



Communications

Discovery of a novel (*R*)-selective bacterial hydroxynitrile lyase from *Acidobacterium capsulatum*

Romana Wiedner^a, Mandana Gruber-Khadjawi^a, Helmut Schwab^{a,b}, Kerstin Steiner^{a,*}

^a ACIB GmbH, Austrian Centre of Industrial Biotechnology, c/o TU Graz, Petersgasse 14/4, 8010 Graz, Austria

^b Institute of Molecular Biotechnology, TU Graz, Petersgasse 14/5, 8010 Graz, Austria

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ABSTRACT

Hydroxynitrile lyases (HNLs) are powerful carbon–carbon bond forming enzymes. The reverse of their natural reaction – the stereoselective addition of hydrogen cyanide (HCN) to carbonyls – yields chiral cyanohydrins, versatile building blocks for the pharmaceutical and chemical industry. Recently, bacterial HNLs have been discovered, which represent a completely new type: HNLs with a cupin fold. Due to various benefits of cupins (e.g. high yield recombinant expression in *Escherichia coli*), the class of cupin HNLs provides a new source for interesting, powerful hydroxynitrile lyases in the ongoing search for HNLs with improved activity, enantioselectivity, stability and substrate scope. In this study, database mining revealed a novel cupin HNL from *Acidobacterium capsulatum* ATCC 51196 (AChNL), which was able to catalyse the (*R*)-selective synthesis of mandelonitrile with significantly better conversion (97%) and enantioselectivity (96.7%) than other cupin HNLs.

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Hydroxynitrile lyases (HNLs) catalyse the reversible stereoselective addition of hydrogen cyanide (HCN) to aldehydes and ketones. Both, (*R*)- and (*S*)-selective HNLs are well-established industrial biocatalysts for the synthesis of chiral cyanohydrins, which are important building blocks for pharmaceuticals and agrochemicals [1–3]. Hydroxynitrile lyases represent a diverse group of mainly plant enzymes with members in several protein folds [4]: FAD-dependent oxidoreductases, α/β -hydrolase family, serine carboxypeptidases, Zn^{2+} -dependent alcohol dehydrogenases, and very recently, bacterial cupins. They descend from distinct ancestors and differ in their enantioselectivity, co-factor dependence and substrate scope [5,6]. For industrial application, in particular, activity and stability at acidic pH and solvent stability are desired to reduce the unselective non-enzymatic background reaction. Further demands made on HNLs are high expression levels in an easy to handle expression host and a broad substrate scope. Although a lot of effort has already been made to improve and tailor HNLs for industrial requirements by protein, substrate and reaction engineering [5,7–9], there is still a clear need for further enhancement of HNLs or discovery of new HNLs exhibiting improved properties. A new door has recently opened with the discovery of bacterial hydroxynitrile lyases with a cupin fold, e. g. GtHNL from *Granulicella tundricola*, by our group [10, 11]. Cupins are small stable beta barrel proteins, which are ubiquitous in nature and very diverse in function [12]. In most cupins metal ions are bound to the active site [13]. Cupin HNLs are characterised by high

expression levels in *Escherichia coli* and an already remarkable stability under various conditions, however, so far relatively low activity compared to other HNLs [6,10,11]. The goal of the present study was to expand the class of cupin HNLs by identifying new members with higher activity and enantioselectivity and broader substrate scope via database search. Out of ten chosen targets, one enzyme, AChNL from *Acidobacterium capsulatum* ATCC 51196 exhibited improved activity and was characterised in more detail.

The basic approach comprised a Basic Local Alignment Search Tool (BLAST, [14]) search with the sequence of GtHNL (Uniprot: E8WYN5, [10]) focused on protein sequences originating from acidophilic and/or thermophilic organisms. The search was restricted to protein sequences with identical metal-binding amino acids as GtHNL (His53, His55, Gln59 and His94). Moreover, only sequences containing His96 and His106 located in the active site (numbering according to GtHNL) were considered, because previous site-directed mutagenesis studies showed that they were essential for activity [10]. Out of hundreds of sequences with high similarity to GtHNL, a set of ten mainly hypothetical proteins was selected (Table S1) originating from acidophilic and/or thermophilic bacteria or bacteria with high resistance to environmental hazards. The respective enzymes share between 38% and 84% sequence identity with GtHNL (Fig. 1).

