000. The version described in this paper is the first version, AGUD01000000.

Quantitative proteome analysis of ibuprofen degrading bacteria

The ^{14}N- and ^{15}N-labelled cells were harvested, lysed and fractionated into a cytosolic and a membrane fraction and mixed in accordance with the forward/reverse-labelling strategy and separated by SDS-PAGE. No major differences were evident by direct visualization when comparing the isolate grown under the two different sets of conditions (Figure 4.4).

For each of the two biological replicates (forward and reverse labelling experiment) the membrane and the cytosolic fraction were nested following statistical analysis. In total, 25 % of all proteins proposed by the genome could be identified. Specifically, 251 unique proteins, identified and quantified, were found to be statistically significant ($p<0.05$). Of these, 251 proteins, 99 proteins were shared between the forward and the reverse labelling experiments, when both biological replicates were considered (supplementary material Figure 7.3).

73 proteins were exclusively present in the forward labelling experiment whereas 79 proteins were exclusive to the reverse labelling experiment (Figure 7.3). For all of the 251 quantified proteins, full length protein sequences retrieved from functionally annotated genome of *Patulibacter* sp. strain I11 were blasted against the UniRef100 database, yielding the closest protein homologue. Proteins (closest homologues) were categorized based upon Gene Ontology Categories (GOC) revealing similar distributions between the forward and reverse labelling experiments (Figure 7.3).

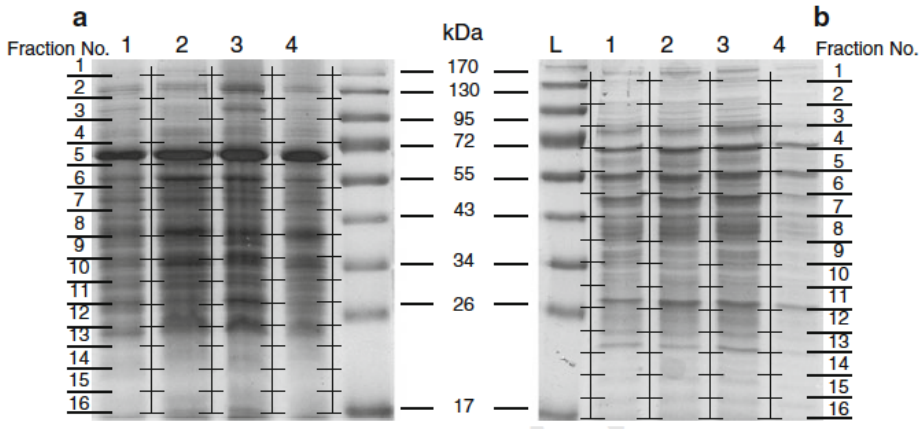

Figure 4.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Patulibacter* sp. I11 cultured in OD2-medium supplemented with ibuprofen.

a Membrane and **b** cytosolic associated fractions, from culture grown in: **1** 15N-OD2 with ibuprofen; **2** 14N-OD2 without ibuprofen; **3** 15NOD2 without ibuprofen and **4** 14N-OD2 with ibuprofen.

Up-regulated proteins in the presence of ibuprofen

Comparative analysis revealed that in total, 72 proteins (nesting forward and reversed labelled replicates) were differentially up-regulated, i.e. defined as having a log2 ratio above 0.9, in response to ibuprofen feeding. Of these 72 proteins, 64 were unique. Out of the 64 unique up-regulated proteins, 12 were observed in both the forward and the reverse labelling experiment (supplementary material Figure 7.4 A). 25 proteins were exclusive to the forward labelling experiment whereas 15 were exclusive to the reverse labelling experiment.

Proteins were functionally characterised using 3 levels of Gene Ontology: biological process, molecular function and cellular component. "Cellular processes" was found to be the predominant biological process, with 75 % of the GO annotated proteins falling within this category. 44 % of these proteins were annotated as being associated with the ribosome. As for molecular function annotation, "Binding" and "Catalytic activity" accounted for 40 % and 38.3 % of the GO annotated proteins, respectively.

Proteins which had a log2 ratio above 1.5 were dominated by putative uncharacterized proteins. In an attempt to elucidate potential biological relevance of these proteins, function prediction was attempted by blasting the sequences of the hypothetical proteins of *Patulibacter* sp. strain I11 against the UniRef100 database, using UniProt BLAST. However, no function could be inferred based upon protein homology, with this approach. Function prediction was also attempted with ProtFun 2.2 (http://www.cbs.dtu.dk/services/ProtFun/), a tool that performs *ab initio* prediction based upon sequence. Of the total uncharacterized up-regulated

proteins, D3FA23 was predicted as having a possible lyase function and D3F6B1 as a possible stress response protein (supplementary material Table 7.2). The tool used for the prediction was developed for eukaryotic cells and was expected to have a lower accuracy of prediction for prokaryotes. However, it has been demonstrated that the tool performs better on prokaryotes than expected, and is able to yield meaningful results (Jensen et al., 2002).

Within the group of proteins categorized as being involved in metabolic processes, several proteins related to the degradation of aromatic hydrocarbons were identified. A number of proteins were highly up-regulated in response to ibuprofen feeding (log2 ratio > 1.5): Aminotransferase class III (E6SEB9), Acyl-CoA synthetase (AMP-forming) (J2XUN5), protein containing a Rieske (2Fe-2S) iron-sulphur domain (D3F3V3), Enoyl-CoA hydratase/isomerase (D3F114), nuclear export factor GLE1 (D3F2P4) as well as several uncharacterised proteins (Table 4.1). Proteins involved in catalytic processes represented an abundant group of up-regulated proteins with a possible correlation with ibuprofen degradation and were composed of cytochrome b and c, several dehydrogenases and oxidoreductases (Table 4.1). Proteins involved in fatty-acid metabolism and energy metabolism were found to be up-regulated as well, and are possibly related to energy gain response. Growing the isolate I11 in the presence of 65 µg L^{-1} of ibuprofen does not appear to have triggered a cellular stress response; only two proteins related to stress stimulus, the 60kDa chaperonin (D3F2T2) and the predicted stress response protein (D3F6B1), were found up-regulated.

Table 4.1. Differentially expressed proteins (Log2 ≥0.9) of the forward and the reversed (numbers in brackets) labeled biological replicates of *Patulibacter* sp. I11 grown in presence/absence of ibuprofen.

GI number[A]	Accession[B]	% Identity[C]	Description[D]	Log2 ratio[E]	Protein Score[F]	#[G]	Fraction[H]
				Replicate: Forward (Reverse)	Replicate: Forward (Reverse)	Replicate: Forward (Reverse)	Replicate: Forward (Reverse)
367468470	D3F114	59	Enoyl-CoA hydratase/isomerase	3,7	145	2	C
367471080	Q47N65	34	Putative uncharacterized protein	2,7 (1,8)	235 (219)	4 (4)	M (C)
367471428	D3F178	34	Putative uncharacterized protein	2,4	482	24	M
367470191	E6SEB9	57	Aminotransferase class-III	2,4	96	8	C
367468134	J2XUN5	51	Acyl-CoA synthetase (AMP-forming)	2,3	314	6	M
367468777	D3F6B1	36	Putative uncharacterized protein	2,3 (1,9)	311 (292)	7 (14)	M (C)
367467720	D3FBK3	38	Collagen triple helix repeat protein	2,1 (1,3)	412 (316)	26 (21)	M (M)
367471354	D3FA23	36	Putative uncharacterized protein	2,1 (2,0)	174 (382)	6 (10)	M (M)
367467801	D3F2P4	34	Nuclear export factor GLE1	1,9 (3,3)	335 (388)	17 (25)	M (M)
367468099	D3FFG0	33	Putative uncharacterized protein	1,8 (1,7)	2195 (1785)	45 (97)	M (M)
367470501	D3F3V3	61	Rieske (2Fe-2S) iron-sulphur domain protein	1,4 (0,9)	313 (366)	13 (13)	M (M)
367469421	F1YMM4	33	Glycosidase	1,4	749	16	M
367469148	D3F293	33	Putative uncharacterized protein	1,4 (1,7)	747 (723)	40 (46)	M (M)
367470912	D3FEF4	33	PpiC-type peptidyl-prolyl cis-trans isomerase	1,3	958	64	M
367471028	D3EZ72	27	Putative uncharacterized protein	(1,3)	(881)	(46)	(M)
367470919	D3FEE9	65	Putative uncharacterized protein	(1,3)	(252)	(8)	(C)
367470503	D3F3V1	58	Cytochrome b subunit of the bc complex-like protein	1,2 (1,0)	191 (154)	9 (3)	M (C)
367470155	D3FEU2	74	Elongation factor G	1,2	1109	24	M
367467802	B0CFJ3	29	Putative uncharacterized protein	1,1 (1,6)	113 (110)	4 (6)	M (M)
367469262	D3FFG0	34	Putative uncharacterized protein	1,1 (0,9)	1702 (1687)	58 (97)	M (M)
367469831	D3FBI3	67	Daunorubicin resistance ABC transporter ATPase subunit	1,1 (0,9)	509 (473)	8 (10)	M (C)
367469604	Q8XZ05	57	Probable dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (E2) protein	1,1	104	2	C
367469495	D3F0K9	70	Carbamoyl-phosphate synthase L chain ATP-binding protein	1,1	453	8	M
367467866	D3EZD5	73	Alanine dehydrogenase	1,1	321	11	M

GI number[A]	Accession[B]	% Identity[C]	Description[D]	Log2 ratio[E]	Protein Score[F]	#[G]	Fraction[H]
				Replicate: Forward (Reverse)	Replicate: Forward (Reverse)	Replicate: Forward (Reverse)	Replicate: Forward (Reverse)
367467388	D3FDE9	67	Oxidoreductase alpha (Molybdopterin) subunit	(1,1)	(113)	(7)	(M)
367469830	D3FBI2	59	ABC-2 type transporter	1,0	161	4	M
367471405	D3EZ73	32	Putative uncharacterized protein	1,0 (1,2)	160 (253)	6 (7)	M (M)
367469164	D3FBH9	65	Extracellular solute-binding protein family 1	1,0	369	8	M
367470502	D3F8X9	43	Putative uncharacterized protein	1,0	130	4	M
367469765	D3F3V2	65	Cytochrome b/b6 domain protein	1,0	128	4	M
367467620	D3F8Q5	73	Aminotransferase class-III	1,0	130	3	M
367469524	D3FAW8	68	6-phosphofructokinase	(1,0)	(417)	(8)	(C)
367467800	D3F2P4	33	Nuclear export factor GLE1	(1,0)	(292)	(13)	(M)
367467449	C9W357	50	Amino acid/metabolite permease	(1,0)	(181)	(9)	(M)
367470156	D3FEU3	87	Elongation factor Tu	1,0	821	104	M
367471419	D3F251	35	Putative uncharacterized protein	0,9	607	12	M
367467099	D4KJY6	31	Putative uncharacterized protein	0,9	222	5	M
367471018	D3F5M2	67	Enoyl-[acyl-carrier-protein] reductase [NADH]	0,9	463	16	M
367467622	Q1AWV4	42	Tfp pilus assembly protein PilE	0,9	260	14	M
367469219	E4M2D6	45	Phosphate ABC transporter substrate-binding protein, PhoT family	0,9	113	4	M
367467446	Q1AR79	68	Betaine-aldehyde dehydrogenase	0,9	275	6	C
367469269	D3F5Z5	75	Acyl-CoA dehydrogenase domain protein	(0,9)	(189)	(5)	(M)
367469961	D8TVG4	27	Putative uncharacterized protein	(0,9)	(594)	(34)	(M)
367469169	Q3K4T7	43	Transcriptional regulatory protein AlgP	(0,9)	(204)	(6)	(M)

Duplicate proteins have been eliminated based upon number of quantitated peptides used for protein quantitation (#) and protein score. Ribosomal proteins were excluded from the list.

a NCBI GenBank accession number

b UniProt accession number of the closets protein homologue, obtained by blasting the translated sequence retrieved from GenBank using UniProt blast

c % identity of the closest protein orthologue

d Description of the closest protein orthologue

e Log2 ratio obtained from the quantitative proteomics analysis

f Protein Score obtained from the quantitative proteomics analysis

g The number of quantitated peptides upon which the quantitative value (log2 ratio) was determined

h Fraction in which the protein was observed. M: Membrane and C: Cytosolic

Several proteins related to the up-take and degradation of other aromatic acids (i.e. benzoate and phenylacetic acid) were found up-regulated in the presence of ibuprofen: enoyl-CoA hydratase/isomerase (D3F114), Acyl-CoA synthetase (AMP-forming) (J2XUN5) and Rieske (2Fe-2S) iron-sulphur domain protein (D3F3V3). Similarly proteins related to transport and binding of extracellular compounds were up-regulated in the presence of ibuprofen and might be related to the uptake of this compound: ABC-2 type transporter (D3FBI2), daunorubicin resistance ABC transporter ATPase (D3FBI3) and an extracellular solute-binding protein type 1 family (D3FBH9).

