New Phylogenetic Molecular Markers in Bacteria of the Genus *Bacillus*: Fibrinolytic Proteases

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Abstract: The fibrinolytic proteases developed by bacteria have become substances of medical interest, as they have been recognized as antithrombotic substances in the blood. It is in this context that a study has been carried out with the aim of studying the fibrinolytic proteases produced by bacteria isolated from food in the Congo. Four strains of bacteria of the genus *Bacillus* isolated from crushed and cooked squash seeds, namely *Bacillus sp strain ASM7*, *Bacillus sp strain CRK*, *Bacillus pumilus strain ASM5*, and *Bacillus subtilis strain ASM1*, identified by their rDNA16S, were tested positive for the production of fibrinolytic proteases. The fibrin box technique was used. The diameters on the fibrin boxes prove a significant production of fibrinolytic proteases. The genes coding for these fibrinolytic proteases were amplified by PCR and 1% Agarose gel electrophoresis shows that the size of the amplicons for the four strains is between 1200-1450bp. The sequences of these coding genes have been for the four strains studied submitted to GenBank and the assigned accession numbers are respectively: *Bacillus sp strain ASM7 MT743004*, *Bacillus sp strain CRK MT743005*, *Bacillus pumilus strain ASM5 MT743006*, and *Bacillus subtilis strain ASM1 MT743007*. These genes show a high degree of similarity of almost 99.50% with their counterparts in the databases, are all coding and show some observable differences. The translation of these genes in coherent reading frames confirms the amino acids already known in the active sites relating to their fibrinolytic role. The fibrinolytic protease CFE1 (Id=QNJ60181) is for *Bacillus sp strain ASM7*, the fibrinolytic protease CFE2 (Id=QNJ60182) is for *Bacillus sp strain CRK*, the fibrinolytic protease CFE3 (Id=QNJ60183) is for *Bacillus pumilus strain ASM5*, and the fibrinolytic protease CFE4 (Id=QNJ60184) is for *Bacillus subtilis strain ASM1*. All these fibrinolytic proteases show a strong similarity (99.51-99.76%) with the *Bacillus AprX* serine protease, reference sequence. The phylogenetic inference test based on these fibrinolytic proteases shows that these proteases form a highly conserved characteristic group in bacteria of the genus *Bacillus*. This allowed us to retain these fibrinolytic proteases as an important phylogenetic molecular marker.

Keywords: Fibrinolytic Proteases, Sequencing, Phylogenetic Molecular Markers, *Bacillus*

1. Introduction

Proteolytic enzymes are ubiquitous in all living organisms and are essential for cell growth and differentiation. Extracellular proteases have commercial value and have multiple applications in different industrial sectors. Although there are many microbial sources available to produce proteases. But among all the contenders for the production of microorganisms, the genus Bacillus is unanimously recognised as a potential commercial producer [1]. Fibrin, responsible for
blood coagulation, is activated after fibrinogen degradation by thrombin, and can be lysed (fibrinolysis) by plasmin [2, 3]. Normally, fibrin formation and fibrinolysis are kept in equilibrium [4, 5], however, an unbalanced situation occurs when fibrin cannot be hydrolyzed and can cause cardiovascular diseases, such as thrombosis, due to the accumulation of fibrin in the blood vessels [6].

Intravenous administration of urokinase and streptokinase has been widely used for thrombosis, but these enzymes have low fibrin specificity and are expensive. T-PA (tissue plasminogen activator) has been developed for the treatment of thrombosis because of its efficacy and greater affinity for fibrin [7, 5]. However, to prevent thrombosis and other related diseases, daily consumption of fibrinolytic enzymes from dietary sources is increasingly recommended. Many fermented foods offer such fibrinolytic activity, including examples such as the Korean Chungkook-Jang food [6, 4], the Chinese Duchi [8], Japanese natto [9], Japanese shiokara [10], Korean Doen-Jang [11], Asian fermented shrimp paste [12] and Indonesian tempeh [13], fermented cassava leaves: Ntobambodi in Congo [14].

