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Identification of a New Peptide Deformylase Gene From *Enterococcus faecium* and Establishment of a New Screening Model Targeted on PDF for Novel Antibiotics[1]

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Objective To identify a new peptide deformylase (PDF) gene (Genebank Accession AY238515) from *Enterococcus faecium* and to establish a new screening model targeted on PDF. **Methods** A new PDF gene was identified by BLAST analysis and PCR and was subsequently over-expressed in the prokaryotic expression host *E.coli* Bl21(DE3). Over-expressed protein was purified for enzymatic assay by metal affinity chromatography and a new screening model was established for novel antibiotics. **Result** A new PDF gene of *Enterococcus faecium* was identified successfully. Ten positive samples were picked up from 8000 compound library and the microbial fermentation broth samples. **Conclusion** A new PDF of gene *Enterococcus faecium* was first identified and the model had a high efficacy. Positive samples screened may be antibacterial agents of broad spectrum.

Key words: *Enterococcus faecium*; Peptide deformylase (PDF); Antibacterial gents of broad spectrum

INTRODUCTION

Vancomycin is the drug of the last resort for treating resistant Gram-positive bacterial infections, and the emergence of vancomycin-resistance *Enterococci* (VRE) presents a serious threat to public health[1]. Till now there has been no effective drugs for treating VRE infection, and therefore searching for new antibiotics with novel mechanisms and potent activity against VRE has become an urgent need. As PDF is an essential enzyme for the growth and multiplication of bacteria and is absent from mammalian cells, it is regarded as one of the most promising targets for the discovery of new antibiotics[2]. To establish a screening model targeted on the PDF of *Enterococci*, we cloned and identified a new peptide deformylase gene from *Enterococcus faecium.* First of all, the amino acid sequences of peptide deformylase from *E.coli, Str. pneumoniae* were used in BLAST search at NCBI with

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E. faecium genome[3,4] on the basis of their homologue sequence. The amino acid sequence of PDF of *E. faecium* has a 40% similarity with *E.coli* and a 68% similarity with *Str. pneumoniae* (Fig. 1).

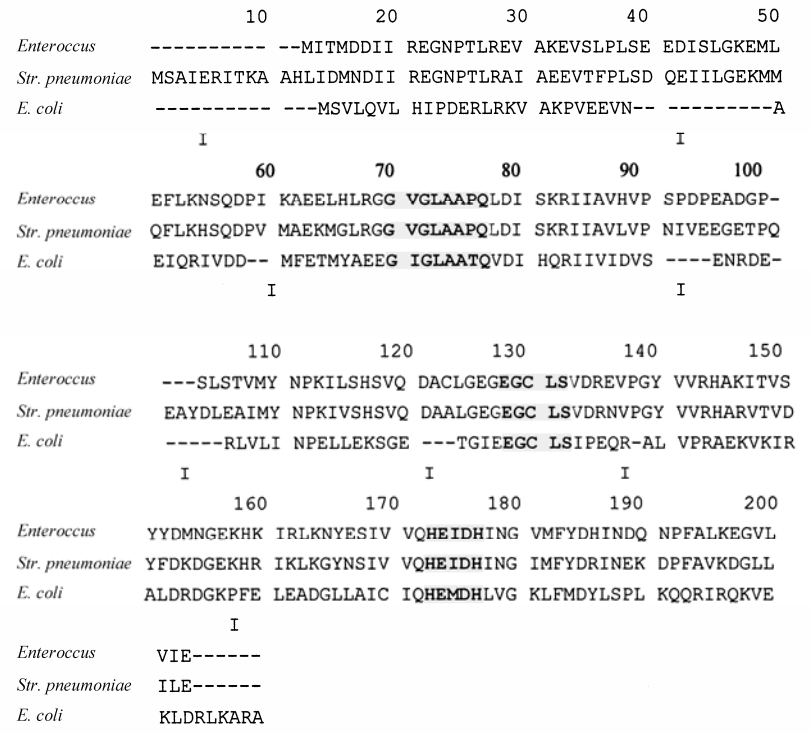

FIG. 1. Comparison of amino acid sequences of peptide deformylases of *E. faecium, Str. pneumoniae* (Gene bank Accession No. AY014509), *E.coli* (Gene bank Accession No. X77800) by clustraw software. Black background indicates three conserved motifs. I indicates the insertion amino acids. The sequence identity between *E. faecium* and *E.coli* was 40%, 68% between *E. faecium* and *Str. pneumoniae*, and 32% between *Str. pneumoniae* and *E.coli.*

MATERIALS AND METHODS

Strains, Plasmids and Media

E. faecium ATCC6057 was obtained from the Institute of Microbiology, Chinese Academy of Sciences and routinely grown at 37℃ overnight in MH broth media modified. *E.coli* DH5α, BL21 (DE3) and plasmid pET-30a (+) for protein expression were purchased from Novagen Company. T-vector for sequencing was purchased from Promega Company. *E.coli* DH5α and BL21 (DE3) were grown at 37℃ overnight in Luria-Bertani (LB) media.

