Systematic and Applied Microbiology 36 (2013) 457–466

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

# Genomic organisation, activity and distribution analysis of the microbial putrescine oxidase degradation pathway

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ARTICLE INFO

*Article history:*
Received 5 April 2013
Received in revised form 25 June 2013
Accepted 28 June 2013

*Keywords:*
Putrescine degradation pathway
Amine oxidase
Dehydrogenase
Phylogenetics

ABSTRACT

The catalytic action of putrescine specific amine oxidases acting in tandem with 4-aminobutyraldehyde dehydrogenase is explored as a degradative pathway in *Rhodococcus opacus*. By limiting the nitrogen source, increased catalytic activity was induced leading to a coordinated response in the oxidative deamination of putrescine to 4-aminobutyraldehyde and subsequent dehydrogenation to 4-aminobutyrate. Isolating the dehydrogenase by ion exchange chromatography and gel filtration revealed that the enzyme acts principally on linear aliphatic aldehydes possessing an amino moiety. Michaelis–Menten kinetic analysis delivered a Michaelis constant ($K_M$ = 0.014 mM) and maximum rate ($V_{max}$ = 11.2 μmol/min/mg) for the conversion of 4-aminobutyraldehyde to 4-aminobutyrate. The dehydrogenase identified by MALDI-TOF mass spectrometric analysis (*E* value = 0.031, 23% coverage) belongs to a functionally related genomic cluster that includes the amine oxidase, suggesting their association in a directed cell response. Key regulatory, stress and transport encoding genes have been identified, along with candidate dehydrogenases and transaminases for the further conversion of 4-aminobutyrate to succinate. Genomic analysis has revealed highly similar metabolic gene clustering among members of *Actinobacteria*, providing insight into putrescine degradation notably among *Micrococcaceae*, *Rhodococci* and *Corynebacterium* by a pathway that was previously uncharacterised in bacteria.



## 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 [29]. Putrescine (PUT), as the major polyamine in bacteria [28,74], serves to stimulate both RNA and protein synthesis [18,48]. Stringent regulation of this diamine is required to ensure an optimal intracellular environment for cell viability and proliferation, a balance that must also encompass broader functional requirements such as response to external stressors [7,26,30,74]. 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 [62], uptake and excretion through antiporter and ATP dependent transport proteins [20,24,25,28], as well as degradation [27,59] and corresponding feedback mechanisms [20,78]. These pathways, including the constituent enzymes and processes, are not universal and it is 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 of 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 [16,68]. Three major catabolic mechanisms for PUT degradation have been proposed, *i.e.* γ-glutamylation, direct oxidation and acetylation [27,33,59]. 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 *E. coli*, PUT degradation is mediated by the dual action of γ-glutamyl intermediates with a corresponding oxidase in addition to direct deamination utilising a transaminase with oxoglutarate co-substrate [27,49,59]. Studies on *Pseudomonas aeruginosa* [8] and *Arthrobacter* sp. TMP-1 [66,76] have identified a similar derivative with pyruvate as the transaminase co-substrate in the direct deamination of PUT.

The experimentally verified PUT degradation pathways, confirmed by strains possessing all the necessary enzymes, are shown in Fig. 1. We have also included a postulated microbial oxidase pathway (represented by dashed arrows) that acts directly on PUT where all applicable enzymes have been individually characterised [5,14,22,45,49] but not, to our knowledge, within the

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http://dx.doi.org/10.1016/j.syapm.2013.06.008