Down-regulated proteins in the presence of ibuprofen

Proteome analysis revealed the down-regulation of a total of 103 proteins of which 96 proteins were unique; 35 and 27 were exclusive to the forward and reverse labelling experiment, respectively; 17 were common to both (Figure 7.4 B).

The majority of these proteins are related to binding of extracellular compounds (41 %) and catalytic activities inside the cell (41 %). The most prominent down-regulated proteins, with a log2 ratio between -4 to -1.5 were proteins involved in transport, mainly as binding proteins – Htaa domain protein (D3FBZ2), several periplasmic binding proteins (B5H4G4, D3M8C8, D3F9P9) and PKD domain protein (D3F790) - catalytic activity like NADH-quinone oxidoreductase subunit I (D3FDL4), NADH dehydrogenase I-D subunit (D3FDK9), acyl-CoA dehydrogenase domain protein (D3F5Z5), and several putative uncharacterized proteins (Table 4.1). Other down-regulated proteins were involved in: TCA cycle (citrate synthase (D3FCP7), isocitrate dehydrogenase, NADP-dependent (A1SMP0), malate dehydrogenase (C6WLQ8)

and succinyl-CoA ligase [ADP-forming] subunit alpha (D3F0L5), translation/post translational modifications (30S and 50S ribosomal proteins), transport systems (ABC transporter related protein (D3FCG2), predicted cobalt transporters CbtA (F2RKH7), and some proteins related to stress response like peroxidase (A6G286) and chaperone protein DnaK2 (D3FAG2).

Genomic analysis

The genome sequence of *Patulibacter* sp. strain I11 was analysed for the presence of putative orthologous genes known to be involved in aromatic acid degradation. The focus was on those previously hypothesized to be involved in the biodegradation of ibuprofen (Kagle et al., 2009) and in two other structurally similar aromatic hydrocarbons, phenylacetic acid (Ferrandez et al., 1998) and benzoate (Craven et al., 2009).

Sphingomonas strain Ibu-2 was previously isolated from activated sludge and characterized for its ability to use ibuprofen as a sole carbon source (Kagle et al., 2009). In that study, genetic and biochemical analysis revealed a cluster of genes (*ipfABDEF*) that were proposed to be involved in ibuprofen catabolism. A search in the strain I11 genome revealed five orthologous genes whose products share 33-40 % amino acid sequence similarity with those found in *Sphingomonas* Ibu-2 (Supplementary material Table 7.3). Only one of these genes, *ipfF,* which sharing 33 % amino acid similarity, was found to be up-regulated in this study encoding for Acyl-CoA synthetase (AMP-forming) (J2XUN5). Interestingly, a protein containing a Rieske (2Fe-2S) iron-sulphur domain (D3F3V3) was found up-regulated in ibuprofen-grown cells sharing similar annotated function (dioxygenase) to the protein encoded by *ipfA*.

In the same line*, Acinetobacter* strain ADP1 was shown to degrade benzoate as the sole carbon source (Craven et al., 2009). Two clusters of genes, *ben* genes (*benABCDEKPM*) and *cat* genes (*catABCDFIJM*) have been described to be involved in the metabolism of aromatic hydrocarbons including benzoate (Craven et al., 2009). Protein blast of the two clusters of genes against the predicted proteome of *Patulibacter* sp. I11 yielded a 24-43 % identity (Table 7.3).

Finally, 13 catabolic enzymes (*paaABCEFGHIJKLMN*) were reported in the aerobic degradation of phenylacetic acid in *E.coli* strain - K12 (Ferrandez et al., 1998). Fifteen of the genes encoding these enzymes were recognized in the *Patulibacter* sp. I11 genome with 25-54 % amino acid sequence identity (Table 7.3).

Transcription analysis

Incubation of I11 in OD2-medium under the same conditions used for the proteomic approach was conducted to analyse an early response in ibuprofen induction genes. qPCR was employed in order to confirm the proteomic data and to investigate to which extent the ibuprofen up-regulated proteins found in this study correlated with its transcript levels. Transcript levels of the six genes encoding proteins whose levels appeared to increase in the presence of ibuprofen in the differential proteomic approach and the *ipf* genes previously proposed to be involved in ibuprofen degradation (Kagle et al*.,* 2009) were measured (Table 4.2 and 4.3). Based on the change in transcript levels prior to addition of ibuprofen and 1h after ibuprofen addition to the medium positive correlations were observed for 5 of the 6 chosen target genes (Table 4.2).

Table 4.2. Transcript levels of the six genes encoding proteins up-regulated in the presence of ibuprofen in the differential proteomic approach.

GI number[A]	Accession[B]	% Identity[C]	Description[D]	Log2 ratio[E]	Fold change[F]
				Replicate Forward (Reverse)	
367468470	D3F114	59	Enoyl-CoA hydratase/isomerase	3,7	1.21 ± 0.10
367468134	J2XUN5	51	Acyl-CoA synthetase (AMP-forming)	2,3	1.60 ± 0.14
367470501	D3F3V3	61	Rieske (2Fe-2S) iron-sulphur domain protein	1,4 (0,9)	-1.49 ± 0.09
367469831	D3FBI3	67	Daunorubicin resistance ABC transporter ATPase subunit	1,1 (0,9)	1.24 ± 0.14
367469830	D3FBI2	59	ABC-2 type transporter	1,0	1.22 ± 0.10
367469164	D3FBH9	65	Extracellular solute-binding protein family 1	1,0	1.43 ± 0.15

a NCBI GenBank accession number
b UniProt accession number of the closets protein homologue, obtained by blasting the translated sequence retrieved from GenBank using UniProt blast
c % identity of the closets protein homologue
d Description of the closets protein homologue
e Log2 ratio obtained from the quantitative proteomics analysis
f Fold change in target gene relative to untreated as measured by qPCR and analysed using the C_T method

These positive correlations were corrected for sampling and analysis biases by adjusting to the levels of I11-16S rRNA gene and the increase in fold was found to range between 1.21 ± 0.10 and 1.60 ± 0.14 (n=3). Negative correlation was observed only for D3F3VE (-1.49 ± 0.09; n=3). Elevated transcript levels were detected for the 4 of the 5 tested *ipf* genes with a fold increase ranging from 1.53 ± 0.12 and 2.9 ± 0.16 (n=3) (Table 4.3). Interestingly, only IpfF that shared 51 % amino acid similarity with J2XUN5 (up-regulated at the proteome level) was not up-regulated at the transcript level. None of the other *ipf* gene products were detected at the proteome level in this study.

Table 4.3. Transcript levels of *Patulibacter* sp. I11 genes, similar *Sphingomonas* Ibu-2 *ipf* genes (*ipfABDEF*) previously proposed to be involved in ibuprofen degradation (Kagle et al., 2009)

Name (orf no.)[A]	Organism	Function	E-value	Identity (%)	Accession number[B]	Fold change
PAI11_21070	*Sphingomonas* Ibu-2	Putative dioxygenase hydroxylase component	5E-91	40	A1E031	1.69 ± 0.22
PAI11_21080	*Sphingomonas* Ibu-2	Putative dioxygenase component	4E-34	46	A1E030	1.53 ± 0.12
PAI11_02050	*Sphingomonas* Ibu-2	Thiolase	3E-21	36	A1E029	2.05 ± 0.16
PAI11_25140	*Sphingomonas* Ibu-2	Hydroxymethylglutaryl-CoA synthase	0.02	33	A1E028	2.09 ± 0.16
PAI11_13130	*Sphingomonas* Ibu-2	AMP-dependent synthetase and ligase	10E-61	33	A1E027	-1.00 ± 0.10

a NCBI accession number of *Patulibacter* sp. strain I11 genes
b UniProt accession number of *Sphingomonas* Ibu-2 genes
c Fold change in target gene relative to untreated as measured by qPCR and analysed using the C_T method

Discussion

This work describes the first characterization of the changes in the proteome of a bacterial strain isolated from activated sludge in response to ibuprofen degradation, using a combination of genomic and proteomic techniques. A pure culture of *Patulibacter* sp. strain I11 was obtained from activated sludge and showed capable of degrading ibuprofen in M9 medium supplemented with another carbon source and in rich media such as OD2-medium under different initial concentrations of ibuprofen with up to 92 % removal within 90 h. Using isotopic labelling relative quantification of protein expression levels in the presence/absence of ibuprofen was carried out.

Metabolic labelling incubations were carried out at the mid-logarithmic growth phase with similar ibuprofen reduction in all tested cultures. Comparison of the proteomes obtained from the exponential growth stage ensured that events related to oxidative stress, cell lysis and death were kept at a minimum. In the present study, only a few stress-related proteins were identified.

The genome of the isolate I11 was sequenced and used as a reference sequence for identifying proteins from the MS data. The closest related genome available to this strain was from *C. woesie* (CP001854), which was used in the genome annotation. Sequence analysis of the 16S rRNA gene of this isolate revealed a 96 % similarity with *P. minatonensis* (AB193261). *C. woesie* and *Patulibacter minatonensis* are both gram-positive soil-isolated members of the class *Actinobacteria*. Other ibuprofen degrading microorganisms found in literature are the proteobacterial *Sphingomonas* Ibu-2 (Murdoch and Hay 2005) and the actinobacterial *Nocardia* sp. (Chen and Rosazza, 1994) although the latter does not grow on ibuprofen. Interestingly, this was the first time that a member of the genus *Patulibacter* was isolated from activated sludge and reported as being able to remove ibuprofen.

A sub-fractionation of the soluble (cytosolic proteins) and non-soluble (membrane proteins) protein fractions was carried out prior to 1D-gel based shotgun-proteomics in order to increase proteome coverage and resolution. In total 251 unique proteins were identified and quantified, corresponding to an approximately 5 % coverage of the proteome of the *Patulibacter* sp. strain I11. A bias toward high-abundance proteins exists, affecting protein identification and protein quantitation, and resulting in limited proteome coverage. A large number of proteins within the group of highly up-regulated proteins were classified as putative uncharacterized proteins. Function prediction of these

uncharacterized proteins resulted in the identification of two of them: a putative lyase and a stress response protein. Several lyases are known to participate in the degradation of aromatic compounds (Nadeau et al., 2003). Proteins related to metabolism of ibuprofen may not be among the most abundant proteins and might therefore lead to difficulties in identifying some of the proteins involved in ibuprofen degradation pathway. Nevertheless, among the quantified and identified proteins, several are likely to be involved in ibuprofen degradation. Some of these proteins were identified as having a possible role in the actual degradation whereas others are likely related to the ibuprofen uptake mechanism. Most notably, proteins that are related to the degradation of aromatic hydrocarbons (e.g. phenylacetic acid and benzoate) were up-regulated in the presence of ibuprofen. General consensus was observed between the up-regulated proteins found in the proteomic analysis and the transcript levels of the corresponding genes as estimated by qPCR. Among these proteins is an AMP-forming synthetase that is known to be essential for the aerobic catabolism of aromatic hydrocarbons in other microorganisms, such as *Pseudomonas putida* U (Luengo et al., 2007; Martinez-Blanco et al., 1990). This type of enzyme is commonly involved in the first step of CoA-dependent degradation pathway, catalysing the formation of an acyl-CoA ester with the release of AMP (Pérez-Pantoja et al., 2009). In the present study, Acyl-CoA synthetase (AMP-forming) (J2XUN5) was up-regulated in the presence of ibuprofen with a log2 ratio of 2.35, as suggested earlier in the ibuprofen degradation pathway by *Sphingomonas* Ibu-2 (Kagle et al., 2009), which strongly indicates that it has a similar role in *Patulibacter* sp. strain I11. However, qPCR results indicated that the orthologous gene *ipfF* in I11 is not up-regulated at the transcription level. Thus it is not possible to conclude if the Acyl-CoA synthetase (AMP-forming) (J2XUN5) found up-regulated in this study is involved in ibuprofen degradation and further studies are needed to address

this question. Similarly, one protein containing a Rieske (2Fe-2S) iron-sulphur domain was up-regulated in the presence of ibuprofen, having a log2 ratio of 1.4. Several Rieske-domain containing proteins are well known for participating in the initial oxidation of aromatic ring structure by the addition of dioxygen. Bacterial hydrocarbon dioxygenases, are an example of proteins that belong to a large family of Rieske non-heme iron oxygenases (Gibson and Parales 2000; Pérez-Pantoja et al., 2009). However, the qPCR results did not confirm an increase of this gene in response to ibuprofen degradation. Lastly, enoyl-CoA hydratase/isomerase was highly up-regulated; this enzyme is well known for catalysing the hydroxylation of double bonds after the opening of the aromatic ring (Pérez-Pantoja et al., 2009). In the case of phenylacetic acid catabolism by *E.coli* and *Pseudomonas putida* U, an ortholog to this enzyme was shown to be involved in the last step of the catabolic pathway of this aromatic compound (Pérez-Pantoja et al., 2009; Luengo et al., 2007).