Cloning and Expression

Genomic DNA was extracted from cells of *E. faecium* ATCC6057 by CTAB methods. Pairs of PCR primers were designed on the basis of the homologue sequence of *E. faecium*: sense primer: 5′-GCCATATGATTACAATGGATGAT-3′ (underlining indicating restriction enzyme *Nde* I site), anti-sense primer: 5′-TACTCGAGCATGAGCTGTGGTT-3′ (underlining indicating restriction enzyme *Xho* I site). DNA fragment was amplified by PCR using *Pfu*

polymerase from *E. faecium* genomic DNA. The target DNA was inserted into T-vector for sequencing, and then sub-cloned to pET-30a (+) for constructing expression vectors pET-30a-*def*. *E.coli* BL21 (DE3) transformed by pET-30a-*def* was grown in a 250 mL wide mouth flask containing 50 mL LB media with 50 mg/L Kanamycin (Km) and incubated overnight at 28℃ on a rotary shaker (250 rpm). Five mL of the culture was transferred to a 500 mL flask containing 100 mL LB media with 50 mg/L Km and incubated at 28℃ for 4 h, then induced by 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 6 h. The over-expressed cells were harvested by centrifugation at 5000×g for 5 min.

Enzyme Purification

The high-level expression of the protein greatly benefited subsequent purification of the protein. All steps of purification were performed at 0℃ to 4℃ unless otherwise indicated. Over-expressed proteins with C-terminal hexahistidine tag were purified by column chromatography of BD TALONTM metal affinity resins (Clontech Company). The harvested cells from 1 liter culture were resuspended into 50 mL of extraction buffer (pH 8.0) containing 20 mmol/L imidazole, 50 mmol/L sodium phosphate and 300 mmol/L sodium chloride and lysated by sonication 3×30sec in the presence of 10 μg/mL catalase. The lysate of cells was centrifugalized at 20 000×g for 20 min and the debris was discarded. The supernatant was applied on the column of affinity resins previously equilibrated with binding buffer (identical to extraction buffer) followed by washing with binding buffer. The target proteins were eluted with pH 7.0 washing buffer containing 300 mmol/L sodium chloride, 50 mmol/L sodium dihydrogen phosphate, 100 mmol/L imidazole, and the eluted sample was dialyzed in 20 mmol/L Tris-Cl (pH 8.0 1:500) overnight. The enzyme concentration was determined using Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. The enzyme PDF activity was assayed in the purification procedure.

Assays for PDFs Activity

The activity of PDF enzyme was assayed on the basis of the principle that PDF deformylated the N-formylmethionine of the substrate For-Met-Ala-Ser, and the free amino group explored was detected by fluorescamine (Sigma) (0.2 mg/mL in dry dioxane). Fluorescence was quantified with polarstar plate reader at an excitation wavelength of 375 nm and an emission wavelength of 495 nm[5]. The characterization of PDF was examined at different pH, temperature and concentration of substrate and inhibitor.

Establishing a High Throughput Screening Model Targeted on PDF

Based on the analytic mechanism of PDF active assay, purified PDF was used to establish high throughput screening molecular model for novel antibacterial agents *in vivo*. Actinonin, a known inhibitor of PDF, was used as positive control. With the inhibition of PDF activity as a screening index, 8000 samples from our compound library and microbial fermentation broths were examined, and 10 positive samples among them were picked up, further studied by inhibiting bacteria, and assayed in the inhibited MMP.

RESULTS

Identification of Peptide Deformylase From Enterococcus Faecium

A BLAST search using the amino acid sequence of PDF of *E.coli, Str. pneumoniae*

identified a homolog sequence. PDF had three motifs: motif 1 {GXGXAAXQ}, motif 2 {EGClS}, motif 3 {HEXDH}[6]. PDF of *E. faecium* belonged to Class II[7] (Fig. 1), and by DNA sequences analysis, we found that the PDF gene had SD-sequence and initiation codes ATG and stop codes TGA, indicating that it had a whole open reading frame (Fig. 2).