The genes were codon optimised for *E. coli* and ordered as synthetic DNA, recloned into the pET26b(+) expression vector and transformed into *E. coli* BL21 (DE3) Gold (see Supplemental information). Preliminary activity screenings were performed on colony level towards (*R/S*)-mandelonitrile. As GtHNL was found to be dependent on manganese [10], the activities of the selected enzymes

* Corresponding author at: ACIB GmbH, c/o TU Graz, Petersgasse 14/4, 8010 Graz, Austria. Tel.: +43 316 8739346; fax: +43 316 8739302.
E-mail address: kerstin.steiner@acib.at (K. Steiner).

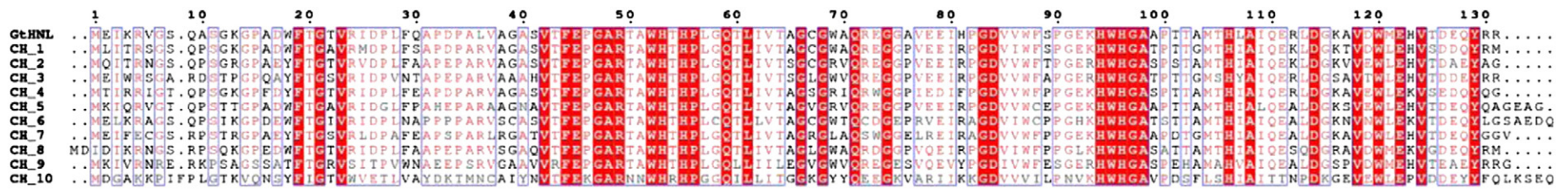


Fig. 1. Multi sequence alignment of CH_1: *Methylocella silvestris*, CH_2: *Acidobacterium capsulatum* ATCC 51196, CH_3: *Acidiphilium cryptum* JF-5, CH_4: *Paenibacillus* sp. JDR-2, CH_5: *Deinococcus deserti*, CH_6: *Burkholderia phymatum* STM815, CH_7: *Sinorhizobium medicae* WSM419, CH_8: *Bradyrhizobium* sp. WSM471, CH_9: *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* Tc-4-1, and CH_10: *Caldicellulosiraptor saccharolyticus* DSM 8903 with GtHNL. The alignment was generated with Clustal W [15] and visualized with the programme ESPript 3.0 [16].

expressed with and without various bivalent ions in the expression media were compared. As expected, all enzymes were able to cleave the substrate in the presence of $MnCl_2$ (Fig. 2), however to a different extent. The addition of other metal ions (Cu^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+} , Fe^{2+}) to the media did not show any impact on the activity (data not shown). In comparison to *GtHNL* especially the enzyme from *A. capsulatum* ATCC 51196 (CH_2) developed stronger blue spots on the HCN-sensitive detection paper after the same period of time.

In accordance with the screening results, all target enzymes except CH_8 were expressed in high yield as soluble proteins in *E. coli* (~50% of total soluble protein) (Figure S1).

As the chemical background reaction is significantly reduced at low pH, an “ideal” HNL should exhibit high stability and activity at low pH. Hence, the cyanogenesis activity of *E. coli* lysate containing the expressed enzymes was tested at pH 3.5, 4.0, 4.5 and 5.0 in 100 mM sodium oxalate buffer or citrate phosphate buffer or at pH 5.6 in 100 mM MES oxalate buffer. All HNLs (except expectedly CH_8) were active at pH 5.0 or higher to variable degrees and also at pH 4.5 the enzymes exhibited low activity; especially CH_2 showed a promising signal at pH 4.5. No activity was detected at pH 3.5 (data not shown).

Subsequently, the stability of CH_2 at different pH values was examined by incubation of cleared CH_2 lysate in the respective buffers. In accordance with the results from the assay, the SDS-PA gel revealed that at pH 4.5 most of CH_2 was still found in the soluble fraction (Fig. 3); the amount, however, decreased significantly at lower pH values.

