AMYLOLYSIN, A LANTIBIOTIC PRODUCED BY *BACILLUS AMYLOLIQUEFACIENS* GA1.

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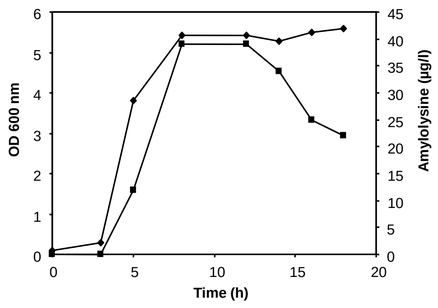
INTRODUCTION :

Genome analysis of *Bacillus amyloliquefaciens* GA1 highlights a high potential for antibiotic biosynthesis. Among them, amylolysin, a peptide antibiotic features activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*. Several experimental evidences suggest that this peptide belong to the group of class I bacteriocin: the so-called lantibiotics (lanthionine-containing peptide antibiotics) are post-translationaly modified peptides interacting with the peptidoglycan precursor LipidII, resulting in the inhibition of peptidoglycan biosynthesis.

1. Amylolysin production and purification.

For amylolysin production, strain GA1 was incubated at 37 °C for 8 h in LB medium in a 80 litres bioreactor. Purification was performed as follows : (i) concentration and semi-purification of the culture supernatant by hydrophobic chromatography on XAD16 resin, (ii) final purification by RP-HPLC using C18 column.

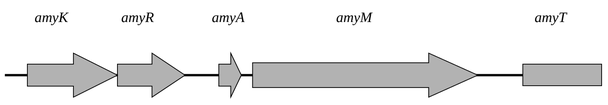


Amylolysin was produced during the early exponential growth phase (♦). After 8h of culture, the concentration of amylolysin in the supernatant was 40µg/L (▪).

2. Gene cluster involved in amylolysin biosynthesis

Part of amylolysin biosynthetic gene cluster was identified and sequenced. Several lantibiotic specific motifs were found in structural (*amyA*) and modification (*amyM*) genes.

A



B

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  1 TTGGGATTAA CGGAAGAGTT AACCCGATTT TTTTTATAAC CTAAAATTCG GGAACGCGCA AAAAAAAAAG 70
 71 CGCTTTTCCG AATATGACTT TTCGGTGCCT TTGGGATTAA CGGAAGAGTT AACCCGATTT TTTTTTATAA 140
                                                                        -35
141 CTAAAATTCG ATAGGTAAAA GATCGTTTAT TAGGTTTATA GAAATATATG GATCAAAATC CAATATATTT 210
                                                  -10
211 CAAAAATCAA GGGGAGGAAT ATTAATGAAT GAGAAAATGT ATCGTTTTGC CGGTGATTTA AGAGAAGAAC 280
              RBS        M  N  E  K  M  Y  R  F  A  G  D  L  R  E  E  L
281 TTGAGGAAAT TTCTTTAAGT GAATTCAGCG GAGGAGGAGG CGCAGAACAG CGAGGGATTT CTCAAGGAAA 350
     E  E  I  S  L  S  E  F  S  G  G  G  G  A  E  Q  R  G  I  S  Q  G  N
351 TGATGGTAAG CTTTGTACGT TAACTTGGGA ATGCGGTCTT TGTCCTACTC ACACTTGCT GGTGTTAAAAC 420
    D  G  K  L  C  T  L  T  W  E  C  G  L  C  P  T  H  T  C  W  C  *
421 AACCCCAAATA AGAAACCCAT TTCCATGCCC CCCCCCCCCC CCCCGTCTCC CTCTTTTTT TCCCGGAGATG 490
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A) Partial amylolysin gene cluster. Regulation (*amyR* and *amyK*), structure (*amyA*), modification (*amyM*) and transport (*amyT*) genes have been found in *B. amyloliquefaciens* GA1.

B) Amino acid sequence of amyA prepropetide (60 aa) contains the lipidII binding motif CTLTXEC. This highly conserved motif is strictly conserved in Type-B lantibiotic.

C) The conserved amino acids involved in the formation of the lanthionine ring were identified in the N-terminal sequence of *lanM*.

