The Heterogeneity of Amine Oxidase within

*Rhodococcus opacus*

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Abstract

Four native amine oxidases have been identified from *Rhodococcus opacus* to reveal phenotypic plasticity and catalytic activity with respect to structurally diverse natural and synthetic amines. Altering the amine growth substrate enabled tailored and targeted oxidase upregulation, facilitating catalytic differentiation and isolation. Each enzyme was purified over 80 fold by chromatography, allowing subsequent characterisation. Two oxidases possessed a copper dependent redox co-factor with broad specificity towards monoamines. Michaelis constants ($K_M$) ranged from 0.1 to 0.9 mM for common C1–C5 aliphatic monoamines and <0.2 mM for a range of aromatic amines. The remaining two oxidases by contrast were highly specific for aliphatic diamines, with a Michaelis constants ($K_M$) = 60 µM for putrescine by a third copper oxidase and a ($K_M$) = 190 µM by a flavin dependent oxidase.

MALDI-TOF and genomic analysis has indicated metabolic gene clusters, multiple gene activation, and complex biodegradation pathways. With a consideration of the diamine acting oxidase, a putrescine degradation pathway is confirmed that utilises oxidases in tandem with a 4-aminobutyraldehyde dehydrogenase. The taxonomic distribution of this pathway is further examined utilising phylogenetic analysis. Oxidase regulation and integration into the nitrogen cycle is then considered, with implications in bioremediation and biocatalysis discussed.
Dedication

I would like to dedicate my thesis to my family, who for years have given me belief, encouragement and support. My wonderful wife Melissa who I love and adore, my daughter Samantha whose smile and laughter is the best inspiration a dad could have, and also “Bones” who sadly passed away a few months ago.
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Table 5.4: Range and structure of amines oxidised by amine oxidases in Rhodococcus opacus DSM43250 [9, 11].

Table 5.5: Compilation of aromatic and aliphatic nitriles degraded by nitrilases in Rhodococcus rhodochrous J1 and Rhodococcus rhodochrous K22 [84-86].
List of Publications by Candidate


A. Foster, R. Speight, N. Barnes, M.A. Keane, “Repertoire of Nitrogen Assimilation in Rhodococcus; Catalysis, Pathways and Relevance in Biotechnology and Bioremediation”, Ready for submission.

A. Foster, R. Speight, N. Barnes, M.A. Keane, “Genomic organisation, activity and distribution analysis of the microbial putrescine oxidase” Ready for submission.

## Glossary

### Enzymes

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<td>AO</td>
<td>Amine oxidase</td>
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<tr>
<td>AAO</td>
<td>Amino acid oxidase</td>
</tr>
<tr>
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<td>4-aminobutyraldehyde dehydrogenase</td>
</tr>
<tr>
<td>AbT</td>
<td>4-aminobutyrate transaminase</td>
</tr>
<tr>
<td>CuAO</td>
<td>Copper amine oxidase</td>
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<td>FlavAO</td>
<td>Flavin amine oxidase</td>
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<td>NHase</td>
<td>Nitrile hydratases</td>
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<td>SsD</td>
<td>succinic semialdehyde dehydrogenase</td>
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### Amines

<table>
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**Scope and Organisation of the Thesis**

*Rhodococcus* spp. are highly versatile *Actinobacteria* with a range of enzyme activities that has attracted considerable research attention, especially with regard to application in bioremediation and as biocatalysts. This biotechnological potential, coupled with the increasing prominence of genomic data, has led to an exponential rise in the number of associated publications and patents. Such analysis has revealed an array of enzymes, associated metabolic pathways and sophisticated regulatory networks, which have served to demonstrate the complexity of this genus but also our lack of understanding of precise mechanisms and enzymatic action.

In this context, we take *Rhodococcus opacus*, a species closely associated with two of the best characterised genomes (*Rhodococcus opacus* B4 and *Rhodococcus* RHA1), and subject the amine oxidase class of enzymes to a comprehensive study. These oxidases deaminate the decarboxylation products of amino acids, and from a biotechnological perspective have untapped potential. This can be addressed through a concerted enzyme characterisation that draws on the available genomic information. This thesis sets out to provide a thorough examination of the catalytic action, biochemistry and genetic properties of the amine oxidases within *Rhodococcus*. The opening Chapter considers oxidase isolation and kinetic analysis with a particular focus on those enzymes with specificity to primary amines. Enzymes that selectively act on diamines are the subject of Chapter 2. This activity coupled with genomic analysis is central to the discovery of a degradation pathway for putrescine. Confirmation of this pathway is achieved by characterisation of the subsequent enzyme(s) activities, as demonstrated in Chapter 3, with a phylogenetic analysis of the pathway distribution. The manner in which this species adapts oxidase activity over transient amine conditions is developed in Chapter 4 with a consideration of regulatory mechanisms, monitoring depletion and generation substrates, metabolites and by-products. Oxidase
activity integration into the nitrogen cycle is established in Chapter 5 with a consideration of the major nitrogenous organic compounds as substrates, examining the genomic and molecular basis that enables this organism to thrive in various habitats. Implications for future application in bioremediation and biocatalysis are also discussed.
Foreword

In order to elicit elevated amine oxidase activity, manipulation of growth conditions coupled with substrate screening is necessary. Altering the amine source during cell growth has resulted in differential oxidase activity profiles, indicating the occurrence of multiple enzymes with overlapping specificity. High levels of enzyme purity were achieved by precipitation, ion exchange and gel filtration, enabling subsequent characterisation by MALDI-TOF with Michaelis–Menten kinetic analysis. Given the observed broad specificity to a range of structurally diverse natural and synthetic amines, potential exploitation of the enzymatic versatility is discussed in terms of biosensing and bioprocessing.

1.1 Introduction

Amine oxidases (AOs) are an important class of enzyme in both industry and medicine but their widespread use has yet to be fully realised. Variable levels of expression, inconsistencies in enzyme nomenclature, as well as an inability to identify or find enzymes with appropriate levels of activity and specificity have all hindered the selection and isolation of suitable oxidases for a targeted end use. Nevertheless, applications in biocatalysis [1, 2], biosensors [3-5] and therapeutics [6] have been proposed for this heterogeneous group of enzymes, which catalyse oxidative deamination generating the corresponding aldehyde, with ammonia and hydrogen peroxide as by-products [7, 8]

\[
\text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2
\]
These enzymes have been the subject of a number of classical biochemical studies that have assessed substrate activity and redox cofactor (FAD or copper dependent topa quinone), classifying the oxidase based upon differing catalytic mechanism and reaction [9, 10]. For copper dependent oxidases (CuAO) the reaction proceeds in two stages. The initial reductive step a Schiff base is formed between the amine substrate and the topa quinone, which results in the aldehyde product. In the second oxidative stage, ammonia and hydrogen peroxide released however the role of copper in converting the reduced enzyme back to its initial state remains controversial. [9, 10] A consensus regarding the catalytic action of flavin dependent amine oxidases (FlavAO) in the degradation of amines has not emerged in the literature. However, in common with CuAO the co-factor is crucial for the dehydrogenation of the -CH₂-NH₃ group and conversion of O₂ to H₂O₂ [11]

Structurally these two groups of enzymes differ significantly. CuAO are dimers typically composed of three or four domains with two embracing arms as shown Fig 1.1. Whilst the other domains largly form the periphery of the enzyme the fourth domain has considerable influence upon substrate accessibility and specificity. Forming a narrow channel leading to the substrate active site the fourth domain provides the the interface between the subunits, in addition to the linking arms which stabilise the two monomers [12]. Structural analysis of FlavAO from Rhodococcus erythropolis (Fig. 1.1) attributed substrate specificly to negatively charged carboxyl group which acts as an anionic anchor for a second amino group of the substrate. The formation of “aromatic cage” between flavin and two aromatic side chains has also been suggested to perform a steric role in substrate binding and access to the flavin cofactor, as well as increasing the substrate amine nucleophilicity [8, 13].
Such structural analysis has recently greatly enhanced our understanding of catalytic mechanisms in a field which has been hampered by combination of inconsistent and ambiguous enzyme classification, coupled with the difficulties of inferring catalytic properties based on genomic information alone.

It is often difficult to explicitly identify enzymes that has actually been examined [14]. Consequently, there remains little correlation between sequence information and AO attributes, including catalytic behaviour, metabolic pathways and biotechnological relevance. This lack of correlation has limited practical implementation to a few well characterised oxidase, notably those from *Aspergillus* spp. [1-3], *Arthrobacter* sp. [15],...
pea seedling [3] and bovine serum [6, 16]. As the number of fully characterised oxidases increase, the possibilities for commercial exploitation should escalate.

Genomic analysis has revealed the presence of several putative AOs in the gram positive soil bacterium *Rhodococcus opacus* [17, 18], an organism that has been identified as particularly suited to industrial biocatalysis [19]. This genus has found application in fossil fuel biodesulphurisation, steroid biotransformation and acrylamide and acrylic acid production [20-22]. Such a level of utilization results from a combination of facile growth with stability in chemical reaction systems [22]. Moreover, the associated metabolic versatility and biochemical diversity spans a range of structural groups, including halogenated aliphatics and aromatics, substituted benzenes and quinolines [23]. Despite current and potential commercial importance, and progress in recent years [17] the *Rhodococcus* spp. remain poorly characterised in terms of enzymatic action. In this Chapter, we report the isolation, characterisation and identification of two novel copper AOs. We establish the catalytic action of these AOs in the oxidative deamination of a range of aliphatic and aromatic amines and identify potential biotechnological applications.

1.2 Materials and Methods.

1.2.1 Bacterial strain and culture conditions

*Rhodococcus opacus* DSM 43250 was used throughout this investigation. The bacterium was cultured in M9 minimal media with glucose as the carbon source. For induction experiments, ammonium chloride was replaced in the medium with 10 mM of the appropriate amine. For oxidation expression experiments 50 mL of bacteria were grown in 100 mL flasks at 30° C for 3 d under continuous shaking at 130 rpm. For oxidase purification, growth was scaled up to 250 mL in 1 L flasks. The cells were then centrifuged for 20 min at 4° C and 2500 g, the supernatant removed and the cell pellet frozen at -20° C.
1.2.2 *Purification of amine oxidase*

The frozen cell pellet was re-suspended in ice cold TES buffer (pH 7.5) at a concentration of 0.4 g/mL and centrifuged for 30 min at 4 °C and 20000 g. On removal of the supernatant, the cells were resuspended in TES buffer (0.4 g/mL) and twice passed through a French press at a pressure of 138 MPa. In order to ensure complete lysis, cells were sonicated in 30 s intervals on ice for a further 10 min. The cell debris was removed by centrifugation (4°C and 60000 g) for 1 h and passed through a 0.2 µm syringe filter. The filtered lysate was initially purified by a two cut ammonium sulphate precipitation, the first at 30% saturation with the second at 70%. The precipitated protein was re-suspended in TES buffer and dialysed overnight against TES buffer with a cellulose membrane (MWCO 12,000) before purification by FPLC. The majority of the remaining protein was removed using an anion Hi-16 10 Q FF column (1.6 cm × 13 cm) with 50 mM Tris-hydrochloride (pH 7.5) and a linear 1 M sodium chloride gradient. Active fractions were pooled and diluted 2 fold with running buffer to reduce the salt concentration. The protein was then run on a Mono Q 5/50 GL column (0.5 cm × 5 cm) using the same Tris-hydrochloride running buffer and a Tris-hydrochloride/sodium chloride elution buffer. Finally, selected active fractions were purified by gel filtration utilising a gel Superose 12 10/300 GL column (1 cm × 30 cm).

1.2.3 *AO Gel activity staining*

AO activity staining was performed using a peroxidase-coupled system after electrophoresis on a native polyacrylamide gel, as previously described by Lee [24]. The native PAGE was equilibrated twice for 20 min in potassium phosphate buffer (pH 7.5). The gel was then transferred to the substrate solution containing 50 mL potassium phosphate buffer (pH 7.5), 10 mg 3-amino-9-ethylcarbazole and 5 mM amine substrate. After 5 min, 200 µl horseradish peroxidase (5 mg/mL) was added and the gel was gently shaken in the dark for 5-20 min, depending on the observed band intensity.
1.2.4 In-gel digestion of protein spots and protein identification

Protein identification was performed using native gels stained for AO activity, and purified enzyme on SDS-Tris-tricine gels. Gels were rinsed in Millipore filtered water (0.2 µm) three times for 5 min followed by staining with Coomassie Brilliant Blue for 1 h. Gels were de-stained using Millipore water and protein bands excised manually with a scalpel. The remaining stain was removed by incubating the gel slice at 37°C twice with 0.2 mL 100 mM ammonium bicarbonate/50% acetonitrile for 45 min. The wash was removed and replaced with 100 µl 100% acetonitrile for 5 min and dried in Speed Vac for 15 min. Gel slices were rehydrated in 10 µl Trypsin Gold (Promega) (20 µg/mL) and 40 mM ammonium bicarbonate/10% acetonitrile at room temperature for 1 h. An additional 90 µl of 40 mM ammonium bicarbonate/10% acetonitrile was added to prevent complete evaporation before incubation overnight at 37°C. The gel slices were further diluted with 100 µl Millipore water and mixed by vortexing for 10 min. The liquid was then transferred to a microcentrifuge tube and the gel slice digest extracted twice for 60 min by vortexing with 50% acetonitrile/5% trifluoroacetic acid. These extracts were pooled and dried in Speed Vac at room temperature for 2 h. Samples were reconstituted in 10 µl 0.1% trifluoroacetic acid, pipetted and expelled three times with ZipTips (Millipore) which had been preconditioned with 10 µl 100% acetonitrile followed by three washes with 0.1% trifluoroacetic acid. Contaminants were removed by washing ZipTips containing the bound protein a further three times with 0.1% trifluoroacetic acid before eluting 0.3 µl spots directly onto the MALDI plate using 1.5 µl matrix consisting of 70% acetonitrile/0.1% trifluoroacetic acid and 10 mg/mL α-cyano-4-hydroxycinnamic acid. Peptide spectra were generated using an Ettan Matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI TOF MS). The identified peaks automatically selected by the peak seeker algorithm were searched against the NCBI database using MSFIT
Those with a high Mowse score, i.e. a weighted measure of how closely the spectrum matches a protein, were subsequently confirmed using the MASCOT programme (www.matrixscience.com), which generates an “expected” value, i.e. the number of (random) proteins that could achieve the same score for the given spectrum. Proteins with an expectation score ($E$-value) of 0.05 and lower (95% confidence), a minimum sequence coverage of 10% and at least three independent peptides matches were taken as positive identifications.

1.2.5 Enzyme assay

A colorimetric assay was performed in order to determine enzymatic activity. The assay is based on the formation of the hydrogen peroxide by-product, which in the presence of 4-aminoantipyrine, and 2,4,6-tribromo-3-hydroxybenzoic acid is converted in equimolar amounts by an added peroxidase to produce a quinoneimine dye [25]. The reaction was performed in 96 well plates containing 10 µl enzyme and 100 µl freshly prepared assay solution (200 mM potassium phosphate buffer pH 7.6, 1.5 mM 4-aminoantipyrine and 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid). In addition, 20 µl amine substrate and 70 µl 1.4 mg/mL peroxidase from horseradish were added to give a final volume of 200 µl. Absorbance was measured at 510 nm and 30°C using a plate reader (VERSAmax, Molecular Devices). All results were normalised against total protein using Bradford’s reagent method [26]; readings were performed in triplicate and the results fell within a 5% relative standard deviation.

1.2.6 Enzyme inhibition

Enzyme inhibition was achieved using non-competitive inhibitors: semicarbazide; isoniazid; aminoguanidine; cuprizone; neocuprizone pargyline; clorgyline. The stock solutions contained 50 mM Tris-hydrochloride (pH 7.5) and 0.1 mM of the inhibitor. The purified oxidases were incubated on ice in triplicate with 0.1 mM of the inhibitor. After 30 min, the inhibitor-enzyme solution was measured for
oxidase activity using the colorimetric assay with either 10 mM benzylamine (BEN) or phenylethylamine (PHE). The final concentration of inhibitor in the assay was 5 µM.

1.2.7 UV spectra analysis

Spectra were recorded in 50 mM Tris-hydrochloride (pH 7.5) at 25°C on an Eppendorf BioSpectrometer-kinetic. From a cuvet containing 50 µM purified AO, 50 µM BUT was added and the absorbance spectrum was recorded from 600 to 350 nm.

1.3 Results and Discussion

1.3.1 Amine oxidase induction

As AOs are ubiquitously distributed in nature, oxidase specificity and activity within microorganisms are considered to vary substantially [27]. This diversity has classically been explored by limiting the nitrogen source to specific amines in order to induce an enzymatic response. Notable examples include the oxidase of the filamentous fungi Aspergillus niger and the yeast Hansenula polymorpha when grown on butylamine (BUT) and methylamine (MET) [28, 29]. In bacteria, phenylethylamine (PHE) has been identified as an effective inducer of AO in Escherichia coli and Arthrobacter globiformis [30, 31]. The latter has also been observed to possess inducible oxidases after culturing with either MET or histamine (HIS) [32]. These amines along with the structural analogues and non-physiological substrate benzylamine (BEN) [33-35] have demonstrated an ability to promote oxidase expression within a variety of microorganisms [36, 37].

In this study, in order to optimise the detection of oxidase activity within Rhodococcus opacus, MET, BUT and PHE were selected as amines to induce activity, while prevalent natural substrates that may arise primarily by decarboxylation or amination of simple aldehydes and ketones by higher plants [38], were utilized as substrates for oxidase screening. These substrates are identified in Table 1.1 and include
aliphatic monoamines (C₁ to C₅), diamines (putrescine (PUT) and cadaverine (CAD)), heterocyclic amines (HIS and tryptamine (TRY)) and catecholamines (PHE and tyramine (TYR)). Oxidase expression in *Rhodococcus opacus* grown under induction with MET displayed a substantial increase in substrate activity when compared with the control, i.e. ammonium chloride as the sole nitrogen source (see Table 1.1). The cells grown with inorganic nitrogen exhibited some aliphatic diamine activity, notably with respect to PUT, but no detectable catalytic response for the other substrates; detection limit = 0.1 μM/min. The MET induced cells displayed a ten-fold increase in PUT activity but a lesser activity for the longer chain CAD diamine. A comparable low activity level was recorded for the aliphatic monoamines but this was measurably greater than that observed for the aromatic or heterocyclic substrates. The enzymatic response exhibited by BUT induced cells was typically superior, with the exception of PUT and MET as substrates where the MET pre-treatment delivered higher activity. The expressed oxidases resulting from BUT treatment, principally acted on aliphatic monoamines, as shown in Table 1.1, with specific rates for the conversion of C₂ - C₅ amines that were 5-30 times greater those recorded for MET induction. In addition, significant activity was also observed with respect to aromatic and heterocyclic amines. Oxidase expression for cells cultured with PHE was similar to that observed for BUT. There are, however, distinct differences in terms of the level of induction and preferred substrates, notably higher activity for the catechol, heterocyclic and C₄/C₅ aliphatic amines.
Table 1.1: Substrate activity of lysed *Rhodococcus opacus* after inducing oxidase expression by limiting the nitrogen source to methylamine (MET), butylamine (BUT) and phenylethylamine (PHE): specific activity measured in µmole/min/g of total protein; oxidase activity relative to that recorded with ethylamine (ETH) as substrate is given in parentheses (as a percentage). Deviation <5% for each measurement.

<table>
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<th>Substrate</th>
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<th>Activity µmole/min/g of total protein</th>
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<tr>
<td></td>
<td></td>
<td>(NH₄Cl) control</td>
</tr>
<tr>
<td>Putrescine (PUT)</td>
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<td>Cadaverine (CAD)</td>
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<td>Amylamine (AMY)</td>
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<td>Phenylethylamine (PHE)</td>
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<td>Tyramine (TYR)</td>
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<td>Histamine (HIS)</td>
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<td>&lt;0.2</td>
</tr>
<tr>
<td>Tryptamine (TRY)</td>
<td>-NH₂</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

The observed variation in relative levels of activity is possibly indicative of the presence of multiple enzymes within the cell with differing levels of expression. While limiting the nitrogen source to specific amines increased global oxidase expression...
within *Rhodococcus opacus* as anticipated, the results suggest that there is a selective expression of AOs that is dependent on the growth substrate. For instance, the enhancement of activity with respect to aromatic amines after pre-treatment with PHE is a specific upregulation of a corresponding oxidase necessary for growth, which additionally is capable of acting upon other related substrates. However, such is the broad response to the three inducers that cellular stress is a likely contributing factor. Indeed, increased AO levels have been detected under conditions of limited carbon and nitrogen in other organisms [39, 40]. The varying level of cellular response to MET, BUT and PHE treatment must be influenced by the efficiency and specificity of the oxidases (or other enzymes) to degrade these amines, along with transport across the cell membrane and possible toxic effects due to the substrate and resulting aldehyde [41]. Cellular influence on oxidase activity coupled with oxidase heterogeneity (in terms of specificity), constrains the explicit value of the information that can be gleaned from substrate specificity alone. However, a consideration of nitrogen limited conditions can lead to some insight into multiplicity and possible overlap of enzyme activities. For example, there are appreciable differences in AO activity with respect to the C$_2$ to C$_5$ aliphatic monoamines (Table 1.1), which could be expected (as a first approximation) to share the same oxidase [42-44]. The observed variation of substrate preference within this amine grouping suggests that the recorded activity is a composite resulting from a contribution of more than one AO acting on the same substrates to different degrees. Conversely, a common proportionate difference in rate for either catechol or heterocyclic amines (activity for HIS is 1.8-2.0 times that of TRY, activity for PHE is 4 times greater than TYR for BUT and PHE induced cells), points to differing levels of induction. The latter response suggests the observed activity is primarily due to the action of a single oxidase or multiple enzymes with similar specificity.
The results presented in Table 1.1 indicate that the three sets of induction conditions generated three separate cellular responses. With MET as inducer, sub-optimal induction is achieved with relatively low levels of non-specific activity and measurably higher activity with respect to PUT. BUT also provides a broad cellular response but typically elevated activity, notably an order of magnitude increase in the case of C₂-C₄ aliphatic amines. The action of the aromatic PHE inducer also shows a degree of specificity, particularly for the longer chain monoamines and delivered the highest recorded rates. Given that *Rhodococcus* growth was largely unaffected by substrate, as shown in Table 1.2, the observed metabolic shift represents considerable flexibility to adapt as conditions dictate.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Dry cell weight (mg)</th>
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</tr>
<tr>
<td>Methylamine (MET)</td>
<td>25</td>
</tr>
<tr>
<td>Butylamine (BUT)</td>
<td>24</td>
</tr>
<tr>
<td>Phenylethylamine (PHE)</td>
<td>21</td>
</tr>
</tbody>
</table>

### 1.3.2 Purification of oxidases post induction

Prior to any purification, a distinct protein band at a molecular weight of *ca.* 70 kDa was in evidence after culturing with either BUT or PHE, as shown in the SDS-PAGE presented in Fig. 1.2. The intensity of this band corresponded qualitatively with enzyme activity and was poorly defined after culturing with either MET or NH₄Cl. The associated molecular weight is close to the values calculated from the isotopic mass for the unmodified amino acid sequence of the four CuAO present in the *Rhodococcus* genome (Table 3) [17,18,45].
The five other AOs, which utilise flavin as a redox co-factor and catalyse the same deamination reaction, have a predicted molecular weight in the range 49-55 kDa. The four copper oxidases exhibit similar characteristics, with molecular weights of the monomers prior to post-translational modification ± 1.4 kDa and an isoelectric point estimated by the pK of the amino acid sequence ± 0.3 pI [45]. Indeed, such is the sequence similarity that the oxidases may have occurred as paralogs with evolutionary selective pressures leading to divergent selectivity of the duplicated gene. Given the degree of structural commonality and overlapping substrate specificity, alternative oxidase induction conditions were required to facilitate enzyme separation and circumvent any inadvertent misidentification or erroneous characterisation. As MET provided low levels of oxidase induction for the given substrates and a similar substrate preference to that observed with BUT treated cells, the work was focused to consider the contrasting activities resulting from BUT and PHE induction. In order to differentiate between possible AOs, PHE was selected as both inducer and substrate due to the observed reaction specificity and high levels of activity with respect to aromatics.
Table 1.3: Possible oxidases whose action directly liberates ammonia (oxidoreductases acting on the CH-NH₂ group of donors) that have been identified within the Rhodococcus RHA1/Rhodococcus opacus B4 genomes [17, 18]. Corresponding homologues shown by protein BLAST score, with the corresponding molecular weight and pI of the monomer subunit as estimated by Compute pI/Mw [45].

<table>
<thead>
<tr>
<th>Rhodococcus sp. RHA1 GI number</th>
<th>Rhodococcus opacus. B4 GI Number</th>
<th>Identities and BLAST E value</th>
<th>Estimated Molecular weight range (kDa)</th>
<th>Estimated pI range</th>
<th>Co-Factor</th>
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<td>111019599</td>
<td>226361748</td>
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<td>71.7-71.9</td>
<td>4.8</td>
<td>Copper</td>
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<tr>
<td>111022563</td>
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<td>96% E=0</td>
<td>73.0-73.1</td>
<td>4.9-5.0</td>
<td>Copper</td>
</tr>
<tr>
<td>111019825</td>
<td>226240457</td>
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<td>111022572</td>
<td>226365080</td>
<td>97% E=0</td>
<td>49.3-49.5</td>
<td>4.9</td>
<td>Flavin</td>
</tr>
<tr>
<td>111019548</td>
<td>226361698</td>
<td>89% E=0</td>
<td>48.9-49.0</td>
<td>5.7-5.8</td>
<td>Flavin</td>
</tr>
<tr>
<td>111018768</td>
<td>226360855</td>
<td>91% E=0</td>
<td>48.9-49.1</td>
<td>5.4-5.5</td>
<td>Flavin</td>
</tr>
<tr>
<td>111022672</td>
<td>226365184</td>
<td>88%, E=0</td>
<td>50.4-50.7</td>
<td>5.2-5.4</td>
<td>Flavin</td>
</tr>
<tr>
<td>111019880</td>
<td>226359955*</td>
<td>95%, E=0</td>
<td>49.5-55.4</td>
<td>8.9</td>
<td>Flavin</td>
</tr>
</tbody>
</table>

*Initial 54 residues not observed in predicted protein. Taking the full region from Rhodococcus opacus B4 genome identities = 87%

Taking the results presented in Table 1.1, BUT as substrate generated a wide range of activities for the induced cells, suggesting overlapping substrate specificity. Coupling BUT induced enzyme purification with BUT as test substrate would not facilitate an explicit enzyme identification. Although the shorter chain aliphatic amines were not subject to such wide variation, they exhibited the drawback of relatively low activity. The non-physiological substrate benzylamine (BEN) was chosen as a suitable substrate as a high level of specific activity has been established for a number of AOs [28, 35, 40]. Additionally, the shorter alkyl chain (relative to PHE) linking the amino group to the benzene ring can hinder deamination for a number of oxidases. Murooka and co-workers [34] in analysing microbial oxidase distribution found only a single organism capable of oxidative conversion of BEN, while enzymes from diverse bacterial genera readily catalysed deamination of PHE derivatives, including TYR.

The results of the sequential purification steps are summarised in Table 1.4. The level of enzyme purity was monitored (after each purification) by gel electrophoresis (Fig 1.3) and measurement of protein by Bradford's method [26] and enzyme activity with respect to BEN and PHE as substrates.
Table 1: Sequential purification of amine oxidases (AOs) from *Rhodococcus opacus* on a series of FPLC columns. Analysis of the enzymes purified from (A) BUT induced culture (AO1) and (B) PHE induced culture (AO2).

<table>
<thead>
<tr>
<th></th>
<th>Crude Extract</th>
<th>NH4SO4 ppt</th>
<th>Hi Prep Column</th>
<th>Mono Q Column</th>
<th>Superose Column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Protein (mg)</td>
<td>Total Volume (mL)</td>
<td>Protein (mg/mL)</td>
<td>Specific BEN activity µmole/min/g</td>
<td>Enzyme Recovery %</td>
</tr>
<tr>
<td>(A)</td>
<td>Crude Extract</td>
<td>67.01</td>
<td>400</td>
<td>0.168</td>
<td>39</td>
</tr>
<tr>
<td>BUT induced Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>6.9</td>
<td>134</td>
<td>0.20</td>
<td>3.8</td>
<td>65</td>
</tr>
<tr>
<td>93.6</td>
<td>13.2</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>9.6</td>
<td>8.7</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>4.4</td>
<td>8.0</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(B)</td>
<td>Crude Extract</td>
<td>62.04</td>
<td>400</td>
<td>0.155</td>
<td>144</td>
</tr>
<tr>
<td>PHE induced Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>161.5</td>
<td>61.0</td>
<td>400</td>
<td>0.155</td>
<td>144</td>
<td>56</td>
</tr>
<tr>
<td>54.1</td>
<td>13.2</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>81.8</td>
<td>8.7</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>4.7</td>
<td>8.0</td>
<td>1342</td>
<td>0.096</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

15
AO1 from BUT induced Cells

AO2 from PHE induced Cells

Figure 1.3: SDS-PAGE from sequential purification of amine oxidases (AOs) upon a series of FPLC columns. Analysis of the enzymes purified from BUT induced culture (AO1) and PHE induced culture (AO2). (A) Cell Free extract, (B) (NH4)2SO4 ppt, (C) Hi Prep Column 16/10 Q FF, (D) Mono Q 5/50 GL Column, (E) Superose Column 12 10/300 GL.

Following ammonium sulphate precipitation that increased specific activity by a factor of 1.4-1.7, the Hi-Prep ion exchange column separated the bulk of the protein from the AO component for both BUT and PHE induced cells with a 9-19 fold increase in specific activity relative to the crude extract. The active fractions (taking PHE induction as a representative case) displayed a normal distribution as shown in Fig. 1.4. The six fractions with the highest activity were pooled (denoted by the magnified area), before separation using the Mono-Q column.