The enzymes were investigated for their ability to catalyse the synthesis of mandelonitrile from benzaldehyde and HCN (Table 1). Only CH_6 did not show any conversion of benzaldehyde after 24 h. Three candidates, CH_1, CH_3 and CH_4 were slightly better than *GtHNL* and reached about 97% conversion with enantiomeric excesses (*ee*) between 90.2 and 93.1%. Remarkably, CH_2 showed nearly full conversion (99.5%) after 24 h with a very high *ee* (*R*) of 96.7%. Furthermore, a high conversion of benzaldehyde (94%) and an *ee* of 96.6% were achieved already after 8 h reaction time. Importantly, the *ee* was stable during the whole course of the reaction.

The structure of *GtHNL*, which was solved recently [10,18], indicates that the active site of the enzyme is located in a large cavity, which should provide enough space to bind bulky substrates. Thus, the new cupin-HNLs were examined in the synthesis of 3-phenoxybenzaldehyde cyanohydrin. Seven out of nine enzymes showed weak or no activity in the synthesis of 3-phenoxybenzaldehyde cyanohydrin (data not shown). But, very interestingly, two enzymes exhibited notable activity (Table 2). Although the conversion was higher with CH_7, excellent enantioselectivity was only obtained with CH_2 (>99.9% after 1 and 4 h).

As for none of the chosen target proteins, 3D structures are available, models were build using the SWISS-MODEL protein structure homology-modelling server [19] and the structure of *GtHNL* as template [10] to be able to identify differences in the active site. Structural alignment in Pymol and cavity analysis using parts of the programme VASCO [20] and a LIGSITE algorithm [21] revealed that there are hardly any

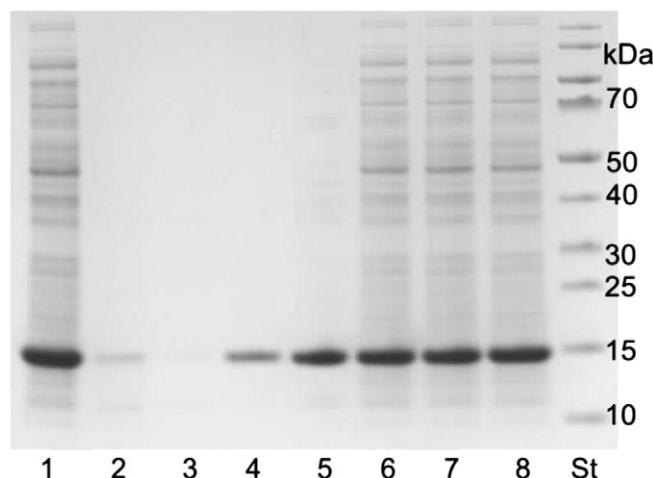


Fig. 3. SDS-PA gel of cleared lysates (~10 µg) of CH_2 (pH 7.0, lane 1 as reference) and soluble fractions of CH_2 after incubation of the cleared lysates in 100 mM citrate phosphate buffer for 30 min and subsequent centrifugation, pH 3.0 (lane 2), 3.5 (lane 3), 4.0 (lane 4), 4.5 (lane 5), 5.0 (lane 6), 5.5 (lane 7) and 100 mM MES oxalate buffer pH 5.6 (lane 8). (CH_2 monomer: 14.1 kDa), St.: PageRuler Prestained Protein Ladder.

significant differences in the size and the shape of the cavities in the different target proteins. This is not unexpected as many of the active site amino acids (V42, F44, L61, H96, H106 and W120, numbering refers to *GtHNL*) are identical (Fig. 1, and S2A). The positions A40 and Q110 are less conserved. Moreover, CH_2 contains a valine at position 25, where an isoleucine is found in most other cupin HNLs creating a little bit more space for bulky substrates. In CH_7 a leucine is found at the respective position (Figure S2B).

Considering the results obtained so far, it was decided to choose CH_2 from *A. capsulatum* ATCC 51196 (subsequently renamed AchNL) for a more detailed analysis concerning its substrate scope. For this purpose the industrially relevant substrates 2-chlorobenzaldehyde, 3-phenylpropanal and 3-phenylprop-2-enal were tested. However, only low conversion (10–20% above the background reaction after 24 h) and no or very low *ee* (<20%) were observed (data not shown).

