



## DETECTION OF SYNTHETASES GENES INVOLVED IN NON RIBOSOMAL LIPOPEPTIDES (NRLPS) BIOSYNTHESIS FROM *BACILLUS* SPECIES BY BIOINFORMATICS AND PCR DEGENERATED PRIMERS AND ESTIMATION OF THEIR PRODUCTION

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### ABSTRACT

Non Ribosomal Lipopeptides NRLPs produced by *Bacillus* spp. were firstly detected by bioinformatics in 54 sequenced genomes available from NCBI, which revealed the presence of the four lipopeptides families; surfactin, fengycin/plipastatin, iturin and kurstakin in 19 different strain, fusaricidin from (*Paenibacillus*) *B. polymyxa* SC2 and unknown PKS-NRPS lipopeptide from *B. safensis* FO-36b . Also the absence of NRLPs synthetases genes were detected in 14 different strain, while the 21 other strains harbor different NRP synthetases genes. Secondly, we used the degenerated primers approach to detect the synthetases genes involved in lipopeptides biosynthesis in 9 *Bacillus* strains. This technique detected non ribosomal synthetases genes for kurstakin in *B. thuringiensis israelienne* NRRL HD-522, surfactin and plipastatin synthetases genes in *B. subtilis* ATCC 21332 and unknown PKS-NRPS synthetases genes in (*Lysinibacillus*) *B. sphearicus* 23268 T. Thirdly, production levels were estimated for these 9 strains by HPLC. The production of *B. amyloliquefaciens* FZB42 were 294, 62 and 210 mg.L<sup>-1</sup> surfactin, fengycin and mycosubtilin, respectively. *B. amyloliquefaciens* S499 were 872, 103 and 103 mg.L<sup>-1</sup> surfactin, fengycin and bacillomycin respectively. *B. subtilis* ATCC 21332 were 1060 and 226 mg.L<sup>-1</sup> of surfactin and plipastatin respectively. While, *B. subtilis* 168 has no production. The production of *B. licheniformis* ATCC 14580 strain was 358 mg.L<sup>-1</sup> of lichenysin, and *B. pumilus* was produced 642 mg.L<sup>-1</sup> of pumilacidin. Also, the strains of *B. thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522 were produced 102 and 62 mg.g<sup>-1</sup> cells respectively of kurstakin.

**KEY WORDS:** Lipopeptides families, *Bacillus* spp., degenerated primers, NRLPs, lipopeptides production.



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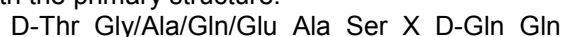
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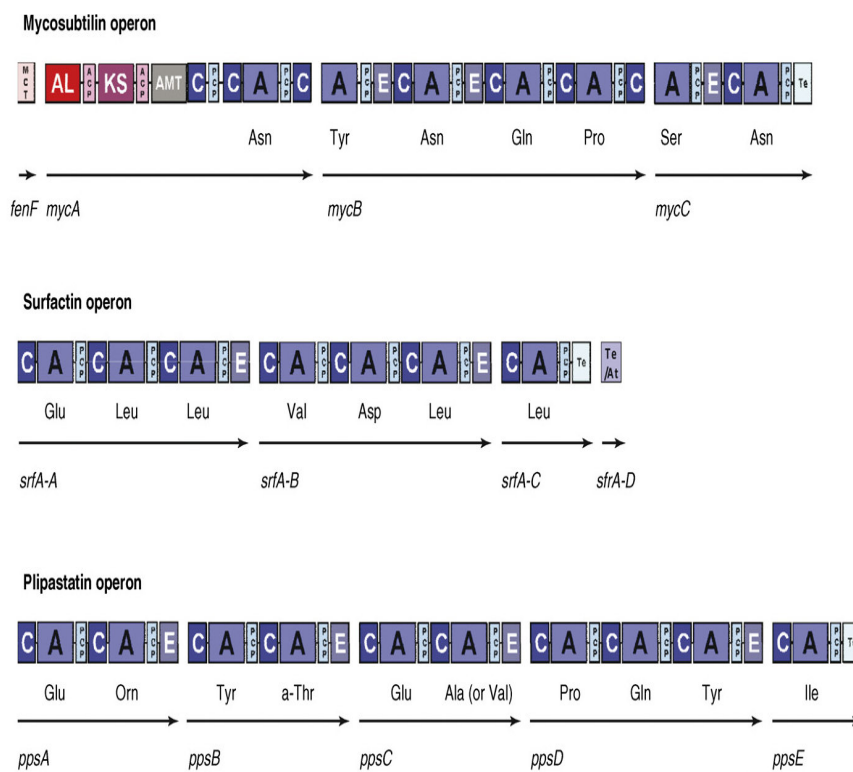
## INTRODUCTION

