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Cloning and heterologous expression of pro-2127, a gene encoding cold-active protease from *Pseudoalteromonas* sp. QI-1

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Abstract The psychrotropic bacterium, *Pseudoalteromonas* sp. QI-1, which produces extracellular cold-active protease, was isolated from Antarctic seawater. The genomic DNA of this bacterium was used to construct a plasmid genomic library with the goal of screening cold-active protease genes. Gene pro-2127 with an open reading frame of 2127 bp encoding protease PRO-2127 was cloned and sequenced. Alignment of amino acid sequences suggested that the precursor of PRO-2127 was a member of subfamily S8A, and that it might contain four domains: a signal peptide, an N-terminal prosequence, a catalytic domain and a C-terminal extension. Amino acids Asp185, His244 and Ser425 might form a catalytic triad. PRO-2127 showed some structural features common to psychrophilic enzymes, such as a decrease in Arg residues and the Arg/(Arg+Lys) ratio. Heterologous expression of pro-2127 in *Escherichia coli* BL21 (DE3) by pColdIII was also successfully observed in this study.

Keywords **Antarctic, *Pseudoalteromonas*, cold-active protease, gene cloning and expression**

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0 Introduction

Cold environments have been successfully colonized by numerous organisms, especially bacteria, yeasts, unicellular algae and fungi. Organisms that have colonized such environments have developed various mechanisms enabling them to adapt to the adverse effects of low temperature. Enzymes are essential targets for the adaptation of an organism to cold environments[1]. Enzymes from psychrotropic and psychrophilic microorganisms have recently received increased attentions owing to their high catalytic activity at low temperature, low thermostability and unusual specificity[2]. Furthermore, fundamental issues concerning the molecular basis of cold activity and the interplay between flexibility and catalytic efficiency are of importance in the study of structure-function relationships in proteins. Such issues are often approached through comparison with the mesophilic or thermophilic counterparts, if available, and/or mutagenesis[2–4].

Proteases (protein hydrolases) are enzymes that catalyze amide (peptide) bond hydrolysis in protein or peptide substrates. Although cold-active proteases from some deep-sea strains of *Pseudoalteromonas* [5–8] and *Pseudomonas* [9] have been studied, further investigations might identify new proteases that can be used for basic research and future technological applications [10].

We isolated dozens of cold-adapted microorganisms that produce protease at low temperature from polar regions. Strain QI-1 was selected as a promising producer of cold-active protease, and was identified as a species belonging to the genus *Pseudoalteromonas*. The almost-complete 16S rDNA (FJ560733) sequence of strain QI-1 consisting of 1353 bp was analyzed using Blastn. The results showed that Q1 shared more than 99% homology with many different species belonging to the genus *Pseudoalteromonas*, including *Pseudoalteromonas elyakovii* BSi20670 (DQ517879), *Pseudoalteromonas* sp. M12-3 (FN377726), and *Pseudoalteromonas* sp. L-10 (FJ386506). A preliminary study of the characteristics of the crude protease showed that *Pseudoalteromonas* sp. QI-1 excreted cold-active protease[11]. Here, we report the

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cloning and sequencing of a protease gene from strain *Pseudoalteromonas* sp. QI-1 and its heterologous expression in *E. coli* BL21 (DE3).

1 Materials and methods

1.1 Materials

Restriction enzymes and T4 DNA ligase were obtained from Takara (Takara Biotechnology Co., Ltd., Dalian, China). DNA polymerase was obtained from Tiangen (Tiangen Biotech Co., Ltd., Beijing, China). Agarose and Tris were purchased from MD Bio (MD Bio Inc., Qingdao, China). X-Gal, IPTG and Amp were obtained from Takara (Takara Biotechnology Co., Ltd., Dalian, China). Casein was obtained from Sigma (Sigma-Adrich Co., USA). Tryptone, yeast extract and granulated agar were purchased from Oxoid (Oxoid Ltd., England). All other reagents and solvents used were of analytical grade.

1.2 Medium

Luria-Bertani (LB) medium was used for the cultivation of *Escherichia coli* cells. A casein plate, which was used to detect the proteolytic activity, was prepared using 1% casein, 1% tryptone, 0.1% yeast extract, 1% NaCl and 1.5% agar.