Figure 1.4: PHE (■) and BEN (●) oxidase activity purification profile for PHE induced cells after purification on the Hi-Prep 16/10 Q FF ion exchange column. Selected fractions for further purification are indicated by the magnified area.
The higher resolution obtained with the second ion exchange column provided a crucial separation of two AOs displaying differing specificity, as shown in Fig. 1.5a and 1.5b for the BUT and PHE induced cells, respectively. Several fractions showed activity towards BEN with no overlapping activity for PHE following BUT induction with the reverse response for the PHE induced cells. Although complete separation (to baseline) in terms of enzyme activity was not achieved, the level of purification was sufficient to isolate fractions associated with the two distinct activities, as denoted by the areas highlighted in Fig. 1.5.

Figure 1.5a

![Graph showing oxidase activity and absorption A280](image)

Figure 1.5b

![Graph showing oxidase activity and absorption A280](image)

Figure 1.5: PHE (●) and BEN (■) oxidase activity purification profile for (a) BUT and (b) PHE induced cells after purification on the Mono Q 5/50 GL column. Magnified area shows fractions with no overlapping activity.
Additionally a possible third oxidase displaying low PHE activity was also detected (Fig. 1.5b). The oxidase (denoted as AO1) that displayed activity towards BEN was eluted prior to the oxidase (AO2) with activity towards PHE. As a fraction of total enzyme activity, the oxidase that eluted first (AO1) was expressed in greater quantities following BUT induction. In the case of purification of PHE induced cells (Fig. 1.5b), the oxidase showing activity with respect to PHE was dominant with a far lesser fraction exhibiting active towards BEN. A single fraction that displayed no detectable activity overlap (Fig. 1.5) was taken in each case, resulting in a low enzyme recovery during the final purification by gel filtration on the Sephadex column (Table 1.4). Despite losing a significant proportion of the total starting enzyme, at the final stage of purification the relative activity was 162 and 91 times greater than that of the crude extract post BUT and PHE induction, respectively.

1.3.3 Enzyme characterisation

AO1 isolated from BUT induced cells with a distinct specificity towards BEN (relative to PHE), was found to act on a broad range of aliphatic monoamines (C₁-C₅). AO2 isolated from PHE treated cells displayed activity towards PHE as well as TYR and heterocyclic amines but negligible conversion of BEN and short chain aliphatic amines. Neither AO1 nor AO2 exhibited any measurable activity when using diamines (PUT or CAD) as substrate. These observations also apply to cells induced with BEN and TYR (data not shown), where enzymes AO1 and AO2 were again isolated. Both oxidases were further characterised by native gel electrophoresis (Fig. 1.6), staining the dimers (ca. 140 kDa by size-exclusion chromatography) directly on the gel with BUT as substrate (see section 1.2.3), where two distinct oxidation bands are evident at slightly different migration points. The overlap of the bands is consistent with overlap in terms of oxidase activity, as shown in Fig. 1.5a.
As AOs are subject to inhibition by an array of compounds, this enables some
differentiation and, with substrate specificity, can serve as a basis for enzyme
classification [46].

AOs that possess a copper co-factor are inhibited by reactive carbonyl reagents
such as semicarbazide, isoniazid and aminoguanidine. As the redox role of copper
during the oxidative catalytic cycle remains somewhat controversial [47], AO1 and
AO2 were also treated with chelating agents (cuprizone and neocuproine). Flavin
dependent monoamine oxidases are not sensitive to these compounds at low
concentrations but are inhibited by parglyline and clorgyline, which have no effect on
AOs with a copper co-factor [48]. Neither enzyme displayed any detectable inhibition
with either parglyline or clorgyline (Table 1.5).

Table 1.5: Inhibition of purified oxidases from BUT (AO1) and PHE (AO2) induced cells. Activity
of the cells incubated (30 min on ice) with selected inhibitors is presented as a percentage of that
obtained for the untreated cells.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>BUT induced cells (AO1)</th>
<th>PHE induced cells (AO2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% BEN activity</td>
<td>% PHE activity</td>
</tr>
<tr>
<td>Pargyline</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Clorgyline</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Isonazid</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Cuprizone</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Neocuproine</td>
<td>98</td>
<td>99</td>
</tr>
</tbody>
</table>
Both enzymes were, however, inhibited by the carbonyl reagents and cuprizone, a response that is consistent with CuAO. All detectable activity was irreversibly inhibited by semicarbazide. Treatment with isoniazid and aminoguanidine, which are known to preferentially (but not selectively) inhibit diamine oxidases [49], also acted to significantly (but not totally) inhibit both AO1 and AO2. Application of cuprizone (cupric chelating agent) served to suppress catalytic action whereas neocuproine (cuprous inhibitor) had no significant inhibitory effect. When both enzymes were subjected to UV spectra analysis, a broad peak was observed at 480 nm (Fig 1.7). This absorption band is characteristic of the oxidized form of the copper dependent topaquinone cofactor.

![UV spectra of purified AO1 (-----) and AO2 (----) in 50mM Tris-hydrochloride (pH 7.5)](http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/sup2008/)

As both AOs displayed activity towards monoamines and possess a copper cofactor, they can both be classified in terms of the IUBMB (International Union of Biochemistry and Molecular Biology) nomenclature as primary amine oxidases (EC 1.4.3.21) [46] see supplement 14 (http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/sup2008/). Based on our results, we can discount classification as diamine oxidase EC 1.4.3.22 (due to substrate preference), monoamine oxidase EC 1.4.3.4 and polyamine oxidase EC 1.5.3.11 (due to co-factor).
1.3.4 Proteomic identification and genomic analysis

The enzyme bands were extracted from the native activity gel and identified by MALDI–TOF analysis. The results were matched to the proteins associated with *Rhodococcus* RHA1 and *Rhodococcus opacus* B4, which are genomically similar and display synteny conservation [17, 18, 50]. Matching at least seven tryptic digest fragments, analysis confirmed the identification of two oxidases based on an E-value (expectation value, see section 1.2.4) ≤ 0.05. Additional induction conditions that resulted in similar phenotypes validated further the matching identification of the oxidases; the results are summarised in Table 1.6.

Table 1.6: Amine oxidases expressed by *Rhodococcus opacus* after inducing the cells with four different amines. All proteins were identified by MALDI-TOF MS analysis and matched to the *Rhodococcus RHA1* using both MSFIT and Mascot.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>GI Number</th>
<th>M_r (Da)</th>
<th>PI</th>
<th>% sequence coverage/peptides</th>
<th>Mowse Score</th>
<th>MSFIT E-value</th>
<th>Mascot E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUT</td>
<td>111019599</td>
<td>71695</td>
<td>4.6</td>
<td>29/11</td>
<td>1.5e+8</td>
<td>9.1e-06</td>
<td></td>
</tr>
<tr>
<td>BEN</td>
<td>111019599</td>
<td>71695</td>
<td>4.6</td>
<td>23/9</td>
<td>3.8e+7</td>
<td>1.2e-05</td>
<td></td>
</tr>
<tr>
<td>TYR</td>
<td>111019825</td>
<td>72051</td>
<td>4.9</td>
<td>23/12</td>
<td>15758</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>PHE</td>
<td>111019825</td>
<td>72051</td>
<td>4.9</td>
<td>12/12</td>
<td>70024</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

AO1 was matched to GI 111019599 (E-value in the range 9.1e-06 - 1.2e-05) and AO2 identification was consistent with GI 111019825 (E-value in the range 0.009-0.021) from two separate induction conditions. These highly significant expectation values were calculated from the entire NCBI database, which was not limited by organism, PI or size. As shown by the representative MALDI–TOF spectrum (for AO1) in Fig. 1.8, over twenty distinct peaks in the m/z range 500-3000 were observed, only three of which were not matched to this oxidase. One of these represents a low molecular weight (m/z = 569) and a second (m/z = 2386) exhibited low intensity. The high percentage of matching peptide peaks, coupled with high sequence coverage, (Table 1.6) provides a high confidence for positive identification. With the exception of the near identical oxidases from *Rhodococcus opacus* B4, the protein with greatest homology (by BLAST) to AO1 is AO2 (63% “identities”, 75% “positives”).
Figure 1.8: MALDI-TOF Mass spectrum of AO1 digested with trypsin. Detected peaks using the peakseeker algorithm are displayed, with corresponding matches within 1 Da to the predicted enzyme (at m/z = 741.2, 1180.4, 1185.5, 1258.4, 1266.3, 1466.4, 1503.4, 1648.4, 1802.4, 1818.3, 1877.3, 2030.4, 2500.4, 2513.1, 2599.2, 2613.1, 2876.9). Unmatched peaks (at m/z = 569.2, 1523.5, 2383.9) denoted by *. Possible post-translational modifications: Phosphorylation; Dihydroxylation; Dimethylation.

The majority of the sequence variation between the homologues of AO1 and AO2 occurs on the structural domains (such as those that encode the domains 2 and 3 Fig 1.1) that lie on the periphery of the globular protein. The enzyme component that forms the active site, and therefore has a considerable influence on specificity, exhibited 72% “identities” and 83% “positives”. The closest characterised enzyme to both AOs occurs in another gram-positive soil bacterium (Arthrobacter globiformis) that shares 57 and 64% “identities” with AO1 and AO2 homologues respectively. It should be noted that Arthrobacter sp. possesses two CuAO with high homology and specificity towards PHE or HIS [32].

The catalytic response recorded in this study provides some insight into possible implications for the surrounding genes, given the small evolutionary distance between the species [51] and implicated regions are conserved (>95% identities by BLAST) within both Rhodococcus RHA1 and Rhodococcus opacus B4. AO1 is clustered among genes that are predicted to be involved in the metabolism of a range of xenobiotics [17].
A gene encoding an aldehyde dehydrogenase (GI 111019601) is separated from AO1 by a single amino acid permease and, as such, is likely to act on the resulting generated aldehyde. The possible involvement of this enzyme is significant in that while numerous micro-organisms are capable of oxidising BEN, few can utilise the resulting benzaldehyde due to its high toxicity [35]. To date, only *Pseudomonas putida*, *Paracoccus denitrificans* IFO 12442 and *Mycobacterium* sp. JC1 DSM3803 have shown significant growth with BEN as the sole substrate [35] *Mycobacterium* is the only example where this action is mediated by a copper amine oxidase [35]. In our investigation, *Rhodococcus opacus* was also found to grow on BEN as the sole energy source. We can propose a possible contribution due to nearby padR (GI 111019609), the gene encoding a known repressor in the transcriptional control of catabolic pathways of aromatic compounds [52]. AO2 is found in close proximity to a group of previously studied PAA genes [53]. Phenylacetaldehyde generated from PHE oxidation is further degraded via a phenylacetic acid pathway, as demonstrated by knockout mutagenesis studies on *Rhodococcus* RHA1 [53]. When either PAAN, a putative ring-opening enzyme, or PAAF phenylacetyl-CoA ligase were mutated, no growth was observed with PHE [53]. This observation suggests that PHE is the main substrate for AO2 and phenylacetaldehyde is catabolised by nearby genes as part of the phenylalanine degradation pathway.

**1.3.5 Kinetic Analysis**

Kinetic analysis was undertaken using a standard Michaelis-Menten approach over an extended concentration range in order to quantify the catalytic action of the purified AOs. Crucial for adaptation and survival, the range of substrates was extended beyond those presented in Table 1.1 to include structural analogues that are synthetic or less naturally abundant; these are identified in Table 1.7. As molecular surface topology and charge considerably influence catalytic efficiency of AOs [54], their action is
determined by substrate chain length, steric hindrance and charge distribution. Therefore, taking AO1, additional aliphatic mono-amines and BEN derivatives were also tested (Table 1.7). These included ethanolamine (ETO) possessing a polar functionality, apolar branched sec-butylamine (sec-BUT), aromatic methylbenzylamine (MBEN) and halogen (4-fluorobenzylamine (FBEN)) and methoxy (vanillylamine (VAN)) congeners.

Table 1.7: Additional amines selected for a Michaelis-Menten kinetic analysis of the catalytic action of AO1 and AO2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolamine (ETO)</td>
<td><img src="structure.png" alt="Ethanolamine structure" /></td>
</tr>
<tr>
<td>sec-Butylamine (sec-BUT)</td>
<td><img src="structure.png" alt="sec-Butylamine structure" /></td>
</tr>
<tr>
<td>Benzylamine (BEN)</td>
<td><img src="structure.png" alt="Benzylamine structure" /></td>
</tr>
<tr>
<td>4- Fluorobenzylamine (FBEN)</td>
<td><img src="structure.png" alt="4-Fluorobenzylamine structure" /></td>
</tr>
<tr>
<td>Methylbenzylamine (MBEN)</td>
<td><img src="structure.png" alt="Methylbenzylamine structure" /></td>
</tr>
<tr>
<td>Vanillylamine (VAN)</td>
<td><img src="structure.png" alt="Vanillylamine structure" /></td>
</tr>
<tr>
<td>Amphetamine (AMP)</td>
<td><img src="structure.png" alt="Amphetamine structure" /></td>
</tr>
<tr>
<td>Octopamine (OCT)</td>
<td><img src="structure.png" alt="Octopamine structure" /></td>
</tr>
<tr>
<td>Phenylpropanolamine (PPA)</td>
<td><img src="structure.png" alt="Phenylpropanolamine structure" /></td>
</tr>
<tr>
<td>1-Methyl-3-phenylpropylamine (MPP)</td>
<td><img src="structure.png" alt="1-Methyl-3-phenylpropylamine structure" /></td>
</tr>
<tr>
<td>Dopamine (DOP)</td>
<td><img src="structure.png" alt="Dopamine structure" /></td>
</tr>
</tbody>
</table>
The catalytic response for AO2 was probed by considering substituted phenethylamines as substrates, which included amphetamine (AMP), 1-methyl-3-phenylpropylamine (MPP) and encompassed hydroxyl substituent(s) on the benzene ring (octopamine (OCT) and dopamine (DOP)) and the carbon chain linking the amino-group to the ring (phenylpropanolamine (PPA)). Representative activity/substrate concentration plots for oxidative deamination promoted using AO1 and AO2 are presented in Fig. 1.9a and 1.9b, respectively. An increase in activity was observed with increasing concentration to attain activity maxima in the range 0.1-1 mM, with typically a subsequent decrease at higher concentrations, suggesting substrate inhibition. Substrate inhibition was not observed for the shorter chain amines where catalytic efficiency was lower in AO1. This extended to the conversion of AMY and HIS by AO2, where relative catalytic efficiency and substrate affinity were poor relative to the preferential substrates. Similar observations have been reported for Porcine AO, where a decrease in activity at higher concentrations was attributed to the binding of more than one substrate molecule to the active site [55]. In the case of Arthrobacter globiformis, it was reasoned that two amines attach during the reductive cycle of the reaction where the second amine binds near the active site or interacts with the post-translated amino acid residue (TPQ) that is required for electron transfer [56]. In common with those studies, inhibition of AO1 and AO2 was consistent with a simple one-site binding model, where

$$V = V_{max} [S]/(K_M + [S] + [S^2]/K_I),$$

as shown in Fig. 1.9.
Figure 1.9A

Figure 1.9B

Figure 1.9: Initial reaction rate as a function of substrate (PRO (□), BUT (▲), AMY (●), PHE (■) and TYR (▲), DOP (★)) concentration for oxidative deamination promoted using (A) AO1 and (B) AO2. Note: curves generated by fitting the data to the expression \( V = \frac{V_{\text{max}} [S]}{K_{M} + [S] + [S^2]/K_I} \).

1.3.6 Analysis of AO1 kinetics

From a consideration of the Michaelis (\(K_M\)) and rate (\(k_{\text{cat}}\)) constants given in Table 1.8, it is clear that the oxidase catalytic action is strongly dependent on the nature of the substrate. Simple aliphatic amines are characterised by \(K_M\) values that, with the exception of MET, were inversely related to chain length. This can be attributed to an increased stabilization and substrate positioning afforded by an enhanced interaction with the enzyme. In the case of bovine serum AO, substrates were proposed to bind to
two different regions, depending on structure and charge distribution of the substrate [57]. The lower $K_M$ observed for MET as well as the aromatic substrates (an order of magnitude lower for BEN and VAN) suggest different interaction/docking at the AO1 active site that is sensitive to amine structure and aromaticity. Amine recognition within this class of enzyme is partially achieved by a “substrate channel” that governs access to the active site within the protein structure. This channel is composed of amino acid side chains that facilitate the catalytic reaction by directing and positioning the amino group of the substrate [58].

Table 1.8: Kinetic parameters obtained from a Michaelis-Menten treatment of oxidative deamination promoted by AO1 and AO2; “ND” denotes where no inhibition was detected.

<table>
<thead>
<tr>
<th>AO1</th>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s^{-1})</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
<th>$K_I$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylamine (MET)</td>
<td>0.18</td>
<td>0.4</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ethylamine (ETH)</td>
<td>0.86</td>
<td>7.2</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Propylamine (PRO)</td>
<td>0.49</td>
<td>18.9</td>
<td>39</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>Butylamine (BUT)</td>
<td>0.32</td>
<td>15.3</td>
<td>48</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Amylamine (AMY)</td>
<td>0.11</td>
<td>5.9</td>
<td>54</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine (ETO)</td>
<td>2.05</td>
<td>3.3</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Benzylamine (BEN)</td>
<td>0.04</td>
<td>12.1</td>
<td>303</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>Vanillylamine (VAN)</td>
<td>0.02</td>
<td>1.0</td>
<td>50</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>4-Fluorobenzylamine (FBEN)</td>
<td>0.13</td>
<td>17.3</td>
<td>133</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AO2</th>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s^{-1})</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
<th>$K_I$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenethyethylamine (PHE)</td>
<td>0.02</td>
<td>20.9</td>
<td>1045</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Tyramine (TYR)</td>
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<td>16.1</td>
<td>322</td>
<td>0.4</td>
</tr>
<tr>
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<td>Octopamine (OCT)</td>
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<td>3.5</td>
<td>116</td>
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<tr>
<td></td>
<td>Dopamine (DOP)</td>
<td>0.10</td>
<td>1.9</td>
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<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Histamine (HIS)</td>
<td>0.28</td>
<td>13.2</td>
<td>47</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Butylamine (BUT)</td>
<td>0.32</td>
<td>4.3</td>
<td>13</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Amylamine (AMY)</td>
<td>0.16</td>
<td>12.7</td>
<td>79</td>
<td>ND</td>
</tr>
</tbody>
</table>

In the case of ETO, the polar hydroxyl moiety, as opposed to any steric influence, is the likely source of the considerably higher $K_M$ and lower associated $k_{cat}$. It must be noted that there was no detectable conversion of $sec$-BUT as substrate where steric hindrance, arising from branched C-NH$_2$, must inhibit the oxidation reaction; this
response extended to MBEN. The highest $k_{cat}$ values were obtained with PRO and BUT, which may reflect their abundance in the natural environment, as both these amines are associated with the degradation of vegetation and animal waste [38, 59]. The highest catalytic efficiency, in terms of low $K_M$ and high $k_{cat}$, was achieved with the aromatic BEN substrate ($k_{cat}/K_M = 303 \text{ mM}^{-1} \text{s}^{-1}$, see Table 1.8). The presence of fluorine as a ring substituent in the para-position (FBEN) served to increase both $K_M$ and $k_{cat}$ relative to BEN. Similar observations have been reported for both mouse and human SSAO where $k_{cat}$ increased from 0.18 s$^{-1}$ (BEN) to 0.23 s$^{-1}$ (FBEN) and 0.45 s$^{-1}$ to 0.71 s$^{-1}$, respectively [60]. It was proposed that the smaller BEN substrate was more readily accommodated in the active site but hindered release of the aldehyde product to a greater extent than the bulkier FBEN [60]. To the best of our knowledge, this is the first instance of FBEN oxidative deamination using a micro-organism. The presence of hydroxyl and methoxy groups on the benzene ring (in VAN) did not significantly alter $K_M$, but resulted in an appreciably lower $k_{cat}$.

The heterogeneity that characterizes AOs makes comparison with published work difficult as the studies that have considered variations in both specificity and activity have been limited with respect to the range of substrates that were considered. There are several instances where oxidases with low $K_M$ have been recorded for individual substrates but the response does not appear to apply to a broad spectrum of amines. For instance, $K_M$ in the range 0.2-2 mM for C$_1$-C$_4$ amines was achieved with the methylamine oxidase of Arthrobacter P1 [42] and can be compared with 0.24-1.6 mM for C$_3$-C$_6$ amines using Aspergillus niger [43] and 0.23-1.5 mM for C$_1$-C$_5$ amines with Candida boidinii [36]. When these AOs were tested for aromatic activity, Candida boidinii did not display any activity with respect to BEN [36], the oxidase of Arthrobacter P1 exhibited poor substrate affinity ($K_M = 3.76 \text{ mM}$) while BEN readily underwent oxidative conversion by Aspergillus niger due to two separate AOs; $K_M =$
0.24 mM, $k_{cat}$ of 37 s$^{-1}$ for the copper oxidase and $K_M = 0.75$ mM, $k_{cat}$ of 100 s$^{-1}$ for FAD dependent oxidase [42, 43]. The observed deviation in $K_M$ presumably provides an innate advantage for a variety of environments and must be partially responsible for the isolation of *Rhodococcus* in diverse conditions [51]. The enzymatic and cellular characteristics provide opportunities for biotechnological implementation of AOs as medical sensors and in bioprocessing. AO1 can potentially be utilised in the detection of phospholipids if coupled with phospholipase D and an assay has been developed to detect phosphatidylethanolamine, a prominent phospholipid in mammals that is linked to apoptosis, cell signalling and coagulation [61]. The *Arthrobacter* oxidase utilised to detect phosphatidylethanolamine displayed a $K_M = 15$ mM and $k_{cat} = 1.2$ s$^{-1}$ with respect to ETH. The sensitivity can be improved with AO1, which exhibited a significantly lower $K_M$ and higher $k_{cat}$, as shown in Table 1.8. An alternative application is in vanillin production, a commercially important flavouring agent in foods/beverages and in pharmaceutical processes [62]. Currently, the vast majority of vanillin is chemically synthesised, as natural forms are expensive and limited by plant supply. A demand, however, remains for natural vanillin, providing an opportunity for microbial conversion as demonstrated by the successful but still relatively expensive approach that utilises ferulic acid [63]. Peppers and capsicums are low cost sources of VAN, which can be formed *via* cleavage of the secondary metabolite capsaicin by proteases. Application of AO from *Escherichia coli* and *Aspergillus niger* has been considered where immobilisation of the more active *Aspergillus niger* oxidase resulted in a significant drop (by ca. 80% from $k_{cat} = 1.0$ s$^{-1}$) in enzyme activity, possibly due to structural changes resulting from enzyme binding to the cellulose support [1]. AO1 exhibited an equivalent VAN conversion rate in its unbound form but shares a relatively low sequence homology with the *Aspergillus* oxidase. Although further study is required to assess AO1 in an immobilised form, the results generated in this study
suggest that it is a viable alternative, especially given the presence of eugenol oxidase in *Rhodococcus*, a second enzyme that can oxidise VAN, providing an additional vanillin bioconversion pathway [64, 65].

1.3.7 *Analysis of AO2 kinetics*

As was the case with AO1, AO2 did not exhibit any activity with branched amine substrates, including sec-BUT, PPA, MPP or AMP. It should be noted that the CuAO of *Klebsiella oxytoca* has been reported to promote a low but measurable oxidation of AMP [66]. The highest $k_{cat}/K_M$ ratio was delivered by PHE, supporting the genomic analysis that this is the preferred substrate. The presence of a para-positioned hydroxyl substituent on the ring (TYR) lowered catalytic efficiency, with an increase in $K_M$ and lower $k_{cat}$, which we ascribe to steric hindrance and polar effects. This was further compounded in the case of DOP that bears a second hydroxyl substituent. The presence of a hydroxyl substituent on the side chain (in OCT) did not significantly affect $K_M$ but resulted in a significantly lower $k_{cat}$. The importance of the phenyl ring to influence substrate positioning and enhance substrate affinity in AO2 is illustrated in the case of HIS where the associated $k_{cat}/K_M$ was lower by a factor of 22 relative to PHE. A similarly low catalytic efficiency was recorded for the aliphatic amines. Lower activity with respect to heterocyclic HIS relative to PHE and decreased affinity for aliphatic amines that was inversely proportional to chain length has been noted in the literature [43, 44, 61]. The AOs of *Aspergillus niger, Klebsiella oxytoca* and *Arthrobacter* sp. have all been reported to act on aromatic, aliphatic and heterocyclic amines [43, 61, 66]. However, this capacity is not as prevalent in bacteria where the work of Murooka *et al.* is notable in that 16 strains were found to show catecholamine activity but only one was active with respect to HIS [34]. The oxidase from *Aspergillus niger* exhibited $K_M = 0.12$-0.6 mM for aromatic amines [43], appreciably higher than that measured for AO2 but the $k_{cat}/K_M (= 1142 \text{ s}^{-1} \text{ mM}^{-1})$ for PHE obtained with *Aspergillus niger* is close that
recorded for AO2 (Table 1.8). In contrast, the oxidase of *Arthrobacter glomerulus* exhibited a lower $K_M$ for PHE (0.003 mM) and TYR (0.017 mM) and comparable $k_{cat}$ values 8.9 s$^{-1}$ and 25.2 s$^{-1}$ respectively, but the highest substrate concentration tested (0.033 M) due to substrate inhibition was much lower than that used in this study (Fig. 1.6) [61]. The oxidases of *Klebsiella oxytoca*, on the other hand, possessed lower $K_M$ (0.029-0.003 mM) but similar $K_I$ (0.7-2.6 mM) values [66].

In terms of potential applications, AO2 specificity towards pathophysiological amines that are associated with food spoilage can be exploited to control amine levels in food [67]. In an investigation of biogenic amine degradation from 166 food strains, complete degradation of 0.54 mM HIS and 0.58 mM TYR after 24 hours from a starter culture was only achieved with *Rhodococcus* sp. [67]. Other oxidases with similar specificity have found use in biosensing to determine quality and freshness of fish [4, 5] and other foodstuffs [13] where there is still considerable scope for improvement in terms of specificity and activity. As amines are also environmental pollutants, the ability of *Rhodococcus opacus* to act on a range of aromatic amines as presented here, in addition to a number of other harmful compounds such as naphthalene, herbicides and PCBs [51] serves to highlight the potential of this organism not only in biotechnological applications but also in bioremediation of contaminated waters and soils.

1.4 Conclusions

Our study demonstrates that *Rhodococcus opacus* is a catabolically rich strain, with the capacity to act on an array of amine substrates. In the approach that we have taken by limiting the nitrogen source to inorganic nitrogen (NH$_4$Cl), MET, BUT or PHE, the contrasting phenotypic response in terms of AO expression provided the means of resolving enzymatic multiplicity and facilitating isolation. Coupling of enzyme purification with use of BEN and PHE as test substrates
and the action of inhibitors has enabled the isolation and identification of two copper oxidases (EC 1.4.3.21) that we label AO1 and AO2 and which share 63% homology with overlapping specificity. Despite the similarities, the two oxidases appear to participate in alternative degradation pathways as indicated by gene clustering. The prevalence of *Rhodococcus opacus* in multiple environments can, in part, be attributed to its large genome and considerable catabolic potential. Functional expression, broad substrate affinity and turnover of multiple AOs with their subsequent catabolic pathways exemplify genomic capacity, enabling adaptation and survival. A Michaelis-Menten analysis of AO1 has revealed that this enzyme preferentially deaminates aliphatic amines and aromatic amines where the amino group is attached to the benzene ring via a short alkyl chain (*eg.* BEN). The AO2 oxidase can also act on aliphatic amines but with a preference for aromatic amines that bear a longer alkyl chain linkage (*eg.* PHE or TYR). As both enzymes possess appreciable substrate plasticity (including synthetic amines), coupled with high catalytic efficiency, we believe there is considerable scope for exploitation in bioprocessing and biosensing. Additional oxidases within this organism remain to be characterised. Future work will focus on the identification of these enzymes and their relevance for *Rhodococcus opacus* as part of both the carbon and nitrogen cycle.

### 1.5 References


Chapter 2 - Role of amine oxidase expression to maintain putrescine homeostasis in *Rhodococcus opacus*

**Foreword**

Genomic analysis presented in Chapter 1 has revealed a number of putative oxidases. Further substrate screening is required to probe the catalytic action of these enzymes. Limited success was achieved in targeting FAD dependent oxidases with growth on secondary and branched amines. However, extension of the putrescine and cadaverine tests from Chapter 1 served to elevate activity by an FAD and copper dependent oxidase. The two diamine oxidases are examined by applying the induction/isolation/characterisation approach introduced in Chapter 1. From a consideration of metabolic gene clustering, a previously uncharacterised microbial amine degradation pathway is postulated.

**2.1 Introduction**

*Rhodococci* possess remarkable innate metabolic versatility coupled with a tolerance to stress conditions, which have led to applications in the bioremediation of a range of recalcitrant contaminants, including petroleum hydrocarbons, pesticides, explosives and a diversity of chlorinated, nitrogenated and hydrophobic organic compounds [1-3]. Successful exploitation has been achieved by utilising isolated indigenous strains stimulated with the addition of nutrients and via bioaugmentation through the addition of *Rhodococcus* spp. with a high degradation capacity [3]. Current methodologies are limited as the catabolic complement often dictates microbial selection rather than ecological demands and corresponding phenotypic properties that are critical in enabling the bacterium to remain functionally active in a given environment [4]. For many bacteria such as *Rhodococcus* spp. the success of such microbial intervention is intrinsically linked to polyamine homeostasis, which under
inhospitable conditions plays a key role in cellular viability and growth. Ubiquitously distributed in nature, diamines influence a number of key cellular processes by binding electrostatically or covalently to a range of macromolecules, including RNA and proteins [5-7]. The most prevalent bacterial diamine is putrescine (PUT), which in addition to numerous cellular roles, impacts global transcription and translation by stimulating RNA polymerase expression, stabilising ribosomal structure and modulating translational fidelity [8-10]. Consequently, gene expression is indirectly associated with increased cellular PUT and elaborate regulatory mechanisms are pivotal to ensure a narrow concentration range [10]. Coordinated uptake, synthesis, utilisation and excretion via both positive and negative effectors on enzymes and associated processes [11] provide a cytoprotective role, ensuring viability with respect to a range of stressors associated with environmental pollution, notably low pH, oxygen radicals, ultraviolet light, γ-radiation and antibiotics [12-15]. Disruption or inability to optimally regulate this PUT balance will reduce viability under such conditions, while normal cellular activities including growth, colonisation and microbial competition are impaired as has been illustrated using the rhizobacteria Pseudomonas fluorescens [16]. Consequently, biostimulation with PUT can have a profound positive effect on cellular activity in a contaminated environment and addresses two fundamental ecological barriers: nutrient availability; stress response to toxicity.