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TGAAACACAGTCTTTTAAGAAAGGGGTACATATTCCATGATTACAATGGATGATATCATTCGTGAAGGA
                    SD                      M  I  T  M  D  D  I  I  R  E  G
AATCCGACCTTACGCGAAGTTGCAAAAGAAGTTTCTCTTCCATTAAGCGAAGAAGATATCTCTTTAGGA
 N  P  T  L  R  E  V  A  K  E  V  S  L  P  L  S  E  E  D  I  S  L  G
AAAGAGATGCTTGAGTTCTTAAAAAACAGCCAAGATCCAATCAAAGCTGAAGAACTACACTTACGAGGC
 K  E  M  L  E  F  L  K  N  S  Q  D  P  I  K  A  E  E  L  H  L  R  G
GGTGTTGGCTTAGCTGCTCCTCAATTAGATATTTCTAAAAGAATCATTGCTGTTCACGTACCAAGTCCT
 G  V  G  L  A  A  P  Q  L  D  I  S  K  R  I  I  A  V  H  V  P  S  P
GACCCAGAGGCAGATGGACCTTCTCTAAGTACTGTTATGTATAATCCTAAAATTTTGAGCCATTCTGTT
 D  P  E  A  D  G  P  S  L  S  T  V  M  Y  N  P  K  I  L  S  H  S  V
CAAGATGCTTGTTTAGGGGAAGGTGAAGGCTGTTTATCTGTTGATCGAGAAGTACCAGGATATGTTGTT
 Q  D  A  C  L  G  E  G  E  G  C  L  S  V  D  R  E  V  P  G  Y  V  V
CGCCATGCCAAAATCACTGTGTCTTATTATGATATGAATGGTGAAAAACACAAGATTCGTTTGAAGAAT
 R  H  A  K  I  T  V  S  Y  Y  D  M  N  G  E  K  H  K  I  R  L  K  N
TATGAATCGATCGTCGTTCAACATGAAATCGACCATATCAACGGTGTGATGTTCTATGACCACATCAAC
 Y  E  S  I  V  V  Q  H  E  I  D  H  I  N  G  V  M  F  Y  D  H  I  N
GATCAAAATCCATTTGCACTAAAAGAAGGCGTATTGGTGATCGAGTAG
 D  Q  N  P  F  A  L  K  E  G  V  L  V  I  E-  --
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FIG. 2. DNA sequence of PDF from *Enterococcus faecium*. SD-sequence (Shine-Dalgarno), possible sequence and initiation codes and stop codes.

Molecular Weight of PDFs of Enterococcus faecium

The molecular weight of PDF of *E. faecium* determined by protein analysis was 21.50 kD. (Fig. 3)

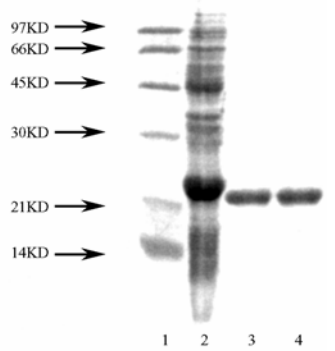

FIG. 3. SDS-PAGE of purified peptide deformylase of *Enterococcus faecium* Lane 1: protein Mark, Lane 2: BL21 (DE) cell extract, Lanes 3, 4: purified his-tag proteins. The proteins were stained with Coomassie brilliant blue.

By testing enzymatic activity, we found that PDF retained full activity from the beginning to the end in its purification procedure. Purified PDF had higher activity than cell lysate, but the total activity was decreased (Table 1).

TABLE 1
Purification of Active PDF to Homogeneity

Purification Step	Total Protein (mg)	Total Activity (U[a])	Activity (U/mg of Protein)	Yield (%)
Crude Extract	469	1200	2.58	100
Ni-Metal Affinity Resins	20	310	15.5	25

Note. [a] One unit is defined as the amount of enzyme capable of hydrolyzing 1 nmol/L of For-Met-Ala-Ser at 37℃, pH 7.5.

Kinetics of PDF From Enterococcus faecium

The three peptides For-Met-Ala-Ser were selected as the substrates for PDF reaction. Km and Vmax values were calculated based on the slope and intercept values of the linear fit in a Lineweaver-Burk plot. The catalytic efficiency of PDF of *E. faecium* was relatively high compared with Kcat/Km values of PDF from *E.coli and Str. pneumoniae*[8,9]. The result revealed that the deformylation rate of PDF presented a hyperbolic curve in the range of 0-13 mmol/L of substrate concentrations and the activity of PDF was inhibited (Fig. 4) by increasing the substrate concentration (13-25 mmol/L), as observed in other PDFs such as from *E.coli*, *Str. pneumoniae*.