It has been confirmed that nattokinase (NK) can directly lyse thrombi in vivo and that its oral administration can improve the activated plasma fibrinolytic effects as well as the production of t-PA [15].

The presence of strains producing fibrinolytic proteases in squash seeds crushed, packaged on leaves and cooked for consumption was first proven in Brazzaville in the study by [16] These strains of bacteria of the genus Bacillus were identified at the molecular level by their 16S rDNA and their phylogenetic classification based on this gene was tested [16].

In this work, four (4) strains of bacteria of the Bacillus genus already identified by their 16S rDNA were explored for their production of fibrinolytic proteases. These strains are respectively: Bacillus sp strain ASM7, Bacillus sp strain CRK, Bacillus pumilus strain ASM5 MK207435.1, Bacillus subtilis strain ASM1 MK193815.1. PCR amplification and sequencing of the genes coding for these enzymes have been carried out. Bioinformatics analysis made it possible to assess the degree of similarity between the different genes and the translation of these genes was carried out by SMS-ORF Finder-NCBI. The fibrinolytic protease sequences obtained were used not only to elucidate some properties, but also for a phylogenetic classification test to verify their role as a reference phylogenetic molecular marker in bacteria of the Bacillus genus.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Four (4) Bacillus strains namely Bacillus sp strain ASM7, Bacillus sp strain CRK, Bacillus pumilus strain ASM5, Bacillus subtilis strain ASM1, isolated from squash.

The study of the growth of the different strains was carried out in 250 ml Erlenmeyers containing 100 ml of medium (LB). A colony was inoculated. Incubation was carried out at 37°C for 48 hours. For each strain, 3 ml of the bacterial suspension are taken under sterile conditions. 2 ml will be used to measure the optical density of the cell culture at 600 nm using a zuzi type spectrophotometer (Model 4211/50) [17, 14, 18].

2.2. Fibrinolytic Proteases ASSAY

Fibrinolytic activity was determined by the Fibrin Box method with modification [19]. The supernatant of the appropriate growth stage of Bacillus cells was used. Succinctly; 25 ml of a 0.5% fibrin solution was mixed with 25 ml of 1% agarose gel in a Petri dish and placed for 30 minutes at room temperature. Wells were made on the gel contained in the fibrin plate. 20 microliters of sample were added to each well and the plate was incubated at 37°C for 16 hours. The fibrinolytic activity was measured by the lytic surface through the diameter of the clear zone. The diameter of the clear zone was measured and used to assess enzyme production [14, 20, 18].

2.3. PCR Amplification of Genes Encoding Fibrinolytic Proteases

2.3.1. Primers Design

We have consulted the primers in the literature on fibrinolytic enzymes, many primers are used. In our previous study [21] an optimization on the primers used and the hybridization temperature for the amplification of genes coding for enzymes or fibrinolytic proteases was made, the results showed a variability at the level of the different strains. In fact, the fibrinolytic enzymes have a zone at the N-terminal end whose amino acids are highly conserved, this zone would be ideal for the choice of primers. This shows that degenerated primers are used to amplify the genes coding for fibrinolytic proteases in bacteria of the Bacillus genus, which illustrates the variability due to genetic mutations in this zone. However, in order to have a complete gene, it is necessary to take one of the F primers upstream. Given the diversity of genes encoding fibrinolytic enzymes, an in silico analysis was carried out in order to select a few primers. Data from the NCBI (National Center of Biotechnology Information) combined with those obtained with BLASTn shed light on the choice of primers. However, the test carried out with the Bacillus primers of [22], optimising the Hybridization temperature gave interesting results. These primers selected were: F5'-CATATGTGTTGGTGCTACCTATAGG-3' and R5'-GGATCCTTATTTGCGGAGCGA-3'. These primers were synthesized by the Society Macron gene France.

