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Characterization of Two Bacterial Hydroxynitrile Lyases with High Similarity to Cupin Superfamily Proteins

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Hydroxynitrile lyases (HNLs) catalyze the cleavage of cyanohydrins. In the reverse reaction, they catalyze the formation of carbon-carbon bonds by enantioselective condensation of hydrocyanic acid with carbonyls. In this study, we describe two proteins from endophytic bacteria that display activity in the cleavage and the synthesis reaction of (*R*)-mandelonitrile with up to 74% conversion of benzaldehyde (enantiopreference *ee* 89%). Both showed high similarity to proteins of the cupin superfamily which so far were not known to exhibit HNL activity.

Hydroxynitrile lyases (HNLs) catalyze the reversible cleavage of cyanohydrins, yielding the respective carbonyl compound and HCN. They are of great relevance in organic synthesis, in which the ability of the enzymes to catalyze the reverse reaction forming C-C bonds in a stereoselective manner is of substantial industrial relevance (9, 11, 19). Hydroxynitrile lyases are quite heterogeneous exhibiting remarkable diversity with respect to their substrate specificity, mass, glycosylation, and amino acid sequence, and their similarity to oxidoreductases, α/β-hydrolases, carboxypeptidases, or Zn^{2+}-containing alcohol dehydrogenases has been reported (3, 4, 8, 17, 18, 19).

Hydroxynitrile lyases are widespread in plants playing a major role in disease suppression, and only recently a bacterial protein with HNL activity in the cyanohydrin cleavage reaction was reported (16). Usually plants are colonized by a range of different bacteria (14). Endophytes in particular live in close association with their host and promote plant growth and health by various mechanisms, including the production of substances with phytohormonal activity or antimicrobial substances such as antibiotics (13, 15, 21). The production of HCN in pseudomonads has been reported (5), for example, but it has also been reported in other bacteria. In contrast to plants, cyanogenesis in bacteria usually follows a completely different biosynthetic pathway involving a HCN synthase (2, 20). In this work, we present the discovery of a new bacterial enzyme class with hydroxynitrile lyase activity initially identified by function-based screening of a gene library of an isolated bacterial endophyte related to *Pseudomonas mephitica* and further investigated with a second highly similar protein from *Burkholderia phytofirmans* strain PsJN.

Gene libraries of different endophytic bacteria isolated from potato were constructed in the pZero-2 vector by standard procedures (see supplemental material) and screened for HNL activity toward (*R/S*)-mandelonitrile using a colony-based colorimetric filter assay [67 mM (*R/S*)-mandelonitrile in 30 mM citrate-phosphate buffer (pH 3.5) at room temperature (RT)] (10). In the gene library of a strain related to *Pseudomonas mephitica*, HNL activity was detected. After subcloning and rescreening, the transformant exhibiting the strongest activity toward mandelonitrile was selected for further characterization. Sequencing revealed that the plasmid contained a 1,659-bp insert, which carried two open reading frames (ORFs) (GenBank accession no. JF937913). The

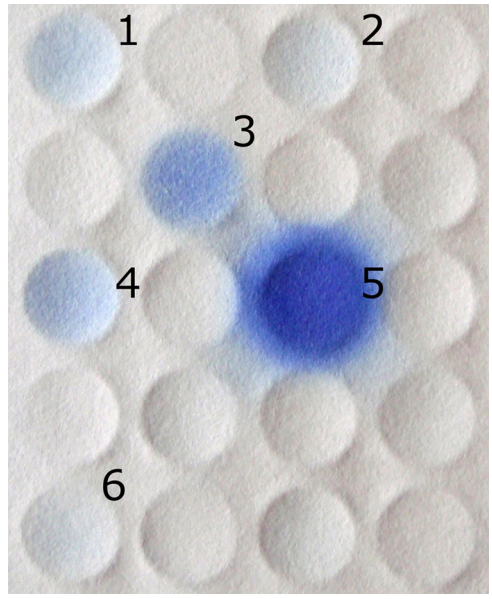

FIG 1 Microtiter plate activity assay with cleared lysates of *Psm*HNL-short, *Psm*HNL-long, and *Bp*HNL. For the different spots, the lysates and times of appearance of the blue spots are shown as follows: spot 1, *Psm*HNL-short and ca. 5 min; spot 2, *Psm*HNL-long and ca. 7 min; spot 3, uninduced *Hb*HNL and ca. 3 min; spot 4, *Bp*HNL and ca. 4 min; spot 5, induced *Hb*HNL and ca. 25 s; spot 6, pEamTA and ca. 12 min. *Hb*HNL was the positive control, and the negative control was the empty vector pEamTA.