1.3 Strains and vectors

Strain QI-1 producing cold-active protease was isolated from the seawater of the Great Wall Bay near Chinese Antarctic Great Wall Station in Antarctica and identified as *Pseudoalteromonas* sp. on the basis of its 16S rDNA (GenBank accession number FJ560733)[11].

E. coli DH5α and BL21 (DE3), which were used as host strains for DNA manipulation and gene expression, respectively, were obtained from Tiangen (Tiangen Biotech Co., Ltd., Beijing, China). Plasmid pUC118 *Hinc*II/BAP (Takara Biotechnology Co., Ltd., Dalian, China) and pColdIII (Takara Biotechnology Co., Ltd., Japan) were used for gene cloning and expression, respectively.

1.4 Cloning of protease gene

Chromosomal DNA of strain QI-1 was extracted using a Genomic DNA Prep Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The chromosomal DNA was partially digested with *Sau*3A I and then electrophoresed, after which 3-kb to 6-kb fragments were purified from the 0.8% agarose gel using the Agarose Gel DNA Purification Kit Ver.2.0 (Tiangen Biotech Co.,Ltd., Beijing, China). These DNA fragments were ligated to pUC118 *Hinc*II/BAP using a DNA Ligation Kit (Takara Biotechnology Co., Ltd., Dalian, China).

The recombinant plasmids were transformed into *E. coli* DH5α. Clones producing proteases were detected based on the formation of clear halos around the colony on LB agar plates supplemented with Amp (50 μg/mL), 0.1 mmol/L IPTG and 1% (*w/v*) casein after incubation at 4℃. The recombinant plasmid DNA from the protease positive clones was extracted and sequenced.

1.5 Construction of expression plasmid pro-2127

Expression vector pColdIII was used to express the protease. Based on the upstream and downstream sequences of the known protease gene, the specific primers for PCR amplification of pro-2127 were designed and synthesized as follows: Primer PF1: 5'-CTGTAGGAGCTCATGACAACT-AGTA AAACTTT-3', which carried a *Sac*I restriction site at its 5' end, and Primer PR1: 5'-CATAAATCTAGAAACTT-TAAATTACGGTTG-3', which carried a *Xba*I site at its 3' end. The PCR conditions of amplification of pro-2127 were as follows: denaturation at 95℃ for 1 min, annealing at 53℃ for 1 min, and extension at 72℃ for 2 min for a total of 30 cycles.

The amplified fragments were purified from a 1% agarose gel. The purified fragments digested with *Sac*I and *Xba*I were ligated into vector pColdIII, which was previously digested with the same two restriction enzymes, resulting in a recombinant plasmid pColdIII-pro-2127. The recombinant plasmid was transformed into *E. coli* DH 5α, and transformants producing proteolytic enzymes were detected by the formation of clear halos around the colony on LB agar plates supplemented with ampicillin (100 μg/mL) and 1% (*w/v*) casein during incubation at 4℃.

1.6 Expression of protease gene in *E. coli*

E. coli BL21 (DE3) transformed by recombinant plasmid pColdIII-pro-2127 was grown overnight with shaking (150 r/min) in 5 mL LB broth. When the optical density of the culture reached 0.5 at 600 nm, the culture solution was kept at 15℃. IPTG was then added to a final concentration of 0.5 mmol/L, after which the culture was incubated at 15℃ with shaking for 24 h. The cells were then harvested and ruptured using constant cell disruption systems (Constant Systems Ltd., England).

1.7 Enzyme assay and SDS-PAGE analysis

Using casein as a substrate, an enzyme assay was conducted at 40℃ using the Folin-phenol method as described by Wang et al.[12] . SDS-PAGE was run essentially as described by Sambrook[13]. Briefly, the sample was dissolved in Laemmli's sample buffer and then heated to 95℃ for 5 min,

after which it was cooled and applied to a gel. The SDS gel was composed of a 5% (*w/v*) stacking gel and a 15% (*w/v*) separating gel. The samples were first subjected to electrophoresis at 80 V on stacking gel and then at 120 V on a separating gel. After electrophoresis, the gel was fixed and stained with 0.1% (*w/v*) Coomassie brilliant blue R-250 (Eastman Kodak). A standard marker obtained from Tiangen was also run on the gel (Tiangen Biotech Co., Ltd., Beijing, China).