The overall up-regulation of the described catalytic proteins might suggest a similar transformation of ibuprofen and ibuprofen metabolites by *Patulibacter* sp. strain I11. Besides the actual degradation, several proteins potentially associated with the uptake of the compound were up-regulated in response to ibuprofen. An ABC transporter related protein, which is likely to be involved in the uptake of ibuprofen, was observed to be up-regulated as a response to ibuprofen presence. The uptake of ibuprofen is the initial step necessary for its degradation, and previous reports have shown that ABC transporter related proteins, cytochromes and NADH-dehydrogenases were up-regulated in cells grown on aromatic hydrocarbons such as benzoate by *Corynebacterium glutamicum* (Haussmann et al., 2009) and *Pseudomonas putida* strain KT2440 (Yun et al., 2011), phenol by *Pseudomonas putida* strain KT244 (Roma-

Rodrigues et al., 2010) and 4-chlorophenol by *Pseudomonas putida* (Cao and Loh 2009).

In an attempt to complement the proteomic analysis, the genome of *Patulibacter* sp. strain I11 was studied in relation to the degradation of ibuprofen by *Sphingomonas* Ibu-2 (Kagle et al., 2009) and by other aromatic hydrocarbons.

Benzoate and phenylacetic acid degradation were well described in other studies and were used as model compounds in several studies focusing on aromatic degradation (Ferrandez et al., 1998; Loh and Cao 2008; Luengo et al., 2007; Patrauchan et al., 2005). Some of the proteins described in this work shared similar functions with the proteins involved in the degradation of such compounds. For this reason *Patulibacter* sp. strain I11 genome was also screened for genes involved in the degradation of benzoate and phenylacetic acid, which share structural similarity to ibuprofen. The presence of these genes in I11 genome could potentially reveal the versatility of this organism towards the degradation of other aromatic compounds. Similar genes were detected that account for the degradation of benzoate by *Acinetobacter* strain ADP1 (Craven et al., 2009), phenylacetic acid by *E.coli* K12 (Ferrandez et al., 1998) and ibuprofen by *Sphingomonas* Ibu-2 (Kagle et al., 2009), but having different percentages of similarity. I11 is phylogenetically distinctly related to previously described ibuprofen degraders, and this could account for the low protein similarity to *ipf* genes previously proposed to be involved in ibuprofen degradation. The most similar genes among this set of genes detected in *Patulibacter* sp I11 were the ones described for the degradation of benzoate. A proteome search for the expression of the genes proposed to be responsible for the degradation of ibuprofen in *Sphingomonas* Ibu-2 (Kagle et al., 2009)

revealed that only a similar protein to IpfF, was expressed. This protein (J2XUN5) shares 51 % identity with IpfF from *Sphingomonas* sp. Ibu-2. However, the transcription of this gene was not induced by the presence of ibuprofen by I11. The *ipfABDE* genes were not found up-regulated at proteomic level but were induced by I11 in the presence of ibuprofen. The expression of these genes by I11 and its previous report in ibuprofen degradation by *Sphingomonas* sp. Ibu-2 supports the possible involvement of these enzymes in ibuprofen degradation by *Patulibacter* sp strain I11.

The differences observed between the proteomic data and the qPCR data, with the negative correlation for D3F3VE and the positive results for the expression *ipf* genes, could be explained partly by fundamental biological differences between the transcription and translation processes, and/or by experimental challenges and the relative low coverage in the proteome (Hedge et al., 2003).

The abundance of putative uncharacterized proteins among the up-regulated proteins illustrates the extent of novelty within functionally important individual proteins, and possibly entire cellular processes/pathways involved in ibuprofen degradation. Four orthologous genes proposed to be involved in the degradation of ibuprofen by *Sphigomonas* sp. Ibu-2 was also found to be present and expressed in response to the presence of ibuprofen in *Patulibacter* sp. I11. Verification of role of the identified proteins requires further analysis such as expression studies and protein characterization.

In summary, this work clearly represents novel information regarding the cellular response of an ibuprofen-degrading microorganism to this important NSAID. The *Patulibacter* sp. described here is the first gram positive isolate with a demonstrated ability to degrade ibuprofen. A number of proteins were

identified and found to be up-regulated in the presence of low levels of ibuprofen. These proteins constitute potential candidates that may be involved in enzymatic degradation pathways for the micropollutant ibuprofen. The details of such pathway(s) and how they are regulated *in situ,* for the biodegradation of this important micropollutant, remain largely unknown. This study demonstrates the value of such a genomic and proteomic approach in providing a better understanding of ibuprofen biodegradation in the environment and in wastewater treatment systems.

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Chapter 5

Microbial characterisation of mercury reducing mixed cultures enriched with different carbon sources

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Almeida B. was involved in all the experimental work presented in this chapter, except for the enrichment of mercury-reducing cultures with different carbon sources that was performed by Joana Fradinho and Adrian Oehmen; and the sequencing of the 16S rRNA gene and *merA* gene, that was sequenced by BaseClear, Leiden, The Netherlands.

Abstract

The use of mixed microbial cultures enriched for biological mercury removal is explored in this paper, focusing on the ecological shifts occurring throughout acclimatization to mercury and on the long-term stability of four microbial enrichments. The 16S rRNA genetic profiles obtained by denaturing gradient gel electrophoresis (DGGE) revealed that the glucose and ethanol cultures had similar profiles, whereas the acetate cultures diverged into a totally dissimilar cluster. Quantification of the *merA* gene copies in each enrichment showed higher values for the glucose culture, followed by the ethanol and then the acetate cultures, which was consistent with the mercury removal performance throughout the study. Isolates were obtained from the four cultures and analyzed with respect to their genetic (16S rRNA gene) and functional (*merA* gene) phylogenies in order to identify mercury-resistant species enriched with different carbon sources. All mercury-resistant isolates obtained from the glucose and ethanol cultures belonged to the *Gammaproteobacteria*, whereas acetate cultures also contained members of other phyla, with differences in *merA* sequences. Higher phylogenetic than functional diversity of the isolates, together with increasing *merA* copies even after culture stabilisation, highlight the role of horizontal gene transfer in the acclimatization process.

Introduction

Mercury is the most toxic heavy metal, with no associated biological function (Wagner-Dobler et al., 2003). Mercury pollution arises from sources such as the chlor-alkali industry, coal burning and gold mining, and is one of the most frequently found heavy metals in industrial wastewater (Jaysankar et al., 2007). Once discharged into the environment, mercury tends to accumulate in the aquatic media and soils, where it can contaminate drinking water aquifers. Therefore, the maximum recommended mercury concentration from discharged wastewater varies on a site-to-site basis, although is generally ≤50 μg Hg L^{-1}.

Wastewater treatment and soil remediation of mercury can be mediated biologically, since the mercury resistance (*mer*) operon confers some microorganisms with the capacity to transport Hg^{2+} and organomercurial compounds across the cell membrane and reduce it to insoluble Hg^{0}. The *mer* operon can be present in chromosomal DNA, but is often associated to mobile genetic elements, such as transposons and plasmids (Osborn et al., 1997). This suggests that, together with growth of mercury resistant species by cell division, horizontal gene transfer plays an important role in the adaptation of a microbial community to mercury contamination, resulting in high diversity of mercury-resistant populations (Osborn et al., 1997). The central part of this operon is the *merA* gene, which encodes for the mercuric reductase or MerA enzyme.

The biological mercury reduction mechanism has been applied for the detoxication of mercury-contaminated wastewater (Chang and Law, 1998; Wagner-Dobler et al., 2000, Wagner-Dobler, 2003) and drinking water

(Oehmen et al., 2006). The reduced form of mercury can then be removed by stripping into the gaseous phase and subsequent adsorption (Deckwer et al., 2004). These studies have generally employed pure cultures (von Castein, et al, 1999; Wagner-Dobler et al., 2000), although mixed cultures are advantageous in wastewater treatment, due to the difficulty in maintaining aseptic conditions. Biological mercury reduction requires an external carbon source to the industrial wastewater in order to promote bacterial growth. Previously, our group compared two mixed cultures fed with either glucose or acetate after applying a short-term swap in the carbon source, and concluded that the glucose-fed culture had a higher mercury removal efficiency than the acetate-fed culture (Oehmen et al., 2009); however, this study did not assess the long-term stability of these mixed cultures, nor did it investigate their community structure beyond determination of the most dominant microbial phyla. This knowledge is needed in order to understand the mechanisms of how mercury-removing communities develop and their dynamics in engineered systems.

The aim of the present study was to obtain effective mercury-reducing mixed cultures and correlate their long-term performance with their microbial community dynamics and *merA* gene content. Four microbial cultures were enriched with different carbon sources that are commonly employed for supplementing wastewater treatment processes (including glucose and acetate, but also ethanol) and were characterized through culture-dependent and -independent microbial techniques. The study was carried out throughout > 500 days in order to assess the long-term population dynamics and stability of the mercury removal performance. Complementary molecular tools (denaturing gradient gel electrophoresis [DGGE] and fluorescence *in situ* hybridization [FISH]) were used to monitor the population structure profile and composition throughout acclimatization. Additionally, the abundance of the *merA* gene in

the enrichments was determined through quantitative polymerase chain reaction (qPCR), which could then be correlated with their mercury removal efficiency. Finally, mercury-resistant pure cultures were isolated in order to identify and compare *merA*-containing species obtained with the different carbon sources.

Materials and Methods

Enrichment of mercury-reducing cultures with different carbon sources

Sludge from a mercury contaminated site in the Tagus River (Lisbon, Portugal) was used to inoculate four shaken flasks, which were fed with a minimal medium supplemented with 3.84 g COD L^{-1} of glucose, ethanol or acetate as the sole carbon source (see Oehmen et al., 2009 for the media composition) and spiked with mercury. The enrichments were carried out at 30 °C. Once every 14 d, half of the mixed liquor was replaced by fresh medium, and a new mercury spike was made. Thus, the hydraulic retention time (HRT) and the sludge retention time (SRT) were 28 days. The glucose and acetate-1 cultures were operated initially with 10 mg Hg L^{-1} (added as $HgCl_2$) for 140 days, and then reduced to 1 mg Hg L^{-1}, because the acetate-1 culture was unable to sustain this level of mercury removal. The ethanol and acetate-2 cultures were always supplied with 1 mg Hg L^{-1}. The cultures were operated between 505 and 645 days. Performance monitoring was carried out by atomic absorption measurements of total mercury (including Hg^0, Hg^{2+} and soluble mercury complexes) in the culture supernatant and volatile suspended solids (VSS) were determined according to standard methods (APHA, 2005). For more information about the chemical analysis see Oehmen et al., 2009.