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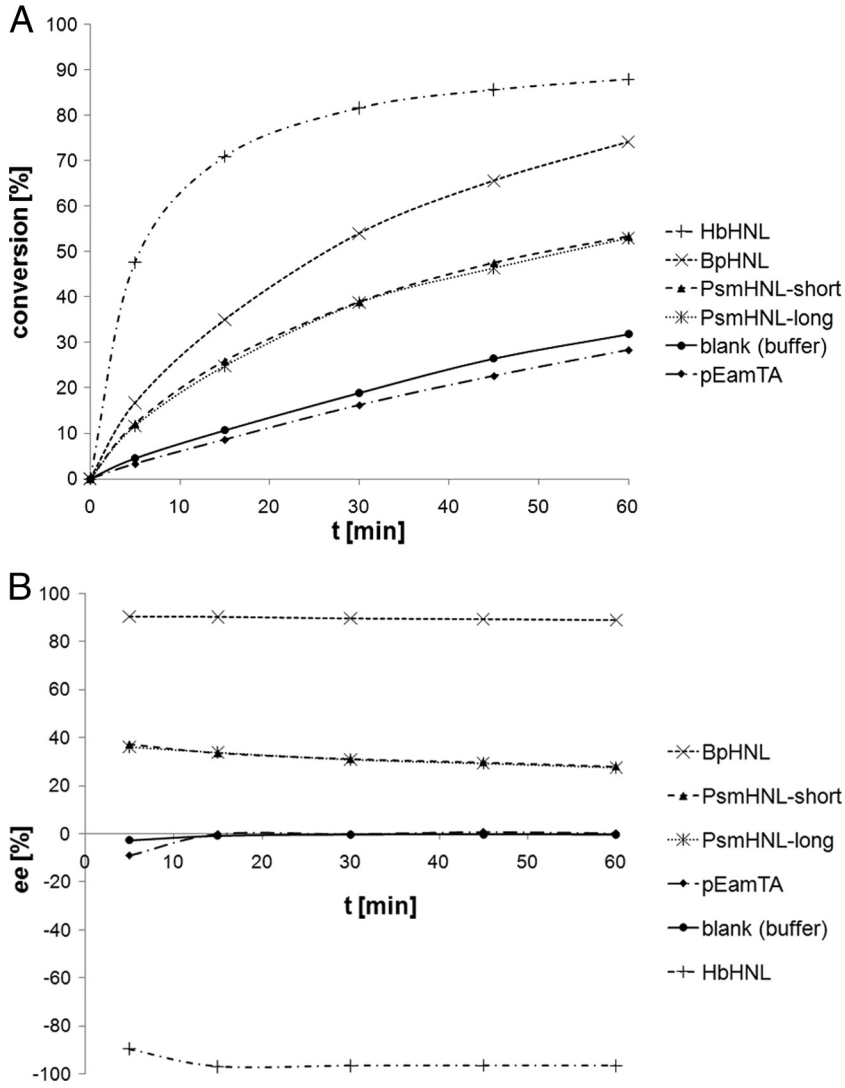

FIG 2 Conversion of benzaldehyde (A) and enantiopreference *ee* (B) of concentrated cleared lysates of *Psm*HNL-short, *Psm*HNL-long, and *Bp*HNL over time (*t*). Both conversion and *ee* are shown as percentages. *Hb*HNL was the positive control. The negative controls were empty vector pEamTA and buffer blank.

ORF1 sequence showed high identity to a protein belonging to the cupin superfamily, while the ORF2 sequence was similar to the sequence of a drug resistance protein. Interestingly, neither ORF sequence showed similarity to known HNL sequences. Two possible start codons were identified for the cupin-like ORF and considered the origin for a 468-bp (ORF1-long) gene and 396-bp (ORF1-short) gene. Upstream of both potential start codons of ORF1 motifs resembling possible Shine-Dalgarno sequences and in front of the second possible start codon of ORF1-short, a putative promoter sequence was identified. ORF1-long, ORF1-short, and ORF2 were cloned into pEamTA (12), and the clones carrying these genes were screened for their performance on (*R/S*)-mandelonitrile. While the drug resistance protein exhibited no activity, the cupin-like protein displayed HNL activity (data not shown). Thus, the protein encoded by ORF1 was named *Psm*HNL.