2 Results

2.1 Cloning of gene pro-2127

Among approximately 20000 recombinant clones from *Pseudoalteromonas* sp. QI-1, one protease positive transformant was identified based on the formation of clear halos around its colony when grown at 4℃, which indicated it might encode protease. Restriction maps of the recombinant plasmid revealed that it contained a DNA fragment of approximately 4.1kb. This fragment was sequenced and an open reading frame (ORF) of 2 127 bp (pro-2127) was obtained using Blastp (GenBank accession number HM047289).

2.2 Sequence analysis of gene pro-2127 encoding protease PRO-2127

Sequence analysis revealed that the gene pro-2127 encoded a polypeptide composed of 708 amino acids residues with a calculated molecular mass of 72.5 kDa. A Shine-Dalgarno ribosome binding site (5'-AGGGA-3') predicted at positions －10 to －6 upstream of the predicted start codon implied that the pro-2127 was of full length.

The sequence of the predicted protein, PRO-2127, is shown in Figure 1. A possible TATA-like promoter site (5'-AATAAT-3') and a -35 box (5'-GTGATC-3') were located 23 bp and 40 bp upstream of the initiation codon for transcription, respectively (Figure 1).

The deduced amino acid sequence of pro-2127 was compared with that of other proteases. The results revealed that it was 96% identical to subtilisin-like serine protease P6 preproprotein from *Pseudoalteromonas* sp. 2-10 (ABS01328), 88% identical to serine protease precursor from *Alteromonadales* bacterium TW-7 (ZP-01613292) and 86% identical to serine protease MCP-03 preproprotein from *Pseudoalteromonas* sp. SM9913 (ABD92880). To group PRO-2127 into a known family, PRO-2127 was used as a query to search the MEROPS database with the BLAST tool available at the website (http://merops.sanger.ac.uk). All of the top 100 retrieved sequences were from subfamily S8A of the serine proteases, indicating that PRO-2127 might be a member of S8A. Homology analysis with other subtilisins revealed that PRO-2127 was likely to be synthesized as a preproprotease, and a putative signal peptide of 27 residues (Met1-Ala27 of the predicted prepeptide) was present at the amino terminus of the deduced protein sequence. Alignment of the amino acid sequences of their precursors suggested that they might share similar domains, a signal peptide sequence, an N-t prosequence, a catalytic domain and a C-t extension[10]. It was assumed that the N-t prosequence of PRO-2127 was composed of 115 amino acid residues (Gln28–Lys142) and had high homology with the N-t prosequences of other subtilisins, especially in the N1 (Ala511-Gly581) and N2 (Gln623-Ala693) motifs that were speculated to be critical for nucleation of the folding process[10, 14]. Therefore, the PRO-2127 N-t prosequence might function as an intramolecular chaperone to guide correct folding, as in other subtilisins[14–15]. Amino acid sequence homology analysis of the catalytic domain in PRO-2127 with other subtilisin-like proteases indicated that the three amino acid residues (Asp185, His244 and Ser425) that likely formed a catalytic triad and the surrounding residues were fully conserved (Figure 1)[16–17]. The C-t extension of the PRO-2127 precursor was characterized by a repeat of the PPC domain (bacterial pre-peptidase C-terminal domain), PPC1+PPC2, which were each composed of 71 amino acid residues.

Analysis of the *Pseudoalteromonas* sp. QI-1 PRO-2127 amino acid sequence composition showed some structural features characteristic of most psychrophilic enzymes. There was a decrease in Arg residues (2.3% versus 3.4%) and the Arg/Arg+Lys ratio (0.37 versus 0.45) relative to the thermostable WF146 protease[18]. In many cases Arg residues stabilized proteins via hydrophobic interaction at the surface. As an ionic positively charged residue, Arg was better adapted to high temperatures than Lys[19].

In cold-adapted proteins, there was a preference for reduction of the Pro content and for an increase in the Gly content[20]. PRO-2127 possessed considerably less Pro residues (4.2% versus 7.0%) and more Gly (11.9% versus 9.1%) residues than the subtilisin-like protease from the hyperthermophilic archaea *Thermococcus stetteri*[21]. Sequence comparisons of PRO-2127 and *Pseudoalteromonas* sp. SM9913 MCP-03 revealed that they contained approximately the same amount of Pro (4.2% versus 4.4%) and Gly residues (11.9% versus 12.1%)[10].