Extraction of DNA from microbial cultures

Biomass samples were collected from each mixed culture throughout the study. DNA was extracted using glass beads and guanidine thiocyanate (adapted from Pitcher et al. 1989), which is summarised as follows: 0.4 g of sludge pellet was homogenized by vortex with 0.5 mL of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 250 mM NaCl, 0.3 % SDS, pH 8.0) and 0.2 mL of glass beads (400-600 microns, Sigma, USA). Cells were lysed by 3 cycles of 5 min incubation on ice followed by 5 min vortexing. This was followed by sequential incubations with RNAse (0.025 mg mL^{-1}, 1h at 37 °C) and 0.5 mL of GES (5M guanidine isothiocyanate, 100 mM EDTA, 0.5 % sarkosyl, pH 8.0, 1h at 65 °C). After centrifugation, the pellet was treated with potassium acetate to a final concentration of 0.5 M. Samples were transferred to ice for 10 min, then centrifuged and the aqueous phase was extracted with chloroform/isoamyl alcohol. DNA was precipitated with cold isopropyl alcohol, pelleted by centrifugation and washed with 75 % ethanol. DNA pellet was resuspended in 0.1 mL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This method was selected for DNA extraction of the mixed cultures since it showed best results with respect to diversity coverage of activated sludge samples as compared to commercial kits (data not shown).

16S rRNA gene amplification by nested-PCR

In the first PCR reaction, a practically full-length 16S rRNA gene was amplified using primers 27f and 1492r_uni (Lane, 1991). The reaction contained 1U of *Taq* DNA polymerase, 0.2 mM of each deoxynucleoside triphosphate, 10x PCR Buffer, 2 mM of $MgCl_2$, 0.5 pmol of each primer and 1µL of template DNA in a total volume of 25 µL. The thermocycler program comprised 1 cycle at 94 °C for

5 min, 20 cycles at 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min, and 1 cycle at 72 °C for 5 min. For the second PCR reaction the primers 968f_GC (Muyzer et al., 1995) and 1492r_bac (Lane, 1991) were used. One microliter of the first PCR reaction was used as template, with 1U of *Taq* DNA polymerase (Invitrogen, USA), 0.2 mM of each deoxynucleoside triphosphate, 10x PCR Buffer, 2.5 mM $MgCl_2$ and 0.5 pmol each primer in a total volume of 25 µL. The thermocycler program comprised 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 2 min, and 1 cycle at 72 °C for 5 min.

DGGE of PCR-amplified 16S rRNA genes

DGGE was performed with the Dcode Universal Mutation Detection System (BioRad, Hercules, CA, USA). Equal amounts of PCR products were loaded onto 8 % (w/v) polyacrylamide gels (40 % acrylamide/bis solution, 37.5:1. Bio-Rad) in 0.5X TAE (20 mM Tris acetate, pH 7.8, 10 mM sodium acetate, 0.5 mM disodium EDTA). Polyacrylamide gels with a denaturant-gradient of 30-60 % (100 % denaturant contains 7 M urea and 40 % formamide) were run at 80 V and 60 °C for 16h. Electrophoresed gels were stained with SYBR Gold (1:10,000 dilution, Invitrogen) for 30 min and the obtained profiles were analyzed using the Bionumerics 5.0 software (Applied Maths, Belgium). Fingerprint patterns of the samples were analyzed using Pearson correlations, which provide pairwise percent similarity values for all fingerprint densitometric curves. Dendrograms relating DGGE profile similarities were automatically calculated by the unweighted pair group method with arithmetic mean (UPGMA) algorithm of the Bionumerics software. The cophenetic correlation of the dendrogram was also determined in Bionumerics and expresses the consistency of the cluster analysis.

The relative change of each microbial population with respect to a common reference, taken in this study as the inoculum, was determined from the matrix of similarities for the densiometric curves of the DGGE profiles, as per Marzorati et al., 2008: % change = 100 - % similarity.

FISH

Sludge samples from each culture were fixed with paraformaldehyde solution at 4 °C according to Amann (1995). The following specific oligonucleotide probes were employed: ALF969 for *Alphaproteobacteria* (Oehmen et al., 2006), BET42a for *Betaproteobacteria* (Manz et al., 1992), GAM42a for *Gammaproteobacteria* (Manz et al., 1992), CF319a for *Cytophaga-Flavobacteria* (Manz et al., 1996), and HGC69a for High GC content Gram-positive bacteria (*Actinobacteria*) (Roller et al., 1994). Each specific probe was applied with a Cy-3 label, together with FITC-labelled EUBMIX probe for all *Bacteria* (EUB338 [Amann et al., 1990] and EUB338-II and III [Daims et al., 1999]). FISH samples were observed using a Leica DRMA2 epifluorescence microscope (Leica, Wetzlar, Germany).

Quantitative PCR analysis of the *merA* gene copy number

The quantification of *merA* gene copies per ng DNA by real-time PCR was performed with an iQ5 thermocycler and real-time detection system (Bio-Rad). The primers used in this study were merA1 and merA5 (Liebert et al., 1997), designed from consensus regions of *mer*A sequences belonging to the *Beta*- and *Gammaproteobacteria*. The 25 µL reaction mixture contained 12.5 µL of iQ SYBR Green supermix (Bio-Rad), 1.5 µL of each primer (0.5 pmol) and 1 µL of template DNA (concentration determined by the spectrophotometer NanoDrop

1000 (Thermo Scientific, Waltham, MA, USA). The thermocycler program was: denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 30 sec, 64 °C for 30 sec and 1 cycle at 72 °C for 3 min. At the end of each PCR run, melting curve analysis was performed from 60 to 95 °C (0.1 ºC s^{-1}) to detect nonspecific PCR product and primer-dimer co-amplification. PCR products were visualized in ethidium bromide-stained 1.2 % agarose gels to verify the size of the amplicons.

DNA standard curves were constructed using the corresponding *merA* PCR products of *Acidithiobacillus ferrooxidans,* DSMZ 14882. Briefly, after *merA* gene amplification, the resulting DNA product was cleaned with UltraClean PCR Clean-Up kit (MoBio Laboratories, Carlsbad, CA, USA) and quantified through spectrophotometric analysis (NanoDrop 1000). The copy number was calculated based on the measured DNA concentration and the molecular weight of the reference *merA* amplicon. Cleaned PCR products were serially diluted in TE (10^9-10^0) and used as template.

Baseline and threshold calculations were performed with the iQ5 software version 2.1, using the fluorescence signals acquired at 94 °C, at which primer dimers completely denatured and did not affect quantification. All real-time PCR quantifications were done in triplicate for both the standards and samples. The results were normalized in respect to the template DNA concentration and given as number of copies ng DNA^{-1}.

Isolation of mercury resistant bacteria and DNA extraction

Aliquots (100 µL) of diluted (0.85 % NaCl) biomass samples were spread on agar plates with 15 mL minimal medium containing glucose, ethanol or acetate and

1 mL $HgCl_2$ solution (1 g Hg L^{-1}). The plates were incubated at 27 °C for 3–4 d. Individual colonies were selected, re-inoculated onto new plates with the respective media and incubated at 27 °C for isolation in pure culture.

Isolates were grown in Luria-Bertani broth (Scharlau, Spain) supplemented with 1 mg L^{-1} of $HgCl_2$ at 27 °C on a rotary shaker. DNA was extracted using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories) and quantified in a NanoDrop 1000 (Thermo Scientific).

Phylogenetic analysis of *merA* and 16S rRNA genes of the isolates

The *merA* gene was amplified by polymerase chain reaction (PCR) using the primers merA1 and merA5, which were designed from consensus regions of *merA* sequences belonging to the *Beta-* and *Gammaproteobacteria* (Liebert et al., 1997). The reaction contained 1 U of *Taq* DNA polymerase (Invitrogen), 0.2 mM of each deoxynucleoside triphosphate, 10x PCR buffer, 2 mM $MgCl_2$, 0.5 pmol of each primer and 1 µL template DNA in a total volume of 25 µL. The thermocycler program consisted of 1 cycle at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 64 °C for 2 min, 72 °C for 3 min, and 1 cycle at 72 °C for 10 min (Liebert et al., 1997). 16S rRNA genes were amplified using the primers 27f and 1492r_bac (Lane, 1991). The reaction contained 1U of *Taq* DNA polymerase (Invitrogen), 0.2 mM of each deoxynucleoside triphosphate, 10x PCR buffer, 2 mM $MgCl_2$, 0.5 pmol of each primer and 1 µL template DNA in a total volume of 25 µL. The thermocycler program was 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 48 °C for 30 s, 72 °C for 2 min, and 1 cycle at 72 °C for 5 min.

Restriction fragment length polymorphism (RFLP) of the PCR products

Amplified products of 16S rRNA and *merA* genes of the isolates were digested with two restriction endonucleases *Hae*III and *Alu*I (Takara, Otsu, Japan). Five microliters of the amplified reaction solution was added to a mixture containing 1 U of enzyme, 2 µL of 10x restriction buffer and 12.9 µL sterile MilliQ water. The mixtures were incubated overnight at 37 °C for digestion. After digestion, the enzymes *Hae*III and *Alu*I were inactivated for 15 min at 70 °C and 60 °C, respectively. Restriction fragments were separated by agarose gel electrophoresis and identical digestion profiles were grouped in the same operational taxonomic unit (OTU). Selected representatives of each OTU were purified with an UltraClean PCR Clean-Up kit (Mo Bio Laboratories) and sequenced by BaseClear (Leiden, The Netherlands).

Phylogenetic analysis

The CLUSTAL W algorithm was used to make sequence alignments with default gap penalties. Phylogenetic trees for the 16S rRNA gene and *merA* gene were constructed by the neighborjoining method. Statistical significance was evaluated by bootstrap analysis with 1,000 repeats. These methods are included in MEGA 4.1 software (http://www.megasoftware.net).

The sequences used for tree construction were deposited in GenBank/NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers HQ650218–HQ650231 and HQ674975–HQ674982, respectively for the 16S rRNA and *merA* genes.

Results and discussion

Mercury removal performance of the mixed cultures

The cultures were operated for between 505 and 645 d using a 14 d cycle (i.e., fresh medium was supplied every 14 d), designed to allow sufficient time for a) mercury removal and b) biomass growth. It has been previously observed that biomass growth is limited at high Hg concentrations due to the inhibitory effect of Hg on the biomass, thus detoxification and growth do not occur simultaneously but rather sequentially (Fortunato et a., 2005; Oehmen et al., 2009; von Canstein et al., 1999). Therefore, microbial survival pressure is imposed by the presence of mercury during the first days after the Hg^{2+} spike, while the community structure is mostly influenced by the carbon source available for growth thereafter.

During the first 140 d, the glucose and acetate-1 cultures were operated with a high mercury load (10 mg Hg L^{-1}). During the two first cycles of operation of these two cultures, low mercury concentrations were found in the effluent (Figure 5.1). Since these cultures were inoculated from sediment, which contains a high amount of inert solids in addition to microorganisms, adsorption may have been the principal removal mechanism during this period. Indeed, a previous study showed that Hg adsorption to solids is a relevant removal mechanism in mixed microbial cultures (Oehmen et al., 2009). The high level of mercury removal observed initially in Figure 5.1 quickly deteriorated into a very poor removal rate, perhaps due to the saturation of the available Hg adsorption sites. On day 139, the effluent Hg concentration at the end of the 14 d cycle was substantially higher in the acetate-1 culture (6349 µg Hg $gVSS^{-1}$) than in the glucose culture (145 µg Hg $gVSS^{-1}$), leading to a very

reduced or even no period of time during the cycle for biomass growth in the acetate-1 culture. After 140 d of operation, the concentration of mercury in the feed was reduced from 10 to 1 mg Hg L^{-1}. This resulted in a notable decrease in effluent Hg concentrations for both cultures (Figure 5.1), particularly for the glucose-fed culture within one sludge age (28 d) after the change in feed. Indeed, in the glucose-fed culture, the Hg concentration was negligible one day after feeding throughout the rest of the study (data not shown).

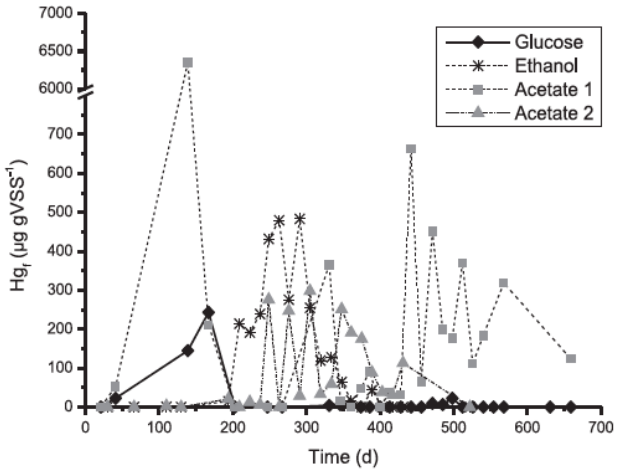

Figure 5.1. Effluent Hg concentration in the four cultures at the end of the 14-day cycle.

