Biodegradation of Nitriles by *Rhodococcus*

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Contents

Abstract Nitriles occur naturally in the environment, are produced by the metabolic pathways of organisms or are released by the chemical and pharmaceutical industries, from agricultural applications or from the processing of fossil fuels. Therefore, a variety of nitrile-converting bacterial species are used to alleviate this toxic effect. Among these bacteria, *Rhodococcus* species have proven to be a superior group for

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the clean-up of pollutants. Nitriles are converted into the less toxic carboxylic acid either by nitrilases or by nitrile hydratase (NHase)/amidase systems. Although NHases, nitrilases and amidases produced by different strains exhibit different catalytic characteristics towards aliphatic nitriles and aromatic nitriles, these enzymes exhibit considerable homology in amino acid sequence or structure. In contrast, the enzymes with different origins present different types of gene organization and regulatory patterns, although the amidase gene is always linked to the NHase gene. Due to the advantage of being resistant to toxic compounds, applications of *Rhodococcus* in pollutant biodegradation and biocatalytic processes are very promising. While studies on the biodegradation of nitrile pollutants focus on the screening and discovery of strains, the industrial application of these enzymes as biocatalysts focuses on engineering combined with immobilization of both *Rhodococcus* cells and enzymes to improve their performance under the adverse conditions in the catalytic process.

1 Introduction

Nitriles are widely distributed in nature and can be divided into three general categories: aliphatic nitriles, aromatic nitriles and heterocyclic nitriles. Nitriles may be produced by a variety of microorganisms and plants and exist as 3-indolylacetonitrile (an auxin precursor), 3-phenylpropionitrile (a growth hormone) and so on. In living organisms, these naturally occurring nitriles may be the product of the following pathway: amino acid → aldoxime → nitriles. Aldoxime dehydratase participates in the formation of nitrile from aldoxime. Nitriles are also products of the agricultural, pharmaceutical and chemical industries (Bhalla et al. 2012). These nitriles are important intermediates for the production of amides, acids and other compounds. Despite the importance of nitriles in organic synthesis, large amounts of nitrile compounds are released to the environment, including to soil, air and water, and are harmful and toxic to human beings; therefore, remediation of the polluted environment is an urgent issue.

Due to the widespread occurrence of nitriles in metabolic pathways and natural environments, nitrile-converting enzymes occur in a wide variety of bacterial species, such as *Pseudomonas* sp., *Bacillus*, *Corynebacterium*, *Nocardia*, *Micrococcus* and *Rhodococcus*. In microorganisms, nitriles are primarily degraded to carboxylic acids, which are then converted to other metabolites. The conversion of nitriles to carboxylic acids occurs via two types of pathways: (1) direct hydrolysis of nitriles to carboxylic acids by nitrilases and (2) cascade catalysis of nitriles by NHases and amidases via the pathway nitriles → amides → carboxylic acids. While some identified nitrile-converting strains have only one of the two pathways, others harbour both the pathways. Nitrilases and NHases produced by different strains also exhibit different catalytic preferences for aliphatic and aromatic nitriles. While

some nitrilases are capable of catalysing aliphatic nitriles efficiently, the enzymes are not efficient at catalysing aromatic nitriles. A similar phenomenon has been observed for different types of NHases. In particular, *Rhodococcus rhodochrous* J1 can produce two types of NHases, namely, high-molecular-mass NHase and low-molecular-mass NHase, which exhibit different specificities for aliphatic nitriles (Komeda et al. 1996b, c).

The diverse catalytic properties of various NHases and nitrilases enable extensive application of these enzymes to transform a vast number of man-made nitriles for the production of many important bulk and fine chemicals or to remove pollutants from waste materials and environment. NHases, which catalyse the hydration of nitriles to the corresponding amides, have been widely used in the chemical industry for the production of acrylamide, nicotinamide and 5-cyanovaleramide. In addition, nitrilases are attractive biocatalysts for the production of various commodity chemicals and pharmaceutical intermediates, such as nicotinic acid, isonicotinic acid, mandelic acid, acrylic acid, glycolic acid and benzoic acid. Among nitrile-converting strains, *Rhodococcus* strains have proven to be the most powerful and successful in the industrial transformation of various nitriles. However, there are some bottlenecks in the application of these enzymes in the transformation of nitriles to the corresponding amides or carboxylic acids, such as activity inhibition at high concentrations of substrates and products, thermal susceptibility and low nitrilase activity. As a result, genetic engineering strategies combined with immobilization were applied to enhance the stability and activities of the *Rhodococcus* cells and enzymes to broaden the industrial application of these enzymes.

2 Nitrile-Degrading Enzymes

Nitrile compounds, which have a $-C\equiv N$ functional group, are ubiquitous in natural environments as intermediates synthesized by chemical processes or biological systems. Nitrile compounds have application in many areas, for example, 2,6-dichlorobenzonitrile is used as an herbicide (Tao et al. 2016), nitrile rubber is used in medical gloves, polyacrylonitrile fibres are used to manufacture clothing, acetonitrile is used in analytical chemistry, and citalopram is used as a pharmaceutical (Fleming et al. 2010). The widespread use of nitrile compounds inevitably causes water pollution, soil contamination and accumulation of nitrile-containing waste. Over the past few decades, nitrile degradation has become a cause for concern worldwide. Biodegradation is more promising than chemical and physical methods due to the complete conversion, low cost and low level of secondary pollution associated with this method (Alexander 2001). Usually, biodegradation is carried out by enzymatic catalysis. The key enzymes used for nitrile degradation are NHase, amidase and nitrilase (Gong et al. 2017). NHase catalyses the hydration of nitriles to the corresponding amides (Ma et al. 2010); amidase catalyses the hydrolysis of amides to the corresponding acids; and nitrilase catalyses the one-step hydrolysis of nitriles to the corresponding acids (Fig. 1).

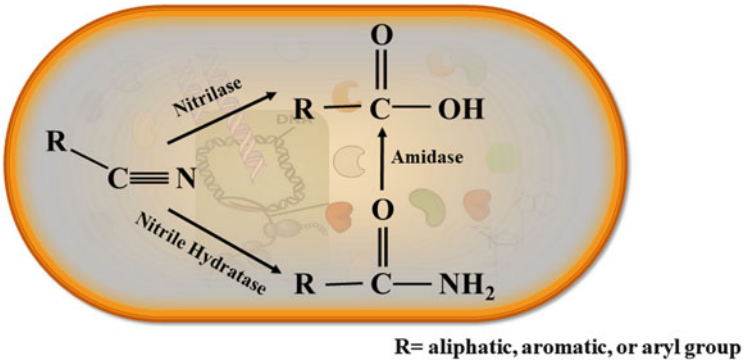

Fig. 1 Nitrile-biodegrading enzymes

2.1 NHase

NHase was first discovered in 1980 (Asano et al. 1980), and over the last few decades, many NHases have been identified and characterized. Various natural NHases are synthesized by plants and bacteria; these enzymes vary widely in length and exhibit structural diversity. However, microbial NHases usually exhibit considerable homology in amino acid sequence or structure (Cramp and Cowan 1999). Microbial NHase components are hetero-oligomers (αnβn), and typically, NHases exist as αβ dimers or tetramers (Cowan et al. 1998). Each αβ unit has a low-spin metal ion at the active centre (Huang et al. 1997), and NHases are classified into Co-type and Fe-type NHases according to the metal ions associated with the enzyme. Co-type NHase exhibits superior thermostability to Fe-type NHase in industrial biochemical synthetic processes (Cowan et al. 1998). Basically, structure determines function. As shown in Figs. 2 and 3, the sequence and structure characteristics of the α and β subunits of both classical Co-type NHases [PDB ID 1IRE from *Pseudonocardia thermophila* (Miyanaga et al. 2001) and PDB ID 1V29 from *Bacillus smithii* (Hourai et al. 2003)] and two Fe-type NHases [PDB ID 2AHJ from *Rhodococcus erythropolis* (Nagashima et al. 1998) and PDB 4FM4 from *Comamonas testosteroni* (Kuhn et al. 2012)] are illustrated.

The α subunits of Co-type and Fe-type NHases have highly homologous amino acid sequences and structures (Fig. 2). The size of the α subunit is 24–28 kDa. Structurally, three cysteine residues and one serine residue (-C-X-X-C-S-C-) in the cysteine cluster region constitute a fully conserved sequence in the α subunit of all Co- and Fe-type NHases (Fig. 2a). The three cysteine thiolates and the two main-chain amide nitrogens form a square-pyramidal geometry as ligands that co-ordinate the Fe or Co ion. The structures of the Co- and Fe-type NHase active centres are the same, except at one site. The sixth site is occupied by a water/hydroxide molecule as the active form in Co-type NHase, whereas a photolabile NO molecule is present at this site in Fe-type NHase (Endo et al. 2001).