The biosynthetic pathway via the decarboxylation of arginine or ornithine has been the main focus of polyamine homeostasis research due to the principal role in regulating intracellular concentration. However, catabolism and excretion are important in eliminating excess PUT, which would otherwise inhibit protein synthesis and impact cell viability [17, 18]. Three bacterial pathways for PUT degradation have been reported, involving aminotransferase enzymes or γ-glutamylated intermediates to form 4-aminobutyrate [19, 21]. Although amine oxidases are essential for the deamination of
\( \gamma \)-glutamylputrescine, none of the verified pathways utilise oxidases that act directly on the diamine [22] to generate the corresponding aldehyde (4-aminobutanal), with ammonia and hydrogen peroxide as by-products [23,24]:

\[
\text{H}_2\text{N} - \text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{H}_2\text{N} - \text{CH} = \text{CH}_2 + \text{NH}_3 + \text{H}_2\text{O}_2
\]

In this chapter, we report the isolation, characterisation and identification of two PUT acting amine oxidases. *Rhodococcus opacus* is particularly relevant as this species is often native to polluted environments and is a viable agent in bioaugmentation and other direct applications where similar strains have been utilised for microbial-assisted phytoremediation [25,26]. The genome has previously been fully sequenced, identifying several putative oxidases of unknown specificity. In addition, the genome possesses a homologue to flavin oxidase (putrescine oxidase, EC 1.4.3.10), which has been only characterised in *Kocuria rosea* (*Micrococcus rubens*) and recombinantly from *Rhodococcus erythropolis* [23,24,27]. A high catalytic efficiency has also previously been demonstrated for this species, with PUT providing the greatest initial growth from 190 carbon sources [28]. The response of oxidase expression to changes in environmental conditions is explored by altering the nitrogen source and is linked to an oxidase degradation pathway, which we propose as the main diamine catabolic route for *Rhodococcus* spp. Implications of such activity in coordinating nutrients, stress and degradation capacity are proposed.

### 2.2 Materials and methods

#### 2.2.1 Bacterial strain and culture conditions

*R. opacus* DSM 42350 was used throughout this investigation. For amine oxidase expression and inhibition studies, the bacterium 50 mL of bacteria were grown in 100 mL flasks in M9 minimal media with 4 mM glucose as the carbon source and 0.5–25 mM ammonium chloride (\( \text{NH}_4\text{Cl} \)), putrescine (PUT), cadaverine (CAD) or
butylamine (BUT) as the sole nitrogen source. For growth experiments, cells were limited to 5 mM PUT, 4-aminobutanal, 4-aminobutyrate or succinate semialdehyde as the sole energy source. Cultures were grown at 30 °C for 3 days with continuous shaking at 130 rpm. Cells were harvested by centrifugation for 20 min at 4 °C and 2500 × g and the cell pellet frozen at −20 °C.

2.2.2 Purification of amine oxidase

The frozen cell pellets were re-suspended in ice cold TES buffer (pH 7.5) at a concentration of 0.4 g/mL and centrifuged for 30 min at 4 °C and 20,000 × g to wash the cells. After removal of the supernatant, the cells were again resuspended in TES buffer (0.4 g/mL) and twice passed through a French press at a pressure of 138 MPa. In order to ensure complete lysis, the cells were sonicated in 30 s intervals on ice for a further 10 min. The cell debris was removed by centrifugation (1 h at 4 °C and 60,000 × g), passing the supernatant through a 0.2 μm syringe filter. The majority of non-specific protein was removed using an anion Hi-Prep 16 10 Q FF column (1.6 cm × 13 cm) with 50 mM Bis-Tris–hydrochloride (pH 6.5) and eluted over a 1 M NaCl gradient. Depending on the amine oxidase, the protein was either purified directly using a pre-activated native affinity resin or fractionated on a high resolution ion exchange resin after a two-fold dilution. In the affinity resin purification, active fractions were run on an EAH Sepharose 4B (GE Healthcare) column (0.8 cm × 1 cm) and eluted in 50 mM Bis-Tris–hydrochloride (pH 6.0) with a 0.4–0.6 mM NaCl gradient. For ion exchange chromatography, active fractions were pooled and run on a Mono Q 5/50 GL column (0.5 cm × 5 cm) using Tris–hydrochloride running buffer (pH 7.0) with a subsequent gel filtration step on a Superose 12 10/300 GL column (1 cm × 30 cm).
2.2.3 In-gel digestion of protein spots and protein identification

The amine oxidases were analysed on a SDS-Tris-tricine gel (ClearPAGE). The gel was rinsed with Millipore filtered (0.2 μm) water three times for 5 min followed by staining with Coomassie Brilliant Blue R250 solution (G-Biosciences) for 1 h. Gels were de-stained with Millipore water and protein bands displaying the correct size were excised manually with a scalpel. The remaining stain was then removed by incubating the gel slice at 37 °C twice with 0.2 mL 100 mM ammonium bicarbonate/50% acetonitrile for 45 min. The wash was removed and replaced with 100 μl acetonitrile for 5 min and the sample dried in a Speed Vac for 15 min. Gel slices were rehydrated in 10 μl Trypsin Gold (Promega) (20 μg/mL) and 40 mM ammonium bicarbonate/10% acetonitrile at room temperature for 1 h. An additional 90 μl 40 mM ammonium bicarbonate/10% acetonitrile was added to prevent drying and incubated overnight at 37 °C. The gel slices were further diluted with 100 μl Millipore water and vortexed for 10 min. The liquid was transferred to a micro-centrifuge tube and the gel slice digest extracted twice with 50% acetonitrile/5% trifluoroacetic acid for 1 h by vortexing. The extracts were pooled and dried in a Speed Vac at room temperature for 2 h. Samples were reconstituted in 10 μl 0.1% trifluoroacetic acid, pipetted and expelled three times with ZipTips, which had been preconditioned with 10 μl acetonitrile followed by three washes with 0.1% trifluoroacetic acid. Contaminants were removed by washing ZipTips containing the bound protein a further three times with 0.1% trifluoroacetic acid before eluting 0.3 μl spots directly onto a Maldi plate using a 1.5 μl matrix consisting of 70% (v/v) acetonitrile/0.1% trifluoroacetic acid and 10 mg/mL α-cyano-4-hydroxycinnamic acid. Peptide spectra were generated using an Ettan Matrix-assisted laser desorption/ionisation time of flight mass spectrometer (MALDI–TOF MS). The peaks automatically selected by the peak seeker algorithm were searched against the NCBI database using MSFIT (http://prospector.ucsf.edu). Those with a high Mowse score, i.e.
a weighted measure of how closely the spectra matches a protein, were subsequently confirmed using the MASCOT programme (http://www.matrixscience.com), which generates an expected value (the number of (random) proteins that could achieve the same score for that spectrum). Proteins with an expectation score \((E\text{-value})\) of 0.05 and lower (95% confidence), a minimum sequence coverage of 10% and at least three independent peptide matches were taken as positive identification.

### 2.2.4 Peroxide assay

A colorimetric assay was used to determine amine oxidase activity. The assay is based on the conversion of hydrogen peroxide, a by-product of the amine oxidase reaction, in the presence of a peroxidase, catalysing the conversion of 4-aminoantipyrine and 2,4,6-tribromo-3-hydroxybenzoic acid to produce a quinoneimine dye [29] that is detectable by spectrophotometry. The reaction was performed in 96 well plates containing 10 \(\mu\)l purified enzyme or cell lysate and 100 \(\mu\)l freshly prepared assay solution (200 mM potassium phosphate buffer pH 7.6, 1.5 mM 4-aminoantipyrine and 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid). A solution containing 20 \(\mu\)l amine substrate and 70 \(\mu\)l 1.4 mg/mL peroxidase from horseradish was added to initiate the enzymatic reaction in a final volume of 200 \(\mu\)l. Absorbance was measured at 510 nm and 30 °C using a plate reader (VERSA\(_{\text{max}}\), Molecular Devices). Oxidase activity was normalised with respect to total protein using Bradford's reagent method; measurements were performed in triplicate and all reported results fell within a 5% relative standard deviation.

### 2.2.5 Enzyme inhibition

Studies on enzyme inhibition were carried out using irreversible selective suicide inhibitors. These included hydrazine derivatives (semicarbazide, isoniazid and aminoguanidine) that act on the TOPA-quinone residue of copper amine oxidase, cuprous and cupric chelating agents (cuprizone and neocuprizone) with inhibitory
properties that are dependent on the metallocentre and propargylamine derivatives (pargyline and clorgyline) that inhibit flavin oxidases by binding covalently to the cofactor [30,31]. Stock inhibitor solutions were prepared in 50 mM Tris–hydrochloride (pH 7.5). The cell lysate or purified amine oxidases were incubated on ice in triplicate with 0.1 mM inhibitor. After 30 min, amine oxidase activity with respect to PUT (10 mM) as substrate was measured using the colorimetric assay described above with a final inhibitor concentration of 5 μM in the assay.

2.2.6 UV spectra analysis

Spectra were recorded in 50 mM Tris-hydrochloride (pH 7.5) at 25°C on an Eppendorf BioSpectrometer-kinetic. From a cuvet containing 50 μM purified AO, 50 μM PUT was added and the absorbance spectrum was recorded from 600 to 350 nm.

2.3 Results and discussion

2.3.1 Regulation of diamine oxidase expression

Amine oxidase activity was investigated by limiting the nitrogen source to specific amines, thereby forcing deamination for cellular survival with an anticipated upregulation of catabolic activity to utilise the essential nutrient [32]. Ammonium chloride (NH₄Cl) was adopted as a benchmark growth substrate and assessed against naturally occurring diamines (Table 2.1), which are produced by putrefactive bacteria and de novo polyamine biosynthesis.

Table 2.1: Dry cell weight of Rhodococcus opacus culture grown in M9 Media (at 30 oC for 3 days) with four nitrogen sources.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Structure</th>
<th>Dry cell weight (mg/mL)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td></td>
<td>25</td>
<td>1.3</td>
</tr>
<tr>
<td>Putrescine (PUT)</td>
<td>H₂N—C—NH₄</td>
<td>25</td>
<td>2.1</td>
</tr>
<tr>
<td>Cadaverine (CAD)</td>
<td>H₂N—C—NH₄</td>
<td>18</td>
<td>1.5</td>
</tr>
<tr>
<td>Butylamine (BUT)</td>
<td></td>
<td>24</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Putrescine (PUT) as the primary and prominent diamine was compared with the longer chain but less abundant cadaverine (CAD) [33]. The ubiquitously distributed butylamine (BUT) [34] as a representative monoamine was also considered as this amine has been reported to competitively inhibit a putrescine oxidase from *R. erythropolis* [24]. These nitrogen sources are associated with decaying matter and frequently introduced (in the form of compost) as biostimulants to accelerate bioremediation [35]. Moreover, many *Rhodococcus* spp. continue to degrade pollutants even when accessible energy sources such as amines are available [36]. When nitrogen availability in the growth medium was limited solely to NH$_4$Cl, a low level of oxidase activity within the cell lysate was observed (Fig. 2.1).

![Graph](Figure 2.1: Oxidative deamination activity with respect to PUT (●) and CAD (■) as a function of increasing NH$_4$Cl concentration used as nitrogen source in *Rhodococcus opacus* cell cultures grown at 30°C for 72 h. Error bars indicate SD.)

This background oxidase action, in the absence of exogenous amines implies either a key role of these enzymes in the conversion of intracellular derived amines and/or selective advantages of constitutive expression in a native environment that typically contains diamines. Catabolic activity in the conversion of PUT and CAD is sensitive to
NH₄Cl concentration with a low but detectable oxidase activity at ≤1 mM and a maximum achieved with cells pre-treated with 5 mM NH₄Cl. Reduced cellular metabolic activity at low concentrations can be linked to limited nitrogen availability as a common bacterial response to such an environmental stress [37]. Conversely, a decrease in activity was observed with NH₄Cl concentrations > 5 mM. It has been noted elsewhere [38] that excess ammonium ions can act to repress amine oxidases and in some instances cause irreversible inhibition [39]. Culturing in PUT enriched media, in the absence of other nitrogen sources enhanced oxidase activity considerably, with increased deamination at higher PUT concentrations (up to 25 mM), as shown in Fig. 2.2.

Figure 2.2: PUT oxidative deamination activity of *Rhodococcus opacus* cells grown (at 30 ºC for 72 h) in the presence of increasing concentrations of PUT (●), CAD (■) and BUT (▼) as the sole nitrogen source. Error bars indicate SD.

A similar response was observed for the oxidative action with respect to CAD (see Fig. 2.3), albeit with a lower overall oxidase activity. This observation demonstrates a directed cell response to eliminate and utilise exogenous diamines through the action of amine oxidases. Growth on CAD further enhanced oxidase activity in the conversion of PUT (Fig. 2.2) and CAD (Fig. 2.3) with an activity maximum when cells were grown in
media containing 5 mM CAD. The difference in oxidase activity in response to growth using the two diamines is significant and is indicative of an altered regulatory mechanism for the two substrates. The greater oxidase activity resulting from CAD relative to PUT pre-treatment can be associated with a lesser capability of the organism to utilise CAD as a nitrogen source, which is indicated by the lower dry cell weight compared with that resulting from growth in NH₄Cl or PUT (Table 2.1).

![Graph showing oxidase activity vs amine concentration](http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/sup2008/)

**Figure 2.3:** CAD oxidative deamination activity of *Rhodococcus opacus* cells grown (at 30 ºC for 72 h) in the presence of increasing concentrations of PUT (○), CAD (■) and BUT (▲) as the sole nitrogen source. Error bars indicate SD.

Cells pre-treated with BUT displayed appreciably lower amine oxidase activity that was insensitive to BUT concentration. Given that a high cell growth is maintained with BUT (see Table 2.1) and that monoamines are typically deaminated by alternative oxidases [40] see supplement (http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/sup2008/), the observed residual activity reflects a maintenance of background/constitutive oxidase activity as seen for cells grown on NH₄Cl.
2.3.2 Inhibitor specificity

The cellular oxidase response was probed further by testing inhibitor sensitivity. Cells cultured with NH₄Cl, PUT or CAD did not exhibit any detectable response to the action of flavin inhibitors (pargyline or clorgyline), as shown in Table 2.2. Moreover, inhibitors which target copper diamine oxidases (isoniazid, semicarbazide and cuprizone) also had a negligible effect on oxidative deamination.

Table 2.2: Irreversible inhibition of oxidative deamination activity with respect to PUT for *Rhodococcus opacus* cells grown using four nitrogen sources (5 mM). Oxidase activity measured after cells were incubated (at 0 °C for 30 min) with selected inhibitors is presented as a percentage of the activity obtained with untreated cells.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>NH₄Cl</th>
<th>CAD</th>
<th>PUT</th>
<th>BUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paragyline</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>Clorgyline</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>99%</td>
<td>99%</td>
<td>98%</td>
<td>73%</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>56%</td>
</tr>
<tr>
<td>Cuprizone</td>
<td>100%</td>
<td>100%</td>
<td>98%</td>
<td>67%</td>
</tr>
</tbody>
</table>

In contrast, cells grown with BUT exhibited lower diamine activity due to the action of these inhibitors, with up to 44% of total activity subject to inhibition. The total diamine activity observed for cells grown on BUT may then represent the activity of a copper dependent enzyme (CuAO) in addition to the enzyme(s) active under NH₄Cl and diamine pre-treatment. Further inhibition was not observed as semicarbazide concentration was increased ten-fold (data not shown), indicating that the observed response was a composite effect due to the induced CuAO and the constitutive insensitive oxidase, accounting for approximately half of the observed total activity.

As the level of oxidase activity is dependent on the growth substrate and concentration (Fig. 2.1, Fig. 2.2 and Fig. 2.3), inhibition by semicarbazide, as the most potent inhibitor, was examined over a range of substrate concentrations; the results are shown in Fig. 2.4. Cells grown on NH₄Cl exhibited low activity that was unaffected by treatment with semicarbazide. This suggests negligible contribution due to CuAO in the catalytic response shown in Fig. 2.1. The CAD cultured cells did not display any
significant inhibition by semicarbazide at lower concentrations but there was a definite response at $[\text{CAD}] = 25 \text{ mM}$. This contrasts with cells grown with PUT that exhibited a significant loss of oxidase activity for PUT concentration $> 10 \text{ mM}$; differences in the degree of inhibition were magnified with increasing amine concentration. As the high level of diamine catabolism shown by cells grown on CAD (Fig. 2.2) is largely immune to inhibition by semicarbazide, this supports the proposal that alternative metabolic processes are involved. The data presented in Fig. 2.4 reveal that growth with PUT, BUT (and CAD to a lesser extent) is subject to concentration related promotion of alternative enzymatic processes. BUT grown cells underline cellular flexibility, maintaining background/constitutive diamine activity with CuAO, in response to the biochemical character (nitrogen source/inhibition) of the environment. Furthermore, CAD grown cells illustrate that the regulatory mechanism is not a simple dose-dependent activity system as the high associated activity exhibited low levels of inhibition by semicarbazide, in contrast to the PUT grown cells.

Figure 2.4: Inhibition of PUT oxidative deamination activity by semicarbazide (0.1 mM) for *Rhodococcus opacus* cells grown (at 30 °C for 72 h) in increasing concentrations of PUT (●), CAD ( ■), BUT (▼) or NH4Cl (▲) as the sole nitrogen source. Error bars indicate SD.
It should be noted that BUT has been reported to inhibit spermidine synthase and ornithine decarboxylase [41,42], the latter a key polyamine regulator involved in PUT synthesis [43]. While few flavin diamine acting oxidases have been studied in detail, it has been established that this class of enzyme exhibits substrate or competitive inhibition under growth conditions that are comparable to those used in this study, e.g. elevated PUT concentrations in a Gram-positive soil bacterium [44], CAD in *Micrococcus rubens* [45] and BUT in *R. erythropolis* [24].

### 2.3.3 Oxidase purification and isolation

Explicit characterisation and identification require separation of the constitutive enzyme activity associated with semiarbazide insensitive oxidases from the adaptive transient cellular response where enhanced deamination is due to CuAO. Culturing with 5 mM CAD as the sole nitrogen source was employed for the isolation of the constitutive oxidase, given the activity maxima (Fig. 2.2 and Fig. 2.3) and insensitivity to the semicarbazide inhibitor (Fig. 2.4). The induced CuAO was purified from cells grown in 25 mM PUT, *i.e.* conditions that resulted in the highest level of oxidase activity (Fig. 2.2) coupled with semicarbazide sensitivity (Fig. 2.4).

The cell lysate (56 mg protein) from CAD cultured cells was first separated using a Hi-Prep Q FF ion exchange column that removed the majority of protein from the enzyme component with a 14-fold increase in specific PUT oxidative deamination activity (from 0.19 to 2.73 μmol/mg/min). This primary separation limited non-specific binding on the subsequent affinity column (Sepharose 4B EAH).

![Figure 2.5: SDS-PAGE of sequential AO purification from CAD grown cells. (A) Cell free extract, (B) Hi-Prep 16/10 Q FF, (C) Sepharose 4B EAH.](image-url)

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Taking the Hi-Prep fraction that exhibited the greatest oxidase PUT activity, the remaining protein (3.4 mg) was applied directly to the affinity column, further increasing specific activity to 26.42 μmol/mg/min, i.e. 139-fold increase relative to the crude extract, representing 62% of the starting oxidase activity, as shown in Fig 2.5. This approach was not suitable for isolating the CuAO from PUT grown cells due to weak binding on the affinity matrix. In order to achieve a high level of purity, the cell lysate (35.9 mg protein) was initially precipitated using ammonium sulphate, which resulted in a 1.7-fold increase in PUT oxidase activity (from 0.18 to 0.30 μmol/mg/min). Further fractionation (of 19.5 mg active protein) using a Hi-Prep Q FF ion exchange column generated seven contiguous fractions that displayed oxidase activity (0.84 μmol/mg/min). These were pooled (giving a total 6.6 mg protein) and separated on the higher resolution MonoQ column; the resultant purification profile is shown in Fig. 2.6.

Figure 2.6: MonoQ purification profile of amine oxidases from *Rhodococcus opacus* cells grown in the presence of 25 mM PUT as the sole nitrogen source. Oxidative deamination of PUT is shown with (×) and without (●) the semicarbazide inhibitor. The two fractions (#7 and #18) containing separate oxidases are denoted by ▼.
The peroxide-based assay was used to determine PUT oxidative deamination activity and two fractions (#7 and #18, see Fig. 2.5) can be taken as representative of two different oxidases within *R. opacus*. Treatment with the semicarbazide inhibitor completely suppressed catalytic activity for fraction #18 while the enzymatic response for fraction #7 was unaltered. Sensitivity to semicarbazide discriminates between constitutive and induced enzyme activity. The relative size of the two peaks correlates with the observed profiles shown in Fig. 2.4, where approximately one third of the activity can be attributed to CuAO. In a final purification step, 0.3 mg protein from fraction #18 (CuAO, exhibiting a specific activity of 9.48 \( \mu \)mol/mg/min) was subjected to gel filtration. This final step increased specific activity by a factor of 83 (14.92 \( \mu \)mol/mg/min) relative to the starting cell lysate, with 0.1 mg of protein exhibiting 25% of total oxidase activity as shown in Fig 2.7.

![Figure 2.7: SDS-PAGE of sequential AO purification from PUT grown cells. (A) Cell Free extract, (B) \((NH_4)_2SO_4\) ppt, (C) Hi Prep Column 16/10 Q FF, (D) Mono Q 5/50 GL Column, (E) Superose Column 12 10/300 GL.](image)

When both enzymes were subject to UV spectrum analysis, the AO isolated *Rhodococcus opacus* cells grown with CAD demonstrates a spectrum typical of a protein containing flavin with absorbance maxima at 375 and 457 nm (Fig 2.8). A yellow supernatant illustrating non-covalently bound FAD similar to oxidases of *Rhodococcus erythropolis* and *Micrococcus rubens* [23, 24]. As with previous copper
oxidases isolated (section 1.3.3) a broad peak was observed at 480 nm for the second AO further confirming the second enzyme has a copper dependent topaquinone cofactor.

![UV spectra of purified AO from CAD grown cells (---) and PUT grown cells (—) in 50mM Tris-hydrochloride (pH 7.5)](image)

**Figure 2.8: UV spectra of purified AO from CAD grown cells (---) and PUT grown cells (—) in 50mM Tris-hydrochloride (pH 7.5)**

In order to validate that two enzymes (semicarbazide-insensitive oxidase and CuAO) were chiefly responsible for the observed diamine oxidase activity, the purification protocol was repeated with cells grown with PUT, CAD and BUT at various concentrations (data not shown). There were no detectable additional oxidases that exhibited PUT deamination.

### 2.3.4 Enzyme identification

The two purified oxidases were subjected to SDS-PAGE electrophoresis and the observed single bands excised (Fig 2.5 and 2.7), enzymatically digested with trypsin, the peptide spectra generated and identified by MALDI–TOF. Bioinformatic comparison was made with the putative proteins encoded in the genomes of *R. opacus* B4 (the same species as target organism whereby both contain large (8 Mb) genomes with considerable extrachromosomal DNA) and *Rhodococcus* RHA1 (a species known to be genomically similar and to display synteny conservation) [27, 46-]
49]. The semicarbazide insensitive oxidase was confirmed as putrescine oxidase EC 1.4.3.10, a flavin dependent enzyme (FlavAO). The match was greatest with respect to *Rhodococcus* RHA1 (GenBank ID: ABG97386.1) and characterised by high sequence coverage (≥24%) with *E*-values in the range 0.01–0.04 for cells grown in PUT and CAD (Table 2.3). Homologues of this enzyme in the publicly accessible databases were only found in Gram-positive actinomycetes, typically those with a soil origin, as shown in Table 2.4. These sequences all contain very similar active site regions and most likely represent orthologues of FlavAO, given the lack of sequence divergence and narrow species diversity. We can flag the close match with *Renibacterium salmoninarum* (*E*-value = 0, “identities” = 75%), a pathogenic fish bacterium [50, 51]. As the immune system within fish is positively modulated by PUT, both in terms of gene expression and cellular action (increasing respiratory burst and phagocytic capacity of leucocytes) [52], this oxidase may confer some of this organism’s pathogenic properties. It should be noted that the putrescine oxidase of *R. erythropolis* (*E*-value = 0, “identities” = 92%) [24] and *K. rosea* (*E*-value = $1.0 \times 10^{-180}$, “identities” = 68%) [23, 53] have previously been characterised and the latter proposed for use in biosensors. We can flag a potential application in the determination of microbial spoilage, providing a significant advantage over plant oxidases typically employed due to enhanced PUT specificity [54, 55]. CuAO was best matched with an oxidase within the *Rhodococcus* RHA 1 genome (GenBank ID:ABG97377.1). This was established under two separate growth conditions with a high degree of confidence, *E*-values in the range 0.002–0.05 (Table 2.3). By comparing the sequence with the databases using the BLAST algorithm [56], no match for this oxidase was found within the genome of *R. erythropolis*, suggesting a differing diamine homeostasis mechanism.
Table 2.3: Amine oxidases expressed by Rhodococcus opacus after inducing the cells with different nitrogen sources. All proteins were identified by MALDI-TOF MS analysis and matched to Rhodococcus RHA1 genome using both MSFIT and Mascot.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Protein Identifier</th>
<th>GenBank ID</th>
<th>Mr (kDa)</th>
<th>pI</th>
<th>Sequence coverage/peptides</th>
<th>Mowse Score (MSFIT)</th>
<th>E-value (Mascot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUT (5 mM)</td>
<td>Putrescine oxidase</td>
<td>ABG97386.1</td>
<td>49.463</td>
<td>4.86</td>
<td>32/11</td>
<td>5.2e+7</td>
<td>9.8e-3</td>
</tr>
<tr>
<td>BUT (5 mM)</td>
<td>Copper oxidase</td>
<td>ABG97377.1</td>
<td>73.186</td>
<td>5.00</td>
<td>16/10</td>
<td>3.9e+7</td>
<td>2.0e-3</td>
</tr>
<tr>
<td>PUT (25 mM)</td>
<td>Copper oxidase</td>
<td>ABG97388.1</td>
<td>98.494</td>
<td>4.96</td>
<td>32/11</td>
<td>2.2e+7</td>
<td>0.05</td>
</tr>
<tr>
<td>BLT (5 mM)</td>
<td>Putrescine oxidase</td>
<td>ABG97396.1</td>
<td>98.463</td>
<td>4.96</td>
<td>32/11</td>
<td>2.2e+7</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2.4: Species, natural environment and BLASTP analysis of the closest sequence homologues of putrescine oxidase (111023572).
The copper amine oxidases that have been characterised and which exhibit diamine specificity are almost entirely limited to higher taxa, notably plant oxidases. Besides the homology to other CuAO genes within *R. opacus*, the only meaningful match (sequence identity > 65%) was to genes from various *Mycobacterium* species with the closest agreement for the oxidase from *Mycobacterium abscessus* ATCC 19977 (GenBank ID: CAM64277.1). While no enzymatic characterisation has been performed, it is significant that the enzyme is located within a cluster of functionally related enzymes that are very similar to that found within the *R. opacus* genome, as shown in Fig. 2.9.

**Rhodococcus RHA1 and Rhodococcus opacus B4**

**Mycobacterium abscessus** ATCC 19977

![Genomic cluster of functionally related genes surrounding both oxidases in Rhodococcus RHA1/Rhodococcus opacus B4 (> 97% amino acid sequence identical) and Mycobacterium abscessus ATCC 19977. Anotation is based on phylogenetic classifications (COGs), multiple sequence alignments and hidden Markov models (Pfam and TIGRFAM). Proteins implicated from this study in a direct catabolic role for diamines are identified by open arrows, functionally related proteins by solid arrows and those of undetermined significance by hatched arrows.](image)

In addition to the oxidase, homology between the species extends to aldehyde dehydrogenase (GenBank ID:CAM64276.1), 4-aminobutyrate transaminase (GenBank ID: CAM64280.1), succinate semialdehyde dehydrogenase (GenBank ID: CAM64281.1) and amino acid permease (GenBank ID: CAM64279.1) with associated *E*-value = 0 and “identities” ≥ 70%, suggesting a conserved region and
interdependence of enzymes. The lack of homologues or known microbial copper diamine oxidases is intriguing with respect to evolution and the environmental pressures that have led to their emergence and possible exploitation. *R. opacus* has exhibited efficient uptake of copper, lead and chromium (biosorption) [57] but at high levels these metals are also phytotoxic and result in excess generation of PUT by plants [58]. As other stressors lead to elevated efflux [59-61] and PUT is a key root exudate for rhizobacteria [16], an altered copper/PUT microenvironment may serve a symbiotic nutrient protective role, which is significant in potential applications such as microbe assisted phytoremediation.

### 2.3.5 Metabolic gene clustering

Published detailed analysis of the putrescine oxidase class of enzyme has focused to date entirely on the activity and structure of those isolated from *K. rosea* and *R. erythropolis* [23, 24]. In contrast to the verified microbial PUT degradation pathways involving the action of aminotransferases or γ-glutamylated intermediates [19, 20, 62] in initial oxidation step(s), the role of oxidases that act directly on PUT and their integration with subsequent catabolic enzymes within a strain is yet to be analysed. The product of FlavAO/CuAO is 4-aminobutanal, which cyclises to give a cyclic imine (1-pyrroline) [23, 63]. Although the fate of 4-aminobutanal remains unclear, it is widely believed to follow the aminotransferase pathway where it is converted by 4-aminobutyraldehyde dehydrogenase (an enzyme common in bacteria [64, 65]) and successively by 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase to give succinate, as shown in Fig. 2.10.
Figure 2.10: Putative polyamine degradation pathway for *Rhodococcus opacus*. The proposed pathway draws on a combination of genomic information and enzyme activity.