The amino acid composition of PRO-2127 was compared with that of the archetype of the subtilase family, subtilisin Carlsberg (Table 1)[22–23]. The calculated pI of PRO-2127 was lower than that of subtilisin Carlsberg owing to the higher contents of acidic amino acids and lower contents of basic amino acids in PRO-2127. Based on a similar observation for subtilisin S41 from a psychrophile, Davail et al.[24] proposed that a high acidic residue content on a protein surface resulted in increased interaction

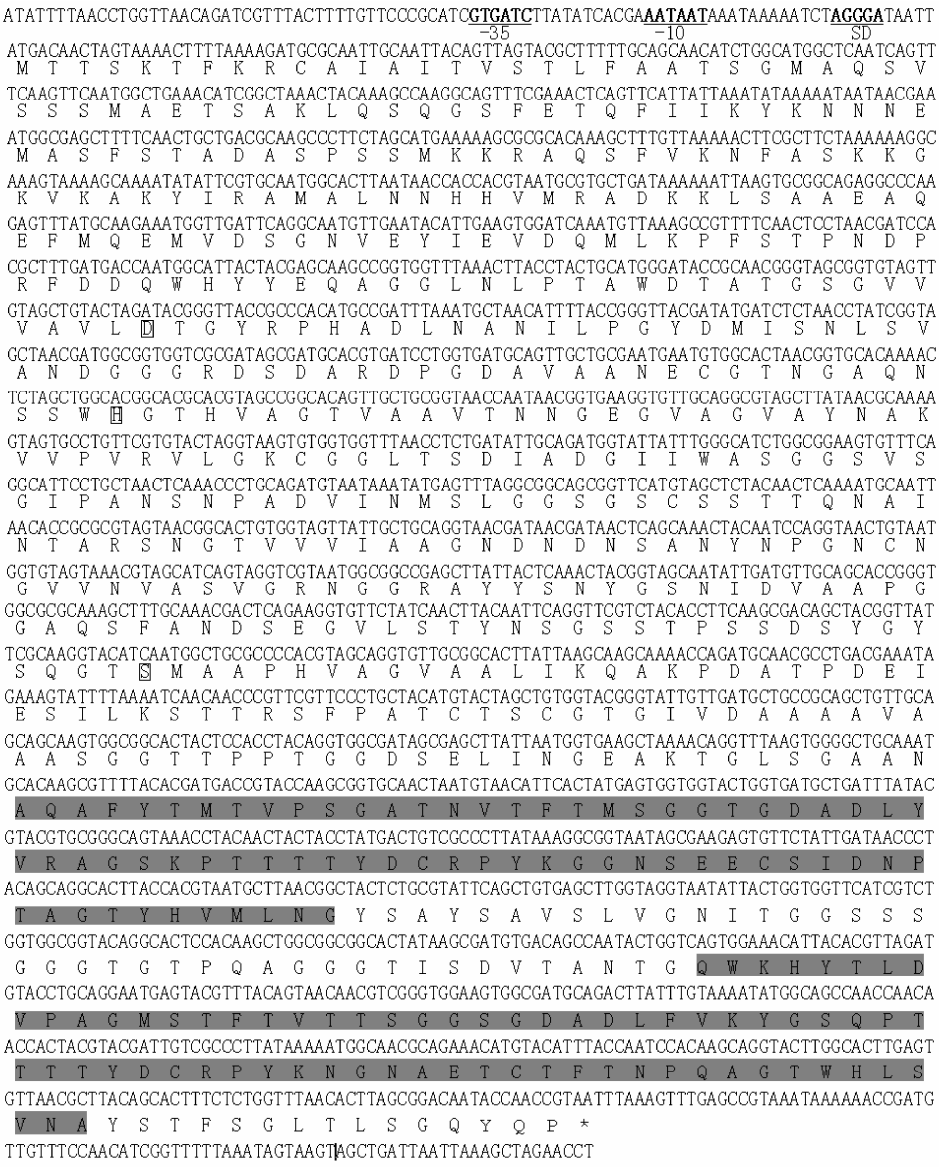

Figure 1 Nucleotide sequence of pro-2127 from *Pseudoalteromonas* sp. QI-1 and its deduced amino acid sequence (GenBank accession No: HM047289). D, H and S might form the catalytic triad of PRO-2127; the amino acids of C-t extension of PRO-2127 in grey indicate the two PPC domains, PPC1 and PPC2, respectively.