For a more detailed analysis, AchNL (expressed in the presence or absence of $MnCl_2$, subsequently named AchNL+Mn and AchNL-Mn) was purified by anion-exchange chromatography and subsequent size-exclusion chromatography. The elution profile of AchNL is identical to *GtHNL* (Figure S3). Thus, we can assume that AchNL is a tetramer as it was observed in the *GtHNL* structure [10]. The metal contents of the purified proteins were determined by inductively coupled plasma/optical emission spectroscopy. Without addition of $MnCl_2$ to the medium 0 mol% manganese and only traces of iron (6.3 mol%) and zinc (4.6 mol%), which are generally present in LB medium, were found per metal-binding site. In contrast, the expression of AchNL in LB medium supplemented with 100 µM $MnCl_2$ led to a loading of 70 mol% manganese per metal-binding site. Both enzyme preparations were tested

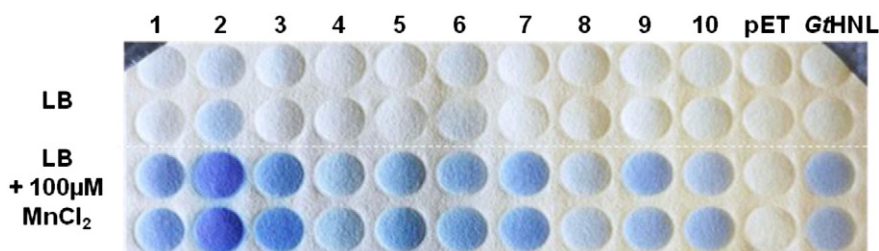


Fig. 2. Influence of $MnCl_2$ addition on the cyanogenesis activity. The assay was performed with an adapted version of the colony HNL-assay developed by Kramer et al. [17] (see supplemental information) using 12 mM (*R/S*)-mandelonitrile in 100 mM citrate phosphate buffer, pH 3.5. Upper two rows: activity of cupin HNLs expressed without $MnCl_2$; lower two rows: activity of cupin HNLs expressed in the presence of 100 µM $MnCl_2$. Negative control: pET26b-vector; positive control: *GtHNL*. The numbering refers to CH_1–10.

Table 1
Stereoselective conversion of benzaldehyde to (*R*)-mandelonitrile in the presence of CH_1–10.

Enzyme ^a	8 h		24 h	
	Conv. [%]	ee [%]	Conv. [%]	ee [%]
CH_1	77.3	93.3	97.6	93.1
CH_2	94.0	96.6	99.5	96.7
CH_3	76.2	92.3	97	92.3
CH_4	77.5	90.8	97	90.2
CH_5	36.9	79.2	75.7	78.5
CH_6	ND	ND	ND	ND
CH_7	53.9	86.7	69.6	81.8
CH_9	75.3	81.7	96.1	81.2
CH_10	5.2	16.7	18.4	11.9
GtHNL	57.3	89.0	91.9	88.9
BpHNL	83.1	95.0	96.6	94.9
pET26b(+)	ND	ND	10.4	0.1

Reaction conditions: Two-phase system comprising 2 mL MTBE containing 2 M HCN and 0.5 M benzaldehyde and 1 mL concentrated cleared lysates (45 mg/mL, pH 4), 1000 rpm, 5 °C.

ND: not determined due to the low conversion.

^a 100 μM MnCl₂ added to expression medium.

for mandelonitrile synthesis. Purified AcHNL+Mn showed 97.3% conversion and 94.7% ee (*R*) after 24 h (Fig. 4). This is significantly higher than values obtained previously for purified GtHNL, for which a large difference in the activity of lysate and purified enzyme was observed (50% conversion with 89.3% ee after 24 h using 18 mg/mL of protein in otherwise identical reaction conditions), which was attributed to the stabilizing effect of the lysate [10]. The only slightly decreased conversion and enantiomeric excess obtained with purified AcHNL in comparison to the concentrated lysate (99.5% conversion and 96.7% ee (*R*) after 24 h) can be also explained by the lower amount of enzyme present in the reaction (~22.5 mg/mL of cupin in cleared lysate, compared to 11.8 mg/mL of purified enzyme). As expected, AcHNL expressed without manganese addition, exhibited only 35.3% conversion and a strongly decreased ee (*R*) of 35.8% after 24 h confirming the manganese-dependence of cupin HNLs.