Homologues for 4-aminobutyraldehyde dehydrogenase, 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase are all found within the same genomic region of the oxidases in *Rhodococcus* RHA1 and *R. opacus* B4. This metabolic cluster of functionally interrelated genes (Fig. 2.6) contains both oxidases separated by just 10,000 bases (6 genes). In addition, agmatine deiminase (GenBank ID: ABG97371.1/BAH53903.1) and an amidohydrolase (GenBank ID: ABG97372.1/BAH53904.1), which are predicted to be involved in the synthesis of PUT, are found in close proximity. One of the genes in the same cluster as CuAO (Fig. 2.6) is an aldehyde dehydrogenase (GenBank ID: ABG97379.1/BAH53911.1) that shows considerable homology to a number of aminobutyraldehyde dehydrogenases. BLASTP analysis against the publicly available databases [56] yields E-values of 0.0 and $2 \times 10^{-167}$ for *R. salmoninarum* and *Acinetobacter baumannii*, respectively. Moreover, subsequent degradative enzymes (4-aminobutyrate transaminase (GenBank ID: ABG97378.1/BAH53910.1) and succinate semialdehyde dehydrogenase (GenBank...
ID: ABG97376.1/BAH53908.1) flank CuAO (Fig. 2.6), which act to convert 4-aminobutyrate sequentially to succinic semialdehyde and succinate [66]. These genes, in addition to functionally related amino acid permease and a universal stress protein, are predicted by the “Database of prOkaryotic OpeRons” (DOORs) to occur within the same operon [67, 68]. Although the biochemical function of universal stress proteins remains unknown, results suggest that they are expressed in response to a variety of environmental insults in order to maintain viability [69]. The genomic association may be related to toxicity of the coupled enzymes or the expression of the predicted operon may be a coordinated stress-related mechanism, given the elevated CuAO activity (Fig. 2.4) and importance of PUT homoeostasis. Co-regulation of successive interdependent enzymes has been reported for the aminotransferase PUT pathway in Pseudomonas where PUT pre-treatment increased expression of several genes [70], notably succinate dehydrogenase and 4-aminobutyrate aminotransferase. Moreover, in the case of a ΔgabT strain of Escherichia coli, there was no observed growth with 4-aminobutanoic acid and the enzymatic expression of 4-aminobutyrate aminotransferase was reliant on the presence of PUT [64].

In order to support the genomic evidence for the proposed degradation pathway, R. opacus was grown on the intermediates identified in Fig. 2.7: 4-aminobutanal; 4-aminobutyrate; succinate semialdehyde (supplemented with NH₄Cl). Dense cell growth (>15 mg/mL dry cell weight) was achieved within 3 days with the exception of 4-aminobutanal, which exhibited slower growth (ca. 4 mg/mL dry cell weight). The capacity to utilise these substrates for growth confirms that R. opacus is enzymatically capable of promoting degradation. Given that the predicted enzymes occur in the genomic vicinity of two diamine acting oxidases where enzymatic expression is considerably enhanced by diamine availability, we propose that this is a
2.3.6 **Substrate specificity and deamination kinetic analysis**

Both isolated enzymes (CuAO and FlavAO) possess narrow substrate specificity, acting principally on diamines. While CuAO displayed a capacity to convert some primary monoamines (benzylamine and phenylethylamine), activity was significantly lower (by an order of magnitude) relative to that recorded for the conversion of PUT. There was no detectable monoamine activity in the case of FlavAO. The oxidative transformation of diamines in *K. rosea* has been proposed to proceed via the binding of one amine group of the substrate at an anionic point near the active site, which enables oxidation of the second amino moiety [42]. Kinetic analysis was undertaken using a standard Michaelis–Menten approach over an extended concentration range (0.02–1.5 mM) in order to quantify the catalytic performance of the two purified oxidases with respect to the Michaelis constant ($K_M$) and maximum turnover ($k_{cat}$). Representative activity/substrate concentration plots for PUT oxidative deamination promoted using CuAO and FlavAO are presented in Fig. 2.11 where the applicability of the Michaelis–Menten treatment can be seen from the fit to the experimental results. FlavAO exhibited some flexibility in terms of catalytic response, promoting the oxidative deamination of longer chain CAD and hexamethylenediamine (HEX) substrates, where associated steric constraints may account for the lower catalytic efficiency ($k_{cat}/K_M$) relative to PUT, as shown in Table 2.5. The decrease in $K_M$ in the sequence HEX > CAD > PUT is consistent with an increased stabilisation and enhanced substrate positioning at the enzyme active site.
Figure 2.11: Initial reaction rate as a function of PUT concentration for oxidative deamination promoted using FlavAO (□) and CuAO (○). Note: curves generated by fitting activity data to $k_{cat}$[PUT]/($K_M$ + [PUT]).

It is instructive to note that conversion of the polyamine spermidine (SPE) is characterised by a lower $K_M$ and higher $k_{cat}$ relative to HEX, which suggests that the distance along the hydrocarbon chain between the two amine groups is a critical factor. No detectable activity was observed for the shorter ethylenediamine (ETH), indicating that the chain length did not facilitate the necessary binding to both the anionic anchor point and flavin co-factor within the active site.

Despite the high degree of sequence similarity between FlavAO isolated in this study and its homologue within R. erythropolis [24], the measured kinetic parameters show significant deviations (Table 2.5). While the $k_{cat}$ values are similar in magnitude, $K_M$ for the R. erythropolis oxidase is significantly lower and catalytic efficiency was an order of magnitude higher in the deamination of PUT [24]. Regardless of the differences in substrate affinity, both enzymes exhibit commonality in terms of substrate preference as demonstrated in Table 2.5, when the $k_{cat}/K_M$ values are normalised with respect to PUT as the favoured substrate. Neither enzyme displayed any measurable catalytic conversion of ETH and exhibited diminished catalytic
efficiency with increasing diamine hydrocarbon chain length (from PUT to HEX). The differences in overall catalytic action must therefore be due to the involvement of CuAO, as neither the gene nor the surrounding metabolic cluster occurs in \textit{R. erythropolis}. Given that \textit{R. opacus} can express a second enzyme capable of deaminating the same substrate, this enables further manipulation and control of PUT homostasis. The CuAO exhibited greater catalytic efficiency relative to FlavAO for the diamines examined (Table 2.5). In contrast to FlavAO, CuAO promoted the oxidative deamination of ETH, illustrating the differing substrate/active site interaction for the two enzymes that leads to reaction. Moreover, CuAO did not present any detectable activity for SPE as substrate, which is in line with the reported inactivity of copper dependent oxidases with respect to triamines [40]. As the generation of hydrogen peroxide in oxidative deamination is stoichiometric, the high $k_{\text{cat}}$ delivered by both oxidases with PUT as substrate is significant for biostimulation. Hydrogen peroxide is utilised as an oxidant in bioremediation, serving to promote the degradation of a number of recalcitrant compounds [71].

In enzyme catalysis this by-product can be utilised by peroxidases in the conversion of phenol and aniline. In this respect, we can flag lignin peroxidase of \textit{Bjerkandera} BOS55 which converts high priority polycyclic and aromatic pollutants [72], in bacteria such lignin peroxidases have only been isolated and identified in \textit{Rhodococcus} RHA1 [73, 74]. Supply of hydrogen peroxide has been identified as a rate limiting factor and a targeted nutrient supplementation to endogenously generate hydrogen peroxide has been proposed as an effective way to enhance catalytic efficiency [72]. We envisage a catalytic synergism involving amine oxidase and peroxidase as a progressive environmental remediation strategy.
Table 2: Kinetic parameters obtained from a Michaelis-Menten analysis of oxidative deamination by purified CuAO and FlavAO. The activity of *Rhodococcus erythropolis* is also given as a benchmark: catalytic efficiency ($k_{cat}/K_M$) relative to that recorded for PUT as substrate is given in parentheses (as a percentage); "ND" denotes that no activity was detected.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>R. Opacus CuAO</th>
<th>R. Opacus FlavAO</th>
<th>R. Erythropolis Putrescine oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (µM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_M$ (s$^{-1}$·µM$^{-1}$)</td>
<td>$k_{cat}/K_M$ (s$^{-1}$·µM$^{-1}$)</td>
</tr>
<tr>
<td>Ethylenediamine (ETH)</td>
<td>410</td>
<td>2.3</td>
<td>5.6</td>
<td>(1.8%)</td>
</tr>
<tr>
<td>Putrescine (PUT)</td>
<td>60</td>
<td>18.2</td>
<td>319</td>
<td>(100%)</td>
</tr>
<tr>
<td>Cadaverine (CAD)</td>
<td>260</td>
<td>5.0</td>
<td>19.5</td>
<td>(17%)</td>
</tr>
<tr>
<td>Hexamethylenediamine (HEX)</td>
<td>350</td>
<td>4.3</td>
<td>39.9</td>
<td>(3.9%)</td>
</tr>
<tr>
<td>Spermidine (SPE)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

"Ethanol is also given as a benchmark: catalytic efficiency ($k_{cat}/K_M$) relative to that recorded for PUT as substrate is given in parentheses (as a percentage); "ND" denotes that no activity was detected."
2.4 Conclusions

Progress in bioremediation has been hampered by a fundamental lack of understanding of the prevailing physiological functions, regulation and processes. This study has examined the role of polyamines in both proliferation and maintainance of cell viability. The cellular response of *R. opacus* in terms of oxidase catalytic action with respect to different substrates and concentrations points to effective environmental regulatory mechanisms and adaptive capabilities that draw on dynamic enzymatic deamination. An appreciation of different physiological demands provides insight not only into evolution in the natural environment but also possible cellular manipulation in bioremediation applications. A full understanding of PUT homeostasis can facilitate microorganism selection to optimise biostimulation of indigenous and introduced *Rhodococcus* and ensure ecosystem stability and resilience, notably in terms of crop productivity and the removal of pollutants through a number of indirect mechanisms, notably the use of peroxidases. While not explicitly tested here, bioaugmentation or direct enzyme application can also be applied in future technologies for odour control of agricultural waste, where PUT and CAD represent pungent volatiles [75]. As Gram-positive bacteria such as *R. opacus* are unable to excrete or store any substantial amounts of PUT [76], our results suggest that CuAO is expressed to maintain an intracellular concentration of polyamines in response to either FlavAO inhibitors, such as BUT, or excess diamine levels. This functional interdependence to maintain a common degradation capacity is perhaps best illustrated in the conversion of PUT with $K_M = 190 \mu M$ and $60 \mu M$, respectively, for FlavAO and CuAO and similar substrate turnover ($k_{cat} = 21.8 s^{-1}$ and $18.2 s^{-1}$). We propose that the catalytic action of both oxidases provide the principal PUT degradation pathway, leading to the generation of 4-aminobutanal, which may be oxidised and further converted to 4-aminobutyrate and succinate semialdehyde. The degradation of PUT ultimately to succinate can be
achieved by enzymes within *R. opacus* and which appear (by functional convergence) to surround both oxidases. Cellular capacity for growth from each of the intermediates as the sole energy source supports the proposed pathway, which has not been characterised in prokaryotes. Further genomic and catalytic studies will aim to explicitly link oxidase activity with the catabolic fate of PUT, unravelling regulatory factors and potential stress related mechanisms that underpin the response to changing environmental conditions and associated taxonomic distribution.

### 2.5 References


Chapter 3 - Genomic organisation, activity and distribution analysis of the microbial putrescine oxidase

Foreword

Putrescine specific amine oxidases acting in tandem with 4-aminobutyraldehyde dehydrogenase are explored to confirm the degradative pathway implied from genomic analysis (see Fig. 2.7 in Chapter 2). By limiting the nitrogen source, increased catalytic activity was induced that facilitated oxidation of putrescine to 4-aminobutyraldehyde and subsequent conversion to 4-aminobutyrate. The associated 4-aminobutyraldehyde dehydrogenase are identified as an enzyme belonging to the same metabolic cluster (see Fig. 2.6 in Chapter 2). Further genomic/phylogenetic analysis has revealed highly similar metabolic gene clustering among members of Actinobacteria, providing new insight into putrescine degradation, notably among other members of Micrococcaceae, Rhodococci and Corynebacteruim.

3.1 Introduction

Polyamines are ubiquitous in nature. Although their influence on prokaryotes remains poorly understood, they are essential for normal cellular growth and function and are implicated in a spectrum of physiological responses and molecular interactions [1]. Putrescine (PUT), as the major polyamine in bacteria [2, 3], serves to stimulate both RNA and protein synthesis [4, 5]. Stringent regulation of this diamine is therefore required to ensure an optimal intracellular environment for both cell viability and proliferation, a balance that must also encompass broader functional requirements such as response to external stressors [2, 6-8]. Current information on PUT homeostasis in prokaryotes is largely based on Escherichia coli (Gram negative), notably with respect to PUT biosynthesis via ornithine and arginine pathways [9], uptake and excretion through antiporter and ATP dependent transport proteins [3, 10-12], as well as degradation [13, 14] and corresponding feedback mechanisms [12, 15]. These pathways,
including their constituent enzymes and processes are not universal and it is therefore of fundamental importance to characterise PUT related mechanisms in other taxa in order to elicit the functional roles of polyamines.

PUT biosynthesis has been the subject to many studies as this is the primary means of cellular polyamine regulation. By comparison, PUT catabolism remains somewhat neglected but plays an important role in eliminating excess PUT that would otherwise inhibit macromolecule synthesis and cell viability [16, 17]. Three major catabolic mechanisms for PUT degradation have been proposed, i.e. γ-glutamylation, direct oxidation and acetylation[13, 14, 18]. These pathways rely on oxidases or aminotransferases, for PUT conversion to 4-aminobutyrate and, ultimately to succinate. It should, however, be noted that acetylation of PUT has yet to be demonstrated in bacteria and archaea. Within Escherichia coli, PUT degradation is mediated by the dual action of γ-glutamatyl intermediates with a corresponding oxidase in addition to direct deamination utilising a transaminase with oxoglutarate co-substrate [13, 14, 19]. Studies on Pseudomonas aeruginosa [20] and Arthrobacter sp. TMP-1 [21, 22] have identified a similar derivative with pyruvate as the transaminase co-substrate in the direct deamination of PUT. These experimentally verified PUT degradation pathways, confirmed by strains possessing all the necessary enzymes, are shown in Fig. 3.1.

A postulated microbial oxidase pathway which acts directly upon PUT is represented by dashed arrows where all applicable enzymes have been individually characterised [19, 23-26] but not, to our knowledge, within the same microbial strain. Support for this pathway can draw on analysis of plant response to a range of stressors where oxidases were induced with the accumulation of the protective 4-aminobutyrate [27]. While aminoaldehyde dehydrogenases are widely accepted as the enzymes chiefly responsible for the formation of 4-aminobutyrate from 4-aminobutyraldehyde, few have been explicitly identified and characterised [28, 29]. Multiple gene homologues,
differing subcellular locations and a common broad substrate specificity have all hindered an explicit identification of dehydrogenase catalytic activity in direct relation with the broader catabolic pathway.

Figure 3.1: The pathways for PUT degradation within prokaryotes as demonstrated by experimental analysis. These include oxidation via aminotransferases and γ-glutamated intermediates as illustrated by solid arrows and the amine oxidase pathway (dashed arrows) investigated in this study.

In the previous Chapter, we isolated and characterised two amine oxidases (AO) from *Rhodococcus opacus* that differed with respect to the redox cofactor, i.e. flavin (FlavAO) and copper dependent (CuAO) [23]. The product of these enzymes *in vitro* was 1-pyrroline (a cyclic imine), which exists in equilibrium with 4-
aminobutyraldehyde, an intermediate in PUT oxidation (Fig. 3.1) [30, 31]. Genomic analysis has revealed sequence homologues of all the enzymes required for this PUT degradation pathway (Fig. 3.2), notably 4-aminobutyraldehyde dehydrogenase (AbD), 4-aminobutyrate transaminase (AbT) and succinic semialdehyde dehydrogenase (SsD).

In this study we provide the first experimental evidence for the direct oxidation route (utilising oxidases) in PUT catabolism in bacteria. We report the isolation, characterisation and identification of AbD and link its expression with the two diamine oxidases (FlavAO and CuAO). The catalytic capacity to act upon further degradative products is additionally probed with biochemical and genomic investigation. Furthermore phylogenetic analysis is applied to determine the probable distribution of this degradative pathway involving amine oxidases, 4-aminobutyraldehyde dehydrogenase and other interrelated enzymes, transcription factors, and transporter proteins.

3.2 Materials and Methods

3.2.1 Bacterial strain and culture conditions

*Rhodococcus opacus* DSM 42350 was used throughout this investigation. For amine oxidase and 4-aminobutyraldehyde dehydrogenase activity, the 50 mL of bacterium was cultured in M9 minimal media (100 ml flasks) with 4 mM glucose as the carbon source and 20 mM putrescine (PUT) as the sole nitrogen source. In enzyme induction experiments analysing capacity to act upon PUT, 4-aminobutyraldehyde, 4-aminobutyrate and succinate-semialdehyde, cells were limited to 5 mM of PUT, butylamine or ammonium chloride (NH₄Cl) as the sole nitrogen source. The bacteria were grown at 30° C for 3 days under continuous shaking at 130 rpm. The cells were then centrifuged for 20 min at 4° C and 2500 g, the supernatant removed and the cell pellet frozen at -20° C.
3.2.2 Enzyme Assays

A colorimetric assay was performed in order to determine catalytic oxidative deamination activity. The assay is based on the generation of H$_2$O$_2$ as by-product, which in the presence of 4-aminoantipyrine and 2,4,6-tribromo-3-hydroxybenzoic acid is converted in equimolar amounts by an added peroxidase to produce a quinoneimine dye [32]. The reaction was performed in 96 well plates containing 10 µl of the cell lysate/purified oxidase and 100 µl freshly prepared assay solution (200 mM potassium phosphate buffer (pH 7.6), 1.5 mM 4-aminoantipyrine and 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid). To start the reaction a combination of 20 µl PUT (100 mM) and 70 µl 1.4 mg/mL peroxidase from horseradish was added to give a final volume of 200 µl. Absorbance was measured at 510 nm and 30 °C using a plate reader (VERSAmax, Molecular Devices). 4-Aminobutyraldehyde dehydrogenase, 4-Aminobutyrate aminotransferase and succinate semialdehyde dehydrogenase activity was assayed by the formation of NADH/NADPH spectrophotometrically at 37 °C by measuring the change in absorbance (340 nm). In the case of 4-aminobutyraldehyde dehydrogenase, the reaction mixture (1 mL) contained 50 µmol tris-hydrochloride (pH 7.8), 1 µmol NAD$^+$, 0.5 µmol mercaptoethanol and reaction was initiated by adding 5 µmol 4-aminobutyraldehyde. 4-Aminobutyrate aminotransferase activity was measured in a coupled assay system utilising succinate semialdehyde dehydrogenase from Pseudomonas fluorescens (Sigma) to generate NADPH. 1 U This enzyme was added to a reaction mixture consisting of 5 µmol 4-Aminobutyrate, 100 µmol Bis-tris propane (pH 8.9) and 3 µmol NADP$^+$. The succinate semialdehyde dehydrogenase assay consisted of 100 µmol Bis-tris propane (pH 9.3), 3 µmol succinate semialdehyde and 3 µmol NAD$^+$; the final pH was 8.9 with the reaction initiated on addition of the cell lysate. The metabolic relationship of PUT oxidatiation followed by aldehyde dehydrogenation was followed by the formation of NADH at 340 nm. The reaction mixture (1 mL) contained 50 µmol tris-hydrochloride (pH 7.8), 1 µmol NAD$^+$, 0.5 µmol
mercaptoethanol and 0.02 to 0.1 mM PUT. Assay was initiated by the addition of 0.092 mg of 4-aminobutyraldehyde dehydrogenase and/or flavin amine oxidase, before incubation for 15 minutes at 37 °C.

All the results were normalised against the total cellular protein in the assay determined by using the Bradford’s reagent method. Readings were performed in triplicate and the individual results all fell within a 5% relative standard deviation.

3.2.3 Purification

The PUT oxidase was purified as described in detail previously [23]. The majority of nonspecific protein was removed using an anion Hi-Prep 16 10 Q FF column (1.6 cm × 13 cm) with 50 mM Bis-Tris-hydrochloride (pH 6.5) and eluted over a 1 M NaCl gradient, as presented in Table 3.1.

Table 3.1: Sequential purification of flavin amine oxidase from *Rhodococcus opacus*.

<table>
<thead>
<tr>
<th></th>
<th>Total Protein (mg)</th>
<th>Total Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>Specific PUT activity (µmol/min/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>56</td>
<td>50</td>
<td>1.12</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>Hi-Prep Column</td>
<td>3.4</td>
<td>5</td>
<td>0.68</td>
<td>1.73</td>
<td>12.6</td>
</tr>
<tr>
<td>Affinity Column</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>26.42</td>
<td>136.1</td>
</tr>
</tbody>
</table>

The fraction with the greatest activity after two fold dilution was purified directly using a pre-activated native affinity resin (EAH Sepharose), resulting in a 136-fold purification. In the case of 4-aminobutyraldehyde dehydrogenase, cells cultured in M9 Media with 20 mM PUT were re-suspended in TES buffer (0.4 g/mL) and twice passed through a French press at a pressure of 138 MPa. In order to ensure complete lysis, the cells were sonicated over 30 second intervals on ice for a further 10 min. The cell debris was removed by centrifugation (4 °C and 60000 g) for 1 h and passed through a 0.2 µm syringe filter. A significant proportion of nonspecific protein was removed by (NH₄)₂SO₄ precipitation, as shown in Table 3.2. Samples were then dialysed overnight with 50 mM tris-hydrochloride (pH 8) and further purified on a
HiTrap DEAE FF column (1.6 × 2.5 cm), eluting over a 200 mM NaCl gradient. The fraction with the most intense peak was separated by gel filtration using a Superose 12 10/300 GL column (1 cm × 30 cm), resulting in a 38-fold purification (Table 3.2 and Fig 3.2).

Table 3.2: Sequential purification of 4-aminobutyraldehyde dehydrogenase from Rhodococcus opacus.

<table>
<thead>
<tr>
<th></th>
<th>Total Protein (mg)</th>
<th>Total Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>4-aminobutyraldehyde dehydrogenase (µmol/min/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract (NH₄)₂SO₄ ppt</td>
<td>45.5</td>
<td>20</td>
<td>2.27</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>HiTrap DEAE FF</td>
<td>30.9</td>
<td>50</td>
<td>0.61</td>
<td>0.31</td>
<td>1.3</td>
</tr>
<tr>
<td>Superose 12/10</td>
<td>2.1</td>
<td>4</td>
<td>0.50</td>
<td>5.25</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>0.92</td>
<td>1</td>
<td>0.92</td>
<td>9.22</td>
<td>38.3</td>
</tr>
</tbody>
</table>

Figure 3.2: SDS-PAGE of sequential purification of 4-aminobutyraldehyde dehydrogenase. (A) Cell free extract, (B) (NH₄)₂SO₄ ppt, (C) HiTrap DEAE FF (D) Superose 12/10.

3.2.4 Gel activity staining

4-Aminobutyraldehyde dehydrogenase activity staining was performed using a NADH coupled stain after electrophoresis on a native polyacrylamide gel, as previously described [23]. The native PAGE was equilibrated twice for 20 min in 150 mM Tris buffer (pH 8). The gel was then transferred to the substrate solution containing 150 mM Tris buffer (pH 8), 5 mM 4-aminobutraldehyde, 1mM NAD⁺, 300 µg/mL Nitro-Blue Tetrazolium, 20 µg/mL phenazine and was incubated for ca. 1 h, depending on the observed band intensity.
3.2.5 In-gel digestion of protein spots and protein identification

The purified 4-aminobutyraldehyde dehydrogenase was excised manually with a scalpel from the native PAGE or corresponding SDS Tris-tricine gel (stained with Coomassie Brilliant Blue R250 solution (G-Biosciences) for 1 h). The gels were rinsed with Millipore filtered (0.2 µm) water followed by incubating the gel slice at 37 °C twice with 0.2 mL 100 mM ammonium bicarbonate/50% acetonitrile for 45 min. The wash was removed and replaced with 100 µl acetonitrile for 5 min and dried in a Speed Vac for 15 min. Gel slices were rehydrated in 10 µl Trypsin Gold (Promega) (20 µg/mL) and 40 mM ammonium bicarbonate/10% acetonitrile at room temperature for 1 h. An additional 90 µl 40 mM ammonium bicarbonate/10% acetonitrile was added to prevent complete evaporation and incubated overnight at 37 °C. The gel slices were further diluted with 100 µl Millipore water and vortexed for 10 min. The liquid fraction was then transferred to a micro-centrifuge tube and the gel slice digest extracted twice by vortexing with 50% acetonitrile/5% trifluoroacetic acid for 1 h. These extracts were pooled and dried in a Speed Vac at room temperature for 2 h. Samples were reconstituted in 10 µl 0.1% trifluoroacetic acid, pipetted and expelled three times with ZipTips, which had been preconditioned with 10 µl acetonitrile followed by three washes with 0.1% trifluoroacetic acid. Contaminants were removed by washing ZipTips containing the bound protein a further three times with 0.1% trifluoroacetic acid before eluting 0.3 µl spots directly onto the Maldi plate using a 1.5 µl matrix consisting of 70% (v/v) acetonitrile/0.1% trifluoroacetic acid and 10 mg/mL α-cyano-4-hydroxycinnamic acid. Peptide spectra were generated using an Ettan Matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI TOF MS). The peaks automatically selected by the peak seeker algorithm were searched against the NCBI database using MSFIT (http://prospector.ucsf.edu). Those with a high Mowse score, i.e. a weighted measure of how closely the spectra matches a protein, were subsequently confirmed using the MASCOT programme (www.matrixscience.com), which generates
an expected value, i.e. the number of (random) proteins that could achieve the same score for that spectrum. Proteins with an expectation score ($E$-value) of 0.05 and lower (95% confidence), a minimum sequence coverage of 10% and at least three independent peptides matches were taken as positive identifications.

### 3.2.6 Genomic analysis

Predicted proteins were annotated by phylogenetic classification (COGs), multiple sequence alignments and hidden Markov models (Pfam and TIGRFAM). Phylogenetic classification of all known actinomycetales with complete genomes was performed by multiple sequence alignment on 16sRNA, utilising MUSCLE [33]. Trees were constructed using the “maximum likelihood” method, based on the Tamura-Nei model [34]. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was below 100 or less than 25% of the total number of sites, the maximum parsimony method was used; otherwise the BIONJ method with MCL distance matrix was employed. The analysis involved 171 nucleotide sequences, where all positions containing gaps and missing data were eliminated, giving a total of 116 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [35].

### 3.3 Results and Discussion

#### 3.3.1 Enzymatic induction

The initial stage of PUT degradation was previously examined in detail by characterising the dual action of the flavin (FlavAO) and copper (CuAO) dependent oxidases which generate 4-aminobutyraldehyde from PUT with the concomitant production of ammonia and hydrogen peroxide as shown below [23].

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{NH}_2
\end{align*}
\]

\[+ \text{H}_2\text{O} + \text{O}_2 \quad \rightarrow \quad \begin{align*}
\text{H}_2\text{N} & \quad \text{O}_2
\end{align*}
\]

\[+ \text{NH}_3 + \text{H}_2\text{O}_2
\]

By limiting the nitrogen source to PUT, cadaverene (CAD) or butylamine (BUT), differential upregulation of oxidase activity was elicited in *Rhodococcus opacus* [23,
36]. In that work, the subsequent fate of 4-aminobutyraldehyde (Fig. 3.1) was hypothesised to be dependent on a genomic cluster of functionally related genes that is found within the same operon as CuAO (Fig. 3.3) and genomically close to the FlavAO.

Figure 3.3: Genomic cluster of functionally related genes surrounding both oxidases in Rhodococcus opacus B4 and Rhodococcus RHA1 [23]. Those proteins implicated in a direct catabolic role in PUT degradation are identified by open arrows, functionally related proteins by solid arrows and those with an undetermined significance are given by hatched arrows.

In order to test this premise, cells were grown with either PUT or BUT as the sole nitrogen source, conditions that promote FlavAO and CuAO, respectively. The resulting cellular activity of dehydrogenases (AbD) that act on 4-aminobutyraldehyde, aminotransferases (AbT) that deaminate 4-aminobutyrate and further dehydrogenases (SsD) that reduce succinate-semialdehyde (Fig. 3.1) were monitored as their action is a prerequisite for the conversion of 4-aminobutyraldehyde (the product of PUT oxidation) to succinate. Taking ammonium chloride (NH₄Cl) as a non-inducing nitrogen control, the results presented in Table 3.3 demonstrate the potential for the complete conversion of PUT to succinate within the organism.

Table 3.3: Total activity of lysed Rhodococcus opacus with respect to PUT, 4-aminobutyraldehyde, 4-aminobutyrate and succinate-semialdehyde (units: µmol/min/mg) after pretreating cells with ammonium chloride, PUT and butylamine (BUT) as the sole nitrogen source.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>PUT oxidase</th>
<th>4-Aminobutyraldehyde dehydrogenase</th>
<th>4-Aminobutyrate aminotransferase</th>
<th>Succinate-semialdehyde dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>1.2</td>
<td>&lt;0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>PUT</td>
<td>184</td>
<td>0.24</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>BUT</td>
<td>5</td>
<td>0.02</td>
<td>0.05</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Cells grown with NH₄Cl show a background level of catalytic activity towards PUT and low but detectable conversion of 4-aminobutyrate and succinate-semialdehyde. This constitutive activity, in the absence of exogenous diamines,
suggests an important cellular role that may include prompt response to changes in exogenous nutrient conditions or continual homeostasis of intracellular diamine levels. There was no significant activity detected with respect to AbD, suggesting the expression of only FlavAO, which is separated from the principal metabolic cluster (Fig. 3.2), possibly coupled with the expression of alternative terminal enzymes or processes given growth as sole energy source can be maintained without CuAO, an enzyme predicted to belong to the same operon as AbD [23].