between the protein and solvent, which destabilized the protein structure[25]. Another characteristic of PRO-2127 was the fact that its hydrophobic amino acid content was lower than that of subtilisin Carlsberg. When the hydrophobicity was estimated using the Grand Average of Hydropathicity (GRAVY) index obtained from http://www.expasy.ch/sprot/protparam.html[26], the value for PRO-2127 was less than the value for subtilisin Carlsberg (Table 1), which indicated that PRO-2127 was much less hydrophobic than subtilisin Carlsberg. The fact that the thermostability of PRO-2127 was lower than that of subtilisin Carlsberg was consistent with the general finding that hydrophobic interactions were important for protein thermostability. Moreover, the aliphatic index calculated from molar ratios and the relative volumes of the Ala, Val, Ile, and Leu residues determined by Ikai's method[27] were lower for PRO-2127 than subtilisin Carlsberg. Ikai observed a correlation between the aliphatic index and protein thermostability, with higher index values being obtained for proteins with greater thermostability[27].

2.3 Expression of the *Pseudoalteromonas* sp. QI-1 protease gene pro-2127

The formation of clear halos around colonies of pColdIII-pro-2127 on the LB casein plate indicated that there was functional expression of protease. The positive transformant displayed secretive protease activity on the

Table 1 Properties of PRO-2127 and subtilisin Carlsberg determined from a comparison of the deduced amino acid sequences

Enzyme	Amino acid frequencies (%) [a]						pI	GRAVY index[b]	Aliphatic index
	Charged amino acids	Acidic amino acids	Basic amino acids	Polar amino acids	Hydrophobic amino acids	Aromatic amino acids			
Subtilisin Carlsberg	17.4	7.4	8.2	24.3	37.9	7.1	8.73	－0.036	83.69
PRO-2127	15.4	7.9	6.1	32.7	33.7	7.3	5.27	－0.300	60.17

[a] The charged amino acids are arginine, lysine, histidine, aspartic acid, and glutamic acid; the acidic amino acids are aspartic acid and glutamic acid; the basic amino acids are lysine and arginine; the polar amino acids are asparagine, cysteine, glutamine, serine, and threonine; the hydrophobic amino acids are alanine, isoleucine, leucine, phenylalanine, tryptophan, and valine; and the aromatic amino acids are phenylalanine, tryptophan, and tyrosine. [b] GRAVY, Grand Average of Hydropathicity.

plate, while a negative control carrying an empty vector, pColdIII, had no detectable protease activity.

Although protease activity of the expression systems was detected on the casein plate, when the specific activity was measured using the Folin-phenol method, the enzyme activity of recombinant *E. coli* harbouring pColdIII-pro-2127 was fairly low. The specific activity of the expression systems of pColdIII-pro-2127 was 4.44 U/mL, although much of the protease expressed by *E. coli* seemed to exist in the soluble form, indicating that the protease PRO-2127 might be in an improper structure. The total proteins from the fermentation culture medium were analyzed by SDS-PAGE (Figure 2), and a new protein band with a molecular mass of approximate 73kDa was observed, which was consistent with the molecular mass deduced from the nucleotide sequence produced by recombinant *E. coli* harbouring pColdIII- pro-2127.