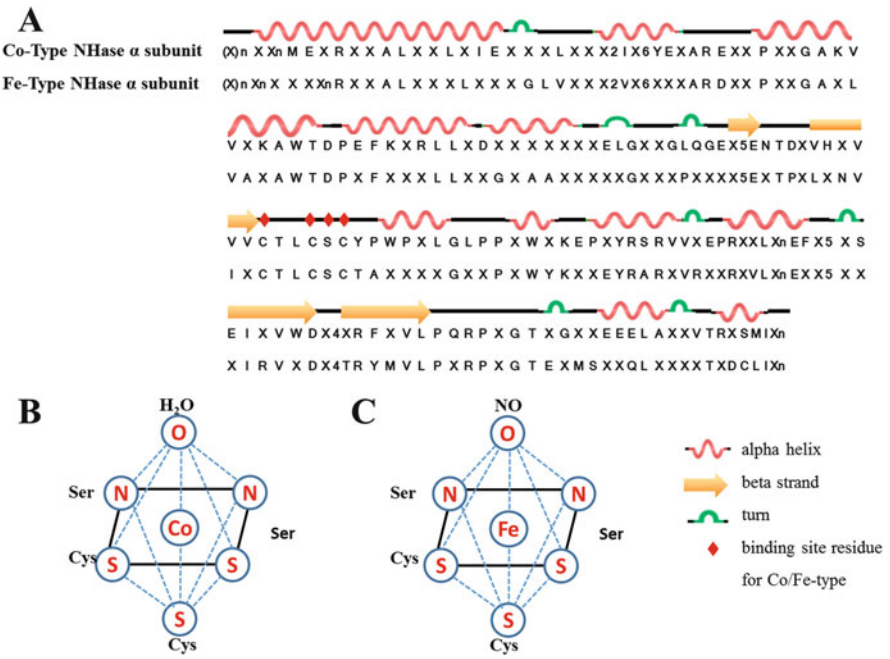

Fig. 2 Partial amino acid sequences and structure of Co- and Fe-type NHase α subunits. Co-type NHase: 1IRE (Miyanaga et al. 2001) and 1V29 (Hourai et al. 2003). Fe-type: 2AHJ (Kuhn et al. 2012) and 4FM4 (Endo et al. 2001). (**a**) Amino acid sequence alignment of Co- and Fe-type NHase α subunits. (**b**) Cobalt active centre of Co-type NHase. (**c**) Structure of the iron active centre of Fe-type NHase in the inactive state

The β subunits of NHases, ranging from 25 to 39 kDa in size, have lower amino acid sequence homology than the α subunits. Two Arg residues in the β subunit are highly conserved in both Co-type and Fe-type NHases (Fig. 3a, b). The guanidine groups of these two Arg residues react with the sulphur atoms of Cys in the α subunit and form a salt bridge network with electrostatic interactions (Fig. 3c). The formation of a salt bridge around the metal ion ensures the binding of the α and β subunits to stabilize the subunit interface. In addition, there exist several structural differences between Co-type and Fe-type NHases (Fig. 3). In particular, one α helix (blue in Fig. 3c, β111–β125) in Co-type NHase is replaced by a long loop (β95–β138 shown in Fig. 3c) in Fe-type NHase. This helix, which is composed in part of these residues (β111–β125), interacted with another helix belonging to the α subunit. This additional interaction may contribute to the better thermostability of Co-type NHase than Fe-type NHase (Miyanaga et al. 2001).

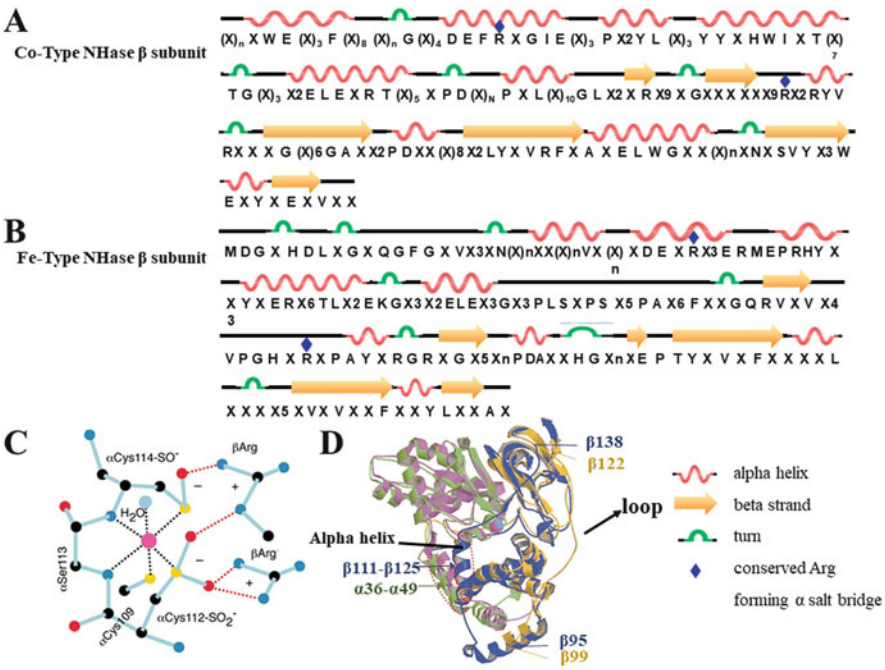

Fig. 3 Amino acid sequences and structures of Co- and Fe-type NHase β subunits. (**a**) Amino acid sequence alignment of the β subunit of Co-type NHase. Co-type NHase: 1IRE (Miyanaga et al. 2001) and 1V29 (Hourai et al. 2003). (**b**) Amino acid sequence alignment of the β subunit of Fe-type NHase. Fe-type NHase: 2AHJ (Kuhn et al. 2012) and 4FM4 (Endo et al. 2001). (**c**) Model of the noncorrin cobalt centre of NHase. Atoms are shown in different colours: pink for Co, black for C, red for O, yellow for S and blue for N. The salt bridge networks formed between the cysteines of the α subunit and the arginines of the β subunit are shown as red dotted lines (Zhou et al. 2008). (**d**) Ribbon diagram of Co-type and Fe-type NHases (Miyanaga et al. 2001)

2.2 *Amidase*

Amidase (EC 3.5.1.4) is widespread in nature and catalyses the hydrolysis of amides into the corresponding carboxylic acids and ammonia. Coupled with NHase, amidase has great potential in the degradation of toxic nitrile compounds. Based on amino acid sequence and structural homology, amidases have been classified into two groups: the nitrilase superfamily and the amidase signature (AS) family (Ohtaki et al. 2010).

The first group of amidases belongs to nitrilase superfamily. These enzymes have low amino acid sequence homology but high structural homology (Andrade et al. 2007; Kimani et al. 2007; Makhongela et al. 2007). Being members of the branch 2 nitrilase superfamily, amidases have the conserved catalytic Glu-Lys-Cys triad, and the amidase monomers have the typical nitrilase superfamily αββα sandwich fold, similar to nitrilase.

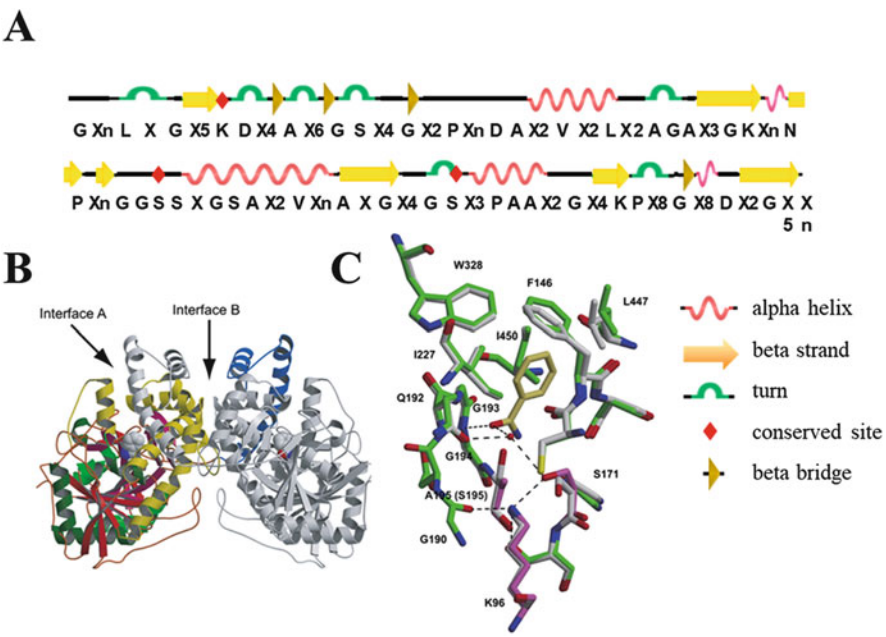

Fig. 4 Amino acid sequence and structure alignment of amidase. (**a**) Structural sequence alignment of the amidase signature (AS) region. The secondary structural elements identified in 3A1I (Ohtaki et al. 2010) are indicated in the top line. (**b**) Dimer structure of RhAmidase (Ohtaki et al. 2010). A monomer is shown in colour. (**c**) Structure of the active site of RhAmidase (Ohtaki et al. 2010)

The other group of amidases belongs to the AS family. AS family members have a highly conserved amino acid sequence, named the AS sequence, and a conserved Ser-Ser-Lys catalytic triad, and these proteins exist as homodimeric or homo-octameric complexes (Fig. 4). The amidase from *Rhodococcus* sp. N-771, namely, RhAmidase, can be taken as an example that has an active homodimeric structure and the active catalytic triad. A single monomer is shown in colour in Fig. 4b, and the two monomers binding crosswise. All helices of the small domain form interfaces A and B to participate in the formation of the homodimeric structure, and these interactions contribute to the formation of a closely packed dimer structure. The conserved catalytic Ser-Ser-Lys triad is located in the large domain. The first Lys96 can be deprotonated and acts as the catalytic base, and this residue also forms hydrogen bonds with Ser171 and Gly190; the second Ser171 is in an unusual *cis* conformation; and the third Ser195 forms a covalent bond with the substrate (Ohtaki et al. 2010; Lee et al. 2015; Valiña et al. 2004).