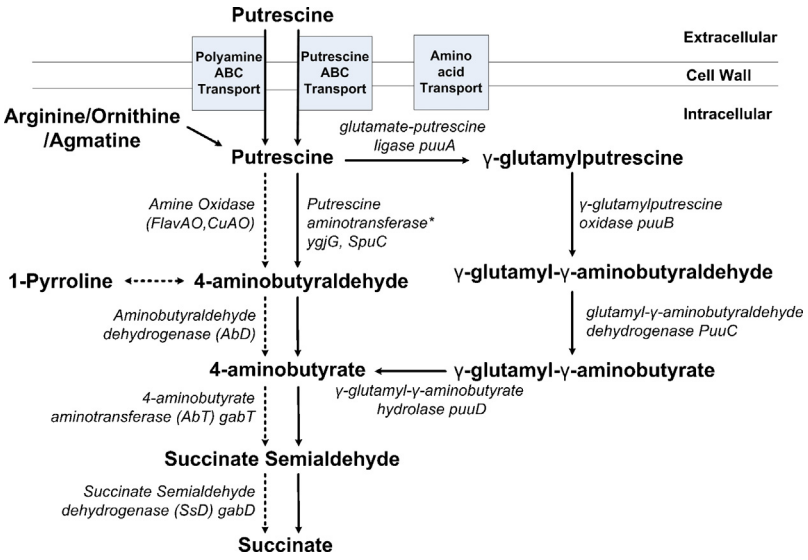


**Fig. 1.** The pathways for PUT degradation within prokaryotes as demonstrated by experimental analysis. These include oxidation *via* aminotransferases and γ-glutamated intermediates as illustrated by solid arrows and the amine oxidase pathway (dashed arrows) investigated in this study; *pyruvate or oxoglutarate cofactor.

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 protective 4-aminobutyrate [72]. While aminoaldehyde dehydrogenases are widely accepted as the enzymes chiefly responsible for the conversion of aminobutyraldehyde to aminobutyrate, few have been explicitly identified and characterised [35,58]. Multiple gene homologues, differing subcellular locations and broad substrate specificity have hindered an explicit identification of dehydrogenase catalytic activity in the overall catabolic process. In previous work, 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) [14]. 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. 1) [10,21]. Genomic analysis has revealed sequence homologues of all the enzymes required for this PUT degradation pathway (Fig. 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 on subsequent degradation products is also probed and phylogenetic analysis applied to determine the genomic distribution of this pathway, involving amine oxidases, 4-aminobutyraldehyde dehydrogenase and other interrelated enzymes, transcription factors and transporter proteins.

## Materials and methods

### *Bacterial strain and culture conditions*

*R. opacus* DSM 42350 was used throughout this investigation. For tests of amine oxidase and 4-aminobutyraldehyde dehydrogenase activity, the bacterium was cultured in M9 minimal media with 4 mM glucose as the carbon source and 20 mM putrescine (PUT) as the sole nitrogen source. In enzyme induction experiments to analyse capacity to act on PUT, 4-aminobutyraldehyde, 4-aminobutyrate and succinate-semialdehyde, cells were limited to 5 mM PUT, butylamine or ammonium chloride ($NH_4Cl$) 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.

### *Enzyme assays*

A colorimetric assay was performed to determine catalytic oxidative deamination activity. The assay is based on the generation of $H_2O_2$ 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 [39]. 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)

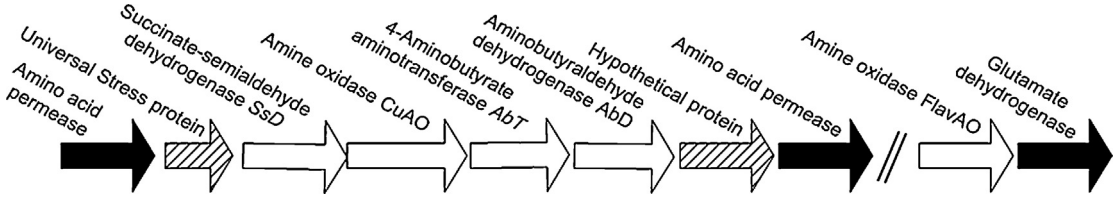


**Fig. 2.** Genomic cluster of functionally related genes surrounding both oxidases in *Rhodococcus opacus* B4 and *Rhodococcus* RHA1 [32]. Those proteins implicated in a direct catabolic role in PUT degradation are identified by open arrows, functionally related proteins by solid arrows and those with an undetermined significance are given by hatched arrows; double dash illustrates a distance of 8.3 kb before the subsequent FlavAO.