Sequence alignments identified several proteins with high similarity to *Psm*HNL. These proteins are predicted to be members of the cupin superfamily, which contains 50 families, including various enzymes like dioxygenases, decarboxylases, hydrolases, isomerases, and epimerases, but also some nonenzymatic proteins (1, 6). Cupins are characterized by a conserved domain, comprising a six-stranded beta-barrel structure, including the motifs $[G(X)_5HXH(X)_{3,4}E(X)_6G]$ and $[G(X)_5PXG(X)_2H(X)_3N]$ together with a 11- to 16-amino-acid (aa) intermotif region (6, 7). These motifs are also present in *Psm*HNL (see Fig. S1 in the supplemental material). All highly conserved proteins originate from sequenced bacterial genomes, but none of these proteins has been biochemically characterized so far. One protein of *Erwinia billingiae* Eb661 showed 100% identity on the protein level and 97% identity on the nucleotide level to *Psm*HNL. A hypothetical cupin-like protein of *Burkholderia phytofirmans* strain PsJN (GeneID Bphy_5714) with an amino acid sequence identity of 64% was chosen to verify the evidence of its functionality as a HNL. Significant activity could be observed in the colony-based filter assay (data not shown), and the respective protein was thus named *Bp*HNL. These data conclude that proteins with HNL activity can be found among cupin family proteins of so far unknown function.

*Bp*HNL, *Psm*HNL-long, and *Psm*HNL-short cloned into the pEamTA vector were overexpressed in *Escherichia coli* TOP10F′ by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density (OD) at 600 nm of 0.8 for 20 h at 21°C. Following cell disruption and centrifugation, SDS-polyacrylamide (SDS-PA) gels confirmed that both *Bp*HNL and *Psm*HNL-short were localized mainly in the soluble fractions with molecular masses of 14.4 kDa and 14.3 kDa, respectively. For *Psm*HNL-long, two bands at 17.2 and 14.4 kDa were observed. Moreover, a large portion of the 17.2-kDa *Psm*HNL-long protein was found in the pellet fraction (see Fig. S2 in the supplemental material), indicating that the second start codon in ORF1 is the correct translation start. High background absorption of the examined concentrated lysates at 280 nm made it impossible to perform a quantitative spectrophotometric activity assay that measures the formation of benzaldehyde from mandelonitrile at 280 nm. For that reason, these lysates were investigated by performing the reactions in microtiter plates and detecting the HCN that formed by the sensitive and fast filter assay, allowing the estimation of relative activity levels by monitoring the time of the appearance of the blue spots (10) (Fig. 1). These assays clearly confirmed that *Bp*HNL (Fig. 1, spot 4) and *Psm*HNL-short (Fig. 1, spot 1) displayed HNL activity toward racemic mandelonitrile; however, they displayed HNL activity to a much lower extent than the positive control, (*S*)-selective HNL from *Hevea brasiliensis* (*Hb*HNL) (Fig. 1, spots 3 and 5) did. *Psm*HNL-long (Fig. 1, spot 2) was found to be only very weakly active, which correlates with the low level of soluble protein expression. The negative control (Fig. 1, spot 6) showed very weak background stain after prolonged incubation due to slow chemical cleavage of the substrate and the release of HCN.

Moreover, *Psm*HNL and *Bp*HNL showed activity with acetone cyanohydrin and Cl-substituted mandelonitrile. Even with the bulky *meta*-phenoxybenzaldehyde cyanohydrin, weak activity was observed (data not shown).

The enzymes were also investigated in the mandelonitrile synthesis reaction. Under the chosen reaction conditions (0.3 M benzaldehyde and 50% *tert*-butylmethylether at pH 6.0 and 15°C; for detailed reaction conditions and chiral gas chromatography [GC] analysis, see supplemental material), the positive control, (*S*)-selective HNL from *H. brasiliensis* showed 88% conversion with an enantiopreference *ee* (*S*) of 97% after 60 min. Both variants, *Psm*HNL-short and *Psm*HNL-long gave conversions of 47% after 60 min. Surprisingly, very good conversion (74% after 60 min) was achieved with *Bp*HNL (Fig. 2A). The nonselective base-catalyzed chemical background reaction occurring proportional to increasing pH values showed 28% (empty vector pEamTA) and 32% (buffer blank) conversion after 60 min, respectively. The enzyme-catalyzed reactions, however, were stereoselective in the synthesis of mandelonitrile. *Psm*HNL-short and *Psm*HNL-long showed an appreciable *R* preference of 28%. *Bp*HNL exhibited remarkable stereoselectivity with an *ee* of 89% in the *R* direction (Fig. 2B). This is especially interesting, as other HNLs usually display higher activity in the cleavage than in the synthesis direction. The enantioselective cyanohydrin synthesis reactions clearly provide evidence that the identified cupin family proteins represent a novel class of hydroxynitrile lyases. As the identified proteins do not show similarity to HNLs that are known so far and whose reaction mechanism has been characterized, it will be interesting to further investigate the amino acids in the putative catalytic domain to be able to postulate a new reaction mechanism.