The ethanol and acetate-2 cultures were fed with a 1 mg Hg L^{-1} load from the beginning of the study; however, the high Hg removal efficiency observed initially in these cultures (Figure 5.1) was also likely due to adsorption. The lower Hg removal observed in the ethanol culture until around day 300 may have been due to the need for the populations to adapt to two potential toxic compounds: mercury and ethanol. Indeed, the low ethanol consumption rate led to gradual ethanol accumulation in this culture during this period (data not

shown). In order to minimize the potential inhibitory effect of ethanol, the accumulated ethanol was washed out of the system on day 300 (by adding an ethanol-free feed during one cycle), which facilitated microorganism growth and resulted in improved mercury removal efficiency.

Overall, the performance of the glucose-fed culture was superior to the others, although the ethanol-fed culture also displayed good Hg removal efficiency after day 350. The two acetate cultures showed a lower and unstable performance. The average mercury concentration in the final effluent (during the period where 1 mg Hg L^{-1} was supplied to all cultures) was 4, 14, 66 and 36 µg Hg L^{-1} for the glucose, ethanol, acetate-1 and acetate-2 cultures, respectively. Only the glucose-fed culture had effluent that was consistently far below the 50 µg Hg L^{-1} discharge limit. The average biomass concentration in the glucose culture (1.77 g VSS L^{-1}) was also higher than the other 3 cultures (0.66, 0.84 and 0.89 g VSS L^{-1} for the ethanol, acetate-1 and acetate-2 cultures, respectively). The high mercury removal efficiency observed in the glucose culture may have led to the increased biomass growth observed in this culture, since Hg^{2+} detoxification and biomass growth occur in distinct, sequential phases.

DGGE analysis of the long-term population dynamics

The microbial community shifts occurring in the cultures were analyzed using PCR-DGGE of the 16S rRNA gene as a fingerprinting technique. Analysis of the profiles acquired throughout the acclimatization showed that two main clusters diverged from the initial inoculum. One cluster (cluster I, Figure 5.2) consisted of the glucose-fed and ethanol-fed cultures, which only shared **<** 5% similarity with the cluster composed of the two acetate-fed cultures (cluster II, Figure

5.2). This shows that the microbial population structure enriched with acetate diverged from those developed in the glucose and ethanol enrichments. Furthermore, the two acetate cultures showed different DGGE profiles, which probably reflects their different histories, since acetate-1 was initially fed with 10 mg Hg L^{-1} until day 140.

The reason for the divergence of the acetate cultures from the glucose and ethanol cultures could be due to the fact that acetate forms complexes with ionic mercury (Gardfeldt et al., 2003), making the toxic pressure lower in the acetate cultures due to a lower bio-available Hg fraction. Indeed, at the working pH and ionic strength of this study, stable mercuric acetate complexes are abiotically produced, particularly the tetra-acetate form, which possibly hinders the transport of Hg^{2+} across the cell membrane. This would also explain the lower removal efficiency by the acetate cultures as compared to the others. Moreover, mercuric acetate complexes can slowly be converted abiotically into methylmercury (Gardfeldt et al., 2003), which is the most toxic mercury form. For these reasons, acetate seems least suited for the enrichment of mercury-reducing bacteria than the other studied carbon sources.

The microbial population structure of the glucose-fed culture stabilized faster than the other enrichments, as shown by comparison of the profile changes with respect to the inoculum (Figure 5.3). The glucose culture showed negligible variation after day 104, whereas the ethanol- and acetate-fed cultures had more dynamic populations. The high initial Hg concentration fed to the acetate-1 culture up to day 140 was possibly responsible for the large divergence from the inoculum, as shown by the high percent change observed in the microbial population immediately following this period (Figure 5.3). The ethanol culture also changed markedly from the inoculum after day 300, as a

result of the reduction in the ethanol concentration in the reactor as discussed above.

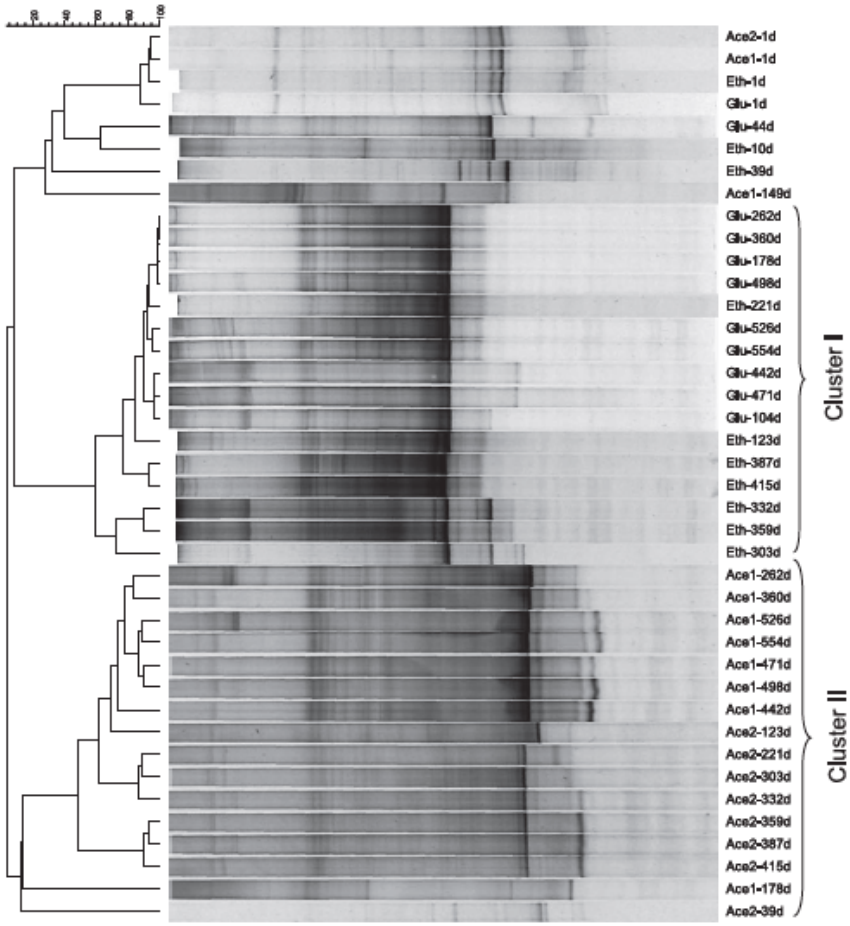

Figure 5.2. DGGE profiles and UPGMA dendrogram showing Pearson correlations of DGGE fingerprint patterns, displaying the similarity of the four microbial communities throughout acclimatization to mercury fed with glucose (Glu), ethanol (Eth) or acetate (Ace-1 and Ace-2).
Cophenetic correlation=0.78. Clusters I (comprising most of the glucose and ethanol samples) and II (most of the acetate samples) are indicated.

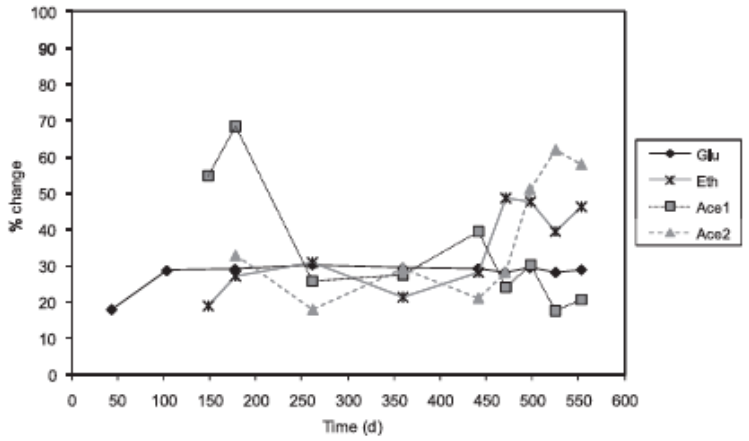

Figure 5.3. Percent change as compared to the inoculum determined from DGGE fingerprinting profiles of the four cultures throughout the study.

FISH analysis

The changes in the proportion of the most predominant bacterial groups during the acclimatization study were monitored through FISH. This analysis was carried out using probes targeting general phylogenetic groups that are commonly found in wastewater treatment systems and environmental communities, such as the *Alpha*, *Beta* and *Gamma* sub-groups of the *Proteobacteria* phylum, *Cytophaga-Flavobacteria* and High G+C content Gram-positive bacteria (*Actinobacteria*). The glucose- and ethanol-fed cultures were clearly dominated by members of the *Gammaproteobacteria* during the whole study (Table 5.1). No major differences were observed in the glucose culture throughout the study, which matches the low variability observed through DGGE analysis of this culture. The high % changes (as compared to the inoculum) obtained for the ethanol culture after day 300 (Figure 5.3) may have been largely due to the changes detected for *Betaproteobacteria*, *Cytophaga*

and *Actinobacteria*, since the abundance of *Gammaproteobacteria* stayed relatively stable.

Table 5.1. Semi-quantitative FISH results for the four cultures at key days of operation.

Culture /days of operation	FISH probe				
	ALF969	**BET42a**	**GAM42a**	**CF319a**	**HGC69a**
Inoculum	+	++	++	(+)	+
Glucose					
170	-	-	+++	-	-
296	-	-	+++	-	(+)
479	(+)	+	+++	-	-
Ethanol					
192	-	(+)	+++	-	(+)
221	-	+	+++	(+)	(+)
332	-	-	+++	-	-
387	-	-	+++	(+)	+
Acetate-1					
216	(+)	-	++	-	+
354	-	+++	(+)	-	-
497	+	++	+	-	-
505	(+)	+++	+	-	-
Acetate-2					
303	+	++	-	(+)	(+)
358	+++	-	-	-	(+)
415	++	-	-	-	++

- not detected; (+) <5%; + 10-20%; ++ 20-30%; +++ >30%

ALF969: *Alphaproteobacteria;* BET42a: *Betaproteobacteria;* GAM42a: *Gammaproteobacteria;* CF319a: *Cytophaga-Flavobacteria*; HGC69a: *Actinobacteria*

The FISH results also showed different population structures for the two acetate cultures, as evidenced by the different DGGE band profiles, where the acetate-1 culture showed higher dynamics with both techniques (Figure 5.3 and Table 5.1). The acetate-1 culture was dominated by *Betaproteobacteria* after day 354, although this group was not initially detected in this biomass. In contrast, the acetate-2 culture was dominated by *Alphaproteobacteria* and had a low abundance of *Betaproteobacteria*. These differences reflect the different

conditions the cultures were subjected to at the beginning of the study, where 10 mg Hg L^{-1} was initially supplied to the acetate-1 culture and not to acetate 2. *Actinobacteria* were sporadically observed in all cultures, always in low numbers, while a few *Cytophaga-Flavobacteria* were found in the ethanol-fed culture during approximately 2 SRT (days 385– 445) and also in the acetate-2 culture on day 303.

Quantitative-PCR of the *merA* gene

The number of copies of the *merA* gene was monitored through quantitative-PCR during acclimatization. In the inoculum, it was found to be below the limit of quantification of the method (10±1), but it increased substantially in all cultures within 5 sludge ages (SRT**=** 28 d), and attained very high numbers towards the end of the study, particularly in the glucose and ethanol cultures (Figure 5.4). Overall, the glucose culture exhibited the highest number of *merA* gene copies, which is in good agreement with the better mercury removal performance of this culture throughout the study (Figure 5.1). The acetate-1 culture always showed a relatively low number of *merA* copies as compared to the other cultures, which is consistent with the lower mercury removal efficiency achieved by this system. The sudden decrease in the number of copies of *merA* in this culture around day 450 could result from underestimation due to primer specificity to *Beta*- and *Gammaproteobacteria*, since this culture contained many *Alphaproteobacteria* at this time according to FISH results.