2.3 Nitrilase

Nitrilase was first described by Thimann and Mahadevan in 1964, and since then, over 200 nitrilases belonging to the nitrilase superfamily have been discovered and sequenced (Thuku et al. 2009). The nitrilase superfamily is characterized by a homodimeric building block with an αββα-αββα sandwich fold and is classified into 13 branches based on amino acid sequence similarity and the presence of additional domains (Thuku et al. 2009; Pace and Brenner 2001).

The nitrilases in branch 1 of the nitrilase superfamily hydrolyse nitrile (RCN) to ammonia and the corresponding carboxylic acid. Moreover, nitrilases are further categorized into aliphatic nitrilases, aromatic nitrilases and arylacetonitrilases according to substrate specificity, although some nitrilases exhibit broad substrate specificity, and the substrate specificity can also be changed by mutating key amino acid residues of nitrilases (Nigam et al. 2017). Natural nitrilases can be synthesized by plants, animals, fungi and bacteria. Among the nitrilases from different sources, microbial nitrilases are often exploited for biochemical syntheses and environmental remediation (Pace and Brenner 2001). The most well-known branch 1 nitrilases are from the most abundant nitrilase source, namely, bacteria of the *Rhodococcus* genus. To better depict their characteristics and structural homology, the sequences of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2007) and the nitrilase-related enzymes from *Agrobacterium* sp. KNK712 (PDB ID 1ERZ) (Nakai et al. 2000) and *Pyrococcus horikoshii* (PDB ID 1J31) (Sakai et al. 2004) are aligned in Fig. 5.

The majority of microbial nitrilases have a subunit size of 30–45 kDa and homomultimers with native compositions such as α_n (with n ranging from 4 to 22) (Thuku et al. 2009). The amino acid sequences of different nitrilases vary greatly, but all members exhibit secondary and tertiary homology upon alignment of their crystal structures (Thuku et al. 2007, 2009; Park et al. 2017) (Fig. 5a). Take the best characterized nitrilase from *R. rhodochrous* J1 as an example. The nitrilase in J1 is known to be inactive as a dimer in solution but to be active as an oligomeric complex (with 10–12 subunits) when subjected to heat treatment or in the presence of nitrile, ammonium sulphate or organic solvents (Thuku et al. 2009; Park et al. 2017). The dimer model of *R. rhodochrous* J1 nitrilase shown in Fig. 5b is built based on structural homology to nitrilase-related enzymes whose crystal structures have been solved (Thuku et al. 2007). The monomer association occurs via two interfaces, namely, the 'A' and 'C' surfaces, and leads to an eight-layered αββα-αββα dimer (Fig. 5b). As seen in Fig. 5a, the length of the amino acid sequence that forms 'C' surface varies much more than that of the sequence that forms the 'A' or 'D' surface (Thuku et al. 2007). The C surface is responsible for spiral elongation. All nitrilase superfamily enzymes have a conserved Glu-Lys-Cys catalytic triad, whereas the active site of the nitrilase in J1 has an extra glutamic acid and comprises the residues C165, K131, E48 and E138 (shown in Fig. 5c). C165 is speculated to initiate a nucleophilic attack on the substrate to form a tetrahedral intermediate, and K131 stabilizes the tetrahedral intermediate (Thuku et al. 2009). The two Glu residues are speculated to play an important role in positioning the substrate. E48 increases the

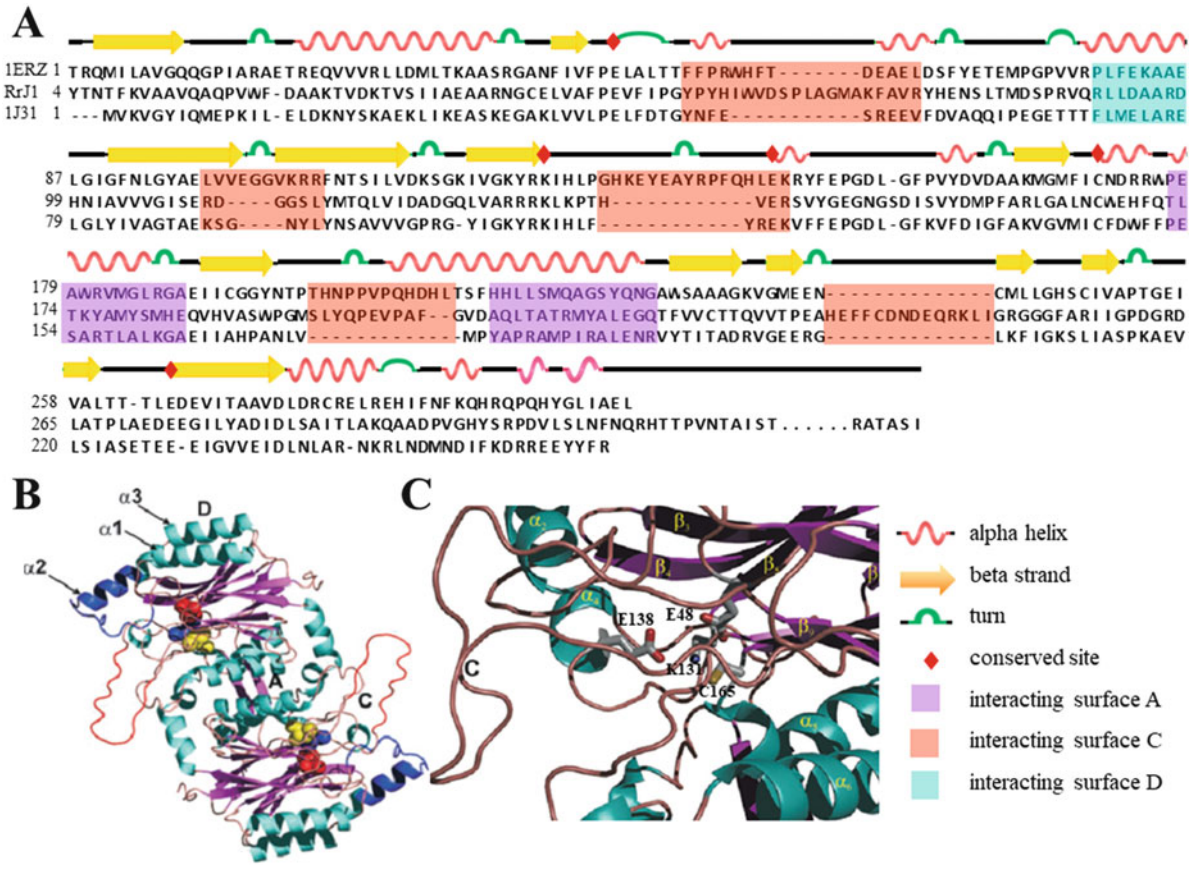

Fig. 5 Amino acid sequence and structural alignment of nitrilase. (**a**) Sequence alignment of the nitrilase from *Rhodococcus rhodochrous* J1 (RrJ1) with two nitrilase-related enzyme homologues [1ERZ (Nakai et al. 2000) and 1J31 (Sakai et al. 2004)]. The symbol in the top line indicates the position in the

nucleophilicity of the cysteine, and E138 is located in a 'C' surface loop, leading to the possibility that association of dimers moves this residue into the correct position for catalytic activity (Thuku et al. 2009).

3 Cluster Arrangement and Expression Regulation of Nitrile-Converting Genes

3.1 *Nitrile-Converting* Rhodococcus *Strains*

Nitrile-converting enzymes (NHases, nitrilases) exist in many *Rhodococcus* strains. Previous studies have reported that Fe-type and Co-type NHases were found in *R. erythropolis* and *R. rhodochrous* species and nitrilase-producing strains mainly belong to the *R. rhodochrous* species (Martínková et al. 2010). However, with the rapid development of next-generation sequencing technology, various *Rhodococcus* species have been revealed to be capable of producing NHase and nitrilase.

A genome-wide search of 283 *Rhodococcus* strains showed that all the *Rhodococcus* strains could produce amidase; 57% of the *Rhodococcus* strains harbour genes encoding NHases; and only 18% of the strains were identified as harbouring genes encoding nitrilases (Fig. 6a). Twenty-five of these strains exhibited NHase, amidase and nitrilase activities (Fig. 6b).