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REFERENCES

1. **Agarwal G, Rajavel M, Gopal B, Srinivasan N.** 2009. Structure-based phylogeny as a diagnostic for functional characterization of proteins with a cupin fold. PLoS One **4**:e5736.
2. **Blumer C, Haas D.** 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. **173**:170–177.
3. **Dadashipour M, Asano Y.** 2011. Hydroxynitrile lyases: insights into biochemistry, discovery and engineering. ACS Catal. **1**:1121–1149.
4. **Dreveny I, Andryushkova AS, Glieder A, Gruber K, Kratky C.** 2009. Substrate binding in the FAD-dependent hydroxynitrile lyase from almond provides insight into the mechanism of cyanohydrin formation and explains the absence of dehydrogenation activity. Biochemistry **48**:3370–3377.
5. **Duffy B, Keel C, Defago G.** 2004. Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. Appl. Environ. Microbiol. **70**:1836–1842.
6. **Dunwell JM, Purvis A, Khuri S.** 2004. Cupins: the most functionally diverse protein superfamily? Phytochemistry **65**:7–17.
7. **Gane PJ, Dunwell JM, Warwicker J.** 1998. Modeling based on the structure of vicilins predicts a histidine cluster in the active site of oxalate oxidase. J. Mol. Evol. **46**:488–493.
8. **Hasslacher M, Kratky C, Griengl H, Schwab H, Kohlwein S.** 1997. Hydroxynitrile lyase from *Hevea brasiliensis*: molecular characterization and mechanism of enzyme catalysis. Proteins **27**:438–449.
9. **Holt J, Hanefeld U.** 2010. Enantioselective enzyme-catalysed synthesis of cyanohydrins. Curr. Org. Synth. **6**:15–37.
10. **Krammer B, Rumbold K, Tschemmernegg M, Pöchlauer P, Schwab H.** 2007. A novel screening assay for hydroxynitrile lyases suitable for high-throughput screening. J. Biotechnol. **129**:151–161.
11. **Purkarthofer T, Skranc W, Schuster C, Griengl H.** 2007. Potential and capabilities of hydroxynitrile lyases as biocatalysts in the chemical industry. Appl. Microbiol. Biotechnol. **76**:309–320.
12. **Reisinger C, Kern A, Fesko K, Schwab H.** 2007. An efficient plasmid vector for expression cloning of large numbers of PCR fragments in *Escherichia coli.* Appl. Microbiol. Biotechnol. **77**:241–244.
13. **Reiter B, Pfeifer U, Schwab H, Sessitsch A.** 2002. Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atroseptica.* Appl. Environ. Microbiol. **68**:2261–2268.
14. **Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN.** 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol. Lett. **278**:1–9.
15. **Sessitsch A, Reiter B, Berg G.** 2004. Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Can. J. Microbiol. **50**:239–249.
16. **Sulzbacher Caruso C, et al.** 2009. α-Hydroxynitrile lyase protein from *Xylella fastidiosa*: cloning, expression, and characterization. Microb. Pathog. **47**:118–127.
17. **Trummler K, et al.** 1998. Expression of the Zn^{2+}-containing hydroxynitrile lyase from flax (*Linum usitatissimum*) in *Pichia pastoris*—utilization of the recombinant enzyme for enzymatic analysis and site-directed mutagenesis. Plant Sci. **139**:19–27.
18. **Wajant H, Mundry KW, Pfizenmaier K.** 1994. Molecular cloning of hydroxynitrile lyase from *Sorghum bicolor* (L.): homologies to serine carboxypeptidases. Plant Mol. Biol. **26**:735–746.
19. **Winkler M, Glieder A, Steiner K.** Hydroxynitrile lyases: from nature to application. *In* Carriera E, Yamamoto H (ed), Comprehensive chirality, vol 7: Synthetic methods VI—enzymatic and semi-enzymatic. Elsevier, in press.
20. **Zagrobelny M, Bak S, Moeller B.** 2008. Cyanogenesis in plants and arthropods. Phytochemistry **69**:1457–1468.
21. **Zinniel DK, et al.** 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. **68**:2198–2208.