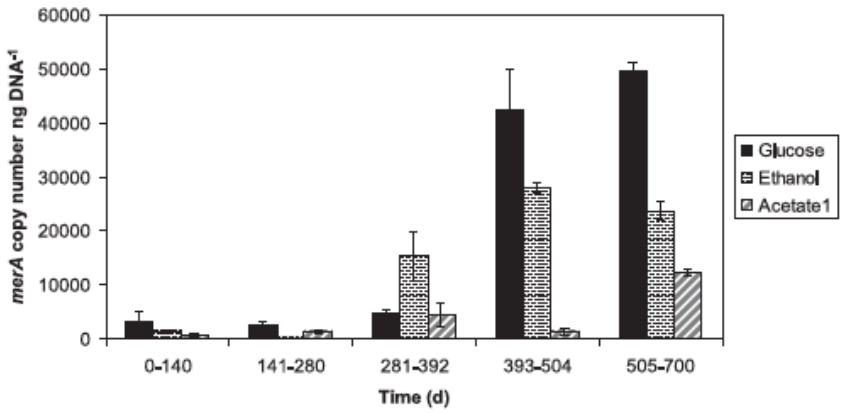

Figure 5.4. Copy number of the *merA* gene determined by qPCR in the glucose-, ethanol- and acetate-fed cultures throughout acclimatization.

Nevertheless, other primers covering *Alphaproteobacteria* and Gram-positive bacteria are degenerated (Chadhain et al., 2006; Oregaard and Sorensen, 2007), and thus should not be applied for qPCR (Polz and Cavanaugh, 1998; Ramond et al., 2009). Furthermore, this decrease coincides with a decrease in mercury removal performance (Figure 5.1), suggesting that the microbial community was not favorable for biological mercury reduction at this stage. Similarly, the improvement in mercury removal performance of the ethanol-fed culture after day 300 (Figure 5.1) was also consistent with a substantial increase in the *merA* gene copy number, which increased from 16 copies ng DNA^{-1} on day 221 to 15,365 copies ng DNA^{-1} on day 332 (Figure 5.4).

The *merA* gene copy number was also assessed in activated sludge samples from three different WWTPs receiving domestic and industrial wastewater, where it was found to be detectable in only one plant, and at a very low level (39±8). This is similar to the low abundance observed in the inoculum, despite the high level of mercury contamination at the collection site (see Canario et

al., 2005). These low values support the need for bioaugmentation or acclimatization (using an appropriate carbon source) when aiming at carrying out biological mercury removal.

The increased number of *merA* gene copies per ng DNA observed soon after inoculation (3,320 ± 1,820, 1,398 ± 284 and 780 ± 311 *merA* gene copies ng DNA^{-1} in the glucose, ethanol and acetate-1 cultures, on days 44, 123 and 90, respectively, Figure 5.4) was an expectable response to exposure to mercury stress conditions (Nazaret et al., 2003; Ramond et al., 2008; Rasmussen and Sorensen, 2001). During acclimatization, an increased number of *merA* copies was observed in all cultures, suggesting that it is an important factor in the acclimatization process. The increase in *merA* copies was possibly induced by horizontal gene transfer during exposure to mercury, but also by subsequent growth of the transconjugants during the mercury-free culture operation phase, which usually accounted for **>** 12 d out of a 14 d cycle. This increase in *merA* occurred independently of the change in microbial community, where the number of gene copies continued to increase even after the population had stabilized.

Characterization of the isolates

While FISH and DGGE enable monitoring of the microbial populations enriched in each culture and their evolution throughout the study, these techniques do not allow the mercury-resistant bacteria in the cultures to be identified.

Therefore, after a stable performance and population structure was observed in the cultures (Figure 5.1 and 5.3), pure cultures were isolated on agar plates containing mercury. This process was repeated three times during the final 100

d of the study, resulting in 101 isolates. The isolates were grouped into 13 different operational taxonomic units (OTUs) by combined analysis of RFLP patterns obtained with their *merA* and 16S rRNA gene amplicons using two restriction enzymes (data not shown). Representatives of each 16S rRNA or *merA* OTU were sequenced and their phylogenetic affiliation was investigated through similarity analysis with 16S rRNA and *merA* genes sequences (GenBank/NCBI) of bacteria previously reported to possess the *merA* gene (Figure 5.5 A and 5.5 B, respectively). This approach aimed to identify mercury-resistant (*merA* positive) organisms enriched in each culture. Additionally, the diversity of the 16S rRNA and *merA* genes obtained from the isolates was compared to study the transfer mechanism of the resistance determinant in the cultures developed in this work.

The 16S rRNA OTU with the highest number of isolates (36) was very similar to three other minor OTUs (adding up to 47 isolates) and were closely related to *Enterobacter* and *Klebsiella* species (Figure 5.5 A). These members of the *Gammaproteobacteria* include numerous faecal contamination-related bacteria, which have been reported to have the *merA* gene (Ramond et al., 2009). Another 16S rRNA OUT cluster with 34 isolates (mainly from one OTU with 33 isolates), was closely affiliated with *Alcaligenes* and *Bordetella* species, i.e., *Betaproteobacteria*. A smaller OTU cluster, representing 13 isolates, was related to *Stenotrophomonas maltophilia* and other members of the *Xanthomonadaceae*, also *Gammaproteobacteria*. Finally, 6 isolates were affiliated to *Pannonibacter*, belonging to the *Alphaproteobacteria*, and one isolate was related to the *Actinobacteria* (Gram-positive) *Nocardiopsis* and *Streptomyces*. In a study comparing the isolates obtained from soil after contamination with mercury (Nazaret et al., 2003), also the *Alpha-*, *Beta-* and

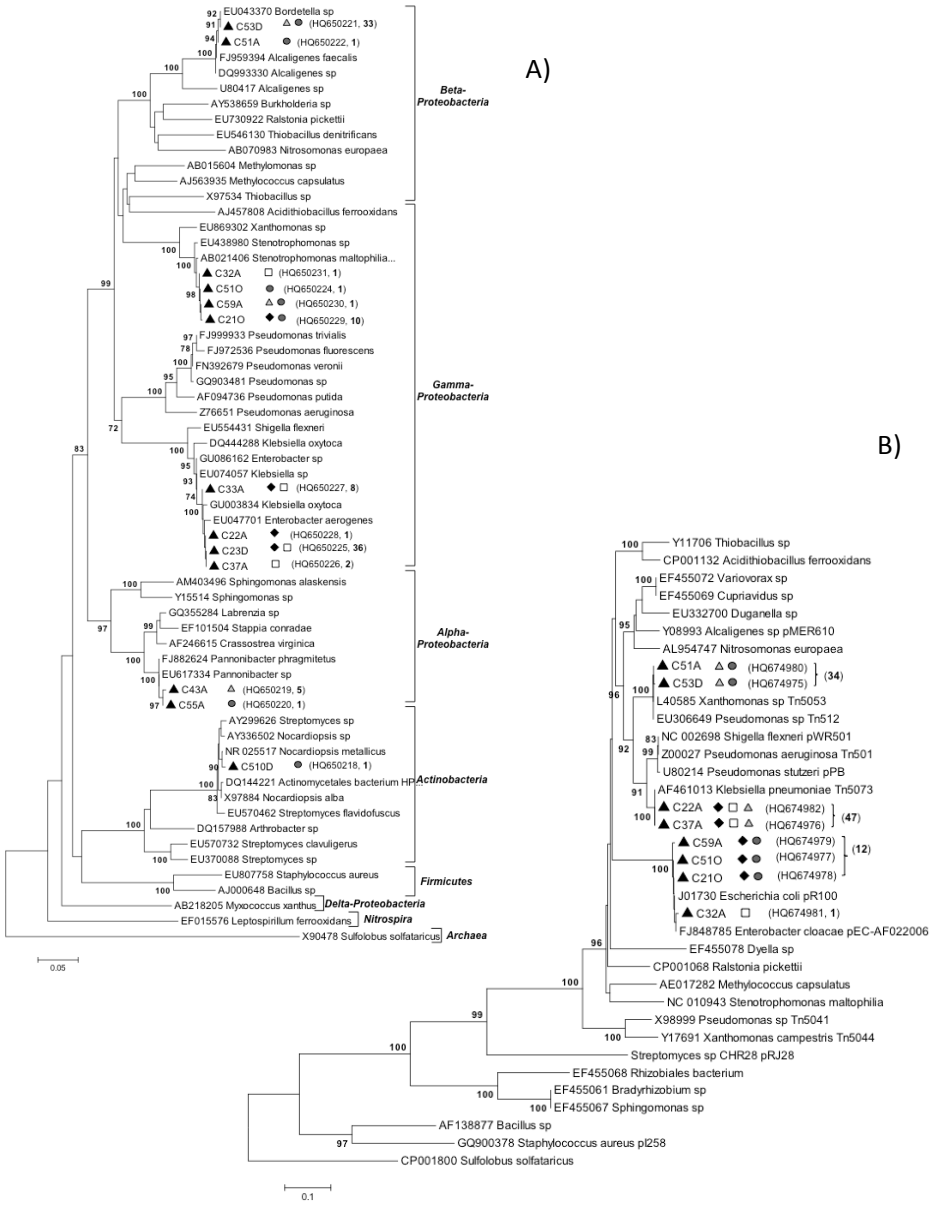

Figure 5.5. Neighbor-joining trees showing the phylogenetic relationship of 16S rRNA gene and (B) *merA* gene sequences obtained from GenBank at NCBI and from this study.

Black triangles indicate OUT representatives of the isolates obtained in this study. The corresponding GenBank accession numbers are given between brackets, followed by the number (in bold) of isolates in that OTU. Other symbols correspond to the cultures from which that OTU was obtained (◆ glucose; ethanol; ●acetate-1 and ▲ acetate-2). Bootstrap values above 70% are shown at each branch point.

Gammaproteobacteria (including *Xanthomonas*) also became dominant, with only one isolate belonging to the high G+C Gram-positive bacteria group.

Interestingly, all isolates obtained from the glucose- and ethanol-fed cultures belonged to the *Gammaproteobacteria*, whereas members of the *Alpha-*, *Betaproteobacteria* and *Actinobacteria* were also obtained from acetate-fed cultures (see symbols in Fig. 5.5 A). Despite the limitations associated with cultivation, these results corroborate those observed with FISH, where the acetate cultures showed higher diversity than the glucose and ethanol cultures throughout the study.

The *merA* OTUs could be grouped into three clusters. A cluster with 47 isolates was closely related to the *merA* gene of transposon Tn5073 in Klebsiella pneumoniae (Figure 5.5 B). These isolates were the same as those affiliated with *Klebsiella* and other *Enterobacteriaceae* through 16S rRNA gene analysis and were only found in the glucose- and ethanol-fed cultures. A second *merA* OTU cluster included 13 isolates and was related to the *merA* found in plasmids of *Enterobactercloacae* and other members of the *Enterobacteriaceae* family. These isolates were all phylogenetically affiliated to the *Xanthomonadaceae Stronotrophomonas* (Figure 5.5 A) and were present in all cultures. Finally, another OTU of 34 isolates matched the *merA* in a transposon observed in *Xanthomonas* (*Gammaproteobacteria*), while through 16S rRNA gene analysis they belonged to the *Betaproteobacteria*. These isolates were only found in acetate-fed cultures. All three *merA* OTU clusters shared relatively high similarity (> 75%), even among bacteria of different genera, families and groups, which is not surprising regarding the high mobility of this gene (Osborn et al., 1997). One hypothesis is that the *merA* gene was originally found in the inoculum in *Gammaproteobacteria* (of the family *Enterobacteriaceae* or

Xanthomonadaceae) and then horizontally transferred to other organisms, including members of the *Betaproteobacteria*.

It was not possible to amplify the *merA* gene of the three isolates belonging to *Alphaproteobacteria* and *Actinobacteria*, despite their growth in mercury-containing media, which suggested that they possess a mercury-resistant determinant. This was likely due to the specificity of the *merA* primers employed (Liebert et al., 1997), which were developed to target mostly *Beta*- and *Gammaproteobacteria*. Nevertheless, the low number of isolates found outside these groups (7 out of 101 isolates) suggested that the selected primers covered the majority of bacteria in the cultures.

Conclusions

In this study, a clear shift in the microbial profiles of engineered cultures for biological Hg^{2+} reduction was observed shortly after mercury contamination (2–4 SRT), with a clear divergence of the two acetate cultures with respect to the glucose- and ethanol-fed cultures, which clustered in higher similarity. The glucose culture displayed a very stable microbial community, with the highest abundance of *merA* gene copies and the best Hg removal performance. After an initial period of unstable mercury removal due to ethanol accumulation, the ethanol culture also achieved good performance and increasing *merA* copy numbers. The acetate cultures showed more dynamic microbial profiles, as well as lower mercury removal efficiency, which may have been due to lower selection pressure on the culture caused by the formation of mercuric acetate complexes. The diversity in the *merA* sequences from the isolates obtained in this study was lower than their corresponding 16S rRNA diversity. This suggests that horizontal gene transfer of *merA* had an important role during

acclimatization of the cultures. This was further supported by the very high increase in *merA* gene copy number by qPCR during acclimatization, which largely occurred after the microbial populations had stabilized, regardless of the community composition.