Except for a large number of strains not classified at the species level (designated *Rhodococcus* sp.), NHase could be produced by all the 36 strains of *R. equi*, 19 strains of *R. erythropolis* species, 8 strains of *R. opacus* and 7 strains of *R. qingshengii*. However, most of the NHases from these species have never been reported, except a number of NHases from *R. erythropolis* strains with high similarity and one from *R. equi* TG328-2, all of which bear the Fe^{3+} cofactor (Rzeznicka et al. 2010; Martínková et al. 2010). Co^{3+}-type NHases have been reported in *R. rhodochrous* J1 (Komeda et al. 1996b, c), *R. rhodochrous* M8 (Pogorelova et al. 1996; Pertsovich et al. 2005) and *R. ruber* TH (Ma et al. 2010). Specifically, in *R. rhodochrous* J1, two NHase subtypes were identified, i.e. high-molecular-mass NHase (H-NHase) and low-molecular-mass NHase (L-NHase), which exhibit different substrate specificities (Komeda et al. 1996b, c). In contrast, only H-NHase was identified in *R. ruber* TH, exhibiting 98% identity with the H-NHase from *R. rhodochrous* J1. A new type of NHase containing three types of metal ions (Co, Cu and Zn) was discovered and characterized in *Rhodococcus jostii* RHA1, which, however, shares no homology with the well-known Fe- and Co-type NHases (Okamoto and Eltis 2007).

Fig. 5 (continued) homologues. The approximate regions of the interacting surfaces A, C and D are indicated in purple, red and blue, respectively. The secondary structural elements identified in 1ERZ (Nakai et al. 2000) are indicated in the top line. (**b**) Stereo view of a dimer model of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2007). (**c**) Close-up view of the 'C' surface loop and the active site of the model of the nitrilase from *R. rhodochrous* J1 (Thuku et al. 2009)

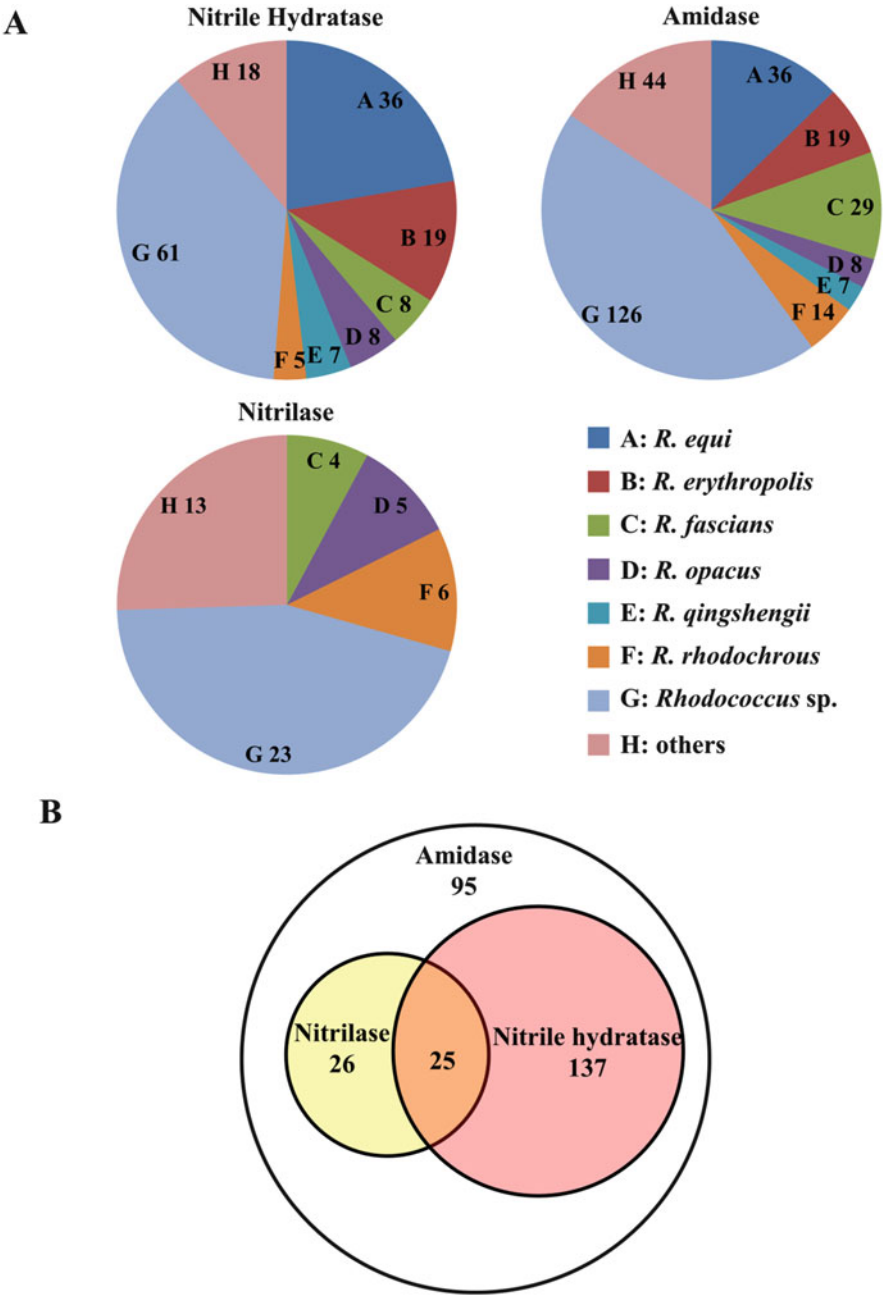

Fig. 6 *Rhodococcus* strains harbouring nitrile-converting enzymes. (**a**) NHase, amidase and nitrilase distribution in different *Rhodococcus* species. (**b**) Relationship of NHase-, amidase- and nitrilase-producing *Rhodococcus* strains

Unlike NHases, which can be produced by many *Rhodococcus* species, nitrilase-coding genes were detected in only a few species, mainly *R. fascians*, *R. opacus* and *R. rhodochrous*. In addition, many other nitrilase-containing strains have not been classified at the species level (*Rhodococcus* sp.). Nitrilases from different *Rhodococcus* strains possess individual preferences for aromatic or aliphatic nitriles. While most nitrilases prefer aromatic nitriles as substrates, the nitrilase from *R. rhodochrous* K22 exhibits significant activity towards aliphatic nitriles (Gong et al. 2012; Martínková et al. 2010).

3.2 NHase-Amidase Gene Organization and Regulation

Generally, genes that share a generalized function are often located in a gene cluster. In the metabolic pathway of nitrile degradation, nitrile is converted to amide by NHase and then transformed to carboxylic acid by amidase, so generally, the amidase gene is linked to the NHase gene, which has been detected in the majority of *Rhodococcus* strains regardless of whether the NHase is a Fe- or Co-type NHase. Despite this characteristic, Fe- and Co-type NHases represent different types of gene organization and regulation patterns.

3.2.1 Fe-Type NHase

The genes involved in the aldoxime-nitrile-amide-carboxylic acid pathway containing the Fe-type NHase are typically organized as shown in Fig. 7a, as seen in the *R. erythropolis* PR4 genome (GenBank: AP008957.1). NHase is expressed by *nha1* and *nha2*, coding for the α and β subunits, which are linked to the amidase gene (*ami*). The *nhr3* gene codes for an NHase activator, which activates the NHase via the oxidation of the iron centre and incorporation of an Fe ion into the protein (Nojiri et al. 2000). This activator gene is also essential for the function of NHase when expressing *nha1* and *nha2* genes in *E. coli*. The expression of NHase is probably regulated by *nhr4*, *nhr2* and *nhr1*, which encode regulators; however, to date, there has been no report regarding the exact functions of these genes. The gene coding for aldoxime dehydratase (*oxd*) was also found to be close to the *nha2*, *nha1* and *ami* genes in *R. erythropolis* PR4. Similar gene structures and organizations were also observed in *R. erythropolis* A4 (GenBank: AM946017.1), *R. globerulus* A-4 (GenBank: AB105912.1), *Rhodococcus* sp. N-771 (GenBank: AB016078.1) (Kato et al. 2004; Endo et al. 2001) and *Rhodococcus* sp. N-774 (Martínková et al. 2010). The NHase-amidase gene cluster was also identified in the *R. erythropolis* strains AJ270 and AJ300, whereas a copy of the insertion sequence IS1166 was present within the *nhr2* gene (O'mahony et al. 2005). In *R. jostii* RHA1, the genes coding for amidase and NHase share 79% and 90% homology, respectively, with those in *R. erythropolis* PR4; however, the regulatory genes *nhr1* and *nhr2* are absent, while the genes *oxd* and *nhr4* were detected (GenBank: CP000431.1).