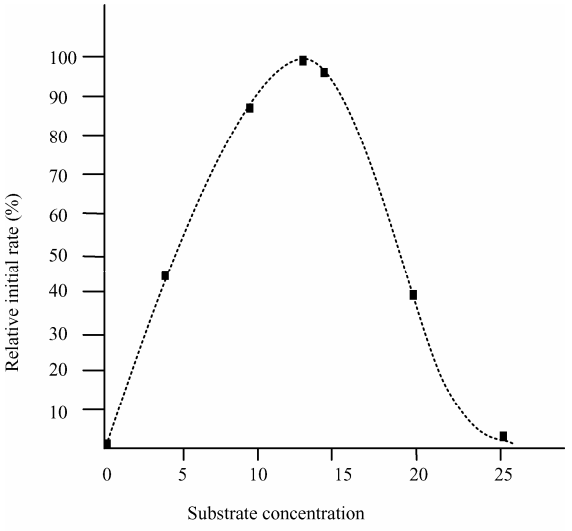

FIG. 4. Effect of substrate concentration on the deformylation rate of PDF.

A relative rate of 100 assigned to the value obtained the highest activity in the range of 1-25 mmol/L For-Met-Ala-Ser at 37℃, pH 7.5.

Effects of Temperature and pH on Enzyme Activity

The effects of pH and temperature on PDF activity were examined. The enzymatic

activity was decreased when pH value was below 7.0, however it was nearly a constant when pH was 7.4 to 11 (Fig. 5b). The enzyme had a broad thermal stability[8,9]. The initial rate raised gradually from 25℃ to 70℃, then dropped when the temperature was higher than 70℃ (Fig. 5a). It was also noticed that the PDF of *Enterococcus faecium* was still active when the temperature was 90℃.

A relative rate of 100 assigned to the value obtained the highest activity in the range of pH 5.0-11.0, in the presence of 8 mmol/L For-Met-Ala-Ser and at 37℃.

A relative rate of 100 was assigned to the value obtained the highest activity in the range of 20-90℃, in the presence of 8 mmol/L For-Met-Ala-Ser and at pH 7.5. The reactive buffer of different pH: Sodium acetate (pH 5.5); HEPES-HCl pH 6.0-8.0, N, N-bis (2-hydroxyethyl) glcine-NaOH pH 8.5-9.5; 3-(cyclohexylamino) propane sulfonic acid-NaOH pH 9.7-11.0.

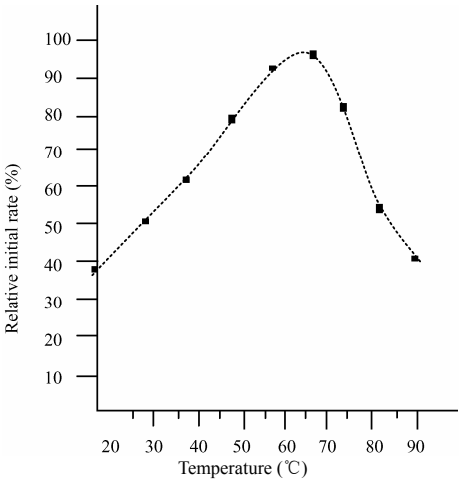

FIG. 5a. Effect of temperature on the initial rate of hydrolysis of peptide deformylase.

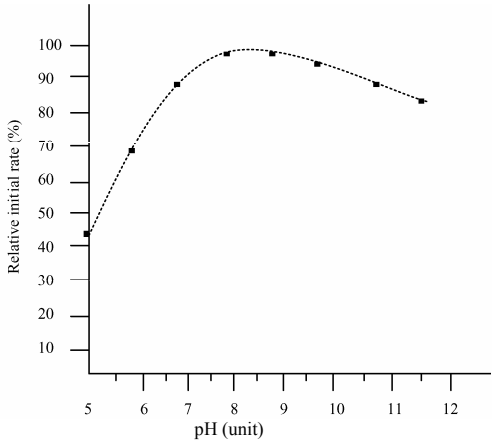

FIG. 5b. Effect of pH on the initial rate of hydrolysis of peptide deformylase.

Actinonin produced by *Streptomyces sp.*c/2 (NCIB)[5] contains a hydroxamate moiety in its structure and is active against Gram pos. bacteria including mycobacteria. It was

identified as the first inhibitor of PDFs[10-12]. As shown in Fig. 6, actinonin inhibited PDF enzymatic activity in a dose dependent manner.