2.3.2. Reaction mixture and PCR Conditions

PCR was carried out according to the literature [22], but modified. The mixture was prepared in a total volume of 50 µl containing 31µL sterile distilled water, 20 ng DNA (2 µl), 200 mmol/L of each primer (2 µl), 0.2 mmol/L Dntp (1 µl), 1.5 mmol/L MgCl2 (1 µl), 10µL of one Taq polymerase enzyme buffer and 2.5 U Taq (1 µl).

A first denaturation at 95°C for 5 min. 35 cycles each comprising: denaturation at 95°C for 30 sec., hybridization at
65°C for 30 sec. and elongation at 72°C for one minute. And finally a final synthesis at 72°C for 7 minutes.

2.4. Agarose Gel Electrophoresis of PCR Products

2.4.1. Gel preparation and Sample Processing

(i) Gel Preparation

In a 250 ml Erlenmeyer flask, we suspended 1g of agarose in 100ml of TBE buffer (Tris 89mM, Boric Acid 89 mM, EDTA 2.5 mM and pH8.3), then heated until the agarose was completely dissolved, then allowed to cool to a temperature of 50 to 60°C. After placing the comb in the mould, the gel was poured evenly into the mould and we waited until the gel polymerized. The comb has been previously filled with TBE buffer solution so that the gel is completely immersed in the buffer. DNA samples are deposited in the wells of agarose gel immersed in a TBE buffer (pH) and allowed to migrate under the effect of an electric field (DNA is a polyanion). The DNA is visualized by adding midori (a DNA dye which fluoresces by intercalating between the DNA bases). The speed depends on the size of the DNA molecule. A size marker is used to determine the size of the fragments.

(ii) Sample Processing

The DNA samples were processed before they were deposited in the wells. The DNA was denatured and concentrated (using 5microliters of DNA, 0.5 microliters of Midori and one microliter of Buffer Dye). Midori is an intercalating agent, it intercalates between the nucleotides and allows the nucleotides to fluoresce and the Buffer Dye (charge buffer), allows the DNA to be deposited and maintained at the bottom of the wells. The 6.5 ml mixture is deposited in each well of the Gel.

2.4.2. Migration in TBE Buffer and gel Visualization

The gel was subjected to a voltage of 100V for one hour, and we allowed it to migrate so that the blue line reached the end of the gel, then the power supply was interrupted. The gel was visualised under a UV lamp, by fluorescence.

2.5. Sequencing of PCR Products

The products of PCR amplification of the genes encoding fibrinolytic enzymes were purified using the NucleoFast 96 PCR plate (Macherey-Nagel EURL, France) and sequenced using BigDye terminator chemistry on an ABI3730 sequencer (Applied Biosystems, Foster City, California, USA). Sequencing was performed by electrophoresis on a 3730xl-Titania DNA analyser (Applied Biosystems) using the same primers. [16].

3. Results

3.1. Fibrinolytic Enzymes Production

The Figure1 below shows the fibrinolytic activity of the four strains isolated from squashes. You can see the difference in their diameters. All the studied strains produce fibrinolytic enzyme. If the halo diameter is more important in the strains Bacillus subtilis ASM1, in the Bacillus subtilis sp. ASM7 and in the Bacillus pumilus ASM5, the halo is smaller than in the first, the smaller diameter is in the Bacillus sp CRK.

![Figure 1. Halos representing the fibrinolytic activity of the four strains.](image)

ASM5=Bacillus pumilus, ASM1=Bacillus subtilis, B. SP=Bacillus sp ASM7, B. CRK=Bacillus sp CRK

3.2. PCR Amplification of Encoding Fibrinolytic Enzymes Genes

Figure 2 shows 1% agarose gel electrophoresis of PCR products of genes encoding fibrinolytic enzymes in isolated strains of squashes, profile displays bands of about 1200 bp in all for strains.
Only four (4) strains were positive. The amplicons obtained after amplification of the 4 strains each showed a clear band of approximately 1200 bp in size. The other strains of the negative controls obviously did not present bands.