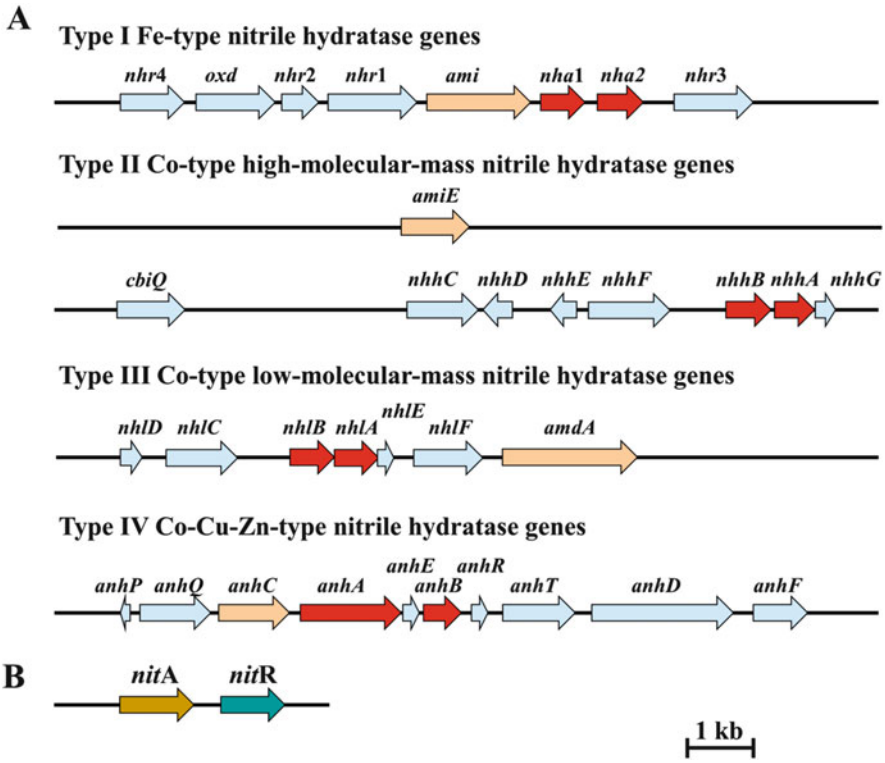

Fig. 7 Organization of nitrile-converting gene clusters from various *Rhodococcus* strains. (**a**) Four types of NHase-amidase gene cluster organizations. (**b**) Nitrilase gene cluster organization

In most cases, the NHase and amidase genes are inducible by amides (reaction products) instead of nitriles (reaction substrates) (Kobayashi and Shimizu 1998), which has been observed mostly with acetamide (Rucká et al. 2014; O'mahony et al. 2005). However, constitutive expression patterns occur in some other strains, such as *Rhodococcus* sp. N-771 and *Rhodococcus* sp. R312 (Prasad and Bhalla 2010).

3.2.2 Co-Type NHase

In contrast to the Fe-type NHase, two types of NHases, i.e. H-NHase and L-NHase, exist in *Rhodococcus* species and were found to coexist in *R. rhodochrous* J1.

The typical gene cluster of H-NHase contains *nhhC*, *nhhD*, *nhhE*, *nhhF*, *nhhB*, *nhhA* and *nhhG*, as determined in *R. rhodochrous* J1 (Fig. 7a) (GenBank: D67027.1) (Komeda et al. 1996b). *nhhB* and *nhhA* code for the subunits of NHase and are positively regulated by the essential *nhhC* and *nhhD* genes. The *nhhG* gene is homologous to *nhhB*, although the exact function of this gene has not been determined. *nhhF* encodes an insertion sequence IS1164, suggesting that horizontal gene

transfer or genetic rearrangement of the H-NHase gene cluster occurred over the course of evolution. *nhhE* encodes a 14.6-kDa protein that does not share significant similarity with any reported protein and is not indispensable for the expression of the H-NHase gene. Genes homologous to *nhhC*, *nhhD*, *nhhB*, *nhhA* and *nhhG* are present in *Rhodococcus* sp. M8 (NCBI Reference Sequence: NZ_MLYX02000005.1), whereas *nhhE* and *nhhF* are absent. Despite the absence of *nhhF*, a gene coding for another IS481 family transposase has been identified immediately upstream of *nhhB*. In addition, the whole-genome sequence of *Rhodococcus* sp. M8 reveals a gene encoding amidase that is located 15.8 kb upstream of the H-NHase gene, which is consistent with the observation that amidase is always found together with NHase (Fig. 7a). As a result, whether amidase is present in the gene cluster of *R. rhodochrous* J1 H-NHase should be re-evaluated by cloning a sequence longer than the current 6555 bp. A gene encoding the cobalt ECF transporter T component (CbiQ) has also been identified upstream of *nhhBA* in *Rhodococcus* sp. M8 and may be involved in cobalt ion transport (Fig. 7a). Homologous H-NHase genes were also found in *Rhodococcus pyridinivorans* (Kohyama et al. 2006).

The subunits of L-NHase are encoded by *nhlB* and *nhlA*, which are clustered with *nhlD*, *nhlC*, *nhlE*, *nhlF* and *amdA* (amidase). *nhlC* and *nhlD* are located upstream of the *nhlBA* genes and code for negative and positive regulators, respectively. *nhlC* may function as an activator to inhibit the repression effect of *nhlD* in the presence of the inducer amide. *nhlE* codes for a protein that is homologous with the β subunit of L-NHase. *nhlF* encodes a markedly hydrophobic protein with eight hydrophobic putative membrane-spanning domains and is considered to be involved in cobalt uptake.

Expression of H-NHase and L-NHase also exhibits different preferences for various types of amide inducers. While H-NHase and L-NHase can both be induced by acetamide, propionamide, acrylamide and methacrylamide, H-NHase can be selectively induced by urea, and L-NHase can be selectively induced by cyclohexanecarboxamide (Komeda et al. 1996b).

3.2.3 Co-Cu-Zn-Type NHase

Other than the classical Fe- and Co-type NHases, a novel NHase that requires Co, Cu and Zn ions for its activity was discovered in the *R. jostii* RHA1 plasmid (Okamoto and Eltis 2007). Although the subunits of this NHase are encoded by *anhA* and *anhB*, similar to the Fe- and Co-type NHases, a gene, named *anhE*, with unknown function is present between *anhA* and *anhB*, and this gene has never been reported in any other *Rhodococcus* strains. The *anhC* gene encoding amidase is located upstream of *anhAB*. Three regulatory genes, namely, *anhP*, *anhQ* and *anhR*, and a possible cobalt transporter gene, namely, *anhT*, are clustered with the *anhAB* and *anhC* genes. A similar gene cluster, containing *anhP*, *anhQ*, *anhC*, *anhA*, *anhE*, *anhB*, *anhR* and *anhT*, has been identified in *R. opacus* 1CP (GenBank: CP009111.1). However, *anhD* is not present in this cluster, and *anhF* is missing in the genome. The expression of NHase and amidase is induced by acetamide.

Comparing the gene clusters of the three types of NHases, NHase genes are always associated with the amidase gene, although the gene arrangement differs; some amidase genes are adjacent to the NHase, while others may be 15.8 kb away from the NHase gene. Expression of NHase and amidase is always regulated by some genes present in the cluster.

3.3 Nitrilase Gene Organization and Regulation

Compared with the complex NHase-amidase gene cluster, the nitrilase gene cluster has been reported in only *R. rhodochrous* J1, which contains a *nitA* gene, encoding nitrilase, and a *nitR* gene, encoding a positive regulator of *nitA* expression (Fig. 7b) (Komeda et al. 1996a). These two genes are co-transcribed as a single mRNA and induced by isovaleronitrile. The nitrilase gene from *R. rhodochrous* K22 has also been found to be inducible by isovaleronitrile, but isobutyronitrile and fumaronitrile are the most favourable inducers for nitrilases from *R. rhodochrous* PA-34 and *R. rhodochrous* NCIMB11216 (Bhalla et al. 1992; Hoyle et al. 1998).

4 Biodegradation of Nitrile Pollutants

Nitriles are important compounds that are widely manufactured and used in the chemical industry and in agriculture. For example, acetonitrile is a commonly used solvent, and acrylonitrile is a precursor of acrylic fibres and plastics. Nitrile herbicides, such as dichlobenil, ioxynil and bromoxynil, are widely used for rice, corn, wheat and berry crops (Kobayashi and Shimizu 2000). These compounds, however, have been extensively released into our environments through industrial waste water or agricultural chemicals. Most nitriles are highly toxic and harmful to humans and the environment, resulting in an urgent need for remediation of nitrile-contaminated soil and water.

Chemical hydrolysis of nitriles requires harsh conditions such as extreme pH and elevated temperatures and leads to the creation of large amounts of by-products and secondary pollutants (Mukram et al. 2016). Bioremediation is a cost-effective technology to restore polluted environments by degrading these compounds into harmless intermediates by using microorganisms. A number of microorganisms, such as *Rhodococcus*, *Nocardia*, *Bacillus*, *Pseudomonas* and *Arthrobacter*, have been reported for the degradation of nitriles (Gong et al. 2012). Among these microorganisms, *Rhodococcus* species harbouring nitrilase or NHase are the most frequently used species. Nitrilase catalyses the hydrolysis of nitriles into their corresponding acids (ammonium salts), while NHase catalyses the hydration of nitriles to the corresponding amides. Mostly, NHase coexists in *Rhodococcus* with amidase, which converts amides into acids (ammonium salts) (Martínková et al. 2010). Nitrile pollutants include aliphatic nitriles and benzonitrile herbicides. The

former, including acrylonitrile, acetonitrile, butyronitrile and adiponitrile, are primarily released by the chemical industry, while the latter are found in the residues of agricultural chemicals. The strains capable of degrading these compounds are summarized in Table 1.