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Chapter 6

General Discussion

The detection of micropollutants in water sources is raising global concerns regarding their potential risks to the environment and human health. WWTPs represent the first treatment opportunity for removing these compounds and preventing significant environmental exposure. In biological systems the microbial fraction is known to play an important role in the elimination of pollutants. However, little is known about the microbial populations in treatment systems and their responses to these compounds. The assessment of variations in microbial community structure and functionality are of basic importance for understanding the micropollutant degradation potential of a system, and to optimise the elimination of these compounds during the treatment process.

In the first part of this thesis, the ability of activated sludge from three different WWTPs to degrade two of the most highly detectable NSAIDs in the influents of WWTPs worldwide was investigated and characterised through the use of a mathematical model. Modelling of activated sludge processes is valuable to further improve process optimisation and control in treatment systems aiming to increase micropollutant removal and minimise their occurrence on receiving waters. The model developed in this study effectively characterised the influence of different loading rates and mixtures of compounds on the degradation of both ibuprofen and ketoprofen by activated sludge. The degradation performance was found to be a function of biomass composition and substrate loading rate. Higher loading rates induced higher removal rates. Additionally, mixtures of both compounds did not stimulate nor inhibit the degradation as compared to when

each NSAID was fed individually. Furthermore, a lag phase was observed in all the cases. The overall results contrast with the ones previously obtained by Collado et al. (2012), where a lag phase was not observed and where higher degradation rates were obtained at lower ibuprofen concentrations. The lag phase could possibly be related to the acclimation and growth of degrading microorganisms, as previously demonstrated by Chong et al. (2009). This hypothesis could hardly be explored in activated sludge due to the impossibility of measuring the microbial degraders. For this reason, microorganisms were isolated that could easily allow to monitor the growth/function performance. Twelve new ibuprofen and ketoprofen degraders were isolated and identified from the sludge that showed the best NSAID removal performance. The *in situ* relevance and role of these isolates in treatment systems is still unknown and requires further study. Nevertheless, their identities are of great use in future studies aiming to characterise the microbial populations involved in PhAC degradation in WWTPs. One of the isolates, an ibuprofen degrader, was studied in similar experimental conditions used in the tests with activated sludge to demonstrate the link between biomass growth rate and ibuprofen degradation rate. The degradation of ibuprofen by the isolate began immediately after its addition to the medium without a lag phase showing a different behaviour as compared to the activated sludge. This degradation profile was incorporated in a new model. This isolate was not able to use ibuprofen as the sole carbon source, although degradation was observed in the presence of other carbon sources. The ibuprofen degradation performance was simultaneously dependent on the growth rate as well as the ibuprofen concentration, with higher degradation rates at higher biomass growth rates. Higher biomass growth rates were achieved with increasing concentrations of yeast extract and tryptone. Other studies have also associated the degradation efficiency with the presence of another primary substrate that can sustain cell growth and maintenance (Egli, 2010), and thus this fact should be taken in

consideration when aiming at stimulating the degradation of micropollutants in WWTPs.

The differences in degradation performance observed between mixed microbial cultures and pure cultures under similar environmental conditions highlight the importance of testing the degradation performance for each specific microbial community. Besides, these results emphasised that mathematical models may require adaptation to describe different systems. The applicability of the developed models in this study to predict and describe the degradation of other NSAIDs or PhACs should be further investigated. Future studies should also test technological and operational characteristics of the treatment systems, such as temperature, redox potential, sludge and hydraulic retention time on the degradation of performance of these NSAIDs.

Interestingly, the ibuprofen bacterial isolate for which a model was developed was found to be a new species. A polyphasic taxonomic approach, where both phenotypic and genotypic methods were employed, suggested this isolate as a new species of the genus *Patulibacter*, for which the name *Patulibacter medicamentivorans* sp. nov. was proposed. The new species described here is the first gram-positive bacteria with a demonstrated ability to degrade ibuprofen. The identification of this degrading microorganism is of great importance since it enables to further monitor its presence and function in treatment systems. For example, specific FISH probes can be designed to detect this microorganism allowing its detection and quantification in natural systems and/or in engineered systems aiming to enrich microbial communities for bioaugmentation or bioremediation purposes and correlate it with the removal performance. The isolation of a degrading organism further allows studying the enzymatic

mechanisms of ibuprofen degradation by identifying key enzymes involved in process using a proteomic approach.

In this thesis, the value of the combination of genomic and proteomic approaches was demonstrated, which revealed novel information regarding the cellular responses to this important NSAID. Ibuprofen degradation by *Patulibacter medicamentivorans* was characterised by a metabolic labelling quantitative proteomics. This methodology enabled the identification and quantification of a number of up-regulated proteins in the presence of low levels of ibuprofen, that constitute potential candidates involved in the metabolic degradation pathway of ibuprofen. Furthermore, all the proteins were reliably identified from the genome of *Patulibacter medicamentivorans,* which was specifically sequenced for this purpose. Some of the proteins identified in this study, including those that have been previously described to be involved in the uptake and degradation of aromatic hydrocarbons, were up-regulated in the presence of ibuprofen: Acyl-CoA synthetase (J2XUN5), involved in the first step of CoA-dependent degradation pathway, catalysing the formation of an acyl-CoA; Rieske (2Fe-2S) iron-sulphur domain protein (D3F3V3), involved in the oxidation of aromatic ring structure; enoyl-CoA hydratase/isomerase (D3F114), known for catalysing the hydroxylation of double bonds after opening of the aromatic ring; and an ABC-2 type transporter (D3FB12), daunurobicin resistance ABC transporter ATPase (D3FBI3) and an extracellular solute-binding protein type 1 family (D3FBH9), known for participating in the uptake of aromatic hydrocarbons. Additionally, the genome of *Patulibacter medicamentivorans* revealed the presence of similar genes known to be involved in the degradation of other aromatic hydrocarbons, benzoate and phenylacetic acid, which reveals the versatility of this microorganism towards the degradation of other compounds, making it a strong candidate to degrade other micropollutants.

The genes previously reported as involved in ibuprofen degradation (Kagle et al., 2009) were not found in the proteome of *Patulibacter medicamentivorans*, except for a protein sharing 33 % identity with IpfF from *Sphingomonas* sp. Ibu-2. This is not surprising in view of the phylogenetic distance between the two organisms, and supports the hypothesis of a novel metabolic pathway. The verification of the role of the identified proteins requires further analysis such as expression studies and protein characterisation. The identification of metabolites will further provide more definitive links to physiological functions.

The proteins identified in this study can be further used as biomarkers to characterise the ability of a microorganism or a community of microorganisms to degrade ibuprofen or other structural similar compounds. Biomarkers are of great value for biotechnological applications, as they enable to monitor the degradation potential of a microbial community and its dynamics throughout operational/environmental variations in engineered and natural systems. For example, qPCR primers targeting functional genes that encode for the specific proteins identified in this study can be designed and applied for this purpose.

The application of genetic/proteomic knowledge to the investigation of micropollutant-degrading communities was explored in this thesis, to establish a link between mercury degradation performance and microbial community dynamics. This work was focused on the enrichment of mercury-reducing microbial cultures fed with different carbons sources (glucose, ethanol and acetate).

The results showed that higher mercury reduction rates were obtained in the glucose-fed culture and lower in the acetate-fed culture, suggesting that different microorganisms were selected in each culture. This fact was confirmed by the 16S

rRNA gene profiles obtained by DGGE. Additionally, FISH results matched the variability observed with DGGE analysis, in which glucose- and ethanol- fed cultures were dominated by *Gammaproteobacteria* while acetate cultures were dominated by *Alphaproteobacteria*.

The previous knowledge on the biological mercury reduction mechanism allowed assessing the functional changes of the communities through quantification of the *merA* gene copy numbers by qPCR. These results correlated with the corresponding mercury removal efficiency of each culture. Moreover, the taxonomic and functional diversity of the mercury-resistant organisms was investigated through isolation and sequencing of the 16S rRNA and *merA* genes, respectively. Higher diversity of the *merA* gene was observed when compared to the 16S rRNA gene, suggesting that horizontal gene transfer played an important role in the acclimatisation process. This was also supported by the increase in the *merA* copy number during acclimatisation, which occurred after the populations performance had stabilised. Overall, it was demonstrated that glucose positively affected the microbial selection resulting in a very stable microbial community, with the highest abundance of *merA* gene copies and the best mercury removal performance.

Future studies should address other carbon sources such as sludge hydrolysate or molasses due to economic advantages. A similar approach could be carried out to verify the effect of these carbons sources on the microbial communities. Nevertheless, the extrapolation of such studies should be done carefully and should also account for the possible impacts on other important microbial communities that are responsible for the optimal function of WWTPs.

The overall results contribute to the understanding of the micropollutant degrading microbial community structure and function in WWTPs. Biodegradation of micropollutants in WWTPs is possible. However, in order to maximize the potential benefits of microbial community in degrading such pollutants, it is important to have fundamental understanding of the microbial degradation capacity under different operational conditions, its biochemical systems and its molecular biology.

The biological removal of micropollutants in water and wastewater treatment systems remains a great challenge that requires continuous study due to the constant detection of new emerging micropollutants. The work presented in this thesis represents a step forward in the understanding, monitoring and promoting the microbial removal of micropollutants

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Supplementary Material

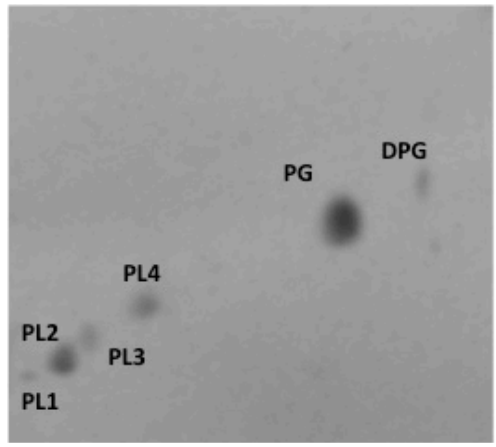

Figure 7.1. Polar lipid profile of strain I11^T after separation by two-dimensional thin-layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol and PL, unknown phospholipid.

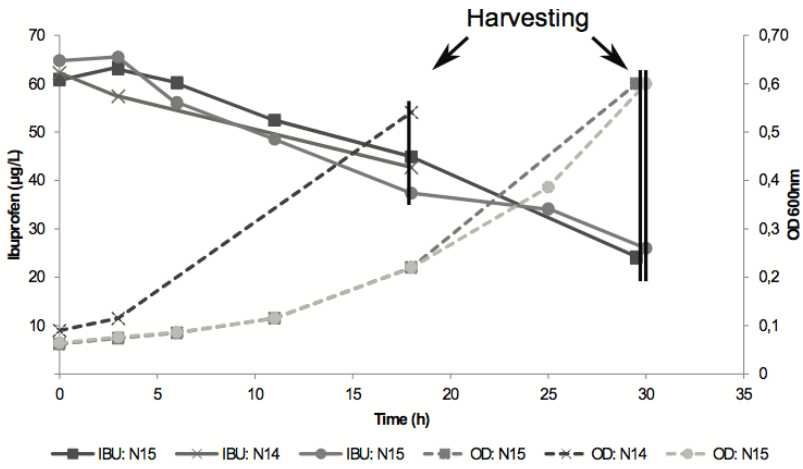

Figure 7.2. Ibuprofen degradation by the bacterial isolate *Patulibacter* sp. I11. The progression of degradation of ibuprofen by the *Patulibacter* sp. I11-strain is shown over 30h in 15N- and 14N-labelled OD2-medium.