### 3.3. Bioinformatic Analysis of the Nucleic Sequences

#### 3.3.1. Multiple Alignment of Nucleic Sequences Encoding for the Fibrinolytic Proteases

Figure 3 on the multiple alignment of the different nucleotide sequences of the genes encoding the fibrinolytic enzymes of strains of bacteria of the genus *Bacillus* isolated from squashes, shows that there are areas where the nucleotides have been strongly conserved. However, some areas are less similar. Indels (insertion-deletions) can be observed in the areas of the following nucleotide positions: 118-119, where two types of sequences can be observed, one with two indels and the other with: AG. Nucleotide positions 167-171 with 5 indels on the one hand and TCTTT on the other hand; Nucleotide positions 212-218 with 7 indels on the one hand and on the other hand TGGCTGG...
3.3.2. Protein Sequence Analysis

From the nucleotide sequences of the genes coding for fibrinolytic enzymes in pumpkin strains, we did the translation and the protein sequences obtained were analyzed by a Blastp.

Table 1 shows that all these proteins act as acid.

Table 1. The different properties of the four fibrinolytic proteases elaborated from bacteria isolated from squashes.

| Fibrinolytic Proteases | Protein GRAVY | Isoelectric point | Molecular weight | Amino acid residues | GenBank accession numbers of related genes | Protein ID |
|------------------------|---------------|-------------------|------------------|--------------------|-------------------------------------------|------------|
| CFE1                   | -0.297        | pH 4.48           | 38.94 kDa        | 366                | MT743004                                  | QNJ60181.1 |
| CFE2                   | -0.342        | pH 4.46           | 40.47 kDa        | 380                | MT743005                                  | QNJ60182.1 |
| CFE3                   | -0.291        | pH 4.57           | 26.16 kDa        | 244                | MT743006                                  | QNJ60183.1 |
| CFE4                   | -0.361        | pH 4.43           | 40.38 kDa        | 379                | MT743007                                  | QNJ60184.1 |

Table 2 shows the similarity levels of each protein to the homologous sequences in the NR library using Blastp-NCBI. The level of identity for all proteins is greater than 99.50%, allowing them to be identified as belonging to the AprX [Bacillus] family of serine proteases. These form a very broad group among fibrinolytic enzymes.

Table 2. Results of bioinformatics analyses on BLASTp of the genes encoding the fibrinolytic enzymes of the four (4) squash strains.

| Strains         | Max score | Total score | Query cover | E. value | Percentage of similarity | Type of enzyme | Accession Numbers of equivalent strains in GenBank |
|-----------------|-----------|-------------|-------------|----------|-------------------------|----------------|--------------------------------------------------|
| Bacillus subtilis ASM1 | 851       | 851         | 98%         | 0.0      | 99.51%                  | Multispecies: serine protease AprX [Bacillus] | WP_041850809.1 |
| Bacillus pumilus ASM3 | 568       | 568         | 100%        | 0.0      | 99.63%                  | serine protease AprX [Bacillus]           | WP_024572446.1 |
| Bacillus sp ASM7 | 844       | 844         | 100%        | 0.0      | 99.75%                  | serine protease AprX [Bacillus]           | WP_041336030.1 |
| Bacillus sp CRK  | 848       | 848         | 100%        | 0.0      | 99.76%                  | serine protease AprX [Bacillus]           | WP_041336030.1 |

3.3.3. Sequences Alignment of Fibrinolytic Proteases

Figure 4 show the amino acid alignment,
Figure 4. Alignment of amino acid sequences of fibrinolytic enzymes in four (4) Bacillus strains isolated from squashes.
These genes have been structured in three (3) parts: a first part called a signal peptide whose number of amino acid residues varies from one strain to another. In Bacillus sp CRK and ASM5 strains, the signal peptide contains 24 amino acids, 26 in B.sp ASM7 and finally 33 amino acid residues for Bacillus subtilis ASM1, a second part called pro-peptide contains about 74 amino acids and finally a third part called mature peptide which contains 310 amino acid residues.