*Bacillus* species produces a broad spectrum lipopeptides known as biosurfactants and has antifungal, antibacterial and antiviral activities. Firstly, three families of lipopeptides were identified in *Bacillus* species; surfactins, iturines, and fengycins or plipastatins families, while kurstakin family was detected recently in *Bacillus thuringiensis subsp. kurstaki* strain HD-1<sup>1</sup>. surfactin was discovered from the culture broth of *Bacillus subtilis*<sup>2</sup>. The surfactin operon consists of four openreading frames (ORFs) coding for four enzymatic subunits; SrfA, SrfB, SrfC and SrfD. Surfactin family harbor also four openreading frames (ORFs) coding for lichenysin synthetases; lchA, lchB, lchC and lchD, respectively and this heptapeptide is linked to a  $\beta$ -hydroxyl fatty acid chain (C12- C16).<sup>3,4</sup> The second lipopeptide family Fengycin and plipastatin which their discovery In 1986 was concomitant by German<sup>5</sup> and Japanese teams<sup>6</sup>. There was only a small structural difference between these two compounds when it's Tyr3 and Tyr9 residues are present in the L- and D-form and till now a doubt still exists about their structure and their biological activities. Plipastatin is a lipodecapeptide which the operon harbor five ORFs (ppsA, ppsB, ppsC, ppsD and ppsE or fenC, fenD, fenE, fenA and fenB)<sup>7,8</sup> and are linked to a  $\beta$ -hydroxyl fatty acid chain (C14-C18). Iturin family is different than both surfactin and plipastatin or fengycin families; it is synthesized by a hybrid of PKS-NRPS<sup>9,10</sup> and their is 38-40 kb in size

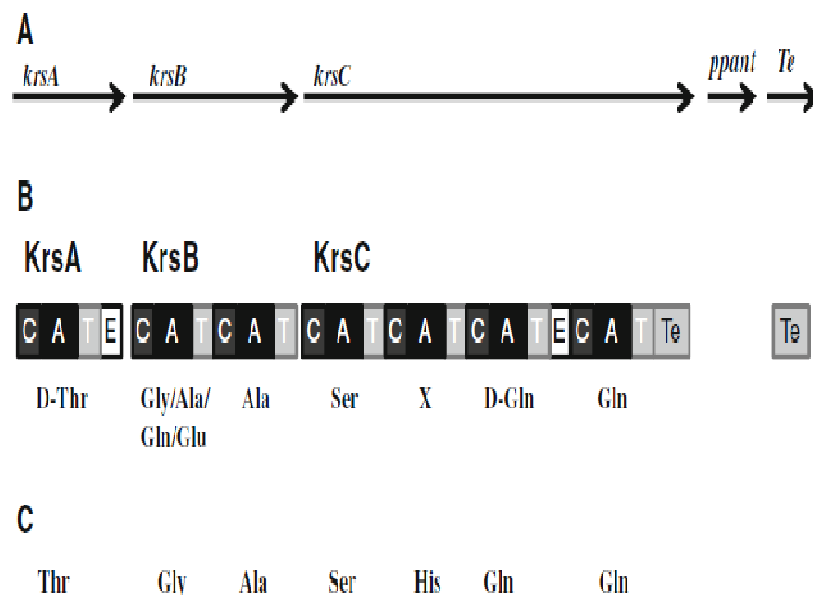
harbor four ORFs; fenF, mycA, mycB and mycC for mycosubtilin or ituD, ituA, ituB and ituC for iturin<sup>11</sup> (Fig 1). This heptapeptide is linked to a  $\beta$ -amino fatty acid chain (C14-C17). The new lipopeptide family kurstakin operon contains three genes (krsA, krsB, and krsC) which encode three large multifunctional proteins (KrsA, KrsB, and KrsC) constituting the complete synthetase with the primary structure:



as mentioned<sup>12,13,14</sup> (Fig 2). The study of the genetic potential considered useful and important in the detection of lipopeptides as in the case of *B. amyloliquefaciens* S499 which was characterised for its ability to produce iturin and surfactin only and later shown to be a producer for another lipopeptide which is fengycin<sup>15,16</sup>. We firstly analyzed 54 *Bacillus* genomes by bioinformatics tools to get an overview about the Non Ribosomal Lipopeptides (NRLPs) potentially produced by these *Bacillus* strains and secondly used the approach of degenerated primers<sup>17</sup> to detect the presence of lipopeptide genes by PCR with degenerate primers based on intraoperon DNA sequence alignment of both adenylation and thiolation domains for lipopeptide biosynthesis enzymes in each family. The aim of this work is to detect synthetases genes involved in NRLPs biosynthesis in *Bacillus* species by bioinformatics and degenerated primers and then determine their production levels after fermentation process by HPLC.



**Figure 1**  
**Operons of mycosubtilin, surfactin and plipastatin synthetases<sup>18</sup>**



**Figure 2**  
*Kurstakin operon synthetases genes*<sup>19</sup>

## MATERIALS AND METHODS

### Microbial strains and culture media

All bacterial strains were grown aerobically in Luria-Bertani [LB] medium and Landy MOPS medium for

lipopeptides production at 30°C. *Escherichia coli* JM109 strain was grown at 37°C in [LB] medium supplemented with 50 µg.ml<sup>-1</sup> ampicillin<sup>20</sup>. All bacterial strains mentioned in (Table 1).

**Table 1**  
*Bacterial strains used in the study.*

Name	Genotype	Source
<i>Bacillus subtilis</i> 168	trpC <sup>2</sup> , sfp <sup>0</sup>	<i>Bacillus</i> stock gentic center
<i>Bacillus subtilis</i> ATCC 21332	Wild type	ATCC
<i>Bacillus amyloliquefaciens</i> S499	Wild type	ProBioGEM
<i>Bacillus pumilus</i>	Wild type	NRC
<i>Bacillus thuringiensis israelienne</i> NRRL HD- 522	Wild type	NRC
<i>Bacillus licheniformis</i> ATCC 14850	Wild type	ATCC
<i>Bacillus sphearicus</i> 23268T	Wild type	NRC
<i>Bacillus thuringiensis kurstaki</i>	Wild type	AGERI
<i>Bacillus amyloliquefaciens</i> FZB42	Wild type	ProBioGEM
<i>Escherichia coli</i> JM 109	recA1, endA1, gyrA96, thi, hsdR17(rK <sup>-</sup> , mK <sup>+</sup> ), relA1, supE44, Δ(lac proAB), [F', traD36, proAB, lacIqZΔM15]	Promega