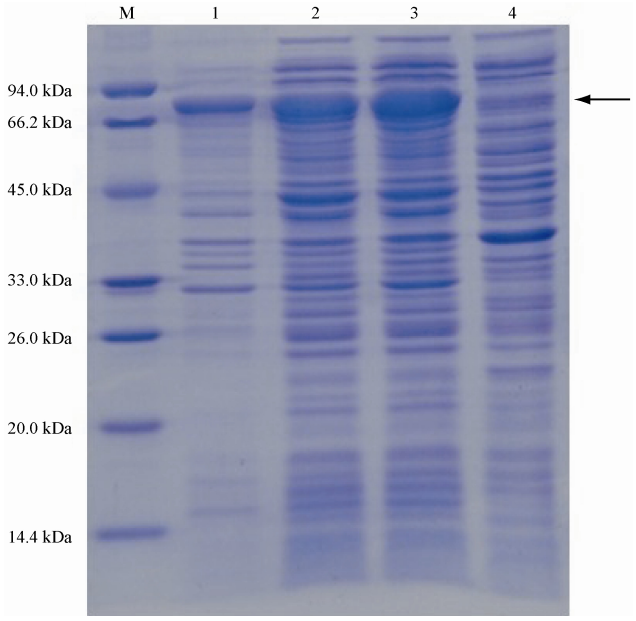

Figure 2 SDS-PAGE analysis of pColdIII-pro-2127 in *E. coli* BL21 (DE3).

M: Standard protein marker, (MP102); Lane 1: The pellet fraction of the recombinant *E. coli* BL21 (DE3) induced cells; Lane 2: The supernatant of the recombinant *E. coli* BL21 (DE3) induced cells; Lane 3: Total proteins of the recombinant *E. coli* BL21 (DE3) induced cells; Lane 4: Total proteins of the recombinant *E. coli* BL21 (DE3) in un-induced cells; The arrow indicates the predicted protein.

3 Discussion

Polar regions are among the most extreme environments on earth, having limited sunlight, extreme temperatures, short growing seasons, sea ice, snow cover, glaciers and permafrost. To adapt to these harsh conditions, bacterial proteases may have special structures and functions that enable protein degradation.

For example, the protease MCP-01 is a cold-adapted enzyme[5] that contains a C-t PKD domain, which enables binding to insoluble proteins to facilitate protein degradation by MCP-01[28]. *Pseudoalteromonas* sp. QI-1 is a psychrotropic bacterium with an optimum temperature for growth of 5℃. The extracellular protease activity of strain Q1 was found to be 106 U/mL using casein as substrate when it was cultured in Zobell 2216E medium[11]. Sequence analysis showed that the gene pro-2127 encodes a multidomain subtilase precursor with four regions, similar to the novel subtilase MCP-03[10].

In the present study, the activity of the recombinant protease produced by the expression systems was relatively low. It was speculated that the protease was not fully and properly processed in *E. coli*[29]. The results demonstrated that PRO-2127 was not properly processed in the *E. coli* recombinants, which might result in the formation of inclusion-bodies in the protein expression systems[30] or an improper structure. However, a study by Yan et al. to investigate deletion mutagenesis showed that the Cterminal PPC domains were unnecessary for enzyme secretion, but had an inhibitory effect on MCP-03 catalytic efficiency and were essential for keeping MCP-03 thermostable[10]. The PPC domains were usually cleaved after secretion, but prior to protease activation. Although their actual function is still not clear, they might aid secretion/localization or inhibit the

protease until needed[31]. Therefore, further research regarding the exact function of the PPC domain is needed.

Peptidase family S8, which is known as the subtilisin family, is the second-largest family of serine proteases. Peptidases in this family are all characterized by an Asp/His/Ser catalytic triad[32]. In subfamily S8A, the active site residues frequently occur in the motifs Asp-Thr/Ser-Gly, His-Gly-Thr-His and Gly-Thr-Ser-Met- Ala-Xaa-Pro. These motifs are also observed in the catalytic domain of protease PRO-2127. Most members of the family are endopeptidases and nonspecific peptidases with a preference to cleave after hydrophobic residues. The tertiary structures of several members of family S8 have now been determined and a typical S8 protein structure usually consists of three layers with a seven-stranded β sheet sandwiched between two layers of helices. However, most of these are predicted proteins derived from corresponding gene or cDNA sequences, the biochemical properties of which are not well known[6, 33]. Accordingly, a strategy should be developed to assist PRO-2127 with processing and folding in *E. coli* and new expression systems in species with efficient protein secretion and correct maturation should be developed to enable over-expression of protease. Additionally, research regarding the purification and biochemical characteristics will be necessary to elucidate the catalytic mechanism and function of each domain of PRO-2127 to enable further use of PRO-2127.

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