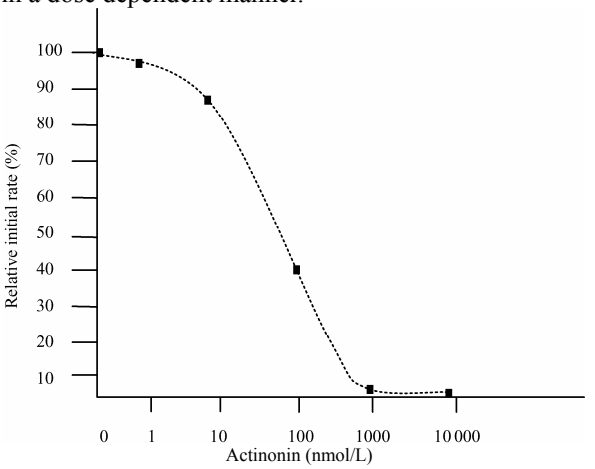

FIG. 6. Inhibition curve of Actinonin on PDF of *E. faecium*.

Establishment of High Throughput Screen Model and Screening

In order to gain very efficient inhibitors of PDF, high throughput screening molecular model for novel antibacterial agents *in vivo* was established and actinonin, a known inhibitor of PDF, was used as positive control. Based on the analytic mechanism of PDF active assay, Polarstar was used to detect the screen, which could finish 96 samples once. Hereon Polarstar could screen about ten thousand samples every day. Ten positive samples were picked up from 8000 samples of our compound library and microbial fermentation broths. Eight samples were from microbial fermentation broth mixture, 2 samples were from compound library. Ten positive samples all could inhibit the growth of bacteria, 10 positive samples were used to assay the activity against MMP. Five PDF inhibitors from microbial fermentation samples had no effect on MMP and the compound samples were lower specific for PDF. Subsequent work was to separate the active compounds from microbial fermentation broths, 2 compounds would be further characterized.

DISCUSSION

Protein synthesis in prokaryote initiates an N-formyl-methio-tRNAf, resulting in N-terminal formylation of all nascent polypeptides. Removal of the formyl group from N-terminal methionine residue is the first and essential step in maturation of proteins. Peptide deformylase catalyzes the removal of the formyl group from nascent polypeptide chains. That is why PDF has been regarded as a good target for the design of new antibiotics[13-17].

In the last decade, many peptide deformylase homologues have been identified[7], but studies on the enzymatic properties of PDF were mostly based on PDF of *E.coli*[18-21]. Recent reports on PDFs revealed that there was a great difference in enzyme properties of PDF from different sources[21,22]. It is essential to identify all kinds of PDFs, especially from resistant bacteria in order to facilitate the design and screen of specific PDF inhibitors.

In this study, we successfully cloned and identified the PDF gene from *E. faecium* and determined the effect of various factors on the activity of PDF of *E. fae-cium*. It was

reported that PDF (including Class I and Class II) had three common conserved motifs: motifs1 {GΦGΦAAXQ}, 2 {EGClS} and 3 {HEΦDH}; where Φ is a hydrophobic amino acid and X is any amino acid[6,7]. PDF of *Enteroccus faecium* belongs to Class II because its amino acid sequence and enzyme properties are closer to that of *S.aure* rather than *E.coli* .

PDF activity was examined by the detection of the free amino group using fluorescamine, which was explored by deformylating the substrate For-Met-Ala-Ser. Fluorescence was quantified with Polarstar plate reader using an excitation wavelength of 375 nm and an emission wavelength of 495 nm[5]. By testing we considered this method to be accurate, simple, sensitive and suitable for a high throughput screening. In examining enzymatic activity, the PDF of *Enterococcus faecium* was found to be resistant to temperature as high as 90℃, that verified the explanation that the 3-D structures of PDFs would be very compact and solid.

The results we got from a large scale of screening showed that those samples from compound library containing hydroxamate group in their structure exhibited inhibition not only on PDF but also on MMP (matrix metalloproteases) present in human cells. However, 5 PDF inhibitors from microbial fermentation samples had no activity on MMP. Although they belong to metallohydrolases family and have common motifs such as {HEXXH}, MMP and PDF have their own specific structures and different inhibition mechanisms[7,23]. Those inhibitors specific for PDF might be promising candidates for new antibiotics.

Although PDF was regarded as a good target for novel broad spectrum antibacterial agents, the intensive research on PDF found that there was counterpart in human cells having PDF activity. Moreover, the selectivity of some PDF inhibitors for metallo-proteases is low, and the resistant mutation to PDF inhibitor is high in some pathogens such as *E.coli*. After these questions are addressed, it is important to identify the function of human PDF in N-terminal protein processing to understand whether PDF is a good target or not.

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