**NRC:** National Research Centre institute, Egypt.

**AGERI:** Agricultural Genetic Engineering Research Institute, Egypt.

**ProBioGEM:** Laboratoire des Procédés Biologiques, Génie Enzymatique et Microbien, France.

### Bioinformatics analyses

Fifty-four sequenced genomes were obtained from the National Center for Biotechnology Information (USA) NCBI (Table 2) and analyzed for the detection of NRLPs synthetases genes using some keywords as; non-ribosomal, synthetase, lipopeptide. The second step were to identify their NRPS genes, annotation, description of modules and domains, specificity

prediction of A domains by PKS-NRPS website (<http://nrps.igs.umaryland.edu/nrps>), potential primary structure of the peptide. The last step was to compare the predicted peptides structures obtained by PKS-NRPS website with Norine database (<http://bioinfo.lifl.fr/norine>) which includes nonribosomal peptides and 1185 peptides to know the predicted peptide is existing molecule or new one<sup>21</sup>.

**Table 2**  
**Bacillus genomes data analyzed for non ribosomal lipopeptides genes detection.**

No	Organisme	Genome Size (Mbp)	GenBank
1	<i>Bacillus amyloliquefaciens</i> DSM7	4.0	FN597644.1
2	<i>Bacillus amyloliquefaciens</i> FZB42	3.9	CP000560.1
3	<i>Bacillus amyloliquefaciens</i> S499	3.9	NZ_CP014700.1
4	<i>Bacillus anthracis</i> str. 'Ames Ancestor'	5.5	AE017334.2
5	<i>Bacillus anthracis</i> str. A0248	5.2	CP001598.1
6	<i>Bacillus anthracis</i> str. Ames	5.2	AE016879.1
7	<i>Bacillus anthracis</i> str. CDC 684	5.5	CP001215.1
8	<i>Bacillus anthracis</i> str. Sterne	5.2	AE017225.1
9	<i>Bacillus atrophaeus</i> 1942	4.2	CP002207.1
10	<i>Bacillus azotoformans</i> MEV2011	4.7	NZ_JJRY00000000.1
11	<i>Bacillus cellulosilyticus</i> DSM 2522	4.7	CP002394.1
12	<i>Bacillus cereus</i> 03BB102	5.4	CP001407.1
13	<i>Bacillus cereus</i> AH187	5.9	CP001177.1
14	<i>Bacillus cereus</i> AH820	5.6	CP001283.1
15	<i>Bacillus cereus</i> ATCC 10987	5.4	AE017194.1
16	<i>Bacillus cereus</i> ATCC 14579	5.4	AE016877.1
17	<i>Bacillus cereus</i> B4264	5.4	CP001176.1
18	<i>Bacillus cereus</i> E33L	5.8	CP000001.1
19	<i>Bacillus cereus</i> G9842	5.7	CP001186.1
20	<i>Bacillus cereus</i> Q1	5.5	CP000227.1
21	<i>Bacillus cereus</i> biovar anthracis str. Cl	5.4	CP001746.1
22	<i>Bacillus clausii</i> KSM-K16	4.3	AP006627.1
23	<i>Bacillus coagulans</i> DSM 1 = ATCC 7050	3.3	CP009709.1
24	<i>Bacillus coahuilensis</i> p1.1.43	3.0	NZ_ABFU00000000.1
25	<i>Bacillus cytotoxicus</i> NVH 391-98	4.1	CP000764.1
26	<i>Bacillus endophyticus</i>	4.8	CP011974.1
27	<i>Bacillus endophyticus</i> 2102	5.1	NZ_ALIM00000000.1
28	<i>Bacillus flexus</i>	5.6	NZ_LFQJ00000000.1
29	<i>Bacillus halodurans</i> C-125	4.2	BA000004.3
30	<i>Bacillus licheniformis</i> ATCC 14580	4.2	CP000002.3
31	<i>Bacillus megaterium</i> DSM 319	5.1	CP001982.1
32	<i>Bacillus megaterium</i> QM B1551	5.5	CP001983.1
33	<i>Bacillus methylotrophicus</i> FZB42	3.9	CP000560.1
34	<i>Bacillus methanolicus</i> MGA3	3.4	CP007739.1
35	<i>Bacillus paralicheniformis</i> ATCC 9945a	4.3	CP005965.1
36	<i>Bacillus pseudofirmus</i> OF4	4.3	CP001878.2
37	<i>Bacillus pumilus</i> SAFR-032	3.7	CP000813.1
38	<i>Bacillus safensis</i> FO-36b	3.7	NZ_ASJD00000000.1
39	<i>Bacillus selenitireducens</i> MLS10	3.6	CP001791.1
40	<i>Bacillus smithii</i>	3.3	CP012024.1
41	<i>Bacillus sonorensis</i> L12	4.5	NZ_AOFM00000000.1
42	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> sp. <i>natto</i> BEST195	4.1	AP011541
43	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> sp. <i>spizizenii</i> str. W23	4.0	CP002183.1
44	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> sp. <i>subtilis</i> str. 168	4.2	AL009126.3
45	<i>Bacillus thuringiensis</i> BMB171	5.6	CP001903.1
46	<i>Bacillus thuringiensis</i> serovar <i>konkukian</i> str. 97-27	5.3	AE017355.1
47	<i>Bacillus thuringiensis</i> str. Al Hakam	5.3	CP000485.1
48	<i>Bacillus tusciae</i> DSM 2912	*3.4	CP002017.1
49	<i>Bacillus weihenstephanensis</i> KBAB4	5.8	CP000903.1
50	<i>Lysinibacillus sphaericus</i>	4.6	CP000817.1
51	<i>Lysinibacillus sphaericus</i> C3-41	4.8	CP000817.1
52	<i>Brevibacillus brevis</i>	6.3	AP008955.1
53	<i>Paenibacillus polymyxa</i> E681	5.4	CP000154.1
54	<i>Paenibacillus polymyxa</i> SC2	* 6.2	CP002213.1