Aliphatic nitrile pollutants, such as acrylonitrile and acetonitrile, can be degraded by *Rhodococcus* effectively via the NHase/amidase pathway or nitrilase pathway (Martínková et al. 2010). Strains harbouring NHases used for the industrial

Table 1 *Rhodococcus* species involved in the biodegradation of nitrile pollutants

Substrate	Organism	Enzyme involved	Product detected	References
Acrylonitrile vapour	*R. ruber* NCIMB 40757	Nitrilase	Ammonium acrylate	Roach et al. (2004)
Acrylonitrile	*R. erythropolis* AJ270	NHase	Amide	Baxter et al. (2006)
Acetonitrile	*R. erythropolis* BL1	NHase/ amidase	Acid; ammonium	Langdahl et al. (1996)
Acetonitrile	*R. pyridinivorans* S85-2; *B. diminuta* AM10-C-1	NHase/ amidase	Acid and amide	Kohyama et al. (2006)
Acetonitrile	*R. pyridinivorans* S85-2; *Rhodococcus* sp. S13-4	NHase/ amidase	Acid and amide	Kohyama et al. (2007)
Propionitrile, butyronitrile, valeronitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Acid and amide	Mukram et al. (2015)
Acetonitrile; acrylonitrile; crotononitrile	*R. rhodochrous* BX2	NHase/amidase (dominant); nitrilase	Ammonium	Fang et al. (2015) and An et al. (2018)
Butyronitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Acid and amide	Mukram et al. (2016)
Bromoxynil; ioxynil	*R. rhodochrous* NCIMB 11215	Nitrilase	Acid	Harper (1985)
Dichlobenil	*R. erythropolis* 9675; *R. erythropolis* 9685	NHase	Amide	Holtze et al. (2006)
Dichlobenil	*R. erythropolis* AJ270	NHase	Amide	Meth-Cohn and Wang (1997)
Bromoxynil; chloroxynil; ioxynil	*R. rhodochrous* PA-34; *Rhodococcus* sp. NDB 1165	Nitrilase	Acid	Veselá et al. (2010)
Chloroxynil; bromoxynil; ioxynil; dichlobenil	*R. erythropolis* A4; *R. rhodochrous* PA-34	NHase/ amidase	Amide; acid	Veselá et al. (2012)
Benzonitrile; indole-3-acetonitrile	*Rhodococcus* sp. MTB5	NHase/ amidase	Amide; acid	Mukram et al. (2015)

bioproduction of acrylamide can be used for the degradation of acrylonitrile in contaminated environments and waste waters (see Table 1). However, the corresponding amides can be highly toxic, for example, acrylamide is a neurotoxic compound with an LD_{50} (p.o.) of 107–203 mg/kg in rats (Martínková et al. 2010). As a result, amidases are required for further degradation of amides into the corresponding acids (ammonium salts). For the most part, the catalytic efficiencies of NHase and amidase in the same microorganism do not match, necessitating the combination of two different microorganisms for increased degradation efficiency. For example, *R. pyridinivorans* S85-2, with high NHase activity, and *Brevundimonas diminuta* AM10-C-1, with high amidase activity, were combined together for the biodegradation of acetonitrile. With the cascade reaction, 6 M acetonitrile could be converted to acetic acid with a conversion rate of over 90% in 10 h (Kohyama et al. 2006). In addition to the NHase/amidase pathway, nitrilases, which catalyse nitrile into acid (ammonium salt) in one step, also have great potential for the biodegradation of nitrile pollutants. Using *R. ruber* NCIMB 40757 immobilized in synthetic silicone polymer rings, Roach et al. achieved an acrylonitrile elimination capacity of over 7.2 kg/m^3/h with a removal efficiency of 90%. The nongrowing biocatalysts could work as long as 70 days, which showed the excellent tolerance of *Rhodococcus* against the toxic nitrile (Roach et al. 2004).

Benzonitrile herbicides include 3,5-diiodo-4-hydroxybenzonitrile (ioxynil), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) and 2,6-dichlorobenzonitrile (dichlobenil) and their ammonium salts and ester analogues (Holtze et al. 2008; Martínková et al. 2010). The utilization of benzonitrile herbicides in agriculture has caused long-lasting pollution in groundwater. Chloroxynil, bromoxynil and ioxynil can be degraded into the corresponding amide or acid by several strains (Table 1). However, the biodegradation of dichlobenil to acid is much more difficult, and 2,6-dichlorobenzamide was considered to be the dead-end product. Though the use of dichlobenil was banned in Denmark in 1997, 2,6-dichlorobenzamide was still the most frequently detected contaminant in the groundwater in 2006 (Holtze et al. 2008). Recently, *R. erythropolis* A4 was reported to degrade 2,6-dichlorobenzamide to 2,6-dichlorobenzoic acid (Veselá et al. 2012). Using *R. erythropolis* A4 resting cells, 0.5 mM dichlobenil was degraded to amide with a conversion rate of 40% and to acid with a conversion rate of 41% in 3 days.

The biodegradation of nitrile pollutants focuses on the screening and discovery of strains, but a few studies have also focused on protein and strain engineering. The activity, substrate specificity and stability of functional enzymes involved in biodegradation can be enhanced by directed evolution and rational design. *Rhodococcus* strains with high tolerance against toxic nitriles and amides can be engineered as efficient and robust hosts for overexpression of NHase/amidase or nitrilase. It is expected that the combination of protein engineering and strain engineering will help improve the performance of *Rhodococcus* in the biodegradation of nitrile pollutants.

5 Bioconversion of Nitriles for Industrial Applications

Bioconversion of nitriles to the corresponding amides or carboxylic acids catalysed by NHase, amidase or nitrilase has been successfully applied in industrial scale. Among these, the most successful case is the application of NHase for production of amides, such as acrylamide and nicotinamide. Under the harsh industrial conditions, however, the natural state biocatalysts in terms of no matter whole cells or enzymes are not stable enough to maintain high productivity and multiple reuses. Hence, diverse cell engineering and enzyme engineering strategies have been applied to enhance the stability/activity/specificity performances of biocatalysts. We can expect that the industrial applications of *Rhodococcus* in bioconversion of nitriles will be further improved and accelerated by various new biotechnologies such as synthetic biology, enzyme rational design and genome editing tools.

5.1 Amide Production Using NHase

Amides are important chemicals due to their wide applications in enhanced oil recovery, nutritional supplements, medical treatment and so on. In particular, acrylamide and nicotinamide are manufactured at the kiloton scale. Owing to the advantages of this process, such as high productivity, high product purity and environmental friendliness, the biotransformation of nitriles to the corresponding amides with NHase is considered to be superior to the conventional chemical process. Currently, NHase is used to synthesize various amides, including acrylamide, nicotinamide, picolinamide, benzamide, indole-3-acetamide and 3-indolacetamide (Prasad and Bhalla 2010). Of these processes, NHase-mediated catalysis of acrylonitrile to acrylamide is the most successful and was the first case in which biotechnology was applied in the petrochemical industry (Kobayashi and Shimizu 1998). Using bioproduction of acrylamide as a typical example, the synthesis of amides with NHase will be discussed here.

Because of the considerable merits of the process, such as simplicity and ease of manipulation, biocatalysis using free resting cells harbouring NHase as the biocatalyst is the main method for the production of acrylamide to date. Many *Rhodococcus* strains have been industrialized to produce acrylamide, including *Rhodococcus* sp. N-774, *R. rhodochrous* J1 and *R. ruber* TH (Yamada and Kobayashi 1996). At the laboratory scale, the highest acrylamide content was obtained by fed-batch biotransformation of acrylonitrile at 10 °C for 10 h with wild-type *R. rhodochrous* J1, which could produce as high as 650 g/L acrylamide (Nagasawa et al. 1993).

However, many problems have emerged in the application of NHase. First, NHases are thermolabile, and their optimal operation temperature ranges from 20 to 35 °C. In contrast, the strong exothermal heat generated upon the conversion of acrylonitrile to acrylamide inhibits NHase activity. Second, NHase is susceptible

to attack by polar organic solvents, i.e. the product acrylamide and substrate acrylonitrile, which hampers the accumulation of high concentrations of acrylamide. The NHase inactivation is enhanced with increasing reaction temperature and acrylamide concentration. Third, cell flocculation and sedimentation are common phenomena in most *Rhodococcus* species, resulting in very low cell density and in the failure of large-scale fermentation for preparation of *Rhodococcus* catalysts (Jiao et al. 2017). In addition, as mentioned above, in almost all *Rhodococcus* strains, inherent amidases are co-expressed with NHase, thus hydrolysing the amide product into carboxylic acid and ammonia, which not only leads to wastage of amides but also increases the cost of the subsequent purification process (Prasad and Bhalla 2010).

To meet the urgent demand for the production of acrylamide at high concentrations, various strategies have been proposed to evolve engineered strains at both the cell level and the enzyme level (Fig. 8). To solve the problem of by-product production, NHase may be heterologously expressed in a host lacking amidase activity; however, this solution is not ideal as amidases exist in all the known superior *Rhodococcus* strains (Prasad and Bhalla 2010). Therefore, amidase activity is generally eliminated via a gene knockout in the parent organism (Ma et al. 2010). In light of the hydrophobic properties of various *Rhodococcus* cells, cell flocculation and sedimentation are associated with the smooth-rough colony dimorphism phenomenon, and the smooth-type cells are preferred for industrial application to prevent cell flocculation in large-scale fermentation (Jiao et al. 2017).