C

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                  N1              N2              N3                  N4
AmyM (164)-LAGETPKEK-(72)-GMGDSHSDGRK-(10)-LIYKPR-(56)-FYQRTGAQIGLLYALKAVDFHSENLIANGSYPVLIDLESL-
LasM ( 73)-MLGNNSSER-(70)-GKGDTHSGKSV-(15)-LLYKPR-(53)-YYYRSGCLLGLFWILGTTDIHSENIITNAGYPIVIDIETL-
MutM  (56)-LIGLTSEEK-(70)-VVGDMHNELAV-( 9)-LFYKPH-(53)-YFSRMGGLIAIAYSLNMTDLHFENIISDGEYPVILDMETI-
MrsM (180)-LRGETSEER-(73)-GVSDTHNKGKT-(10)-IVYKPR-(54)-FYWRIGSYLAILYAMNAVDFHMQNLIADGEYPILVDLESL-
CylM (169)-LKGNDSSKR-(70)-SQGDSHSRGKT-(11)-IVYKPK-(52)-YYERYGKLIGIAFLFNVTDLHYENIIAHGEYPVIIDNETF-
LctM  (56)-LMGNTPEER-(70)-IKGDLHNGKAV-( 9)-LIYKPK-(62)-YYRKIGVLLSVAYTLNLTDLHFENVISQGENPCIIDLETM-
           : *  ..::        .* *.   .      :.***       :: : *  :.: : :. .*:* :*:*:..  * ::* *::
                N5                  N6
AmyM (24)-SVRALGLLP-(106)-RFISKPTIKYSALLELSFHP-(364)
LasM (19)-SVLSSCLLP-( 97)-RQLLRDTQVYADFVHALQLP-(503)
MutM (25)-SVLNTGLLP-(103)-RVIFRQTAHYSLMLEVLNSP-(487)
MrsM (22)-SVLRIGLLP-(102)-RQILRGTSRYANLLKISLHP-(523)
CylM (23)-SIMVTGLVP-(105)-RNVIRPTQRYADMLEFSYHP-(468)
LctM (24)-SVVSTGMLP-(103)-RILFRNTMEYSVLLNAAKSP-(503)
          *::   ::*       * : : *  *: ::.    *
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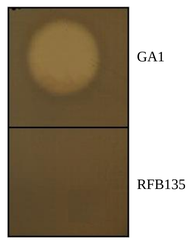
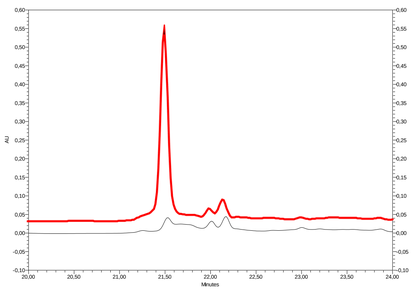
3. Disruption of *amyA*

The structural *amyA* gene, which encode the amylolysin prepropetide was disrupted in *B. amyloliquefaciens* GA1 to yield RFB135. Antibacterial activity of both strains was measured against *Listeria ivanovii*.

A



B

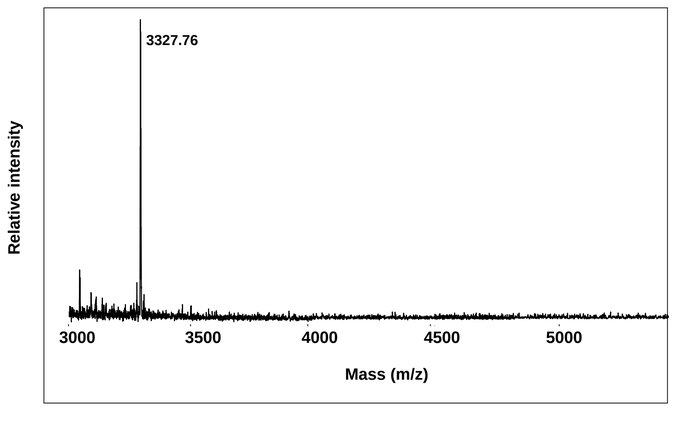


A)Antibiotic activity of XAD16-purified supernatant against *L. ivanovii*.

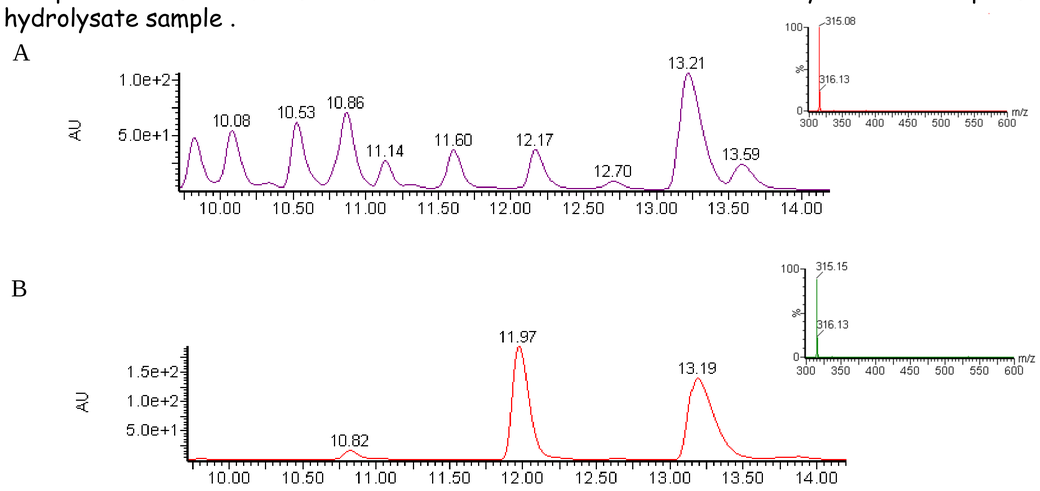
B) HPLC chromatogramm of XAD16-purified supernatant. Red: GA1; Black : RFB135

4.A Structural characterization

Purified amylolysin was analysed by mass spectrometry (MALDI-TOF).