## Molecular biology methods

### DNA isolation, primers and PCR conditions

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit from Promega. Primers were used<sup>17</sup> as described Table (3). Degenerated primers for surfactin, fengycin and mycosubtilin families were designed<sup>17</sup>, while kurstakin was designed<sup>19</sup> by alignment of the nucleic acid sequences identified of adenylation and thiolation domains and using International Union of Pure and Applied Chemistry (IUPAC) code. The PCR conditions started with 94°C for 2 min as initial denaturation step, followed by 35 cycles of three steps; denaturation at 94°C for 30 s; annealing step for 30 s, at 43°C with As<sub>1</sub>-F/Ts<sub>2</sub>-R, at 45°C with Am<sub>1</sub>-F/Tm<sub>1</sub>-R; Af<sub>2</sub>-

F/Tf<sub>1</sub>-R and at 44.4°C with Aks-F/Tks-R; an extension step of 45 s at 72°C except with Ap<sub>1</sub>-F/Tp<sub>1</sub>-R primers (75 s at 72°C) and Aks-F/Tks-R primers (2 min at 72°C). Then final extension step was lanced at 72°C for 5 min.

### Cloning and sequencing conditions

PCR amplified bands were purified from gel with Zymoclean Gel DNA Recovery Kit (Epigenetics, USA). The PCR products were cloned into pGEM-T Easy Vector (Promega), and the heat shock method was followed to introduce plasmids into E. coli JM109 cells as described by the manufacturer's protocol. The transformants were selected on LB agar medium complemented with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-

D-thiogalactopyranoside (IPTG) at a concentration of 100, 200 and 200  $\mu\text{g}\cdot\text{ml}^{-1}$ , respectively. In LB medium complemented with ampicillin at final concentration of 100  $\mu\text{g}\cdot\text{ml}^{-1}$ , the white colonies were picked and grown at 37°C for 24 h. Then, Plasmid miniPREP Kit (GeneDireX) were used to isolate plasmids from the transformed cells. EcoR I (Fermentas) restriction

digestion of the plasmids was performed to check the inserts. Cloned products were sequenced and sequences were compared with the GenBank databases using (BLAST) software provided online by the National Center for Biotechnology Information (USA) NCBI.