Additional efforts have focused on how to enhance the thermal stability and acrylamide tolerance of in vivo NHases. Chaperones are a large family of proteins that have indispensable functions, including aiding the folding of newly synthesized polypeptide chains, oligomeric assembly, responding to the stress denaturation of proteins, assisting proteolytic degradation and transcription factor activation. Therefore, the *E.coli* chaperones GroEL-GroES were introduced to *R. ruber* TH3 to aid NHase folding and stabilize the NHase. Recently, it was discovered that the stress tolerance and cell integrity of *R. ruber* TH3 could be improved by overexpressing the small heat shock protein Hsp16 of *Rhodococcus*, thereby increasing the viability of *R. ruber* cells under high concentrations of acrylamide in the solution. Overexpression of the *Rhodococcus* chaperone GroEL2 was also reported to enhance the organic solvent tolerance of *Rhodococcus* (Takihara et al. 2014). In contrast to rational design with chaperones, a transcriptional regulation strategy by sigma factor random mutation and directed evolution selection was applied to increase the bioconversion productivity of acrylamide (Ma and Yu 2012).

The catalytic performance of the resting cells is ascribed to the activity and stability of the enzyme itself. Compared with the laborious random mutation method, rational design is suitable for enzymes whose crystal structures have been revealed, such as NHase. In addition, the identification of the thermosensitive regions and introduction of stable interactions have been highlighted. By RMSF calculations for thermophilic and mesophilic NHases, three deformation-prone thermosensitive regions were identified and stabilized by the introduction of salt bridges (Chen et al. 2013), and the stabilized C-terminus of the β subunit was the most powerful. In light of the instability of the C-terminus, a salt bridge-based design

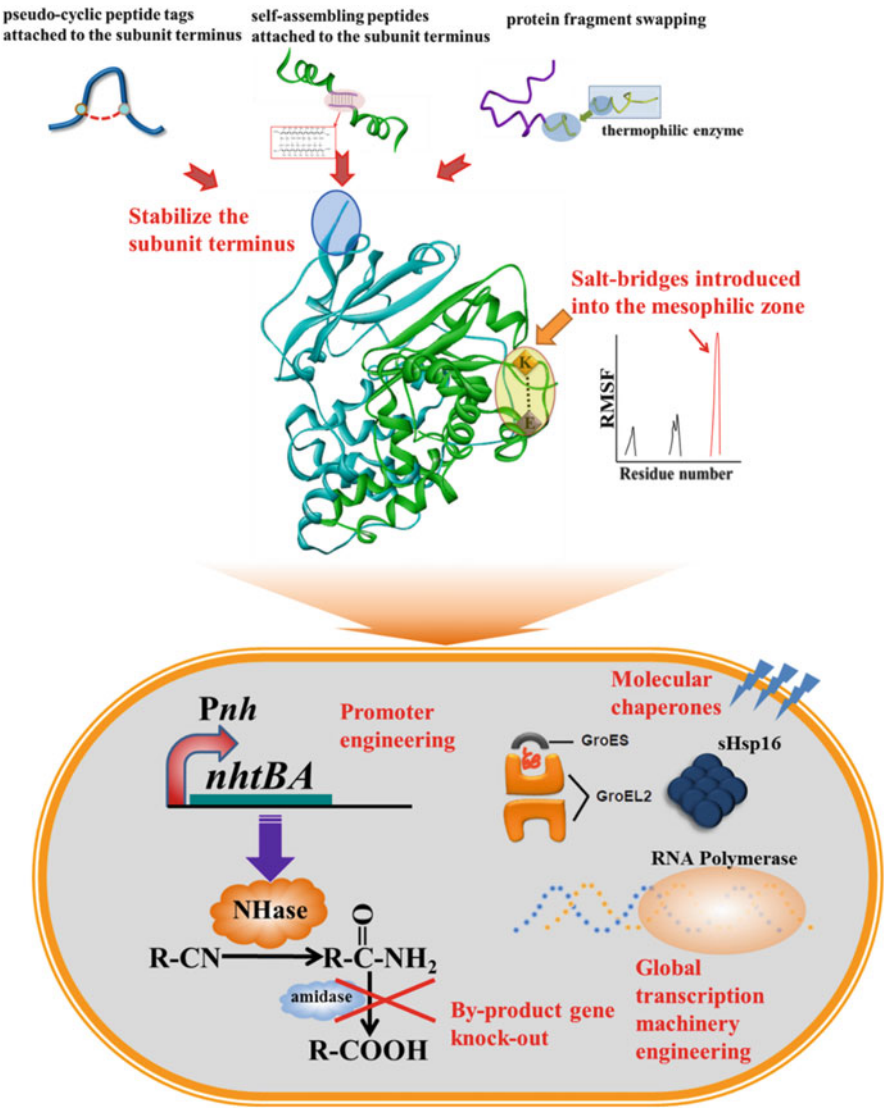

Fig. 8 Strategies used to evolve the engineered NHase-producing *Rhodococcus* strains at both the cell level and the enzyme level

of pseudocyclic peptide tags attached to the subunit terminus was recently applied to the NHase from *R. ruber* TH3, improving the NHase stability significantly without compromising activity. Using STAR (site-targeted amino recombination) software and molecular dynamics to determine the crossover sites for fragment recombination, a homologous protein fragment swapping strategy, involving the swapping of

the mesophilic NHase fragments for the thermophilic NHase fragments, was proposed by Cui et al. (Cui et al. 2014). Similarly, Sun et al. constructed a chimeric NHase by swapping the corresponding C-domains of the β subunit from thermosensitive BpNHase and thermostable PtNHase (Sun et al. 2016b). Regardless of enzyme structure, Liu et al. attached the terminus of the NHase with two self-assembling peptides and obtained enzymes with enhanced stability (Liu et al. 2014). In total, all these efforts involve rigidifying flexible sites to enhance the stability of NHase (Yu and Huang 2014).

Finally, a synergistic evolution strategy for *Rhodococcus* cells and in-cell NHases promises to accomplish the recycling of recombinant *Rhodococcus* cells for production of high concentration acrylamide. Recently, some molecular toolkits for gene expression in *Rhodococcus* strains were developed (Jiao et al. 2018; DeLorenzo et al. 2018), and these may be potentially applied to NHase-producing strains as further modifications to improve catalytic performance.

The successful application of free cells as catalysts notwithstanding, immobilized cells and enzymes confer several benefits and are being used, as previously reviewed in detail (Velankar et al. 2010).

5.2 Carboxylic Acid Production Using Amidase

In addition to its widespread application in amide production, *Rhodococcus* has also been utilized for the synthesis of important carboxylic acids via the NHase/amidase pathway (Ismailsab et al. 2017; Ramteke et al. 2013; Maksimova et al. 2017). For example, whole cells of *R. equi* A4, with NHase and amidase activities, were utilized in the biotransformation of benzonitrile, 3-cyanopyridine, (*R*,*S*)-3-hydroxy-2-methylenebutanenitrile and (*R*,*S*)-3-hydroxy-2-methylene-3-phenylpropanenitrile to the corresponding acids (Kubáč et al. 2006). *R. erythropolis* ZJB-09149, harbouring NHase and amidase, was used to transform 2-chloro-3-cyanopyridine to 2-chloronicotinic acid (Jin et al. 2011). *Rhodococcus* sp. G20 was used for the transformation of β-aminopropionitrile to β-alanine (Liang et al. 2008). The enantioselectivity of NHase and amidase is also of great interest to organic chemists. For example, *R. erythropolis* NCIMB 11540 was found to have a highly active NHase/amidase enzyme system, which can be used to transform α-hydroxynitriles (cyanohydrins) to enantiopure α-hydroxy carboxylic acids. (*R*)-2-Chloromandelic acid and (*R*)-2-hydroxy-4-phenylbutyric acid were prepared at the gram scale with high optical (e.e. >99 and 98%, respectively) and chemical (98%) yields (Osprian et al. 2003). *R. erythropolis* AJ270, harbouring amidase, has been applied in the enantioselective desymmetrization of functionalized prochiral malonamides to afford carbamoylacetic acids with high yield and excellent enantioselectivity (Zhang et al. 2011). Biotransformation of nitrile with *R. erythropolis* AJ270 has been utilized for the enantioselective syntheses of diverse polyfunctionalized organic compounds that are not readily achievable by other methods (Wang 2005).

5.3 Carboxylic Acid Production Using Nitrilase

Recently, a variety of nitrilases from different microorganisms have been discovered to have applications in the fields of biosensing (Roach et al. 2003), bioremediation (Fang et al. 2015; Li et al. 2013) and biocatalysis. In particular, the high substrate specificity, enantioselectivity and regioselectivity of nitrilases make these enzymes attractive biocatalysts for the production of various commodity chemicals and pharmaceutical intermediates at the laboratory scale, such as nicotinic acid, isonicotinic acid, mandelic acid, acrylic acid, glycolic acid and benzoic acid (Bhalla et al. 2018). The biocatalytic reactions that use nitrilases and can be performed in aqueous solutions at moderate temperatures and pH are more efficient, economical and eco-friendly than traditional chemical synthesis methods. To date, *Rhodococcus* species harbouring nitrilase, which is considered to be a potential biocatalyst to hydrolyse nitriles for industrial applications, has been widely investigated (Table 2).