The activity of purified AcHNL+Mn in the cyanogenesis of (*R*)-mandelonitrile was examined spectrophotometrically at pH 4.0, pH 4.5, pH 5.0 (sodium oxalate buffer) and pH 5.5 (MES oxalate buffer) by following the formation of the product benzaldehyde at 280 nm (see Supplemental information and Table S2). The enzyme displayed a specific activity of 0.69 ± 0.02 U/mg at pH 5.5. At pH 5.0, AcHNL exhibited a slightly decreased specific activity of 0.63 ± 0.06 U/mg, whereas a further drop of the pH to 4.5 led to a significant decrease of the specific activity with 0.12 ± 0.02 U/mg which can be attributed to a partial denaturation of the enzyme which was visible as faint cloudiness after the measurement in the wells of the microtitre plate. At pH 4.0 the enzyme was clearly precipitated during the measurement and thus, reliable data evaluation was not possible. Kinetic measurements of purified AcHNL+Mn (0.5 mg/mL) were performed in sodium oxalate buffer, pH 5.0, in a substrate concentration range between 3 and 30 mM, which revealed a *K_m* of 4.2 ± 0.8 mM and a

Table 2
Conversion of 3-phenoxybenzaldehyde.

Enzyme ^a	1 h		4 h		24 h	
	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]
CH_2	10.4	> 99.9	26.0	> 99.9	58.8	93.8
CH_7	18.2	66.1	48.3	90.8	76.3	93.4
GtHNL	2.4	83.5	13.8	86.1	34.5	87.9

Reaction conditions: Two-phase system comprising 1 mL MTBE containing 2 M HCN and 0.5 M 3-phenoxy-benzaldehyde and 0.5 mL concentrated cleared lysates (45 mg/mL, pH 4), 1000 rpm, 5 °C.

^a 100 μM MnCl₂ added to expression medium.

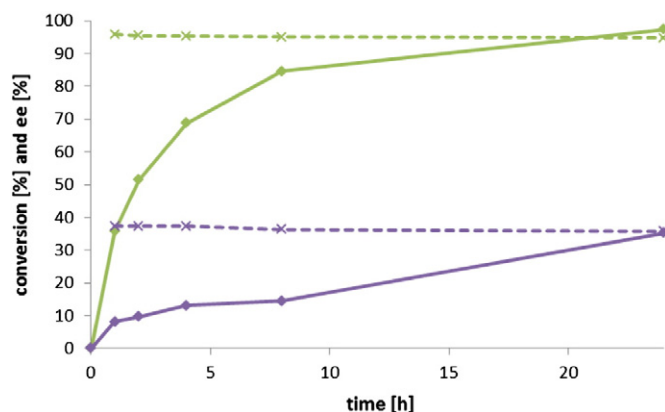


Fig. 4. Conversion of benzaldehyde by purified AcHNL. Reaction conditions: Two-phase system comprising 1 mL MTBE containing 2 M HCN and 0.5 M benzaldehyde and 0.5 mL purified AcHNL expressed ± MnCl₂ (11.8 mg/mL, pH 4), 1000 rpm, 5 °C. Green: AcHNL+Mn, purple: AcHNL-Mn, solid lines: conversion, dashed lines: ee.

turnover number *k_{cat}* of 9.5 s⁻¹, corresponding to a specific activity of 0.71 ± 0.035 U/mg at *v_{max}*.

In summary, a novel manganese-dependent bacterial HNL from *A. capsulatum* ATCC 51196 was discovered by database mining. Compared to other cupin HNLs it shows higher conversion and an excellent enantioselectivity in the synthesis of (*R*)-mandelonitrile.

For additional experimental procedures refer to supplemental information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.csbj.2014.07.002>.

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