3.3.4. Phylogenetic Classification Based on Protein Sequences

Figure 5 shows the phylogenetic relationship between the fibrinolytic enzymes produced by strains of the genus Bacillus isolated from red-coloured squash and some related homologues. The arrangement of the branches clearly shows that all four enzymes have the same common ancestor and are grouped together thus sharing the same node.

The protein sequences of the fibrinolytic enzymes in strains isolated from squash were aligned Figure 4. These sequences were compared with those of other serine proteases with similarity rates (Table 2) ranging from 99.51% to 99.76%. Analysis of the pre-peptide part of these four (4) enzymes clearly shows a difference. For the CRK and ASM5 strains, the first amino acid is a leucine, whereas in B.Sp it starts with a threonine and in ASM1 it is glutamine which is the first amino acid, whereas in the two (2) homologues the pre-peptide starts with a methionine [29, 30]. Considering the mature peptide, the first 11 amino acids of the four (4) fibrinolytic enzymes are respectively LLDTATEASQI. This part is very similar to the two (2) homologues of the same gene type (serine protease AprX Bacillus) which are reference genes [30] but differs from those of subtilisin DFE found in Bacillus amyloliquefaciens CD-4 [31], & and subtilisin BSF1 found in Bacillus subtilis A26 [32] where the first 8 amino acids in subtilisin E (AQSPVYGVSQIKA) Ala1, Gin2, Ser3, val4, Pro5, Tyr6, Gly7, Val8, have been replaced by Leu1, Leu2, Asp3, Thr4, Ala5, Thr6, Glu7, Ala8 in the fibrinolytic enzymes produced by Bacillus bacteria isolated from squashes. In addition, the
catalytic triad consisting of Ser, His and Asp of the four (4) enzymes is preserved. These results are similar to those published by [33, 32]. The four fibrinolytic proteases of the bacterial strains isolated from squashes were recorded in the databases and the Id are recorded in Table 1. All these proteases have very similar characteristics.

Analysis of phylogenetic inference based on fibrinolytic protease sequences indicates that two proteases developed by *Bacillus subtilis* MN41 and *Bacillus licheniformis* MN74 homologues [21] are also in the same group (serine protease AprX *Bacillus*). These two proteases, whose N-terminal end is different from the four proteases developed by the *Bacillus* isolated from squashes in this study, are all in the same phylogeny. All of this supports the hypothesis that since LUCA and over the course of evolution all these fibrinolytic proteases have evolved in the same family, the mutations have not affected the active site whose catalytic triad is always Ser-His-Asp.

All the fibrinolytic proteases CFE1 (Id=QNJ60181) for *Bacillus* sp strain ASM7, the CFE2 (Id=QNJ60182) for *Bacillus* sp strain CRK, CFE3 (Id=QNJ60183) for *Bacillus pumilus* strain ASM5, CFE4 (Id=QNJ60184) for *Bacillus subtilis* strain ASM1 are AprX serine protease. All of them display closely related biochemical properties in concordance with their roles.

### 5. Conclusion

At the end of this study, it should be said that the four (4) *Bacillus* strains isolated from squashes are very promising. The fibrinolytic activity assay has shown that these strains produce fibrinolytic enzymes. Furthermore, amplification by PCR and bioinformatics analysis of these genes has enabled them to be characterized. The four (4) enzymes belong to the family of serine proteases of the Apr type. These four fibrinolytic proteases have been registered in the databases and have an identifier for each one, they display closely related biochemical properties in concordance with their roles. This study has confirmed the use of fibrinolytic enzymes in *Bacillus strains* as phylogenetic molecular markers.

### Conflict of Interest

Authors declare no conflict of interest.

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