5.3.1 Important Aromatic and Aliphatic Carboxylic Acids

Aromatic acids, including nicotinic acid, isonicotinic acid, benzoic acid and hydroxybenzoic acid, have been synthesized using nitrilase as a biocatalyst. Among these syntheses, the biosynthesis of nicotinic acid (Lonza, China) has been successfully applied at the industrial scale (Gong et al. 2012). Free cells of *R. rhodochrous* J1 and *Rhodococcus* sp. NDB1165 were reported as biocatalysts for the conversion of 3-cyanopyridine to nicotinic acid with productivity values of 172 g/L and 196.8 g/L, respectively (Mathew et al. 1988; Prasad et al. 2007).

Among the aliphatic and aryl aliphatic acids, acrylic acid, glycolic acid, 3-hydroxyvaleric acid and mandelic acid have been widely investigated for their nitrilase-based biotransformation processes. Acrylic acid and its derivatives are commonly applied in surface coatings, textiles, adhesives, paper treatment, polymeric flocculants, dispersants and so on (Xiaobo et al. 2006). Currently, most commercial acrylic acid is produced by partial oxidation of propylene, which leads to energy wastage and the formation of by-products. To date, many subspecies of *Rhodococcus* have been isolated from the biosphere with high substrate specificity for acrylonitrile, including *R. rhodochrous* J1 (Nagasawa et al. 1990) and *R. ruber* AKSH-84 (Kamal et al. 2011). Moreover, *R. rhodochrous* tg1-A6, which was obtained after treatment with UV light combined with lithium chlorinate, was used to synthesize acrylic acid. The results exhibited that the highest concentration of acrylic acid obtained was 414.5 g/L during a 10-h continuous reaction (Luo et al. 2006).

However, most of the novel wild-type nitrilases have been found to not be suitable as potential biocatalysts for industrial applications due to their relatively low activity and stability. Immobilization has been considered to be an effective way to enhance the stability and reusability of biocatalysts. Yucai He et al. immobilized the cells of *Rhodococcus* sp. CCZU10-1 using calcium alginate, achieving efficient biocatalyst recycling with a product-to-biocatalyst ratio of 776 g terephthalic acid/g

Table 2 *Rhodococcus* harbouring nitrilase used for conversion of nitriles to the corresponding acids

Product	Substrate	Organism	Catalyst	Conversion (%); volumetric productivity (g/L/days); e.e. (%)	References
Nicotinic acid	3-Cyanopyridine	*R. rhodochrous* J1	Whole cells	100; 159; n.a.	Mathew et al. (1988)
Nicotinic acid	3-Cyanopyridine	*Rhodococcus* sp. NDB1165	Whole cells	94; 430; n.a.	Prasad et al. (2007)
Acrylic acid/ methacrylic acid	Acrylonitrile/ methacrylonitrile	*R. rhodochrous* J1	Whole cells	98.5; 392; n.a. 100; 260; n.a.	Nagasawa et al. (1990)
Acrylic acid	Acrylonitrile	*R. ruber* AKSH-84	Whole cells	63; 109; n.a.	Kamal et al. (2011)
Acrylic acid	Acrylonitrile	*R. rhodochrous* tg1-A6	Whole cells	n.a.; 994.8; n.a.	(Luo et al. 2006)
Acrylic acid	Acrylonitrile	Recombinant *R. ruber TH3dAdN*(*nit*)	Whole cells	n.a.; 2069.4; n.a.	Sun et al. (2016a)
Glycolic acid	Glycolonitrile	*R. rhodochrous* tg1-A6	Whole cells	n.a.; 14; n.a.	Luo et al. (2016)
Benzoylformic acid	Benzoyl cyanide	*Rhodococcus* sp. CCZU10-1	Immobilized cells	>91; 28.32; n.a.	He et al. (2012)
Terephthalic acid/ isophthalic acid	Tetrachloroterephthalonitrile/ isophthalonitrile	*Rhodococcus* sp. CCZU10-1	Immobilized cells	93.5; 64.8; n.a. 92.7; 62.4; n.a.	He et al. (2014)
(*R*)-Ethyl-3-hydroxyyglutarate	Ethyl 4-cyano-3-hydroxybutyate	*R. erythropolis* ZJB-0910	Whole cells	46.2; n.a.; 99	Dong et al. (2010)
(*S*)-2-Cyano-2-methylpentanoic acid	2-Methyl-2-propylmalononitrile	*R. rhodochrous* J1	Whole cells	97; 80; 96	Yoshida et al. (2013)

DCW and 630 g isophthalic acid/g DCW (He et al. 2014). In addition to immobilization, the use of efficient heterologous expression systems and desired variants generated by directed evolution or site-directed mutagenesis are also predominant strategies. Recently, the recombinant strain *R. ruber* TH3dAdN(Nit), overexpressing the nitrilase from *R. rhodochrous* tg1-A6, showed the highest activity to date (187.0 U/mL) and converted the acrylonitrile to acrylic acid with a volumetric productivity of 344.9 g/L/h (Sun et al. 2016a).

5.3.2 Synthesis of Enantiopure Carboxylic Acids

Nitrilases are attractive biocatalysts for the synthesis of chiral intermediates of various pharmaceuticals due to their inherent superiority in terms of enantioselectivity and regioselectivity. (*R*)-(−)-Mandelic acid (Mitsubishi Rayon, Japan; BASF, Germany) has been successfully produced at the industrial scale using nitrilase as a biocatalyst (Schmid et al. 2001). (*R*)-(−)-Mandelic acid is widely used as an optical resolving agent and as an intermediate in the preparation of various pharmaceuticals, such as semisynthetic penicillin, cephalosporin, antiobesity drugs and antitumour agents (Bhalla et al. 2018). Various bacteria have been reported to produce (*R*)-(−)-mandelic acid, such as *Pseudomonas putida* MTCC 5110, *Alcaligenes faecalis* ECU0401 and *Alcaligenes* sp. MTCC 10675.

Many research studies have focused on exploiting the biotransformation process of valuable compounds whose chemical synthesis is difficult. The nitrilase of *R. rhodochrous* J1, when expressed in *Escherichia coli*, enantioselectively hydrolysed 2-methyl-2-propylmalononitrile to form (*S*)-2-cyano-2-methylpentanoic acid (CMPA) with 96% e.e. in fed-batch mode (Yoshida et al. 2013). Optically pure (*R*)-ethyl-3-hydroxyyglutarate is a key precursor for the synthesis of the chiral side chain of rosuvastatin. Whole cells of *R. erythropolis* ZJB-0910 were used to produce (*R*)-ethyl-3-hydroxyyglutarate with a yield of 46.2% (e.e. >99%) (Dong et al. 2010).

In fact, the paucity of available enzymes and the relatively low enzyme activity and stability remain the main limitations of nitrilase-catalysed biotransformation for industrial applications. Virtually, all nitriles are water-insoluble and destructive to cells. In most studies, the reaction is often carried out in fed-batch mode. Nevertheless, this type of operating mode always has several challenges, which arise due to substrate inhibition, production inhibition and a severe scale-up effect in industrial applications. Therefore, the exploration of effective biocatalysts and design of novel bioreactors and downstream processes are two main areas to consider to exploit the full industrial potential of nitrilases.

6 Conclusions

Aerobic and nonsporulating *Rhodococcus* is a genus of Gram-positive bacteria with a relatively fast growth rate (http://en.wikipedia.org/wiki/Rhodococcus). *Rhodococcus* species are of great environmental, chemical and pharmaceutical importance owing to their powerful ability to degrade diverse pollutants in the environment and to produce valuable chemicals such as acrylamide. The level of nitriles in the environment is increasing due to their widespread use in diverse fields such as organic synthesis. Biodegradation of nitriles by *Rhodococcus* has thus become a focus of research over the years. The three major nitrile-degrading enzymes in *Rhodococcus*, i.e. NHase, amidase and nitrilase, with various reaction optima and substrate specificities, have been well described in terms of not only the organization and regulation of gene clusters but also the modification and evolution of enzyme structures and functions.

In comparison with other nitrile degraders, *Rhodococcus* species possess a number of advantages. For example, the species have the advantages of the varied fatty acid composition of their membrane lipids, the existence of several molecular chaperones, the presence of mycolic acids in their cell envelope and their sufficient extracellular polysaccharides (EPS), which lead to an improved resistance to toxic compounds, simple uptake of hydrophobic nitriles and large-scale high-cell-density fermentation without cell flocculation, respectively. Therefore, applications of *Rhodococcus* in pollutant biodegradation and biocatalytic processes are very promising. In particular, the use of nitrile-converting enzymes to produce some valuable chemicals and pharmaceuticals such as acrylamide has been commercialized, and further applications of nitrile biotransformation also have the potential to be scaled up.

The role of *Rhodococcus* in biocatalysis has been increasingly highlighted. These bacteria have been increasingly developed as advantageous biocatalysts, such as for the bioproduction of high-value chiral chemicals. The use of *Rhodococcus* in synthetic biology and metabolic engineering to obtain various interesting compounds and enzymes is also highly desirable. Therefore, new methods and tools for the genetic engineering of *Rhodococcus* are of great interest in the future.

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