**Table 3**  
**Degenerated primers for detection of lipopeptide biosynthesis genes.**

Primers names	Sequence of primers	Expected fragment size (bp)	(NRLPs) identified	References
Ap1- F	AGMCAGCKSGCMASATCMCC	959, 929 ; 893	Plipastatin	17
Tp1- R	GCKATWWTGAARRCCGGCGG			
Af2-F	GAATAYMTCGGMCGTMTKGA	443, 452 455	Fengycins	17
Tf1-R	GCTTTWADKGAATSBCCGCC			
Am1-F	CAKCARGTSAAAATYCGMGG	416, 419	Mycosubtilins	17
Tm1-R	CCDASATCAAARAADTTATC			
As1-F	CGCGGMTACCGVATYGAGC	419, 422, 425, 431	Surfactins	17
Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
Aks-F	TCHACWGGRAATCCAAAGGG	1125, 1152, 1161, 1167, 1173	Kurstakins	19
Tks-R	CCACCDKTCAAAKAARKWATC			

#### **Fermentation process and HPLC quantification**

The produced lipopeptides were extracted after microbial batch fermentation in Landy Mops medium supplied by 0.03  $\text{s}^{-1}$  of volumetric oxygen transfer coefficient ( $k_{\text{La}}$ ) at 30°C for 48 h. The bacterial cells were removed from 48 h fermented culture (stationary phase) by centrifugation at 15.000 rpm at 5°C for 20 min<sup>20</sup>, kurstakin produced strains, cells were collected and sonication for 1 min in low temperature at 6 Watt (Ultrasonic processor, Cole-Parmer Instruments, Illinois, USA). The total yield was collected before analysis by high-performance liquid chromatography (HPLC) in C<sub>18</sub> column (5  $\mu\text{m}$ ; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was isocratic acetonitrile-water-trifluoroacetic acid solvent system (80:20:0.5, 55:45:0.5, 45:55:0.5 and 40:60:0.5 [vol/vol/vol] for surfactin, kurstakin families and fengycins or plipastatins, iturins families, respectively)<sup>22,23</sup>. Samples (20  $\mu\text{l}$ ) were injected and the compounds were eluted at a flow rate of 1  $\text{ml}\cdot\text{min}^{-1}$ . Purified surfactin (Sigma), kurstakin, fengycins or plipastatins, iturins were purchased with purity of 98% as standards. The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millennium Software) of each peak were used to identify the eluted molecules<sup>24</sup>.

## **RESULTS AND DISCUSSION**

#### **Bioinformatics analyses**

Fifty four *Bacillus* genomes were analyzed by PKS-NRPS analysis, NRPS predictor and NCBI web site Table (4). The presence of genes involved in the biosynthesis of members of the four families of lipopeptides were detected [surfactin, pumilacidin, lichenysin from surfactin family, fengycin or plipastatin from fengycin/plipastatin family, bacillomycin and mycosubtilin from iturin family and kurstakin from kurstakin family] and other lipopeptides fusaridin from (*Paenibacillus*) *B. polymyxa* and unknown lipopeptide from *B. safensis* FO-36b. Four *Bacillus* strains (*amyloliquefaciens* DSM7, *amyloliquefaciens* FZB42, *amyloliquefaciens* S499 and *methylophilus* FZB42) showed the presence of three lipopeptides families; surfactin, fengycin/plipastatin and iturin. Three other *Bacillus* strains showed the presence of two lipopeptides families; surfactin and plipastatin from *Bacillus subtilis* 168, surfactin and mycosubtilin from *Bacillus subtilis* spizizenii W23, truncated lichenysin and plipastatin from *Bacillus sonorensis* L12 in addition to; *Bacillus paralicheniformis* ATCC 9945a which harbor plipastatin seems different in the amino acid no 9 (Asp/Asn) and lichenysin. We also mentioned the presence of 14 *Bacillus* genomes harbor no NRLPs synthetases genes as described Table (5).