Culturing under nitrogen limiting conditions with PUT (Table 3.3) served to increase oxidative deamination activity while also enhancing AbD activity. Such co-regulation is consistent with the similar transaminase pathways of *Escherichia coli* K-12 [19], *Pseudomonas* sp. [25, 37] and *Klebsiella aerogenes* [14, 38]. The activity of AbT and SsD was also increased as noted previously [14, 38]. This directed enzymatic response co-regulating all necessary enzymes, supports the contention that these enzymes play a central role in response to exogenous PUT. Within *Escherichia coli* K-12, AbD, AbT, and SsD were subject to cAMP-independent catabolite repression and elevated due to the presence of PUT [14]. Based on the response observed in this study, similar regulatory mechanisms appear to apply to *Rhodococcus opacus*.

In order to test if the observed aldehyde dehydrogenase activity was due to a non-specific upregulation of unrelated enzymes due to the pleiotropic effects of PUT [1], a range of aldehyde substrates were tested (Table 3.4). These included aliphatic aldehydes, with and without an amino group, and the principal products of other prominent oxidases within the organism [23]. It can be seen that enzyme action was directed almost exclusively towards 4-aminobutyraldehyde, with a lower activity for the shorter chain 3-aminopropionaldehyde.
Table 3.4: Aldehyde dehydrogenase activity (as a percentage relative to 4-aminobutyraldehyde) from lysed *Rhodococcus opacus* after inducing activity by limiting the nitrogen source to PUT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dehydrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Aminopropionaldehyde</td>
<td>40</td>
</tr>
<tr>
<td>4-Aminobutyraldehyde</td>
<td>100</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

There was no measurable activity for those aldehydes that can arise from the oxidation of aromatic amines (benzylamine and phenylethylamine), which this strain is known to convert [36]. Moreover, C₁-C₃ aldehydes that did not bear an amino moiety displayed negligible levels of conversion relative to 4-aminobutyraldehyde (or 3-aminopropionaldehyde). The limited specificity of the observed AbD activity suggests an evolved response that is specialised towards the best utilisation of the product of PUT oxidation, particularly in the presence of its precursor.

Culturing the cells with BUT, which promotes CuAO [23], also led to increased general enzyme activity relative to the NH₄Cl culture, with elevated levels of oxidase, AbD, AbT and SsD activity. This effect was appreciably lower than that observed for PUT treatment with, moreover, a difference in the relative activity levels for the four systems. PUT induction delivered an order of magnitude higher AbD activity and a two-fold higher AbT rate (Table 3.3). This observation suggests a differential expression of the same enzyme(s) or, as is more likely from a consideration of the genomic cluster (Fig. 3.3), the occurrence of multiple enzymes capable of acting on the same substrates.

3.3.2 **Characterisation of AbD**

Following purification on the Superose 12/10 column (Table 3.2), the native molecular mass of the principal dehydrogenase that acts on 4-aminobutyraldehyde was estimated to equal ca. 200 kDa. A subunit molecular mass of 50 kDa was determined by
SDS-gel electrophoresis (data not shown), suggesting a multimeric protein consisting of four subunits, similar to that observed in several other bacteria species [21, 37, 39, 40]. A significant loss of enzyme activity (35% of the initial level) was observed after storage for 24 h at 5 °C. Incubation for (15 min at different temperatures) established that AbD activity is retained from 5 to 37 °C with a dramatic loss (by 74%) at 50 °C and no observable activity after treatment at 60 °C. This denaturation at elevated temperature is similar to that reported for *Escherichia coli* [41] and *Arthrobacter* sp [22], while the dehydrogenase of *Pseudomonas aeruginosa*, is reported to be stable over a wider temperature range and for longer periods [42].

![Graph showing the effect of pH on AbD activity](image)

**Figure 3.4:** Effect of pH on AbD activity: sodium acetate (▲), Tris (■), and CHES buffers (●)

The effect of solution pH, using a number of buffers, is shown in Fig. 3.4, where a sharp activity maximum at pH 8 is evident, illustrating the importance of cytoplasmic pH regulation on the function of the dehydrogenase.

Kinetic analysis was undertaken using a standard Michaelis-Menten approach for substrate concentrations up to 1 mM, as presented in Fig. 3.5. Over the range of concentrations tested, there was no detectable substrate inhibition. The apparent
Michaelis-Menten constants \( (K_m) \) were calculated by nonlinear regression and are recorded in Table 3.5.

![Graph](image-url)

**Figure 3.5:** Initial reaction rate as a function of substrate (3-aminopropionaldehyde (●), 4-aminobutyraldehyde (▲)) concentration for oxidoreductase action of AbD. Note: curves generated by fitting the data to the expression \( V = V_{\text{max}} \frac{[S]}{(K_m + [S])} \).

The \( K_m \) value was lowest for 4-aminobutyraldehyde, and was found comparable to that recorded for other isolated bacteria dehydrogenases, including *Escherichia coli* (0.018 mM) [20], *Arthrobacter* sp. (0.065 mM) [22], *Pseudomonas putida* (0.26 mM) [40] and *Pseudomonas* sp. (0.003 mM) [37].

**Table 3.5:** Kinetic parameters obtained from a Michaelis-Menten treatment of aldehyde dehydrogenase purified from *Rhodococcus opacus* pretreated with PUT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) (mM)</th>
<th>( V_{\text{max}} ) (µmol⁻¹ min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>0.146</td>
<td>12.41</td>
</tr>
<tr>
<td>3-Aminopropionaldehyde</td>
<td>0.243</td>
<td>5.52</td>
</tr>
<tr>
<td>4-Aminobutyraldehyde</td>
<td>0.014</td>
<td>11.22</td>
</tr>
</tbody>
</table>

In contrast to *Arthrobacter* sp, which showed a preference for 3-aminopropionaldehyde \( (K_m = 0.003 \text{ mM}) \) [22], the smaller chain aldehyde delivered an appreciably higher \( K_m \) with a much lower maximum rate \( (V_{\text{max}}, \text{Table 3.5}) \). Similar observations [37] have been made for dehydrogenases of *Pseudomonas* sp. with a \( K_m \) variation from 0.003 mM (4-aminobutyraldehyde) to 0.083 mM (3-
aminopropionaldehyde) and lower $V_{\text{max}}$ (0.34-1.5 μmol$^{-1}$ mg$^{-1}$ min$^{-1}$) than recorded in this study.

### 3.3.3 Integration of successive enzymes into pathway

The successive oxidative deamination of PUT followed by the catalytic action of the aldehyde dehydrogenase, as shown in Fig. 3.1, was monitored by using purified FlavAO (Table 1) coupled with AbD (Table 3.2) within a single assay. Monitoring the redox formation of NADH from AbD, the biochemical relationship between the two enzymes is established by the near convergence of 4-aminobutyrate production from 4-aminobutyraldehyde and PUT (Fig. 3.6), where the latter requires the catalytic action of both enzymes.

![Figure 3.6](image)

**Figure 3.6:** The generation of NADH by the reduction of 4-aminobutyraldehyde to 4-aminobutyrate promoted by FlavAO and AbD. The purified FlavAO and AbD were incubated for 15 minutes with varying concentrations (0.02-0.1 mM) of 4-aminobutyraldehyde (■) and PUT (○).

There was no detectable NADH formed when AbD was assayed with PUT in the absence of FlavAO, which clearly demonstrates the successive catabolic process for PUT via 4-aminobutyraldehyde to generate 4-aminobutyrate. 4-Aminobutyraldehyde exists in equilibrium with its cyclic form (1-pyrroline, see Fig. 3.1). The comparable linear increase of NADH resulting from the conversion of the 4-aminobutyraldehyde substrate used in the assay or the derived 4-aminobutyraldehyde from the PUT deamination via amine oxidase suggests that overall activity is not significantly affected
by this equilibrium. The small deviation however can be attributed to incomplete oxidation/reduction. Native-PAGE electrophoresis of the purified ABD coupled with 4-aminobutyraldehyde dehydrogenase staining generated a single band shown in Fig. 3.7 with no indication of overlapping enzymatic action.

![NADH Generation](image)

**Figure 3.7:** Native-PAGE dehydrogenase electrophoresis of purified AbD; activity staining with specificity to 4-aminobutyraldehyde as substrate (and generation of NADH).

The associated protein was excised, extracted, enzymatically digested for MALDI-TOF analysis and confirmed as a homologue to aldehyde dehydrogenase GI 111022563 within the closely related *Rhodococcus RHA 1* genome (Table 3.6).

**Table 3.6: Aldehyde dehydrogenase expressed by *Rhodococcus opacus* after induction with PUT.** Enzyme identified by Maldi-TOF MS analysis and matched to *Rhodococcus RHA1* genome using both MSFIT and Mascot.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Protein Identifier</th>
<th>GI Accession Number</th>
<th>Mr (kDa)</th>
<th>PI</th>
<th>% Sequence coverage/peptides</th>
<th>Mowse Score</th>
<th>MSFIT</th>
<th>Mascot</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUT (25 mM)</td>
<td>Aldehyde dehydrogenase</td>
<td>111022565</td>
<td>50605</td>
<td>4.9</td>
<td>23/9</td>
<td>6.11e+7</td>
<td>0.031</td>
<td></td>
</tr>
</tbody>
</table>

The $E$-value (0.031) and high sequence coverage provide a high confidence of correct identification. It is significant that gene encoding this enzyme is located within the cluster associated with both the CuAO and FlavAO (Fig. 3.3) [23]. This genomic association in tandem with the co-regulation discussed earlier strongly indicates the validity for our proposed pathway.
3.3.4 Distribution of pathway and genomic analysis

The significance of the pathway utilising amine oxidases followed by ABD as key enzymes in the regulation/degradation of PUT in microbes almost certainly extends beyond *Rhodococci*. Although diamine acting oxidases have been reported for a number of species, including *Micrococcus luteus* [43, 44], *Pseudomonas aeruginosa* [45] and several *Candida* sp. [46], few have been explicitly identified, and there is a general lack of knowledge of the distribution as well as principal pathways of PUT catabolism within broader taxa.

Amine oxidases possessing a copper/topaquinone co-factor have typically been investigated in species of eukaryote, including the extensively studied *Porcine* kidney [47, 48] and ATAO1 from *Arabidopsis*, the latter which genomically possess a further eleven putative amine oxidases [49, 50]. Within prokaryotes, CuAOs activity has remained fairly unexplored although one example of a study is our earlier work on *Rhodococcus opacus* [23]. Studies on oxidases acting on diamine that utilise flavin as a redox cofactor are limited to isolates from *Rhodococcus erythropolis* [51, 52], *Rhodococcus opacus* [23] and *Kocuria rosea* (formerly *Micrococcus rubens*) [31].

Genomic analysis of the oxidases that have been isolated can provide insight into the microorganisms that are likely to utilise such enzymes for catabolism, given that over two thousand genomes are now publically available. Little can be gleaned from sequence analysis of CuAO due to substrate plasticity in this class of enzyme and the lack of highly similar prokaryotic homologues. FlavAO, by contrast, has limited substrate specificity [23, 31, 51, 52] with conserved sequence features and domains [52], suggesting that any homologues are likely to possess similar activity and therefore play a common role within the organism. The results of BLAST analysis, given in Table 3.7, reveal twenty six species belonging to ten divergent families with homology (>40% identities, BLAST score <2×10^{-100}) to previously characterised FlavAO of *Rhodococcus* RHA1 and *Kocuria rosea*, providing a basis for further investigation and genetic
analysis. Those with lower homology typically display greater similarity to flavin
dependent monoamine oxidases.

Table 3.7: Species and percentage identity resulting from BLAST analysis of the closest sequence homologues (>36%) of FlavAO of Rhodococcus RHA1 (111022572) and Kocuria rosea (730425).

<table>
<thead>
<tr>
<th>Family</th>
<th>Strain</th>
<th>Identity to R. RHA1 (%)</th>
<th>Identity to K. Rosea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocardiaceae</td>
<td>Rhodococcus RHA1</td>
<td>100</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Rhodococcus opacus B4/PD630</td>
<td>97</td>
<td>69</td>
</tr>
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3.3.5 Genome Mining

The evolutionary pressures which lead to the genomic convergence of functionally related enzymes and proteins, as observed in (Fig. 3.3) supports the assimilation route given in Fig. 3.1. As this cluster of genes are unlikely to have been acquired recently by lateral transfer or utilised for an alternative non-catabolic function,
the genomic region of those bacteria possessing FlavAO homologues were analysed. Within *Arthrobacter aurescens*, *Arthrobacter FB24* and *Renibacterium salmoninarum* (Fig. 3.8), FlavAO is flanked by homologues of apparently functionally related genes, given their corresponding presence in *Rhodococcus* (Fig. 3.3).

![Figure 3.8: Genomic cluster of functionally related genes surrounding both oxidases in *Arthrobacter aurescens*, *Arthrobacter FB24* and *Renibacterium salmoninarum*. Those proteins implicated in a direct catabolic role of diamines are identified by open arrows, functionally related protein by the solid arrow and those with an undetermined significance are given by hatched arrows.]

While the role of amino acid permeases is self-evident in the transport of amino acids and amines across the cell membrane (Fig. 3.1), the precise function of universal stress protein and the transcriptional regulator (tetR) is less clear. Universal stress proteins are triggered by environmental insults in order to enhance viability. Although their mode of action has yet to be resolved, their occurrence is linked to a variety of stress conditions, notably nitrogen and carbon starvation and resistance to hydrogen peroxide, a by-product of oxidative deamination [53]. They have also been shown to affect carbon utilisation under normal conditions with a possible role in modulating carbon use relative to other biosynthetic processes [54]. tetR genes serve to mediate rapid, adaptive responses to signals resulting from changes in the environment by altering either transcription, translation or some other event in oxidase expression [55]. This family of regulators are composed of a signal receiving domain and a DNA-binding domain and are often subject to simple feedback control mechanisms, whereby the presence of the inducer molecule depresses both synthesis of the repressor and regulated enzymes/proteins [55]. In PUT metabolism, TetR action may constitute a fraction of the overall underlying regulatory cascades for nitrogen regulation and PUT homoeostasis. As their most frequent function is in response to osmotic, chemical and antibiotic
stressors, a role in managing environmental insults involving polyamines cannot be discounted, especially given the presence of the universal stress protein. Although subsequent degrading genes were not found in close proximity, multiple candidate genes throughout the genomes were found within all three species. Database sequence analysis of those strains with amino acid homology to FlavAO typically also possessed a homolog to AbD as shown in Table 3.8, these include *Arthrobacter aurescens*, *Arthrobacter FB24* and *Renibacterium salmoninarum*.

**Table 3.8:** Species and percentage identity resulting from protein BLAST analysis of the closest sequence homologues of AbD (E-value= 0) from *Rhodococcus* RHA1 (YP_705537.1), which also possess a homologue for FlavAO of *Rhodococcus* RHA1 (111022572) and *Kocuria rosea* (730425).

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Organism</th>
<th>Identities</th>
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</thead>
<tbody>
<tr>
<td>YP_705537.1</td>
<td><em>Rhodococcus</em> RHA1</td>
<td>100%</td>
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<tr>
<td>YP_002782856.1</td>
<td><em>Rhodococcus</em> opacus B4</td>
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<td><em>Mycobacterium abscessus</em></td>
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<td>ZP_09411742.1</td>
<td><em>Mycobacterium massiliense</em></td>
<td>76%</td>
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<td>YP_832574.1</td>
<td><em>Arthrobacter</em> FB24</td>
<td>70%</td>
</tr>
<tr>
<td>YP_948771.1</td>
<td><em>Arthrobacter aurescens</em> TC1</td>
<td>69%</td>
</tr>
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<td>YP_001625744.1</td>
<td><em>Renibacterium salmoninarum</em></td>
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</tr>
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<td>YP_004242172.1</td>
<td><em>Arthrobacter phenanthrenivorans</em></td>
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</tr>
<tr>
<td>ZP_09281237.1</td>
<td><em>Arthrobacter globiformis</em> NBRC 12137</td>
<td>70%</td>
</tr>
<tr>
<td>YP_002488853.1</td>
<td><em>Arthrobacter chlorophenolicus</em> A6</td>
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<td><em>Rothia dentocariosa</em> DC2201</td>
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<td>YP_001853937.1</td>
<td><em>Kocuria rhizophila</em> M567</td>
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<td>YP_001360593.1</td>
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<td>YP_004493965.1</td>
<td><em>Amycolicoccus subflavus</em> DQS3-9A1</td>
<td>59%</td>
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The biochemical association of these two enzymes within a pathway (Fig. 3.1) highlighted in this study therefore increases likelihood of them acting together in the catabolism for PUT in other species. As gene products may have multiple roles in the metabolism, the lack of complete functional convergence in these strains may reflect genes which are co-expressed, and additionally are subject to different environmental conditions.
Other organisms such as *Amycolicicoccus subflavus* DQS3-9A1 and *Mycobacterium abscessus* ATCC 19977 possess enzymes with high homology to AbD, immediately downstream from FlavAO, as shown in Fig. 3.9.

![Figure 3.9: Genomic cluster of functionally related genes surrounding the flavin dependent oxidase in *Amycolicicoccus subflavus* DQS3-9A1 and *Mycobacterium abscessus* ATCC 19977. Those proteins implicated in a direct catabolic role of diamines are identified by open arrows where protein with an undetermined significance is given by the hatched arrow.](image)

Genomic analysis of *Gordonia effusa*, *Corynebacterium striatum* ATCC 6940, *Corynebacterium variabile* DSM 44702, *Rothia Dentocariosa* and illustrated *Micrococcus luteus* SK58 (see Fig. 3.10) has revealed a more complete cluster of target enzymes that are also common to *Rhodococcus opacus*.

![Figure 3.10: Genomic cluster of functionally related genes surrounding the oxidases in *Micrococcus luteus* SK58. Those proteins implicated in a direct catabolic role of diamines are identified by the open arrows, functionally related protein by the solid arrow and those with an undetermined significance are given by hatched arrows.](image)

The occurrence of AbT and AbD is consistent with the proposed oxidase degradation pathway. Although succinic semialdehyde dehydrogenase (SsD) was absent from the genomic cluster, possibly due to functional redundancy, the central role of SsD in major metabolic routes, including glutamate degradation and the TCA cycle, ensure most organisms are enzymatically capable of further conversion. As genes are subject to significant transfer between the genomes of most prokaryotes, integration of functionally related clusters increases viability, as opposed to the cellular advantages of
co-expression [56]. Genes that are commonly transferred with FlavAO should show a high probability of successful incorporation as a functional module, enabling better integration into the cellular network. Depending on the species origin, such clusters are likely to be incompatible with regulatory sequences and tetR should then play a critical role in the expression strategy, given that the gene is consistently upstream to that encoding the oxidase. Likewise, the involvement of universal stress proteins is indicative of integral mechanisms either related to starvation or hydrogen peroxide generation. Conversely, the absence of genes required for this module to function correctly indicates either a lack of integration or the independent introduction of the required genes. The entries in Table 3.8 are significant in this respect in that most species possess a homologue to AbD even if not found in the cluster.

### 3.3.6 Phylogenetics

Although the majority of bacterial proteins from an evolutionary perspective tend to be confined within a limited taxonomic group [57], key metabolic enzymes such as those involved in energy metabolism, central intermediary metabolism and the processing of amino acids are expected to occur across a broader phylogenetic spectrum than that observed here for AO mediated PUT assimilation [57]. This observation suggests that this catabolic pathway evolved later than many metabolic enzyme families such as the citric acid cycle [58], glycolysis [59] and amino acid biosynthetic pathways [60], which are widely distributed among all taxa and considered to be highly conserved during evolution. Taking homologues of FlavAO distribution is limited to *Acintobacteria*, absent from the many sequenced genomes belonging to *Proteobacteria*, *Cyanobacteria* and *Firmicutes* phyla. Phylogenetics was performed on all complete *Acintobacteria* genomes to reveal the possible distribution within the bacterial class and the results are shown in Fig. 3.11. Three district groups of bacteria possess the target enzyme(s), i.e. the *Micrococcaceae* family (*Rothia, Arthrobacter, Micrococcus* and
Renibacterium) and two major groups within the Corynebacteriaceae family (Corynebacterium and those with an evolutionary association with Rhodococcus, including Tsukamurella and Gordonia). Those members where the oxidase is absent include prominent families of Streptomycetaceae, Frankiaceae, Pseudonocardiaeeae, Bifidobacteriaceae, Promicromonosporaceae and many members of Mycobacteriaceae. These distinct bacterial clusters possess gene homologues to FlavAO in addition to AbD (Table 3.8) with a common gene arrangement (Figs. 3.3, 3.8 and 3.9), suggesting that the enzymes were not simply acquired by recent horizontal gene transfer but evolved with the species from a common ancestor. This can be illustrated by Rhodococcus, where, with the exception of Rhodococcus equi, implicated genes occur in a common genomic order (Fig. 3.3), possessing a high sequence similarly among the genus (Tables 3.7 and 3.8) and all appear crucially to belong to the same evolutionary branch (Fig. 3.11). Rhodococcus equi, which is associated a more stable habitat as a pathogenic species, however may have lost functionally redundant genes or equally not undergone genomic expansion observed with non-pathogenic species which rely upon nutrient-poor and chemically diverse environmental niches.

Both Gordonia effusa and Tsukamurella paurometabola are close evolutionary cousins of Rhodococci, containing a FlavAO homologue but lacking the same AbD gene (Fig. 3.11). While several candidate aldehyde dehydrogenases are present in both species, including YP_003645366 in Tsukamurella paurometabola and ZP_09275589 in Gordonia effusa, the ability to act on 4-aminobutyraldehyde is speculative. The probability of a functional role of FlavAO is likely as both enzymes are located in similar genomic regions to that observed in Rhodococcus, in the case of Tsukamurella paurometabola within the genomic region involved in arginine assimilation (PUT precursor [42] with further related genes including AbT, SsD, universal stress protein and amino acid permease. Such genes are also found in Gordonia effusa which, in
common with *Rhodococcus opacus*, possesses a further CuAO (NZ_BAEH01000088). Many of the *Corynebacterium* that possess the target enzymes have been reported in environments that should be rich in exogenous PUT. *Corynebacterium nuruki* was isolated from a rice wine fermenter [61, 62], *Corynebacterium variabile* from ripened cheese [63, 64], *Corynebacterium genitalium* from patients with bacterial vaginitis [65, 68] and *Corynebacterium striatum* on an odorous chronic wound [67, 68]. However, a larger subset of *Corynebacterium* analysed (eg. *Corynebacterium jeikeium* [69], *Corynebacterium resistens* [70] and *Corynebacterium diphtheriae* [71]) are found in pathogenic environments (bronchial, skin flora, and septicaemia) where PUT assimilation should provide little advantage. It is therefore unclear if these genes are inherited and lost due to their pathogenic nature and genomic shrinkage as in *Rhodococcus equi* or acquired more recently via lateral transfer from related *Rhodococcus* where it shares a high guanine-cytosine content, typically in the 60 to 70% range [62, 63, 72, 73]. A third subset belongs to the *Micrococcaceae* family of bacteria where all but *Rothia mucilaginosa* possess a FlavAO homologue and the majority exhibit a related AbD gene (Fig. 10). In contrast to the other two clusters, *Micrococcaceae* are a relatively evolutionary distant family within *Acintobacteria* but share a soil environment with *Rhodococcus* [74-78].
Figure 3.11: Phylogenetic classification by 16s RNA of all genomes sequenced within Actinobacteria, identifying those organisms that possess a FlavAO homologue (●) or both a FlavAO and AbD homologue (■) to those characterised in Rhodococcus. Major genus where homologues are absent are denoted by (○) including (Streptomyces avermitilis MA-4680, Streptomyces coelicolor A3, Streptomyces flavogriseus ATCC 33331, Streptomyces scabiei 87.22, Streptomyces violaceusniger 4113, Streptomyces bingchengensis BCW1, Streptomyces cattleya, Streptomyces scabiei 87.22); Frankia (Frankia alni ACN14a, Frankia sp. Cc13, Frankia sp. EAN1pec, Frankia sp. Eu11e); Bifidobacterium (Bifidobacterium bifidum, Bifidobacterium animalis sub sp. Lactis B104, Bifidobacterium breve ACS071VSch8b, Bifidobacterium longum DJO10A, Bifidobacterium dentium Bd1, Bifidobacterium adolescentis); Cellulomona (Cellulomonas flavigena DSM 20109, Cellulomonas fimii ATCC 484); Pseudonocardia (Pseudonocardia dioxanivorans CB1190, Pseudonocardia sp 1, Pseudonocardia sp 2). Thirty five Mycobacterium tuberculosis strains were homologues are absent denoted by (○)
The strong correlation of the pathway in all but *Rothia mucilaginosa* among this family member suggests that these strains are direct descendants of a microbial ancestor that assimilated PUT via this oxidase dependent pathway, which was then adopted by *Corynebacteriaceae* through lateral transfer.

These results suggest that the diamine specific FlavAO is a highly specialised enzyme that is limited to select taxa. While the observed taxonomic patterns of enzyme distribution most likely reflect genuine divergent relationships, it is possible that an alternative class of oxidases not yet characterized are present in much broader taxa. Recent microarray analysis of the solvent tolerant *Pseudomonas putida* S12 found that exposure to PUT resulted in the upregulation of three genes encoding putative FAD dependent oxidoreductases [79]. These genes showed little amino acid homology to FlavAO (<30%), lacking key sequence features including the anionic binding point (GLU) that serves to anchor the diamine substrate. The role of two of these oxidoreductases, which have greater homology to amino acid oxidases, was further implicated in PUT degradation from the altered growth characteristics of knock out mutants [79]. It is possible that these oxidases are involved in the direct assimilation of PUT in a similar manner to *Rhodococcus*, albeit with a different form of oxidase. Indeed, the subsequent (AbD, AbT, and SsD) enzymes have putative homologues that were also upregulated in the organism.

### 3.4 Conclusions

We set out to examine the physiological processes and catabolic routes involved in PUT degradation within prokaryotes with a consideration of the constituent enzymes. We have provided experimental evidence that suggests the action of oxidase coupled with dehydrogenase in the assimilation of PUT in *Rhodococcus opacus*. These enzymes can co-express in response to environmental conditions, showing specificity to select substrates. The combined catalytic action of FlavAO and AbD facilitates the
conversion of PUT to 4-aminobutyrate, where associated in genomic metabolic clusters have been confirmed by MALDI-TOF. Furthermore, activity of AbT and SsD necessary for subsequent conversion to succinate altered with diamine availability, with gene candidates within the same genomic cluster. The evolutionary distribution of FlavAO used in this oxidase pathway is limited to Actinobacteria, with homologues within three major taxonomic clusters (Micrococcaeae, Rhodococci and Corynebacteruim), however there were limited additional species beyond these bacteria which may have acquired this enzyme by lateral gene transfer. As adoption of FlavAO (and the associated PUT degradation pathway) is only found in Corynebacterium under niche conditions, its variable presence within the genus suggests a more recent origin.

The close proximity of the universal stress protein to CuAO and FlavAO indicates a possible adverse effect due to the generation of hydrogen peroxide as a by-product of oxidative deamination, or the aldehyde itself. The evolutionary narrow distribution of FlavAO may reflect metabolic disadvantages of such enzymes. We propose that the regulation of amine oxidase dependent pathway requires tetR, found in genomic association with the oxidase. While no similar oxidases are apparent within the broader taxa, it is possible that as yet unidentified (and dissimilar) AOs can contribute to the PUT degradation pathway. Further studies are required to investigate diamine acting oxidases that fall outside the range of organisms examined in this work. Focusing on Rhodococcus, we aim to establish factors that underpin responses to changing environmental conditions, the interplay between constituent oxidases and greater biosynthetic processes. The ultimate goal is to harness these processes in bioremediation and biotechnological applications.

3.5 References


Chapter 4- Plasticity of Putrescine Homoeostasis within

*Rhodococcus opacus*

Foreword

With the establishment of the dynamic activity of two associated diamine oxidases and integration into a catabolic pathway (Chapters 2 and 3), the focus is shifted to cellular regulation, probing metabolic and morphological adaptations during transient amine conditions. Taking diamine (putrescine) deprived cells, the dynamic response to concentration variations (from 2.5 to 30 mM) is examined and the interplay between enzyme induction, catabolite repression and nitrogen control is established. Complete utilisation of the available nitrogen resulted in a cellular metabolic shift with the production and storage of lipids and detection of stored polyphosphates. From a consideration of oxidase expression, generation of metabolites and enzyme by-products, evolutionary advantages over other microorganisms are proposed with a consideration of possible pathogenic effects.

4.1 Introduction

Polyamines are ubiquitous compounds and, with the exception of two orders of *Archaea* [1], are found in all living organisms [2-7]. Evolutionary conservation suggests that polyamines are critical in the cellular growth and differentiation that applies to most taxa [8]. Within prokaryotes, such as *Rhodococcus*, putrescine (PUT) is the most prevalent polyamine [9] and plays a key role in binding charged cellular components, including nucleic acids [10], various proteins and acidic phospholipids [11]. Such interactions extend the contribution of amines beyond that of energy source to influencing global transcription and translation by stimulating RNA polymerase expression, stabilising ribosomal structure and modulating translational fidelity [12, 13]. These effects have guided our understanding of PUT in relation to growth but, conversely, has hindered the detection of other more functional roles [14]. Nevertheless,
it is known that PUT is integral in the response to a number of stress conditions, including pH [15], osmotic effects [16], oxidative environments [17], radiation [18] and temperature [19, 20]. In nature, such physical and chemical conditions are varied and dynamic. As excess or low levels of PUT can interfere with several crucial physiological activities, intracellular PUT concentration must therefore also alter within a defined range, balancing growth and differentiation requirements with other functional roles [9, 14, 21]. Four catabolic pathways for PUT have been reported for microorganisms, notably those that rely on aminotransferases with either 2-oxoglutarate or pyruvate as co-substrate [22-26], oxidases that act on γ-glutamylated intermediates [27, 28] and amine oxidases that directly deaminate PUT with either a flavin or copper dependent cofactor as observed for *Rhodococcus opacus* [29]. Such divergent enzymes, with apparent taxonomic clusters [29, 30], suggest alternate evolutionary pressures and divergent functional roles for polyamines other than those identified for model organisms.