The presence of the modified amino acid lanthionine has been shown by LC-MS on a purified amylolysin hydrolysate sample .



A: LC-MS spectrum of purified amylolysin hydrolysate sample. The mass spectrum corresponds to the 13,21 min peak .
B: : LC-MS spectrum of lanthionine standard sample. The mass spectrum corresponds to the 13,19 min peak .

Discussion: The compound eluted at 13,19 min with a mass of 315 Da in LC-MS analysis of amylolysin corresponds to the modified amino acid lanthionine.

4.B Physico-chemical characterization

Effect of various factor on amylolysin

Factor	Remaining activity (%)	Factor	Remaining activity (%)
Treatment with enzyme		Heat[b]	
Control	100	60 min at 25°C	100
Proteinase K	0	60 min at 55°C	98
Pronase	20	60 min at 65°C	99
		60 min at 75°C	99
pH[a]		60 min at 100°C	93
2	89		
3	96		
4	96		
5	99		
6	96		
7	100		
8	97		
9	93		

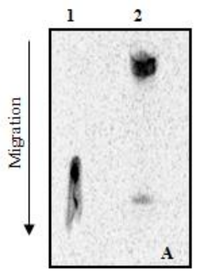
[a] values were normalysed as a pourcentage of the value obtained at physiological pH.
[b] values were normalysed as a pourcentage of the value obtained at 25 °C

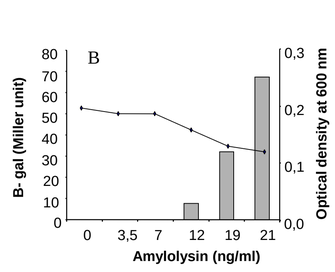
Effects of protease, pH and temperature on amylolysin activity have been determined.

5.Biological mode of action

Interaction with peptidoglycan precursor Lipid II. Bactericidal property of amylolysin and measurement of transmenbrane electrical potential (ΔΨ) have been determined by chromatographic fluorimetric techniques and a reporter gene assay.

I: Interaction with Lipid II



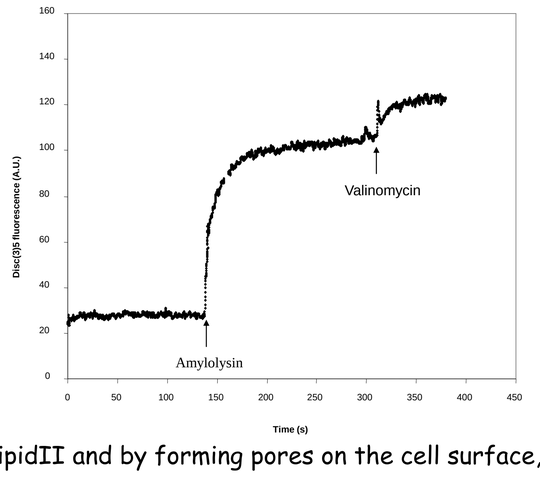


IA: TLC experiment. Delay of lipidid II migration in the presence of amylolysin highlights a direct interaction between the two molecules.

IB: Lipid II cycle interfering antibiotics are sensed by the LiaRS two-component regulatory system. We used a reporter strain (BFS2470) that allows to quantitatively determine lipid II interacting antibiotics via LiaRS system that activates the p*lial-lacZ* reporter fusion in the presence of interfering antibiotics. Measurment of a B-galactosidase activity in the presence of amylolysin confirms the interaction with lipidII.

II: The transmenbrane electrical potential (ΔΨ).

The ΔΨ was determined by fluorescence measurement of the potential sensitive fluorescent probe 3,3'-dipropylthiacarbocyanine [Disc(3)-5]. Upon addition of Disc(3)-5 to the cell suspension, a quenching of fluorescence was observed following the adsorption of the fluorescent probe to the cell membrane due to the ΔΨ. After addition of amylolysin, an increase of fluorescence could be obesrved following the dissipation of the ΔΨ. This membrane depolarisation was due to the pore forming action of the amylolysin on *B. megaterium*. A small dissipasion was observed after addition of the K^+ionophore valinomycin.



Discussion : Amylolysin inhibits cell growth by targetting LipidII and by forming pores on the cell surface, leading to a cytoplasmic leaking.

Conclusions : We demonstrated that amylolysin, a peptide produced by *B . amyloliquefaciens* GA1 inhibited growth of Gram-positive bacteria. We also demonstrated that the gene cluster involved in amylolysin biosynthesis contains motifs strictly conserved in lantibiotics. Interaction of antibitic peptide with LipidII to form pores was also demonstrated. There is ample evidence that amylolysin is the first described lantibiotic produced by a *B. amyloliquefaciens* strain.

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