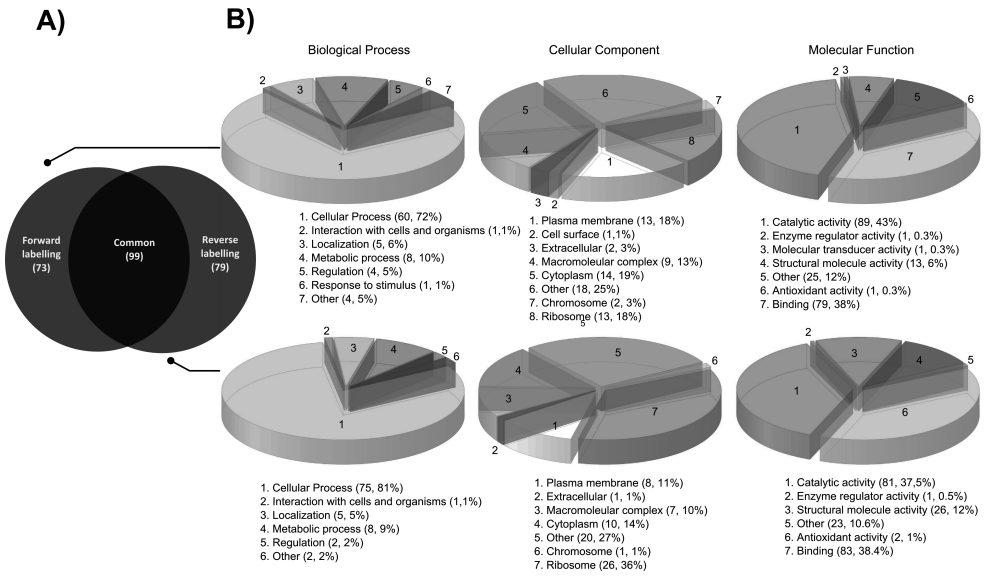

Figure 7.3. A) Distribution of the quantitated proteome of the forward and reverse labelling experiment (only considering unique proteins). 73 proteins were exclusive to the forward labelling experiment, 79 exclusive to the reverse labelling experiment and 99 proteins were common to both. B) Distribution of proteins at three different levels of GO-annotation: Biological process, Cellular component and Molecular function. Each pie slice is labelled with the GO subcategory name, number of GO annotations within the category as well as the percentage fraction of annotations.

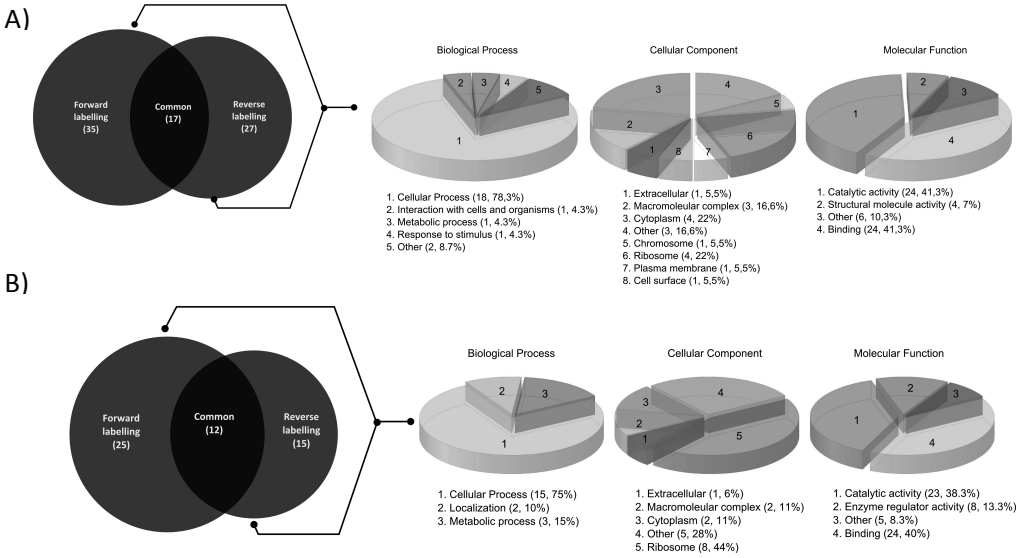

Figure 7.4. Distribution of the upregulated (i.e. log2 ratio >= 0.9) part of the proteome of the forward and reverse labelling experiment (only considering unique proteins) as well as distributions of proteins at three different levels of GO-annotation: Biological process, Cellular component and Molecular function. Each pie slice is labelled with the GO subcategory name, number of GO annotations within the category as well as the percentage fraction of annotations. B) Distribution of the down-regulated (i.e. log2 ratio <= -0.9) part of the proteome of the forward and reverse labelling experiment (only considering unique proteins) and the distributions of proteins at 3 different levels of GO-annotation.

Table 7.1. List of primers used in this study.

Target gene	Primer's name	Sequence (5'-3')	Size (bp)
PAI11_21070*	IpfA2F	CGACTACGTGGTCAAGTCGATCG	170
	IpfA2R	AGAACGTCCACCCGTGGTACG	
PAI11_21080*	IpfB1F	CGGCAGATGTCGGTGATCTTCG	135
	IpfB1R	ATCCAGCAGCTCGACGTTGGT	
PAI11_02050*	IpfD2F	CTTCGAGCCGTTCGGCGAGA	170
	IpfD2R	CCTTCTCGCAGAACCCGAGGT	
PAI11_25140*	IpfE1F	TGACCAACGTCGTCGTCGACT	157
	IpfE1R	CGGGCCTTCCAGAAGTAGTTGTG	
PAI11_13130*	IpfF2F	GACAACGTCATGGCCGGCTAC	150
	IpfF2R	GCCGGTCACGATCATGTCCTTG	
D3F114	P1F2	GCATCGAGATCCCCTTCCGGTA	149
	P1R2	GCCGCGGTTCTACGTCCAGA	
J2XUN5	P2F1	CCTGAACAGCGACCGCTACCT	150
	P2R1	AAGGCGTCGTCGACCAGCAA	
D3F3V3	P3F1	TTCGCCCGCTGGATCGCTT	151
	P3R1	GACGGATACAGGTACTGGCCGAT	
D3FBI3	P4F2	GCAGCAGCGTCGTCATGATCC	142
	P4R2	CGGGGGCTGGTCAAGTCCTT	
D3FBI2	P5F1	ACGGTGAAGCCGATCGCGTT	144
	P5R1	CTTCTCGTTCGGGACCGACGT	
D3FBH9	P6F1	CAAGTCGGTGATCAAGGAGTTCGA	153
	P6R1	TAGTCGGTCAGGACGACGATGTC	
PAI11_13300	I11_16SF2	CACTGGTTAATAGCCAATCGGAGTGA	156
	I11_16SR2	GACTTTCACAGCAGACTTACTGAACC	

* Orthologous genes in *Patulibacter* sp. strain I11 to *ipfABDEF* proposed to be involved in ibuprofen degradation (Kagle et al., 2009).

Table 7.2. *Ab initio* predictions of functions of the putative uncharacterized proteins, based upon the full-length sequence retrieved from the annotated genome of *Patulibacter* sp. I11 using the ProtFun 2.2 server (DTU).

Accession Nº	Functional category	Enzyme/non-enzyme	Enzyme class	GO category
D3F6B1	Cell envelope	Non-enzyme	---	Stress response
D3FA23	Central intermediary metabolism	Enzyme	Lyase	---
Q47N65	Transport and binding	Non-enzyme	---	Immune response
D3F293	Cell envelope	Enzyme	---	Immune response
D3FFG0	Cell envelope	Enzyme	---	Structural protein
B0CFJ3	Cell envelope	Non-enzyme	---	Immune response

Table 7.3. The *Sphingomonas* Ibu-2 *ipf* genes, the *Acinetobacter* sp. (strain ADP1) *cat* and *ben* genes and the *E.coli* K12 *paa* genes with respective align products from *Patulibacter* sp. strain I11.

Genes	Name (orf no.)[A]	Organism	Function	E-value	Identity (%)	Accession[B]
IpfA	PAI11_21070	*Sphingomonas* Ibu-2	Putative dioxygenase hydroxylase component	5E-91	40	A1E031
IpfB	PAI11_21080	*Sphingomonas* Ibu-2	Putative dioxygenase component	4E-34	46	A1E030
IpfD	PAI11_02050	*Sphingomonas* Ibu-2	Thiolase	3E-21	36	A1E029
IpfE	PAI11_25140	*Sphingomonas* Ibu-2	Hydroxymethylglutaryl-CoA synthase	0.02	33	A1E028
IpfF	PAI11_13130	*Sphingomonas* Ibu-2	AMP-dependent synthetase and ligase	10E-61	33	A1E027
benA	PAI11_21070	*Acinetobacter* sp. (strain ADP1)	Putative dioxygenase hydroxylase component	9E-52	33	P07769
benB	PAI11_21080	*Acinetobacter* sp. (strain ADP1)	Putative dioxygenase component	3E-08	29	P07770

benC	PAI11_29360	*Acinetobacter* sp. (strain ADP1)	Methane monooxygenase component C	6E-40	34	P07771
benD	PAI11_02500	*Acinetobacter* sp. (strain ADP1)	3-oxoacyl-[acyl-carrier-protein] reductase	8E-24	30	P07772
benE	-	*Acinetobacter* sp. (strain ADP1)		-	-	P07775
benK	-	*Acinetobacter* sp. (strain ADP1)		-	-	O30513
benK	PAI11_16480	*Acinetobacter* sp. (strain ADP1)	Drug resistance transporter, EmrB/QacA family	5E-04	24	O30513
benP	-	*Acinetobacter* sp. (strain ADP1)		-	-	-
benM	PAI11_29700	*Acinetobacter* sp. (strain ADP1)	Transcriptional regulator, LysR family	4E-39	38	O68014
catA	PAI11_29670	*Acinetobacter* sp. (strain ADP1)	Protocatechuate 3,4-dioxygenase beta subunit	8E-29	33	P07773
catM	PAI11_29700	*Acinetobacter* sp. (strain ADP1)	Transcriptional regulator, LysR family	9E-34	34	P07774
catB	PAI11_29690	*Acinetobacter* sp. (strain ADP1)	Muconate cycloisomerase)	4E-30	30	Q43931
catC	PAI11_29680	*Acinetobacter* sp. (strain ADP1)	Muconolactone isomerase	2E-23	42	Q43932
catI	PAI11_01310	*Acinetobacter* sp. (strain ADP1)	Putative CoA transferase A subunit	2E-03	39	Q43973
catJ	PAI11_05540	*Acinetobacter* sp. (strain ADP1)	Probable 4-hydroxybutyrate coenzyme A transferase	8E-02	37	Q59091
catF	PAI11_29630	*Acinetobacter* sp. (strain ADP1)	Acetyl-CoA acetyltransferase	7E-74	43	Q43935
paaA	-	*E.coli* K12	-	-	-	P76077
paaB	PAI11_33410	*E.coli* K12	Inosine-5'-monophosphate dehydrogenase	0.30	43	P76078
paaC	PAI11_05850	*E.coli* K12	ABC transporter related	0.64	25	P76079
paaE	PAI11_12840	*E.coli* K12	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1	1E-19	26	P76081

paaF	PAI11_02240	*E.coli* K12	Enoyl-CoA hydratase	5E-42	47	P76082
paaG	PAI11_25220	*E.coli* K12	Enoyl-CoA hydratase	3E-39	45	P77467
paaH	PAI11_02250	*E.coli* K12	3-hydroxybutyryl-CoA dehydrogenase	2E-37	35	P76083
paaI	PAI11_01080	*E.coli* K12	Uncharacterized protein, possibly involved in aromatic compounds catabolism	1E-6	30	P76084
paaJ	PAI11_02260	*E.coli* K12	3-ketoacyl-CoA thiolase	8E-112	54	P0C7L2
paaK	PAI11_05290	*E.coli* K12	AMP-binding protein	1E-8	25	P76085
paaL	PAI11_06070	*E.coli* K12	ATP-dependent Clp protease	4E-158	40	P0ABH9
paaM	PAI11_27220	*E.coli* K12	Succinate-semialdehyde dehydrogenase [NADP+]	3E-24	30	P77455
paaZ	PAI11_27220	*E.coli* K12	Succinate-semialdehyde dehydrogenase [NADP+]	3E-24	30	P77455
paaX	PAI11_01070	*E.coli* K12	Phenylacetic acid degradation operon negative regulatory protein PaaX	1E-5	25	P76086
paaY	PAI11_34980	*E.coli* K12	Acetyltransferase	7E-6	33	P77181

[A]NCBI accession number of *Patulibacter* sp. strain I11 align products.

[B]UniProt accession number.