*Rhodococci* are frequently found in the wider environment and possess metabolic versatility coupled with a persistence and tolerance to stress conditions [31]. The ability to exploit a diverse range of inhospitable niches is likely to depend on PUT utilisation, given the associated protective roles for a number of microorganisms [15, 17, 18, 32-34]. We can flag the explicit pathogenic role that PUT serves in *Rhodococcus flascians* [35, 36], the detection of PUT degrading *Rhodococcus* strains in contaminated soil [30], diseased fish [37] and even the atmosphere of a Russian space laboratory [38]. *Rhodococcus* is particularly suitable for further study as several genomes have been sequenced [39,40] and degradation pathways proposed [29]. Moreover, *Rhodococcus* is a viable agent in bioremediation applications [41] where access to polyamines may improve viability. We report here, for the first time, the dynamic response of
Rhodococcus over a wide PUT concentration range. We examine the regulation of metabolic activity and the interplay of growth conditions to determine enzymatic action.

4.2 Methods

4.2.1 Bacterial strain and culture conditions

Rhodococcus opacus DSM 42350 was used throughout this study. 50 mL of the bacterium was cultured in M9 minimal media with glucose (4 mM) as carbon source. Multiple cultures containing 2.5, 5, 10, 20 or 30 mM putrescine (PUT) were inoculated with 0.5 mL cells grown in amine depleted media. The bacteria were grown in 100 mL flasks under continuous shaking (130 rpm and 25°C), the cells centrifuged for 20 min at 4°C and 20000 g. The cell pellet was frozen at -20°C for intracellular enzyme assay or incubated at 95°C to determine dry cell weight. The supernatant was either frozen at -20°C with 20% glycerol for extracellular enzyme assay or heated to 90°C for 0.5 min to ensure enzyme denaturation before metabolite testing.

4.2.2 Extracellular PUT and 4-Aminobutyrate determination

PUT and 4-aminobutyrate concentration was determined by HPLC (Agilent 1100 series), with o-phthalaldehyde derivatisation [42]. Analysis was performed on a Hyperclone column (150 × 4.6 mm) with 10 mM potassium phosphate buffer pH 7 (solvent A) and acetonitrile (solvent B). A 4 μL sample was derivatised with 8 μL borate buffer (0.4 N potassium borate (pH 10)) and 8 μL OPA/BME (50 mg of orthophthaldehyde + 50 μL beta-mercaptoethanol diluted to 5 mL with 0.4 N potassium borate (pH 10)). The derivatisation reagents and sample were mixed in air 10 times and allowed to react for 4 min. 10 μL derivatised amine was injected and eluted at 1.5 mL/min using the following gradient program: 0 to 4 min 95% A, 5% B; 4 to 8 min 75% A, 25% B; 8 to 8.5 min 60% A, 40% B; 8.5 to 10 min 95% A, 5% B. Absorbance
was measured at 338 nm and concentration determined from the linear region of the calibration curve.

4.2.3 **Ammonia Assay**

The concentration of the metabolite ammonia was determined using a diagnostic kit supplied by Sigma. Free NH$_4^+$ reacts with α-ketoglutaric acid and NADPH of L-glutamate dehydrogenase to form L-glutamate and NADP$^+$ where the NH$_4^+$ concentration is proportional to the decrease in absorbance at 340 nm. 1 mL assay reagent containing α-ketoglutaric acid and NADPH was incubated with a 100 μL sample for 5 min at 25°C. Absorbance was measured before the addition of 10 μL L-glutamate dehydrogenase (>12 units). Samples were further incubated for 5 min at 25°C and absorbance again measured.

4.2.4 **Pyrroline Assay**

1-Pyrroline was determined using the ninhydrin method [43] and further validated by the 2-aminobenzaldehyde assay described by Jakoby and Fredericks [44]. 0.8 mL glacial acid was added to 1.2 mL clarified cell suspension and mixed with the addition of 2 mL ninhydrin reagent (50 mg ninhydrin in 60% glacial acid and 40% 6M phosphoric acid). After incubating at 80°C for 2 h, samples were put on ice with the addition of 4 mL toluene and vortexed to remove toluene with absorbance measurement at 515 nm. The concentration was determined using a proline standard calibration curve, the alpha amino acid generating a chromophore of identical extinction coefficient [45].

4.2.5 **Glucose Assay**

Glucose concentration was determined using a commercial glucose oxidase assay kit (Sigma). The hydrogen peroxide by-product reacts with o-dianisidine, forming a stable colour at 540 nm after addition of H$_2$SO$_4$. 1 mL clarified cell suspension was incubated at 37°C for 30 min with 2 mL assay reagent consisting of 25 units glucose oxidase (*Aspergillus niger*), 5 units peroxidase (horseradish), 0.25 mg o-dianisidine
dihydrochloride and buffer salts. Reaction was quenched with the addition of 2 mL 12 N H$_2$SO$_4$, absorbance measured and converted to concentration using the standard calibration curve.

4.2.6 Hydrogen Peroxide Assay

Hydrogen peroxide in clarified cell suspension was quantified by the colorimetric assay described by Wolff [46]. This quantitative method is based on peroxide oxidation of ferrous to ferric iron, which complex with xylene orange producing a colorimetric change at 560 nm. An assay reagent consisting of 0.1 mM xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H$_2$SO$_4$ was mixed in a 9:1 ratio with the cell suspension and incubated at room temperature for 30 min. The absorbance of the reaction mixtures was measured and concentration determined from the calibration curve.

4.2.7 Amine Oxidase Assay

A colorimetric assay was performed to determine enzymatic activity. The assay is based on the formation of hydrogen peroxide as by-product, which in the presence of 4-aminoantipyrine, and 2,4,6-tribromo-3-hydroxybenzoic acid is converted in equimolar amounts by an added peroxidase to produce a quinoneimine dye [47]. The reaction was performed in 96 well plates containing 10 µL enzyme solution (cell lysate/media diluted 100 fold) and 100 µL freshly prepared assay solution (200 mM potassium phosphate buffer pH 7.6, 1.5 mM 4-aminoantipyrine and 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid). 20 µL amine substrate and 70 µL 1.4 mg/mL peroxidase from horseradish were added to give a final volume of 200 µL. Absorbance was measured at 510 nm and 30° C using a plate reader (VERSA$_{\text{max}}$, Molecular Devices). All results were normalised against total protein using Bradford’s reagent method; readings were performed in triplicate and the results fell within a 5% relative standard deviation.
4.2.8 Enzyme inhibition

Semicarbazide as a non-competitive enzyme inhibitor was employed. Purified oxidases were incubated on ice in triplicate with 0.1 mM inhibitor suspended in Tris-hydrochloride (pH 7.5). After 30 min, oxidase activity for the inhibitor-enzyme solution was measured using the colorimetric assay with 10 mM PUT; inhibitor concentration in the assay was 5 µM.

4.2.9 Microscopy analysis and staining of polyphosphates

Cell cultures were fixed to glass microscopic slides by gentle heating, staining polyphosphate inclusions (metabolic storage) with methylene blue [48]. The slides were exposed to Loeffler's methylene-blue solution for 10 min and contacted successively (rinsing with distilled water between each step) with 1% sulphuric acid (5 sec), Lugol's iodine solution (15 sec) and aqueous safranine (2 min). Cells were viewed by light microscopy (1,000× magnification).

4.3 Results and Discussion

The dynamic nature of the environment in which bacteria grow and survive necessitates a capacity to adapt to changing conditions. For a bacterium such as Rhodococcus, this involves a multifaceted sensor-response mechanism, enabling the organism to modulate its phenotype to various environmental cues ranging from fluctuations in nutrients, daily and seasonal cycles to microenvironmental shifts, including the chemical and physical interactions of populations within a habitat [49]. These fluctuations can be instantaneous as in the case of osmolality changes after rainfall or gradual with respect to temperature variations with changing seasons. The ability to exploit a target niche is dependent on both intra- and extra-cellular sensors coupled with a variety of signalling pathways, many of which involve PUT [49]. Exploitation of these organisms requires a full appreciation of the plasticity of the regulatory networks, including polyamine homeostasis, the organisation and
mechanisms of the control systems and the conditions that lead to their emergence. PUT degradation by oxidase promoted deamination appears to exhibit a narrow taxonomic distribution, largely limited to acintobacteria such as *Rhodococcus* [30]. An important feature of *Rhodococcus opacus* is the capacity to utilise alternate oxidases in order to maintain the networks that sustain internal cellular environment, allowing continuous metabolism under a changing environment [29, 30]. In order to assess the plasticity of the cellular response, substrate concentration dependence is analysed in this study with respect to metabolic interconversion, utilisation and storage. A PUT concentration in the 2.5-30 mM range was adopted as the sole nitrogen source, which encompasses concentrations that can result from decarboxylase activity of bacteria [50] and the upper intracellular concentration found in prokaryotes [51].

### 4.3.1 Cellular growth

Temporal cell growth was monitored by measurement of dry cell weight (DCW) at 12 h intervals in order to capture all the growth phases, *i.e.* lag, log, stationary and death. The variation of DCW with time (up to 72 h) is presented in Fig. 4.1 as function of PUT concentration. A lag phase was observed over the first 24 h at each PUT concentration, with limited cell growth as *Rhodococcus* adapted to the change in nitrogen source with activation of relevant genes. Exponential growth was observed over the subsequent 12 h period. It can be noted that cells grown in media containing the lowest PUT concentration (2.5 mM) required a prolonged period to attain the log phase. This can be attributed to low cell stimulation as opposed to kinetic transfer limitations, given the subsequent elevated growth rate over the 36-48 h interval. The 5 and 10 mM cultures provided an enhanced growth/cell stimulation environment with a twofold higher DCW after 36 h relative to 2.5 mM PUT. A diminished growth rate was observed for concentrations ≥ 20 mM, suggesting PUT inhibition and/or toxicity [52, 53].
Figure 4.1: Dry cell weight (DCW) of Rhodococcus opacus as a function of time with varying initial PUT (as the sole nitrogen source) concentration: 2.5 mM (open bars), 5 mM (reverse hatched bars), 10 mM (grey bars), 20 mM (forward hatched bars), and 30 mM (solid bars). Representative growth curve (30 mM) illustrated by the dashed line. Error bars indicate SD.
With the exception of cells exposed to 30 mM PUT, all growth cultures reached the cusp of the stationary phase after 42-48 h. After 60 h, 18-20 mg/mL cell growth was achieved at each PUT concentration as growth was balanced by cell death. The cell cultures that initially contained 2.5 and 5 mM PUT were particularly limited by nutrients and entered death phase before the cultures treated with higher PUT concentrations.

4.3.2 Extracellular PUT concentration

Cell growth is intrinsically linked to PUT consumption. It can be seen from the entries in Fig. 4.2 that the available PUT (at initial concentration ≤ 20 mM) was consumed after 72 h with a ca. 17% of initial value, remaining for the highest starting concentration (30 mM). The initial (0-24 h) low levels of diamine conversion at PUT concentration ≤ 10 mM must be due to the relatively low number of viable cells but there was significant amine consumption at higher concentrations.

Figure 4.2: Temporal variation of PUT and glucose concentration for Rhodococcus opacus grown in different initial concentrations of PUT: 2.5 mM (¶, open bars), 5 mM (●, reverse hatched bars), 10 mM (X, grey bars), 20 mM (○, forward hatched bars) and 30 mM (■, solid bars). Error bars indicate SD.

The observed exponential growth phase (24-36 h, see Fig. 4.1) coincided with the period of greatest PUT consumption with full conversion of the starting PUT (< 10 mM). As little or no PUT is stored in Rhodococcus [54] and Gram positive bacteria in
general [55], this PUT must be catabolised or inter-converted. At higher concentrations (≥ 20 mM), an amine consumption rate of 10-15 μM/min (ca. 0.015 μM/min/mg DCW) was recorded. This represents a 5-fold higher rate relative to Aspergillus niger (ca 0.0031 μM/min/mg DCW), providing a clear selective advantage over a microorganism that often shares the same environment. In addition to PUT utilisation as an energy source for growth, rapid inter-conversion can facilitate better exploitation of the natural environment and reduce nutrient competition. Degradation may generate products of increased toxicity, impacting on the organisms within a nutrient limited niche. This response can play an important role in the phytopathogenic form of Rhodococcus spp., which infects 43 plant families [36]. By disturbing the plant hormone balance with morphogenic signals (mixture of cytokins), an altered PUT metabolism within the plant is triggered [36] that ultimately leads to PUT accumulation, resulting in leafy gall [36, 56]. As the affected tissue does not mature, it serves as a nutrient sink and provides the biotrophic Rhodococcus fascians with an enriched local environment [35]. Exogenous PUT has been found to promote these symptoms, while polyamine biosynthesis inhibitors act to limit this effect [36]. PUT can serve as an essential nutrient for Rhodococcus fascians during initial colonisation (as opposed to long term growth), facilitating increased cell tolerance to the plant immune system. It should be noted that PUT degrading Rhodococcus spp. containing common oxidases [30, 57] are found in salmon [37]. Fish also modulate their immune system by utilising polyamines where PUT enhances phagocytosis activity and the production of reactive oxygen species by head-kidney leucocytes [58]. Effective catalytic PUT degradation must address aspects of virulence, not only in response to oxidative stress [17] but in limiting up-regulation of phagocytosis by reducing the available PUT.
4.3.3 **Extracellular glucose concentration**

In our tests, glucose (4 mM) was included in the media and can act as a preferential carbon source. From a comparison of the entries in Figs. 4.1 and 4.2, it is evident that the consumption of glucose broadly mirrored that of cell growth. The initial lag over the first 24 h was followed by a marked decrease in glucose concentration that coincided with exponential growth. The assimilation of extracellular glucose was largely achieved within 48 h for the 2.5 and 5 mM PUT cultures. The resultant lack of nutrients must ultimately initiate apoptosis, which is seen to occur after 60 h for both cultures (Fig. 4.1). Cells cultured in 10 mM PUT also displayed increased glucose consumption from 24-36 h with a subsequent lower rate over the following 12 h as glucose diminished to residual levels (< 0.065 mM) after 60 h.

Some organisms co-metabolise different carbon sources simultaneously while many prioritise the energy source that is most accessible and permits fastest growth. Such catabolite repression is most frequently observed with glucose where availability down-regulates other enzymes that act on alternative carbon sources and metabolites (such as PUT) [59]. Based on the entries in Fig 4.2, this does not appear to be the case as increased PUT concentrations served to lower the degree of glucose consumption. Indeed, a significant glucose component (0.7-0.8 mM) remained in the media after 72 h in the 20-30 mM PUT cultures (Fig. 4.2). Our results indicate that PUT (or the associated degradation products) are preferentially utilised as carbon source. This observation supports prior analysis of *Rhodococcus opacus* PD630 where, of the 190 carbon sources tested, PUT provided the greatest growth after 44 h, demonstrating enhanced uptake and interconversion relative to carbohydrates, carboxylic acids, oligosaccharides and amino acids [60].
4.3.4 **Intracellular oxidase activity**

Enzymatic degradation of PUT in *Rhodococcus opacus* is governed by two separate amine oxidases that generate 4-aminobutanal (in equilibrium with 1-pyproline) with ammonia and hydrogen peroxide as by-products [29].

\[
\begin{align*}
\text{NH}_2\text{CH}_{2}\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 & \rightarrow \text{CH}_2=\text{CH}_2\text{CH}=:\text{CH}_2 + \text{NH}_3 + \text{H}_2\text{O}_2 \\
\end{align*}
\]

These enzymes, differing principally by co-factor, are dynamically expressed in response to the amine substrate and concentration [29, 30]. We have demonstrated that deamination is primarily achieved by a constitutive FAD amine oxidase (FlavAO) while the reaction is promoted by a copper dependent oxidase (CuAO) in the presence of excess diamine or competitive inhibitors (eg. butylamine) [29]. Little in the way of catalytic information can be gleaned from the initial 36 h culture period due to the minimal cell content and low associated activity. However, differences in the catalytic response at extended times (see Table 4.1) provide an indication of the underlying mechanisms affecting activity of the oxidases *in vivo*.

**Table 4.1:** Total oxidative deamination activity (μmol/min/g) at different times for *Rhodococcus opacus* cells grown in varying PUT concentrations. The response to semicarbazide inhibition is given as a percentage of activity achieved in the absence of the inhibitor.

<table>
<thead>
<tr>
<th>PUT Concentration</th>
<th>42 h</th>
<th>48 h</th>
<th>60 h</th>
<th>66 h</th>
<th>72 h</th>
<th>% activity (+ semicarbazide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM</td>
<td>121</td>
<td>92</td>
<td>162</td>
<td>35</td>
<td>35</td>
<td>99-99%</td>
</tr>
<tr>
<td>5 mM</td>
<td>94</td>
<td>90</td>
<td>179</td>
<td>135</td>
<td>90</td>
<td>95-99%</td>
</tr>
<tr>
<td>10 mM</td>
<td>110</td>
<td>107</td>
<td>144</td>
<td>144</td>
<td>95</td>
<td>94-97%</td>
</tr>
<tr>
<td>20 mM</td>
<td>100</td>
<td>146</td>
<td>238</td>
<td>104</td>
<td>100</td>
<td>83-92%</td>
</tr>
<tr>
<td>30 mM</td>
<td>318</td>
<td>411</td>
<td>420</td>
<td>285</td>
<td>122</td>
<td>68-80%</td>
</tr>
</tbody>
</table>

Taking the lowest PUT concentrations (2.5-5 mM), a decrease in activity was observed at a culture time of 48 h, which can be attributed to the onset of starvation (see Fig. 4.2). A subsequent significant increase in deamination was recorded at 60 h. A higher metabolic enzyme expression in response to limited nutrients has been reported for
many microorganisms [61] and this can result in the observed oxidase peak. Alternatively, the response may be the result of cell death with associated cell shrinkage and expression of only vital components [62], leading to increased enzyme content relative to total protein. At prolonged times, activity declined to the basal level observed in previous studies [29, 30]. Starvation was not as dominant a feature for cells cultured at higher PUT concentrations where activities increased for culture times up to 60 h. Nevertheless, deamination declined at extended times as the cell number per culture volume decreased (Fig. 4.1). The conversion of higher PUT concentrations into ammonia and 1-pyruvyl/4-aminobutanal can result in a down-regulation of oxidase activity either by catabolite repression or global nitrogen regulation. Indeed, NH$_4^+$ ions have previously been demonstrated to diminish oxidase activity in this strain [29].

One striking feature is the enhanced level of oxidase activity achieved with the 30 mM PUT culture. This response is indicative of a channelling of relevant enzymes/cellular resources via expression and activation to minimise adverse effects of excess PUT. We examined the action of semicarbazide that irreversibly inhibits CuAO activity and can facilitate an evaluation of the relative contribution of each enzyme (FlavAO vs. CuAO) to overall activity [29]. Inhibition by semicarbazide was slight for cells cultured with ≤ 10 mM PUT (Table 4.1) and the catalytic contribution due to CuAO can be taken to be negligible. Increased levels of semicarbazide inhibition and, by association, catalysis by CuAO were observed at higher PUT concentration (notably at 30 mM). A stress response mechanism is supported by the presence of the BLAST predicted Universal Stress Protein (Usp, gi|111017022) and succinate-semialdehyde dehydrogenase (gi|111022562) within the same operon as CuAO [30]. Although the biochemical function is largely unknown, USP of similar homology are expressed in response to heat, substrate starvation, exposure to antimicrobial agents and oxidative stress [63,64]. Patrauchan and co-workers have observed an up-regulation of these
proteins with succinate-semialdehyde dehydrogenase during the stationary growth phase for *Rhodococcus* RHA1 under starvation conditions [63]. Such coordination of activity through expression via the same operon may extend beyond metabolism to assume a cytoprotective role.

### 4.3.5 Extracellular oxidase activity

Following centrifugal removal of cells, a significant level of PUT deamination (up to 35% of total activity in culture) was observed due to enzymes in the growth media. All measurements (of PUT and its metabolites, reaction by-products and glucose) therefore required the media to be clarified and any enzymes present denatured by heating (to 90º C). FlavAO was identified by inhibition (semicarbazide insensitive) and MALDI-TOF analyses [63] (data not shown) as the enzyme chiefly responsible for this extracellular catalytic response. There was limited extracellular activity in the 2.5 and 5 mM PUT cultures (Table 4.2) with detectable levels (>0.5 μmol/min) only recorded at prolonged culture times.

**Table 4.2: Extracellular oxidative deamination activity (μmol/min) at different times for *Rhodococcus opacus* cells grown in varying PUT concentration with final pH (after 72 h).**

<table>
<thead>
<tr>
<th>PUT Concentration</th>
<th>12 h</th>
<th>24 h</th>
<th>42 h</th>
<th>48 h</th>
<th>60 h</th>
<th>66 h</th>
<th>72 h</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>0.9</td>
<td>2.1</td>
<td>6.8</td>
</tr>
<tr>
<td>5 mM</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>0.8</td>
<td>0.9</td>
<td>1.9</td>
<td>3.2</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>1.3</td>
<td>1.3</td>
<td>3.6</td>
<td>5.5</td>
<td>7.2</td>
<td>6.7</td>
</tr>
<tr>
<td>20 mM</td>
<td>≤0.5</td>
<td>0.6</td>
<td>1.6</td>
<td>1.5</td>
<td>5.1</td>
<td>10.9</td>
<td>13.5</td>
<td>6.6</td>
</tr>
<tr>
<td>30 mM</td>
<td>≤0.5</td>
<td>0.7</td>
<td>1.8</td>
<td>2.2</td>
<td>5.2</td>
<td>12.4</td>
<td>16.3</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Taking the cultures from 10-30 mM PUT, significant activity was detected after 42 h with a near exponential increase thereafter. Nevertheless, the activity recorded was appreciably lower (by two orders of magnitude) than the level of intracellular deamination. Regular cell lysis is likely to account for a significant proportion of
activity observed at prolonged culture times (> 48 h). The measurable PUT conversion at 12-36 h must be the result of a directed response. Secretory proteins typically possess signal peptides that control transport from the cell and which are cleaved as the protein passes through the cell membrane. However, bioinformatic analysis performed on FlavAO, utilising a signal peptide predictor (SignalP), has suggested that this enzyme does not possess the required N-terminal cleavage site and is unlikely to be subject to conventional secretion [65,66]. Non-classical secretory methods are poorly understood and extracellular FlavAO may arise from directed lysis, shedding of membrane vesicles as reported in other gram positive bacteria, or via the specialised secretion routes proposed in amino acid metabolism by *Bacillus subtilis* [67,68]. Free FlavAO is not subject to the same inhibition or rate limiting conditions that prevail within the cell, such as ADP [57], cellular transport of PUT or hydrogen peroxide generation. Extracellular generation of hydrogen peroxide can provide a selective advantage over microorganisms that are more sensitive to oxidative stress.

### 4.3.6 Extracellular 1-pyrroline and 4-aminobutyrate

We have previously established [29,30] that the fate of PUT in *Rhodococcus opacus* is subject to the action of a genomic metabolic cluster that is induced by diamines. The degradative amine oxidases and aldehyde dehydrogenase possess all the genes necessary for complete PUT catabolism to succinate Fig 4.3.

![Amine oxidase promoted PUT degradation pathway in Rhodococcus opacus.](image)

*Figure 4.3: Amine oxidase promoted PUT degradation pathway in Rhodococcus opacus.*
These enzymes were shown experimentally to promote the oxidative deamination of PUT to 4-aminobutanal/1-pyrroline [44,69] with subsequent oxoreductase catalysis by dehydrogenase to 4-aminobutyrate, as shown in Fig. 4.3. We set out here to probe the utilisation of the principal products and determine time dependent concentration profiles. The 4-aminobutanal/1-pyrroline equilibrium favours 1-pyrroline as principal product [70], which was monitored (Fig. 4.4) in addition to the subsequent catabolic product (4-aminobutyrate, Fig. 4.5).

![Figure 4.4: Temporal variation in extracellular 1-pyrroline concentration at different initial concentrations of PUT: 2.5 mM, ( ), 5 mM ( ) 10s mM ( ), 20 mM ( ) and 30 mM ( ). Error bars indicate SD. (Note: The figure shows the concentration of 1-pyrroline over time for different PUT concentrations.)](image)

There was no detectable pyrroline (detection limit = 0.01 mM) in the 2.5 mM PUT culture and a measurable concentration was only observed in the 5 mM culture at 24 h, i.e. at the onset of exponential growth (Fig. 4.1). As there was no significant extracellular oxidase activity at this time point (Table 4.2), pyrroline generation can be attributed to intracellular FlavAO and subject to transport mechanisms. Higher pyrroline levels were recorded for the 10 mM PUT culture, which can again be attributed to intracellular catalysis where PUT is converted in preference to glucose (Fig. 4.2). An alternative cellular process is evident for the 20 and 30 mM cultures where there was no detectable pyrroline for up to 48 h (Fig. 4.4). These conditions coincide with significant
CuAO activity (Table 4.1), which we have demonstrated is associated with enhanced activity of a 4-aminobutyrate dehydrogenase genomically flanking CuAO that acts to further convert 4-aminobutanal/1-pyrroline (Fig. 4.3) [29]. The detection of appreciable pyrroline at $t \geq 60$ h (Fig. 4.4) correlates with the observed extracellular oxidase activity (Table 4.2) and the stationary growth phase (Fig. 4.1), which may release intracellular pyrroline by cell lysis.

![Graph](image)

**Figure 4.5:** Temporal variation of extracellular 4-aminobutyrate at different initial concentrations of PUT: 2.5 mM, (.), 5 mM (●), 10 mM (×), 20 mM (●) and 30 mM (■). Error bars indicate SD.

The action of the dehydrogenase enzyme is illustrated by the temporal changes to 4-aminobutyrate concentration shown in Fig. 4.5, which mimics the variations in extracellular pyrroline for 2.5-10 mM PUT cultures. This suggests that the implicated enzymes (amine oxidase and 4-aminobutyaldehyde dehydrogenase, Fig. 4.3) are expressed in tandem. In contrast to pyrroline generation, measurable levels of 4-aminobutyrate were detected over the first 12 h at higher PUT concentrations with an increase in concentration with extended times, consistent with a stepwise conversion (PUT→1-pyrroline→4-aminobutyrate). It should also be noted that 4-aminobutyrate has been implicated in cytoplasmic pH regulation in *Escherichia coli* and *Lactococcus lactis*. 
where the metabolite is exported from the cell to reduce internal acidity [71,72]. On the other hand, pyrroline and 4-aminobutanal have been identified as semiochemicals, i.e. compounds that act as messengers within or between species [73] with a multitude of behaviour altering functions that include mating, food detection and warning mechanisms [74]. Pyrroline is secreted by the Mediterranean fruit fly as a sexual attractant and has been demonstrated to enhance fly attraction to synthetic food lures, particularly when coupled with ammonia, methylamine and PUT [75]. Moreover, the attraction of these flies to duck faeces is influenced by the action of bacteria forming allelochemics such as pyrroline, which is the most potent attractant [76]. *Rhodococcus* often enters new environments through faeces and has been isolated as one of the dominant bacterial groups acting on the fly *Wohlfahrtia magnifica* [77], which is attracted to volatiles generated by *Rhodococcus* to a greater degree than other species taken from myiatic lesions [78]. The generation of pyrroline from PUT by *Rhodococcus* may serve a symbiotic relationship, enabling transfer between host environments.

4.3.7 Extracellular nitrogen

At temperatures ≤ 30 ºC and neutral or acidic pH, the metabolite NH₃ is present in solution in ionic form (NH₄⁺) [79]. As shown in Fig. 4.6, there was no detectable extracellular ammonia in 2.5-5 mM PUT media. This observation, with data presented in Figs. 4.2, 4.4 and 4.5, indicate almost complete nitrogen assimilation (94-97%) by *Rhodococcus*. There was no significant ammonia generation at higher PUT concentrations (10-30 mM) until the onset of the stationary phase (Fig. 4.1). Limited cell growth coupled with maintenance of high levels of intracellular enzymes (from the activity measurements in Table 4.1) results in ammonia release in the 42-48 h period (Fig. 4.6). Ammonia acts as a negative regulator of enzyme expression within *Rhodococcus opacus* [29] and amine oxidase in other organisms [80] and may contribute to the decline in intracellular enzyme levels observed at t > 60 h (Table 4.1).
Figure 4.6: Temporal variation of extracellular ammonia and hydrogen peroxide concentration at different initial concentrations of PUT: 2.5 mM, (▲, open bars), 5 mM (●, reverse hatched bars), 10 mM (★, grey bars), 20 mM (●, forward hatched bars) and 30 mM (●, solid bars). Error bars indicate SD.

Taking the free ammonia, extracellular 1-pyrroline/4-aminobutyrate and unreacted PUT, it is estimated that 50-60% of total nitrogen (for [PUT] ≥ 10 mM) was utilised by the cell or converted into undetected products. This represents a significant difference in nitrogen utilisation compared with the lower PUT concentration cultures (≤ 5 mM) although a similar DCW was recorded (Fig. 4.1). The DCW obtained from 2.5-5 mM PUT cultures suggest oleaginous character of Rhodococcus opacus. Indeed, several reports [81-85] have shown that Rhodococci in response to nutritional stress, particularly nitrogen limitation, continue to assimilate carbon sources despite the absence of cell proliferation. In the case of Rhodococcus opacus PD630 grown on fructose or gluconate, the accumulation triacylglycerols accounted for up to 40-76% of the total DCW [81]. Increased hydrophobic character was apparent for lysed cells grown in ≤ 5 mM PUT, where the accumulation of lipids after 60 h in the cell lysate required additional dilution to prevent the formation of insoluble globules in the
colorimetric assays and cleaning detergents were required in chromatographic analysis. In order to confirm stress induced metabolite accumulation, microscopic analysis was undertaken with staining of inorganic polyphosphates (PolyP) that are stored by *Rhodococcus* in response to nitrogen limitation [77].

![Image](image.png)

**Figure 4.7:** Modified Loeffler's methylene blue staining of *Rhodococcus opacus* cultured for 72 h at 30 °C with 5 mM PUT. (A) General view of cells by optical microscopy (1,000 ×). (B) and (C) Magnified views showing dark stained polyP inclusions and lightly stained cytoplasm.

Ubiquitous dark PolyP bodies (see Fig. 4.7) were found in cells grown in ≤ 5 mM PUT (after 72 h) and are identical to those previously reported for *Rhodococcus opacus* PD630 and *Rhodococcus RHA1* [83,84]. The accumulation of high-energy phosphoanhydrides derived from terminal phosphates of ATP (eg. from glycolysis/PUT degradation products entering the TCA cycle) are believed to provide a selective advantage to rapidly changing dynamic environments. The PolyP bodies were not observed for higher PUT concentration cultures or at *t* < 48 h, indicating that their generation represents a global cellular shift to metabolite interconversion and subsequent accumulation. In *Rhodococcus* spp., these bodies are synonymous with cells that generate high levels of triacylglycerols, wax esters, polyhydroxyalkanoic acids and glycogen [83,84]. PolyP and lipid accumulation in *Rhodococci* has been identified as a survival response, enabling endurance during starvation periods and also in dry and hot conditions [84, 85, 86].
4.3.8 Hydrogen peroxide generation and pH

The oxidative deamination of PUT by FlavAO and CuAO produces hydrogen peroxide (H$_2$O$_2$), which can act as a harmful oxidising agent [87]. Extracellular H$_2$O$_2$ increased (up to 50 µM, Fig. 4.6) with increased PUT concentration and culture times. An apparent peak in H$_2$O$_2$ was observed at 36 h, corresponding to the initial stages of the log phase and the highest rate of PUT consumption. Given that negligible extracellular oxidase activity was detected at this point, the associated H$_2$O$_2$ must be generated from the cytoplasm. As H$_2$O$_2$ concentration has been demonstrated to correlate with increased lag phase within Rhododoccus [88], this may contribute to the lower growth rates that were a particular feature of the 30 mM culture (Fig. 4.1). A subsequent increase in H$_2$O$_2$ levels was observed at prolonged times, coincident with the detection of extracellular PUT conversion. We have established (in Fig. 4.2) that negligible PUT is found in the 2.5-5 mM cultures at these times and any increase in H$_2$O$_2$ can either result from cell lysis releasing intracellular constituents into the media or derive from other enzymatic processes (e.g. action of glucose oxidase) that generate H$_2$O$_2$. Peroxide exhibits bactericidal properties and can play a role in reducing competition from sensitive microflora. Indeed, the concentrations recorded in this study are toxic to several bacteria [88]. In addition to oxidative stress, H$_2$O$_2$ can act as a weak acid and, in tandem with 4-aminobutyrate and other acidic oxidative by-products can serve to acidify the cytoplasm. We have recorded a measurable decrease in pH (from 7) of the buffered media over 72 h that is more marked at higher PUT concentration (Table 4.2). Inactivation of FAD dependent oxidases has been reported at pH 6.4 [57] and the narrow applicable pH range for catalytic activity may serve as a self-regulatory mechanism to protect the cell. It is possible that CuAO activation is pH dependent and triggered by deamination of PUT (at high relative concentrations). The natural environment of Rhodococcus does not provide buffering and wide variation in pH may
involve Usp (see section 4.3.4) in a critical protective role, as observed in *Escherichia coli* [64].

### 4.4 Conclusions

Survival in and exploitation of diverse environmental conditions has required *Rhodococcus opacus* to evolve and optimise a number of synergetic components and sub-systems. In this study, we have monitored the co-ordinated cellular response to diamine (PUT) availability. An efficient regulatory system and adaptive strategy are illustrated by effective degradation of an extended PUT concentration range (2.5-30 mM) with minor variations in overall cell growth. Onset of the log phase was observed after 24 h with the majority of PUT assimilated or inter-converted in the subsequent 12 h period for cultures containing ≤ 10 mM PUT. Conversion of ≥ 20 mM PUT required extended culture times. Catalytic action at low PUT concentrations can be attributed principally to FlavAO with a contribution from CuAO at higher concentrations, presumably as a stress related mechanism. The generation of catabolic products such as 1-pyrroline, 4-aminobutyrate (the product of 4-aminobutyraldehyde dehydrogenase), ammonia and hydrogen peroxide with accompanying pH variations serve to significantly alter the organism’s environment. PUT conversion involves both inter- and extra-cellular oxidases where PUT is consumed in preference to glucose. The increased rate of metabolism at higher PUT concentration extends beyond cellular utilisation, providing a potential selective advantage to assimilate energy sources in competition with other microorganisms. Carbon storage with high energy inorganic polyphosphates is observed at low concentrations to enable rapid response when nitrogen becomes available. Future studies will pinpoint the conditions that lead to the expression of CuAO with a full characterisation of lipid and glycogen storage compounds to establish the mechanisms employed by *Rhodococcus* to counter dynamically changing environments.
4.5 References


Chapter 5 - Repertoire of Nitrogen Assimilation in *Rhodococcus*; Catalysis, Pathways and Relevance in Biotechnology and Bioremediation

**Foreword**

In order to understand the multifaceted role and influence that these oxidase enzymes play, a full appreciation of the global metabolic composition of *Rhodococcus* is necessary. This can then explain how this organism competes in niche environments, including exposure to harmful xenobiotics. In addition facilitates exploitation in bioremediation and biocatalysis. Chapter 5 is organised to examine each of the major organic compound involved in nitrogen assimilation (amines, amides, amino acids, nitriles and nitroaromatics) in turn with a critical analysis of the literature. In this way, the considerable repertoire of *Rhodococci* is established. Drawing on detailed enzyme analysis and genomic characterisation, the molecular and cellular basis for these organisms to thrive in polluted environments which typically are harmful to most organisms is examined. The metabolic gene clusters observed for major pathways are identified, noting how this can inform future directions for *Rhodococcus* spp. in terms of enzymatic optimisation and metabolic engineering.

**5.1 Background and Overview**

The genetic and metabolic versatility of *Rhodococcus* spp. allows this genus to thrive in habitats ranging from tropical, arid and arctic soils to marine and deep sea sediments. The capacity to inhabit multiple niche environments has meant that these non-sporulating aerobic bacteria are among the most frequently isolated organisms in terrestrial and aquatic studies [1-5]. Application in bioremediation follows from the observed persistence in response to stressors coupled with an ability to degrade and transform herbicides [6], pollutants and other xenobiotics [7]. As nitrogen is the major limiting substrate for growth in nature, the underlying cellular stimulus for many of
these transformations is the requirement for assimilation and mineralisation [8]. Such evolutionary pressures have lead to a diverse array of enzymes frequently with a plasticity to act on chemically related substrates and periodically prevalent energy sources and essential nutrients [6, 9]. Full exploitation of *Rhodococcus* spp. in bioremediation and biotechnology consequently requires a fundamental understanding of these processes from both a genomic [10] and enzymatic perspective, [9, 11-13] and an appreciation of their role within the organism as part of the nitrogen cycle and associated regulatory aspects (repression and induction). We evaluate here the physiological and genomic basis for catalytic activity/selectivity and identify candidate genes in nitrogen assimilation. Taking the major organo-nitrogen compounds (amino acids, amines, nitriles, amides and nitroaromatics), we establish the considerable catalytic repertoire of *Rhodococcus* with related pathways implied from metabolic gene clusters. The implications with respect to biotechnological application, placing particular emphasis on bioremediation, are addressed.

5.2 *Rhodococcus* and Bioremediation

The process of controlled degradation of waste products by either addition of organisms (bioaugmentation) or other materials (biostimulation) is still a technology in its relative infancy with mixed success to date [14]. Ecological parameters, such as available nutrients, temperature, pH, aeration, co-contaminants and competition with native organisms, influence overall efficiency. Nevertheless, bioremediation using bacteria such as *Rhodococcus* spp. provides a number of advantages over physico-chemical methodologies, facilitating complete mineralisation of xenobiotic pollutants that is eco-friendly and enjoys wide public acceptance [15]. However, the process must become more systematic and methodical, drawing on genomic and enzymatic data to predict the response in specific environments. *Rhodococcus* spp. are recognised among the most promising bioremediation bacteria, and has been utilised to remove
contaminants from soil, water and air [16]. As this genus is readily isolated and grown in the laboratory (as opposed to 99% of bacteria), this provides a significant practical benefit in the preparation of inocula that exhibit known activity. Moreover, only two *Rhodococcus* sp. exhibit pathogenicity, which is an important consideration in bio- and environmental safety [16]. Despite exhibiting a high level of competition with other bacteria under crowded and nutrient limited conditions, *Rhodococcus* spp. are unlikely to produce toxins or antimicrobial compounds that could adversely affect co-metabolism of pollutants [16].

Numerous physiological and genetic adaptations occur to ensure survival under dynamic ecological conditions. Of relevance to bioremediation, this can include weak catabolic repression by more accessible carbon sources [17] with the continuous induction of degradative enzymes for extended periods even in the absence of stimulant pollutants. This ensures a higher degradative capacity than bacteria limited by a critical biomass/activity balance in organic rich environments [18]. *Rhodococci* can endure starvation in extreme environments (hot/cold/dry) through a number of mechanisms such as PolyP and lipid accumulation, which allow rapid response under changing conditions [19-22]. In the presence of xenobiotic or detrimental agents, the thick Gram positive cell wall is critical where the long chain fatty acid composition may be modulated to alter permeability and protect the cell. Contaminant degradation rate has been demonstrated to correlate with cell surface hydrophobicity and the lipid cell wall facilitates access to hydrophobic pollutants such as found in hydrocarbon-contaminated sites [23]. Other biophysical adaptations include generation and adhesion to biofilms that enhance cell aggregation and resistance relative to free living cells [16, 24], and biosurfactants (such as glycolipids) are frequently generated in response to organic media, leading to a significant reduction in surface tension and enhanced bioavailability [25].
5.3 Genomic Aspects of Degradation Activity

*Rhodococcus* survival is directly related to its genomic versatility. Biotransformations with potential biotechnological application (primarily xenobiotic reactions) as recorded in the Minnesota Biocatalysis/Biodegradation Database (http://umbbd.msi.umn.edu/)[26], assigns 6% of reactions to *Rhodococcus* spp., a scale of activity that is second only to the *Pseudomonas* genus. This level of metabolic activity is achieved through numerous catabolic pathways and their homologues by recombination over many millennia[10]. *Rhodococcus* spp. possess complex genomes that are among the largest sequenced in prokaryotes. The best characterised belongs to *Rhodococcus* RHA1 with 9.7 Mb distributed in one chromosome and three large linear plasmids, *i.e.* pRHL1 (1100 kb), pRHL2 (450 kb) and pRHL3 (330 kb)[10]. Catalytic action has been established for 203 oxygenases coding for the degradation of aromatic compounds and steroids, more than twice that identified in other actinomycetes and ecologically related bacteria[10]. These oxygenases provide the basis for 8 core pathways in arene assimilation, as well as a further 26 “peripheral” routes, which amplify diversity and transform compounds into substrates suitable for central catabolic pathways. The genomes of other sequenced *Rhodococcus*, including *Rhodococcus opacus* B4 (8.8 Mb)[27], *Rhodococcus erythropolis* P4 (6.8 Mb)[28] and *Rhodococcus equi* 103s (5.3 Mb)[29], all display similar characteristics with multiple gene homologues and functional redundancy. Differing to *Rhodococcus* RHA1 there is evidence of more recent horizontal gene transfer in relation to anthropogenic xenobiotics that can contribute to flexibility with additional enzymatic activity, of note is the large number of catabolic genes found upon the plasmids[10, 30].

These genomes are invaluable tools not only for characterising the organism but in discovering new catabolic pathways. One of the first examples in *Rhodococcus* spp. is a cluster of genes encoding a taurine-pyruvate degradation pathway shown in Fig. 5.1. [13].
When utilised as a nitrogen source, assimilation is achieved through a genomic cluster consisting of a taurine regulator (tauR), aminotransferase (tpa) and alanine dehydrogenase (ald) in consort with a sulfoacetaldehyde acetyltransferase (xsc) separated by 20 kbp from this group of functionally related genes [13]. This is distinct from the action of taurine dioxygenase, which generates ammonia and is utilised by *Rhodococcus* principally for sulphur assimilation [13, 31]. Genomic and proteomic analyses have also identified a new class of acetonitrile hydratase [32] and a putrescine degradation pathway that utilises amine oxidases [11].

In addition to enzymes that have been characterised, metabolic clusters of genes found throughout the genome provide insight into probable reaction mechanisms in the bacterium. Taking urea, a major metabolite that frequently leads nitrogen catabolite repression, the genes responsible for degradation within *Rhodococcus* RHA1, are encoded by distinct clusters, coding for two separate pathways. Urease, which generates ammonia and CO$_2$ in a single step (Fig. 5.2(a)), is encoded by ureA (gamma), ureB (beta) and ureC (alpha) subunits, similar to those observed in *Bacillus* sp. TB-90, *Helicobacter pylori* and *Klebsiella aerogenes* [33]. The predicted proteins appear in succession genomically (Fig. 5.2(a)) with putative accessory proteins (ureF, ureG and ureD) known in other species to interact with the apoenzyme, inserting Ni$^{2+}$ into the urease active site as part of the assembly process [33].
A second two step pathway, common in yeast and algae but also characterised in bacteria [34], involves the combined action of urea amidolyase/carboxylase and an allophanate hydrolase. Genes encoding homologues of these enzymes appear in succession in two separate metabolic clusters, shown in Fig. 5.2(b). The first cluster includes two distinct enzymes (urea carboxylase and allophanate hydrolase), the second containing amidolyase with four hydrolase subunits. The seeming functional redundancy with a second catabolic route for deaminating urea may reflect expression at different sub-cellular locations or under alternative environmental conditions such as nickel availability.

Figure 5.2(a)

**Figure 5.2(b)**

Figure 5.2: (a) Degradation of urea to ammonia catalysed by a urease with the enzyme cluster showing the three required subunits of apoenzyme encoded by ureA (gamma), ureB (beta) and ureC (alpha) with additional genes (ureF, ureG and ureD) that participate in the molecular assembly of urease; (b) Urea degradation catalysed by urea amidolyase/carboxylase and an allophanate hydrolase to generate ammonia. The two enzyme clusters have been identified in the Rhodococcus RHA1 genome by BLAST analysis.
5.4 Amino Acid Degradation

5.4.1 Background

Utilisation of protein as the most abundant organic nitrogen source in terrestrial habitats necessitates the catabolic action of extracellular enzymes [35]. The resultant polypeptides, amino acids and nitrogenous compounds can be subsequently metabolised by an array of intracellular enzymes. Extracellular degradation can also convert proteins directly to ammonium species as the preferential nitrogen source for most bacteria [35, 36]. While microorganisms can degrade (and conversely synthesise) all essential amino acids, the direct liberation of the amino component is often limited to select amino acids due to high specificity exhibited by deaminase/lyase enzymes. The associated microbial enzymes include amino acid oxidases (AAO) and NAD-linked enzymes such as dehydrogenases that transform amino acids to the corresponding α-keto acid (and ammonia) and lyases that cleave C-N bonds to generate unsaturated acids [37, 38]. Amino acid utilisation can also involve decarboxylation to produce amines with CO₂ release [36]. Alternatively, transaminases transfer the amino group to a keto acid with sequential binding of the substrates and a “ping-pong (bi-bi) mechanism” to deaminate the amino acid [39].

5.4.2 Genomic and catalytic analysis

There are a number of decarboxylases (eg. RHA1_ro08323, RHA1_ro08323 and RHA1_ro06016), putatively identified by sequence homology within the genome of Rhodococcus RHA1, that may encode enzymes that act on amino acids [10]. Transaminases, including homologues of aspartate and alanine aminotransferase, catalyse reversible transamination of straight chain amino acids to glutamate and the corresponding keto-acid (Table 5.1). Homologues of branched chain aminotransferases (ilvE1, ilvE2 and ilvE3) convert amino acids such as leucine, valine and isoleucine. On
the basis of sequence homology, 4-aminobutyrate a product of (among others) arginine and ornithine decarboxylation are likely subject to class III aminotransferases (RHA1_ro08620, gabt3, gabT2, gabT3) in glutamate coupled reactions (Table 5.1) [10, 40].

Table 5.1: Gene homologues of amino acid transaminase enzymes within the Rhodococcus RHA 1 genome, co-substrates (amino donor and accepting keto-acid) and corresponding products as predicted by sequence homology.

<table>
<thead>
<tr>
<th>Transaminase substrate</th>
<th>Candidate genes</th>
<th>Amino acid&lt;sub&gt;1&lt;/sub&gt; + keto acid&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Amino acid&lt;sub&gt;2&lt;/sub&gt; + keto acid&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Aspartate</td>
<td>aspC, RHA1_ro04781</td>
<td>L-aspartate + 2-oxoglutarate</td>
<td>L-glutamate + Oxaloacetate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-glutamate + pyruvate</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>alaT</td>
<td>L-alanine + 2-oxoglutarate</td>
<td></td>
</tr>
<tr>
<td>Branched chain</td>
<td>ilvE1, ilvE2, ilvE3</td>
<td>L-leucine + 2-oxoglutarate</td>
<td>L-glutamate + 4-methyl-2-oxopentanoate</td>
</tr>
<tr>
<td>amino acids</td>
<td></td>
<td>L-valine + 2-oxoglutarate</td>
<td>L-glutamate + 2-oxovalerate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-isoleucine + 2-oxoglutarate</td>
<td>L-glutamate + 3-methyl-2-oxopentanoate</td>
</tr>
<tr>
<td>4-Aminobutyrate</td>
<td>RHA1_ro08620, gabt3, gabT2, gabT3</td>
<td>4-aminobutyrate + 2-oxoglutarate</td>
<td>L-glutamate + succinate semi-aldehyde</td>
</tr>
</tbody>
</table>

Gene homologues with putative glutamate dehydrogenases, acting on glutamate to release ammonia, can facilitate nitrogen assimilation from a variety of amino acids, as given in Table 5.2. Alanine dehydrogenase (RHA1_ro01495, ald1, ald2) and aspartate ammonia lyase (aspA1, aspA2, aspA3), with corresponding transaminases, provide alternative ping-pong (bi-bi) systems. Specificity and cellular catalytic efficiency is extended by a number of genes encoding asparagine dehydrogenase, arginine, cysteine, histidine and serine ammonia lyase (Table 5.2).
Table 5.2: Gene homologues of amino acid deaminating enzymes within the Rhodococcus RHA 1 genome with enzyme specificity predicated by sequence homology.

<table>
<thead>
<tr>
<th>Amino acid substrate</th>
<th>Deaminating enzyme</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>RHA1-ro03288, ghdA4, gdhA1, dghA,</td>
<td>ammonia + oxoglutarate</td>
</tr>
<tr>
<td></td>
<td>gdhA</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>ansA, RHA1_ro04451</td>
<td>ammonia + aspartate</td>
</tr>
<tr>
<td>Ammonia Lyase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>RHA1_ro2963, RHA1_ro6842</td>
<td>ammonia + glutamate</td>
</tr>
<tr>
<td>Cysteine</td>
<td>RHA1_ro06506</td>
<td>ammonia + pyruvate</td>
</tr>
<tr>
<td>Histidine</td>
<td>HutH, RHA1_ro02588</td>
<td>ammonia + urocanate</td>
</tr>
<tr>
<td>Serine</td>
<td>RHA1_ro01828, sdaA, RHA1_ro05336</td>
<td>ammonia + pyruvate</td>
</tr>
<tr>
<td>Aspartate</td>
<td>aspA1, aspA2 aspA3</td>
<td>ammonia + fumarate</td>
</tr>
</tbody>
</table>

The production of extracellular surface-bound amino acid oxidases (AAO) has been investigated in several fungal and bacterial species, including *Rhodococcus opacus* DSM 43250 and *Rhodococcus* sp. Z-35-1 [38, 41-43]. AAO catalyse oxidative deamination to the keto-acid with ammonia and hydrogen peroxide release (Fig. 5.3).

![Amino Acid Oxidase Reaction](image)

**Figure 5.3:** Conversion of amino acids by the catalytic action of amino acid oxidase

These homo-dimeric enzymes are non-covalently bound to an FAD molecule, which acts as a redox co-factor [44]. While some AAO have been characterised by narrow substrate specificity, those of *Rhodococcus opacus* DSM 43250 were found to act on a broad range of amino acids, converting over 50 aliphatic, aromatic, cyclic and sulphur containing amino acids, a selection of which are identified in Table 5.3 [38].
### Table 5.3: Range and structure of amino acids oxidised by AAO in *Rhodococcus opacus* DSM 43260 [38]

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aliphatic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-alanine</td>
<td><img src="image" alt="L-alanine structure" /></td>
<td>L-Valine</td>
</tr>
<tr>
<td>L-leucine</td>
<td><img src="image" alt="L-leucine structure" /></td>
<td>L-Leucine</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td><img src="image" alt="L-isoleucine structure" /></td>
<td>L-Isoleucine</td>
</tr>
<tr>
<td>L-2-amino-4,4-dimethylvaleric acid</td>
<td><img src="image" alt="L-2-amino-4,4-dimethylvaleric acid structure" /></td>
<td>L-2-amino-4,4-dimethylvaleric acid</td>
</tr>
<tr>
<td>L-valine</td>
<td><img src="image" alt="L-valine structure" /></td>
<td>L-Valine</td>
</tr>
<tr>
<td>L-leucine</td>
<td><img src="image" alt="L-leucine structure" /></td>
<td>L-Leucine</td>
</tr>
<tr>
<td>L-3-(1-naphtyl)alanine</td>
<td><img src="image" alt="L-3-(1-naphtyl)alanine structure" /></td>
<td>L-3-(1-naphtyl)alanine</td>
</tr>
<tr>
<td>L-histidine</td>
<td><img src="image" alt="L-histidine structure" /></td>
<td>L-Histidine</td>
</tr>
<tr>
<td>L-tryptophane</td>
<td><img src="image" alt="L-tryptophane structure" /></td>
<td>L-Tryptophane</td>
</tr>
<tr>
<td>DL-homophenylalanine</td>
<td><img src="image" alt="DL-homophenylalanine structure" /></td>
<td>DL-Homophenylalanine</td>
</tr>
<tr>
<td>L-phenylglycine</td>
<td><img src="image" alt="L-phenylglycine structure" /></td>
<td>L-Phenylglycine</td>
</tr>
<tr>
<td>L-3-(1-pyridyl)alanine</td>
<td><img src="image" alt="L-3-(1-pyridyl)alanine structure" /></td>
<td>L-3-(1-pyridyl)alanine</td>
</tr>
<tr>
<td>D-1-norleucine</td>
<td><img src="image" alt="D-1-norleucine structure" /></td>
<td>D-1-Norleucine</td>
</tr>
<tr>
<td><strong>Cyclic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-homoserine lactone</td>
<td><img src="image" alt="L-homoserine lactone structure" /></td>
<td>L-Homoserine lactone</td>
</tr>
<tr>
<td>L-homocysteine thiolactone</td>
<td><img src="image" alt="L-homocysteine thiolactone structure" /></td>
<td>L-Homocysteine thiolactone</td>
</tr>
<tr>
<td>L-4-chlorophenylalanine</td>
<td><img src="image" alt="L-4-chlorophenylalanine structure" /></td>
<td>L-4-Chlorophenylalanine</td>
</tr>
<tr>
<td>L-4-fluorophenylalanine</td>
<td><img src="image" alt="L-4-fluorophenylalanine structure" /></td>
<td>L-4-Fluorophenylalanine</td>
</tr>
<tr>
<td>L-4-nitrophenylalanine</td>
<td><img src="image" alt="L-4-nitrophenylalanine structure" /></td>
<td>L-4-Nitrophenylalanine</td>
</tr>
<tr>
<td>L-2-chlorophenylalanine</td>
<td><img src="image" alt="L-2-chlorophenylalanine structure" /></td>
<td>L-2-Chlorophenylalanine</td>
</tr>
<tr>
<td>L-4-aminophenylalanine</td>
<td><img src="image" alt="L-4-aminophenylalanine structure" /></td>
<td>L-4-Aminophenylalanine</td>
</tr>
<tr>
<td><strong>Sulphur containing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-methionine</td>
<td><img src="image" alt="L-methionine structure" /></td>
<td>L-Methionine</td>
</tr>
<tr>
<td>L-cysteic acid</td>
<td><img src="image" alt="L-cysteic acid structure" /></td>
<td>L-Cysteic acid</td>
</tr>
<tr>
<td>L-cystine</td>
<td><img src="image" alt="L-cystine structure" /></td>
<td>L-Cystine</td>
</tr>
<tr>
<td><strong>Aromatic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td><img src="image" alt="L-phenylalanine structure" /></td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td><img src="image" alt="L-tyrosine structure" /></td>
<td>L-Tyrosine</td>
</tr>
<tr>
<td>L-4-chlorophenylalanine</td>
<td><img src="image" alt="L-4-chlorophenylalanine structure" /></td>
<td>L-4-Chlorophenylalanine</td>
</tr>
<tr>
<td>L-4-fluorophenylalanine</td>
<td><img src="image" alt="L-4-fluorophenylalanine structure" /></td>
<td>L-4-Fluorophenylalanine</td>
</tr>
<tr>
<td>L-4-nitrophenylalanine</td>
<td><img src="image" alt="L-4-nitrophenylalanine structure" /></td>
<td>L-4-Nitrophenylalanine</td>
</tr>
<tr>
<td>L-2-chlorophenylalanine</td>
<td><img src="image" alt="L-2-chlorophenylalanine structure" /></td>
<td>L-2-Chlorophenylalanine</td>
</tr>
</tbody>
</table>

### Additional substrates included: L-arginine; L-lysine; L-glutamine; L-asparagine; N\(^{\epsilon}\)-acetyl-L-lysine; L-2,3-diaminopropionic acid.
5.4.3 Cellular strategies and possible applications

As oxidases act on a broad range of substrates, this increases the diversity of applicable nitrogen and carbon sources, providing a direct route for incorporating newly synthesised protein. Energy is conserved as there is no longer a requirement for specific amino acid transporters and nitrogen may be assimilated by the constitutively expressed ammonia transporters [45]. The generation of toxic hydrogen peroxide as by-product can account for extracellular catalysis. Expression outside the cell serves to suppress microbial competition, as observed in the case of Streptococcus oligofermentans [46]. Genes that encode this group of enzymes are repressed by inorganic nitrogen sources (such as ammonia) and expression of AAO is induced by amino acids, indicating a primary role in cellular survival [9]. Although the biological function is yet to be conclusively established, extracellular deamination of amino acids must confer critical advantages over intracellular enzymes. The dearth of specific assays for soil oxidase activity has limited our understanding of associated activities, regulation and relative contribution in degradation. As AAO appear in several ectomycorrhizal basidiomycetes and ascomycetes [41, 42, 47] with genes conserved in all Rhodococcus genomes sequenced, we can propose that they contribute to the ammonia generated in those assays measuring glutamine, asparagine, histidine and aspartate deamination in soil [48, 49]. A significant commercial application of oxidases draws on the observed regioselectivity that facilitates deracemisation and production of chiral chemical and pharmaceuticals [50]. The catalytic action of AAO in tandem with aldehyde dehydrogenase in Rhodococcus sp. Z-35-1 is notable in converting N-α-benzyloxycarbonyl-L-lysine to N-α-benzyloxycarbonyl-L-amino adipic acid via N-α-benzyloxycarbonyl-L-amino adipate-D-semialdehyde, both precursors to β-lactam antibiotics and building blocks for pharmaceuticals and active peptides [38, 43]. The
rate of production of these chiral intermediates was three times greater than that recorded for *Aspergillus niger* AKU 3302 [43].

5.5 Amines

5.5.1 Background

Natural amines are formed primarily *via* amino acid decarboxylation and are therefore frequently associated with microbes acting on organic matter, as outlined above. Other sources involve animation of simple aldehydes and ketones by higher plants [51]. Amines are widely utilised as azo dyes and intermediates in the production of photographic chemicals, pharmaceuticals, and agricultural chemicals [52]. They are established carcinogens with recorded incidences of human cancer linked to the industrial production and handling of amines [52, 53]. It is recognised that bioconversion of amines has a critical role to play in environmental remediation [54], treatment of livestock waste [55], wine, fish and meat spoilage [56-58] and the enantioselective synthesis of fine chemicals [59]. While bacteria can utilise amines through the action of amine dehydrogenases or catabolic routes found in *Pseudomonas* [60] and *Paracoccus denitrificans* [61], deamination in *Rhodococcus* is normally promoted by amine oxidases. These enzymes possessing a redox co-factor of FAD or copper dependent topaquinone liberate ammonia generating corresponding aldehyde with hydrogen peroxide as a by-product (Fig. 5.4) [62, 63].

![Figure 5.4: Conversion of amines by the catalytic action of amine oxidase.](image-url)
5.5.2 Genomic and catalytic analysis

Genomic analysis of *Rhodococcus* has revealed the presence of at least eight putative amine oxidases within *Rhodococcus* RHA1 and *Rhodococcus opacus* B4 [9]. In contrast, there are two putative genes in the pathogenic (*Rhodococcus equi*) and marine (*Rhodococcus erythropolis*) varieties of the species [3]. Multiple oxidases in *Rhodococcus* spp. can be linked to substrate specificity, facilitating effective amine utilisation at low available nitrogen concentration [64]. Recent work has established three copper dependant oxidases in *Rhodococcus opacus* DSM 43250, induced by pre-treatment with aliphatic monoamines, diamines and primary aromatic amines as sole nitrogen source [9, 11]. van Hellemont and co-workers have identified a homologue (92% identities) of a FAD dependent putrescine oxidase isolated from *Rhodococcus erythropolis* [62].

The FAD and copper dependent oxidases exhibited reaction specificity for the range of amine substrates given in Table 5.4. Microbial diamine specific oxidases have been explicitly characterised in *Rhodococcus* spp. and *Micrcococcus rubens* [65] but a wider distribution has been proposed [66]. These oxidases act to modulate intracellular putrescine, an amine that alters transcription and growth, ensuring viability in response to a range of stressors that impact on pollution treatment strategies, such as pH, oxygen radicals, ultraviolet light, γ-radiation and antibiotics [11]. The FAD putrescine oxidase is highly specific to diamines where one amine group binds to an anionic point near the enzyme active site, facilitating deamination of the second amino function [62]. A copper dependent diamine acting oxidase, found in the same metabolic cluster, provides greater substrate flexibility, acting on longer and shorter chain diamines and monoamines (to a limited extent) [11]. The results of genomic analyses support a complete oxidase degradation pathway for *Rhodococcus opacus*, shown in Fig. 5.5 for putrescine [11]. This differs from mechanisms in other species that utilise transaminases or di-amine modifications (γ-glutamylation or acetylation) to facilitate oxidation [67, 68].
Aminobutyraldehyde dehydrogenase has been identified within the cluster that converts 4-aminobutanal, the product of putrescine oxidation, to 4-aminobutyrate [69]. Gene homologues of successive enzymes (4-aminobutyrate transaminase and succinate-semialdehyde dehydrogenase) are postulated to complete metabolism to succinate [70]. Two copper oxidases were shown to convert monoamines but were inactive with respect to branched, secondary and tertiary amine substrates [9, 11]. These enzymes share 63% homology and may have occurred as paralogues, evolving to provide differing catalytic efficiency/functionality while maintaining activity for a number of common substrates. One of the oxidases exhibited preference for aliphatic monoamines with lower deamination rates for benzylamine and derivatives [9]. The second acted principally on catechol amines (Table 5.4) and contributes to the phenylalanine (phenylacetic acid) degradation pathway as demonstrated by knockout mutagenesis studies [9, 71].

Figure 5.5: Proposed putrescine degradation pathway for *Rhodococcus opacus* with the corresponding gene cluster within the genome [11].
Table 5.4: Range and structure of amines oxidized by amine oxidases in *Rhodococcus opacus* DSM43250.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Structure</th>
<th>Substituent/R function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic diamines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylenediamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadaverine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexamethylenediamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermidine</td>
<td></td>
<td></td>
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<td>4-Fluoro benzylamine</td>
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<td>3,4-Dihydroxybenzylamine</td>
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<tr>
<td>R₁ = CH₂, R₂ = NH, R₃ = Ph</td>
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*No growth observed but serves as enzyme substrate.*

Additional substrates include ethanolamine, cyclobutylamine, cyclohexylethylamine, cyclopentylamine, and spermidine.
5.5.3 Cellular strategies and possible applications

Several roles, beyond increasing the catalytic repertoire in nature, have been proposed for amine oxidases. Pathogenic activities have been noted in both plant and fish, whereby the host immune response increases expression of putrescine as a defence mechanism. The high levels of deamination in *Rhodococci* may reduce the potency of immune activity and aid colonisation [9, 11, 66]. In bioremediation, we can highlight the potential for *Rhodococcus* to fully degrade toxic benzylamine, used in paint production and synthetic textiles [52]. Whilst priority pollutant aniline is subject to steric hindrance by oxidases, it can be converted by a known dioxygenase, promoting simultaneous deamination and oxygenation to generate catechol [72]. In addition to the direct deamination of pollutants, amine oxidation generates hydrogen peroxide (Fig. 5.4), which can be harnessed as a biostimulation strategy where the peroxide serves as oxygen supplement and a powerful oxidant, converting a range of recalcitrant compounds [73]. Moreover, this by-product can be utilised by peroxidase enzymes, which act on phenols and anilines [74]. As hydrogen peroxide availability has been identified as the rate limiting factor [75], targeted nutrient supplementation using amines and the corresponding oxidases has been proposed [11]. It should be noted that *Rhodococcus* RHA1 possesses a lignin peroxidase highly similar to one known to degrade polycyclic and aromatic pollutants [76]; this enzyme has not been explicitly identified or characterised in other bacteria. Alkaloid biosynthesis through metabolic engineering is an exciting avenue of research. We note that amine oxidase from *Rhodococcus opacus* DSM 43250 has exhibited a two-fold higher dopamine oxidation rate than recorded for *Escherichia coli, Aspergillus niger* or *Arthrobacter glomerulus* [9]. The product (4-dihydroxyphenylacetaldehyde) spontaneously forms norlaudanosoline, which is a key rate limiting intermediate in morphine biosynthesis [77]. Oxidation of methylputrescine, a potential target for diamine oxidases, can
generate (N-methyl-Δ1–pyrrolium), this is a central metabolite for the generation of tropane alkaloids or nicotine biosynthesis [78].

5.6 Nitriles

5.6.1 Background

Nitriles are ubiquitous in soil environments, arising from both natural and anthropogenic sources [79, 80]. Naturally derived nitriles, synthesised by plants, fungi, bacteria, algae and insects typically occur as amino acid derived cyanoglycosides but can also include cyanolipids, phenylacetonitrile and ricinine [79]. Extensive use of substituted benzonitrile derivatives as herbicides has increased environmental exposure to these toxic, mutagenic and carcinogenic compounds [6, 81]. This is further exacerbated by the widespread implementation of nitriles in chemical industries [82], ranging from solvents (eg. actyonitrile), polymers (eg. adiponitrile and acrylonitrile) to fine chemical syntheses (eg. enantiopure carboxylic acids and amides) [83, 84]. Environmental degradation of nitriles by microbes is achieved through two principal metabolic pathways, i.e. direct hydrolysis by nitrilase [85, 86] or combined action of nitrile hydratase [87] and amidase, as shown in Fig. 5.6, generating ammonia and the corresponding carboxylic acid.

![Figure 5.6: Metabolic pathways for nitrile degradation by nitrilase and nitrile hydratase/amidase](image)

Over 25% of the nitrile degrading isolates in publicly available strain collections belong to the *Rhodococcus* genus [88] where enzymatic activity applies to either or both pathways. Diversity in the degradation of natural and synthetic nitriles is illustrated by the entries Table 5.5.
Table 5.5: Compilation of aromatic and aliphatic nitriles degraded by nitrilases in *Rhodococcus rhodochrous* J1 an and *Rhodococcus rhodochrous* K2.

<table>
<thead>
<tr>
<th>Substance/Function</th>
<th>Nitriles</th>
<th>Structure</th>
<th>Substrates</th>
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<tbody>
<tr>
<td>Benzamides</td>
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<tr>
<td>Malonamides</td>
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Additional substrates include Z-phenylacetonitrile, phenylacetonitrile and isobutylamine.

Deamination occurring with both meta- and para-substituted analogues.

Additional substrates include 2-furanocarbonitrile, phenylacetonitrile and isobutylamine.
5.6.2 Genomic and catalytic analysis: Nitrilases

Nitrilases can be categorised with respect to substrate affinity into those which preferentially act on (hetero-)aromatic nitriles, aliphatic nitriles or arylacetonitriles. In contrast to nitrile hydratases, nitrilases do not possess a metal co-factor or prosthetic group and catalyse direct bond cleavage yielding the corresponding acid (and ammonia) [89, 90]. The majority of microbial nitrilases are inducible, forming helices of multiple subunits (each subunit ca. 30-45 kDa) [91]. One exception is the unsequenced monomeric nitrilase of *Rhodococcus rhodochrous* PA 34 [92]. It was initially proposed that nitrilases only catalysed reactions involving aromatic nitriles [85] where enzymes from *Rhodococcus* sp. ATCC39484 [93], *Rhodococcus rhodochrous* NCIMB 11216 [94, 95] and *Rhodococcus rhodochrous* J1 converted cyanopyridines, benzonitrile and *meta-/para-* substituted analogues [85]. However, when *Rhodococcus rhodochrous* J1 was incubated with ammonium sulphate or glycerol, the purified subunits exhibited substrate specificity for aromatic/heterocyclic nitriles [87], notably this aromatic nitrilase also had a high degree of specificity to acrylonitrile and crotononitrile [96]. Kobayashi et al [97] have demonstrated that nitrilase from *Rhodococcus rhodochrous* K22 is strongly induced by isovaleronitrile, does not exhibit any aromatic activity but promotes the degradation of unsaturated (acrylonitrile, crotononitrile, isobutyronitrile) and saturated nitriles (acetonitrile and glutaronitrile) and dinitriles (adiponitrile, succinonitrile). [97] Nitrilase expression is governed by a positive transcriptional regulator of the AraC family, which was identified as nitR within *Rhodococcus rhodochrous* J1 and genomically precedes the nitrilase gene [98].

5.6.3 Genomic and Catalytic analysis: Nitrile hydratase (and amidase)

Nitrile hydratases (NHase) are two component metalloenzymes that can be classified according to the metal ion at the catalytic centre. Enzymes possessing iron have been isolated from *Rhodococcus* R312 (formerly *Brevibacterium* R312, one of
the first non-heme iron enzymes with a low spin Fe$^{3+}$ ion) [99, 100], Rhodococcus sp. N-771, [101] *Rhodococcus erythropolis* A4 [102] and *Rhodococcus erythropolis* AJ280 [103]. NHase that possess a non-corrinoid cobalt atom were the first isolated in *Rhodococcus rhodochrous* J1 [104] and have subsequently been detected in *Rhodococcus erythropolis* SP YH3-3 [105] and *Rhodococcus ruber* CGMCC3090 [106]. Although these two classes of enzymes share appreciable amino acid homology, they differ in biotransformation activity and substrate specificity. The iron NHase, *in vivo* and *in vitro*, display unique photosensitivity [101] with increased catalytic activity in response to exposure to light. Endogenous nitric oxide was found to act as a negative regulator, binding to the non-heme iron while photo-dissociation by near UV radiation activates the enzyme [101]. The genes that encode iron NHase are highly conserved, with α- and β-subunits each ca. 23 kDa (nha1 + nha2) that form the enzyme within the same operon, as shown in Fig. 5.7.

![Figure 5.7: Catalysis of nitriles by the iron nitrile hydratase in *Rhodococcus erythropolis* PR4 / *Rhodococcus globerulus* A4: nhr4 = transcriptional regulator of AraC family; oxd = aldoxime dehydratase; nhr2 = regulator; nhr1 = regulator; ami = amidase; nha1 = α-subunit of nitrile hydratase; nha2 = β-subunit of nitrile hydratase; nhr3 = nitrile hydratase activator. [107,108]](image)

Despite the genetic similarity, wide phenotypic variation is observed in specificity to aromatic, aliphatic, alicyclic and branched nitriles [103, 107]. Functionally associated genes in nitrile biosynthesis and degradation are clustered in all strains cloned and characterised, including *Rhodococcus erythropolis* PR4 (Fig. 5.7), *Rhodococcus globerulus* A4, *Rhodococcus* sp N771 and *Rhodococcus* RHA1 [107, 108]. The action
of aldoxime dehydratase (oxd), a lyase that cleaves the hydroxyl function (Fig. 5.7), is critical in tandem with the amidase genes (ami) and associated regulators/activators (nhr1, nhr2, nhr3). Cobalt containing NHases have been isolated in *Rhodococcus rhodochrous* J1 [86]. When the media was supplemented with cobalt chloride, NHase activity was expressed independently of nitrilase’s that act on similar nitriles and subject to alternative inducers, such as crotonamide [104, 109]. Further screening of substrates to enhance expression led to the detection of additional cobalt NHase, differing in molecular weight and specificity. The high molecular weight (520 kDa) H-NHase with a preference for aliphatic nitriles was isolated from cells grown in urea and the lower molecular weight (130 kDa) L-NHase, showing an affinity for aromatic nitriles, was isolated from cells grown in cyclohexane carboxamide [87, 104, 110]; both NHases were induced in the presence of crotonamide and cobalt. The gene organisation for each NHase is quite distinct, as illustrated in Fig. 5.8.

![Figure 5.8(a)](image)

**Figure 5.8(a)**

![Figure 5.8(b)](image)

**Figure 5.8(b)**

**Figure 5.8:** (a) Cobalt H-NHase genes in *Rhodococcus rhodochrous* J1: nhhC = regulator; nhhD = regulator of MarR family; nhhE = unknown function; nhhF = transposase; nhhB = β-subunit of NHase; nhhA = α-subunit of NHase; nhhG = β-subunit of H-NHase.109 (b) Cobalt L-NHase genes in *Rhodococcus rhodochrous* J1: nhlD = repressor; nhlC = activator; nhlB = β-subunit of NHase; nhlA = α-subunit of NHase; nhlE = β-subunit of H-NHase homologue; nhlF = cobalt transporter; amdA = amidase. [109]

In the H-NHase gene cluster (Fig. 5.8(a)), nhhC and nhhD are positive regulatory genes for H-NHase gene expression. In the L-NHase cluster (Fig. 5.8(b)), nhlC and nhlD play positive and negative regulatory roles, respectively, while nhlF serves as a cobalt transporter (for the enzyme metallocentre). A new class of NHase (acetonitrile hydratase) has been reported for *Rhodococcus* RHA1 that promotes hydration of aliphatic nitriles [32]. In common with the systems described above, this NHase is a
heterodimeric metalloenzyme but exhibits an atypical metal content, including cobalt (which is catalytically essential) with copper and zinc but the function of latter ions remains unknown [32]. Moreover, the α- and β-subunits display an unusually low homology to known enzymes and the size of these subunits (63 and 56 kDa) is considerably greater than that which typically characterises nitrile hydratases (26–35 kDa). In addition, this NHase has a pronounced specificity for smaller aliphatic nitriles, notably acetonitrile and acrylonitrile. The enzyme was first identified genomically before characterisation of the catalytic response; the reaction scheme and associated metabolic cluster are shown in Fig. 5.9.

![Catalysis of nitriles by Co-Cu-Zn type NHase isolated in Rhodococcus RHA1: genes include anhP = transcriptional regulator; anhQ = transcriptional regulator; anhC = amidase; anhA = α-subunit of NHase; anhE = unknown function; anhB = β-subunit of NHase; anhR = transcriptional regulator; anhT = cobalt transporter; anhD = acetyl CoA hydrolase; anhF = transporter [32].](image)

The operon encoded a NHase (anhA + anhB) that oxidises acetonitrile to acetamide where an amidase (anhC) promotes deamination to acetic acid. Further predicted proteins include acetyl-CoA hydrolase (anhD), which is proposed to transform the resulting acid, three transcriptional regulators (anhR, anhP, anhQ) and one probable cobalt transporter (anhT) [32].
5.6.4 Genomic and catalytic analysis: Amides

Hydrolysis of amides to carboxylic acid (and ammonia) is achieved in prokaryotes through the action of amidases, which are normally linked, regulated and expressed with a NHase [82, 111]. Enzymatic transformation of amides cannot be readily categorised given the range of applicable enzymes and wide spectrum of activities exhibited by amidotransferases, acyltransferases, acid transferases and estertransferases. Sharma et al [112] have proposed a classification or subdivision on the basis of phylogenetic relationship and substrate specificity. The amidase of *Rhodococcus rhodochrous* J1 [113] and *Rhodococcus erythropolis* MP50 [114] belong to a group of signature enzymes that possess a conserved GGSS motif with glycine, aspartic acid and serine residues [115]. This group of amidases shows specificity to aliphatic and aromatic amides and the amides of α-substituted carboxylic acids [112]. We should note that the “aliphatic amidases” possess a conserved cysteine that acts as a nucleophile during catalysis and is among the conserved motifs [116]. In contrast to the signature amidases exhibiting homo-dimeric or homo-octameric character, aliphatic amidases in the active form are homo-tetrameric or homo-hexameric [112]. Substrate specificity for short-chain aliphatic amides has been demonstrated for *Rhodococcus* sp. R312 with an appreciably greater activity in the conversion of propionamide relative to butyramide [107, 117].

5.6.5 Cellular strategies and possible applications

Viable application of *Rhodococcus* in nitrile biocatalysis and bioremediation can draw on the observed tolerance to high nitrile concentrations and cell wall permeability that facilitates degradation with survival in nutrient-poor environments. [118, 119] Indeed, introduction of isovaleronitrile or caprolactam has resulted in nitrilase expression accounting for up to 35% of total soluble protein [120, 121]. We can flag the application of H- and L-NHases (from *Rhodococcus rhodochrous* J-1) in the conversion
of acetonitrile to acrylamide and 3-cyanopyridine to nicotinamide [122, 123]. Bioprocessing at Mitsubishi Rayon with nitrile hydratases generated 30,000 tons of acrylamide per annum [122]. Moreover, laboratory scale testing has identified the commercial potential of nitrile hydrolysis in the production of antibiotics and anti-inflammatory agents [83]. Mayaux and co-workers [124, 125] were the first to demonstrate enantioselective oxidation of α-methylphenylacetamides and α-methoxyphenylacetamides using enzymes from two Rhodococcus strains, [126, 127]. While can also point to studies that have established NHase and amidases activity in the production of α-amino-3-hydroxy-5-methyl-4-isoxolepropionic acid (a receptor antagonist) and α-arylpropionic acids (non-steroid anti-inflammatory agents) [107, 128, 129].

With respect to bioremediation, benzonitrile derivatives (chlorixynil, bromoxynil, ioxynil) used as herbicides are of increasing concern due to the accumulation of persistent metabolites. Nitrilase enzymes (from Rhodococcus rhodochrous PA-34 and Rhodococcus sp. NDB 1165) can be applied to convert these herbicides into the corresponding substituted benzoic acids, which are readily mineralised [107, 130]. Treatment with NHase-amidase coupled reactions may not be applicable, as the amide intermediate, as in the case of the herbicide dichlobenil (2,6-dichlorobenzonitrile) can represent a more serious contaminant [107, 131]. Nevertheless, amidases from Rhodococcus strains have been applied in the complete mineralisation of the banned atrazine herbicide [132, 133].

5.7 Nitroaromatics

5.7.1 Background

There are few biologically derived nitroaromatic compounds in nature and microbial exposure has largely been limited to recent anthropogenic release [134].
Benzene, toluene and xylene (BTX) as vehicular pollutants can undergo photochemical conversion in the troposphere to produce a range of nitroarenes, which enter terrestrial and aquatic environments during rainfall [52]. Other sources include emissions associated with industrial production of dyes, pesticides, herbicides and medical products [135, 136]. A number of species isolated from nitroaromatic production sites have exhibited a capacity to utilise these synthetic compounds as either a carbon or nitrogen source [137]. As de novo evolution of fully functional pathways is unlikely given the relatively short period of exposure, previously unrelated genes must act to facilitate degradation [138, 139]. Enzymatic transformation of nitroaromatics can proceed via oxidative or reductive routes, involving monooxygenases, dioxygenases, hydride transferases and nitroreductases [137]. It should be noted that the full complement of enzymes in some strains may not be available for complete degradation, while others strains can exhibit alternative enzymatic processes that lead to metabolic dead end products, preventing mineralisation with resultant accumulation in the environment [140]. We can highlight the degradation pathway established for *Burkholderia cepacia* R34 in the catabolism of dinitrotoluene, where denitration was initiated by oxygenases recruited from naphthalene and chloroaromatic reaction pathways with contribution from enzymes associated with amino acid degradation [139]. While assimilation strategies can differ, significant horizontal gene transfer coupled with enzymatic plasticity are crucial in the application nitroarene bioremediation.

### 5.7.2 Genomic and catalytic analysis

The ability of *Rhodococcus* to degrade nitroarenes is established, often with the complete catabolic repertoire for mineralisation [135, 141, 142]. The aromatic character of the substrate significantly hinders biodegradation and xenobiotic properties are enhanced with increasing nitro-group substitution [136]. Oxidative and reductive
biodegradation strategies in the conversion of mono-nitrophenols have been reviewed by Nishino et al. [143]. Many \textit{Rhodococcus} strains can assimilate nitrogen in an analogous manner to \textit{Nocardia} sp, which differentially expresses different oxidative pathways depending on nitro group position on the aromatic ring, e.g. \textit{ortho}- as opposed to \textit{para}- substitution in phenol [141, 144]. In the case of 4-nitrophenol, two aerobic pathways have been identified, i.e. oxidation \textit{via} 4-nitrocatechol with eventual aromatic ring cleavage of the metabolite hydroxyquinol or initial benzoquinone formation with hydroquinone cleavage, associated with Gram negative bacteria (Fig. 5.10).

![Diagram of 4-Nitrophenol degradation pathway](image)

\textit{Rhodococcus sp. PN1}

\textit{Rhodococcus opacus SAO101}

**Figure 5.10**: 4-Nitrophenol degradation pathway with associated genes in \textit{Rhodococcus} sp. PN1 and \textit{Rhodococcus opacus} SAO101 [141,142].

The two component monooxygenase of \textit{Rhodococcus opacus} SAO101 (NpcA and NpcB) and 1,2-dioxygenase (NpcC) were the first enzymes in the hydroxyquinol pathway to be characterised (Fig. 5.11). These can now be taken to apply in \textit{Rhodococcus opacus} AS2 and \textit{Rhodococcus erythropolis} AS3, \textit{Rhodococcus wratislaviensis} J3, \textit{Rhodococcus opacus} J2 and \textit{Rhodococcus sp.} CN6, which all degrade the 4-nitrocatechol intermediate (see Fig. 5.10), releasing nitrite [23, 145, 146]. Subsequent ring cleavage steps appear to be rate limiting as \textit{Rhodococcus sp.} CN6
readily utilises 4-nitrophenol as a nitrogen source but displays limited growth as a carbon source [23]. *Nocardia* and *Rhodococcus* sp. can also convert 4-nitrophenol through the direct action of mono-oxygenases, as illustrated in Fig. 5.10. In an initial catabolic step that is typically associated with gram negative bacteria [147], *Rhodococcus* PN1 converts 4-nitrophenol to benzoquinone with a two-component aromatic ring hydroxylase encoded by nphA1 and nphA2. This metabolite is subsequently oxidised primarily to 2-hydroxy,1,4-benzoquinone to coincide with the hydroxyquinol pathway [141, 148] but also generates hydroquinone as minor product [147, 149]. Both gene clusters (Fig. 5.11) responsible for this pathway are found in *Rhodococcus* PN1 and are genomically separate and independently regulated.

![Figure 5.11: 4-Nitrophenol degradation gene cluster associated with Rhodococcus PN1: npsA1 = 4-nitrophenol hydroxylase; npsA2 = oxidoreductase; npsB = hydroxyquinol 1,2-dioxygenase; npsC = maleylacetate reductase; npsR = LysR-family regulator [141]. 4-Nitrophenol degradation gene cluster associated with Rhodococcus opacus SAO101: npcA+npB = two-component monoxygenase; npcC = hydroxyquinol 1,2-dioxygenase; npcR = LysR-family regulator [142].](image)

A degradation pathway applicable to highly xenobiotic poly-nitroaromatics, taking 2,4,6-trinitrophenol as representative, is shown in Fig. 5.12 (R = OH) [150]. In contrast to the action of oxygenases in the conversion of single nitro-substituents, the aromatic ring is reduced by hydride transfer to generate a Meisenheimer complex. In a series of studies by Lenke and co-workers [151-153], formation of the Meisenheimer complex was demonstrated for *Rhodococcus erythropolis* HL 24-2 with 2,4,6-trinitrophenol as sole energy and nitrogen source where contribution from two F_{420}-dependent hydride transferases resulted in 2,4-dinitrophenol production.
This pathway was subsequently confirmed for six further *Rhodococcus* strains including *Rhodococcus opacus* HL PM-1 [154] where the genomic metabolic cluster was recombinantly expressed. These genes included hydride transferase I (encoded by npdC) and hydride transferase II (encoded by npdI), which form the σ-complexes shown in Fig. 5.12 by successive hydride addition and an F$_{420}$-dependent reductase (encoded by npdG), essential for the transferral of hydrogen ions from NADPH.

### 5.7.3 Cellular strategies and possible applications

Cellular properties such as surface hydrophobicity, modulation of cell membrane composition and generation of biosurfactants are critical in the enzymatic transformation of nitroaromatics. Conversion of soluble nitrophenols (notably 4-nitrophenol) has assumed critical importance in addressing contamination due to pesticides (such as parathion and methylparathion) and widespread implementation of fungicides and insecticides [23, 136, 137, 155]. Given the xenobiotic character of these
substrates, bioremediation is challenging. While *Rhodococcus opacus* strain JW01 has been used to treat 2,4,6-trinitrophenol contaminated water to meet drinking water standards [156], substitution of the hydroxyl function by a methyl group (2,4,6-triaminotoluene (TNT), \(R=\text{CH}_3\) in Fig. 5.12) results in incomplete mineralisation. Exposure of *Rhodococcus opacus* HL PM1 to TNT, a major contaminant found in ammunitions sites, resulted in successive reductive steps, analogous to those shown in Fig. 5.12 but upon the release of nitrite, several secondary diarylamines are formed by condensation leading to further degradation[157]. The limited ability to act upon the dihydride TNT Meisenheimer complex (and resultant diarylamines) must be related to high levels of enzyme specificity. Gene shuffling or directed evolution may overcome this “bottle-neck”, while co-metabolism with other bacteria (yet to be identified) may represent a viable route to complete mineralisation. We should note activity associated with white-rot fungi, in the conversion diarylamines. Although unsuitable for bioremediation due to growth constraints, the action of the lignin peroxidase in converting these compounds is a significant, especially given similarities to an enzyme recently characterised in *Rhodococcus* RHA1 (section 5.5.3)

5.8 Future perspectives

Transformation of nitrogenous compounds by *Rhodococcus* species has untapped potential across the pharmaceutical and fine chemical sectors and particular relevance in bioremediation. We have provided a holistic analysis of catalysis, genome and cellular properties presented in this Review. The challenge is now to make the transfer from laboratory tests to industrial process scale and the treatment of polluted environments where strain selection is critical. With nearly 50 completed or on-going *Rhodococcus* genome projects (www.ncbi.nlm.nih.gov), identification of suitable strains for a given application should become more systematic. The catalytic response examined in this Review has demonstrated the range of enzymes predicted to act on nitrogenous
compounds, ranging from oxidases (amino acids and amines), nitrilases/Nhases (nitiles and amides) and multiple oxygenases, hydride transferases and nitroreductases (nitroaromatics). Such enzymatic diversity represents enormous potential for *Rhodococcus* exploitation but this comes with increasing complexity due to functional redundancy and co-metabolism. Protein and metabolic engineering can enhance the performance of existing enzymes and open new routes/pathways with improved catalytic efficiencies. *Rhodococcus* can serve a rich source of enzymes from enantioseletive oxidases to pollutant degrading nitrilases with considerable scope for directed evolution to enhance stability, specificity and kinetic properties. Furthermore, control mechanisms can be tailored to increase enzyme expression.

Functional clusters of genes encoding enzymes and associated proteins are evident for all the major nitrogenous pathways and this genomic feature can inform our understanding of metabolic pathways. In bioremediation, the modular genetic clusters/pathways are more likely to be adopted by native bacteria. In terms of cellular optimisation “bottlenecks” in chemical transformations can more easily be identified, with genetic engineering hybrid pathways with new composite functions can be incorporated into existing enzymes, regulatory circuits and environmental sensors. It is anticipated that advances in enzymatic and genomic data and analysis will accelerate the development of optimised *Rhodococcus* strains to promote bioconversion of target compounds and bioremediation.

### 5.9 References


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Future Perspectives

Over the past four decades various studies have shown that bacteria belonging to the genus *Rhodococcus* act upon an array of natural and synthetic compounds. The analysis of enzymatic oxidative deamination of amines presented in this thesis further supports the genomic and enzymatic basis for this metabolic plasticity. Moreover, the work has highlighted the considerable potential for biocatalysis and bioremediation. Full exploitation and scope for improvement of such technologies will provide the impetus for further research, analysis and optimisation. Enantioselectivity and alkaloid biosynthesis are the two greatest potential biocatalytic applications of amine oxidases with wide ranging implications across the pharmaceutical and fine chemical sectors. As only FAD dependent oxidases exhibit activity in the conversion of branched and secondary amines, the four remaining uncharacterised gene homologues (Table 1.3 in Chapter 1) should be targeted either by recombinant expression or utilising further induction methods and/or copper amine oxidase inhibitors. Further screening can enable characterisation of a third monoamine oxidase, which displayed weak enatioselective properties (data not shown) and activity/expression that was not consistently replicated. Alkaloid biosynthesis offers an intriguing possible application, particularly in metabolic engineering. High levels of oxidation of dopamine leading to the formation of norlaudanosoline (morphine precursor) is one avenue of potential research. Oxidation of methylputrescine, possibly by either diamine oxidase, can generate a central metabolite (*N*-methyl-∆1–pyrrolium), which can subsequently be converted to tropane alkaloids or utilised in nicotine biosynthesis.

Application in biosensors can also be flagged but use in bioremediation is more immediate given the catalytic and physiological properties of the genus. There are few reported studies that directly assess amine pollutants and none that consider a possible
role in biostimulation. Further research is required to establish explicitly the response of amine addition as a nutrient source, to increase viability to stressors and the effect of hydrogen peroxide generation as by-product, which can be harnessed in degrading pollutants. A key finding of this thesis is the discovery of the oxidase pathway (coupled with subsequent dehydrogenase) for putrescine degradation, which is associated with a narrow taxonomic distribution. This is significant in that *Acinetobacteria* have been identified as feasible Gram positive candidates for bioremediation. We should also note the evolutionary perspective as there are a number of instances of detected, but as of yet unexplored, diamine oxidase activity in other classes of bacteria and fungi, which appears to have evolved independently. Finally, the postulated association of oxidases with pathogenicity warrants examination as the role of putrescine as chemical messenger (for the host) is significant for understanding disease progression of commercially important salmon, as well as crops and other plants. Point mutagenesis of these oxidases eliminating or decreasing enzyme function should provide important insight into such effects.