**Table 4**  
**Peptide sequence of each lipopeptide family from 19 *Bacillus* spp.**

Lipopeptides	Predicted peptide structure	<i>Bacillus</i> spp.
<b>Surfactin family</b>		
Surfactin	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	<i>amyloliquefaciens</i> DSM7
	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	<i>amyloliquefaciens</i> FZB42
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	<i>amyloliquefaciens</i> S499
	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	<i>subtilis</i> subsp. <i>natto</i> BEST195
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	<i>subtilis</i> subsp. <i>spizizenii</i> str. W23
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	<i>subtilis</i> subsp. <i>subtilis</i> str. 168
	D-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp- D-Leu-L-Leu/Ile/Val	<i>methylotrophicus</i> FZB42
Pumilacidin	L-Glu/Asp-L-Leu-D-Leu-L-Phe/Trp-L-Asp-D-Leu-L-Leu	<i>pumilus</i> SAFR-032
Lichenysin	L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	<i>licheniformis</i> ATCC 14580
	L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	<i>Paralicheniformis</i> ATCC 9945a
Truncated lichenysin	L-Glu-L-Leu-L-Ile	<i>sonorensis</i> L12
<b>Iturin family</b>		
Iturin	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-D-Asx-L-Ser	<i>amyloliquefaciens</i> DSM7
Bacillomycin	L-Asx-D-Glu/Asp-D-Asx-Tyr/Trp- L-Pro-D-Ser-L-Thr	<i>amyloliquefaciens</i> FZB42
	L-Asx-D-Glu/Asp- D-Asx- -L-Tyr/Trp- L-Pro-D-Ser-L-Thr	<i>methylotrophicus</i> FZB42
Mycosubtilin	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-L-Ser-L-Asx	<i>atrophaeus</i> 1942
	L-Asx-D-Tyr/Trp-D-Asx-L-Gln- L-Pro-D-Asx-L-Ser	<i>amyloliquefaciens</i> S499
	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-D-Ser-L-Asx	<i>subtilis</i> subsp. <i>spizizenii</i> str. W23
<b>Fengycin family</b>		
Truncated fengycin	L-X-D-Tyr-L-Ile	<i>amyloliquefaciens</i> DSM7
Fengycin	L-Glu-D-Orn-L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-L-Ile	<i>amyloliquefaciens</i> FZB42
	L-Glu-D-Orn- L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-L-Ile	<i>amyloliquefaciens</i> S499
	D-Glu-L-Orn- D-X-L-Thr-D-Glu-L-Val-D-Pro-Glu-L-Tyr-L-Ile	<i>methylotrophicus</i> FZB42
Plipastatin	L-Glu-D-X-L-Tyr-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-L-Ile	<i>subtilis</i> subsp. <i>subtilis</i> str. 168
	L-Glu-D-X-L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Asp/Asn?-L-Ile	<i>Paralicheniformis</i> ATCC 9945a
Truncated plipastatin	L-Tyr-D-Thr-D-X	<i>sonorensis</i> L12
<b>Kurstakin family</b>		
Kurstakin	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Thr-L-Glu/Asp	<i>weihenstephanensis</i> KBAB4
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	<i>thuringiensis</i> BMB171
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	<i>cereus</i> G9842
Truncated kurstakin	D-Thr-L-Gln-L-Gly-L-X-D-Gln-L-Gln	<i>cereus</i> ATCC 14579
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	<i>cereus</i> B4264
<b>Other</b>		
Fusaricidin	L-Thr-D-Val/Ala-Tyr_D-Thr-D-Asx-L-X	<i>polymyxa</i> SC2
Unknown	L-Leu- Pks- pks- D-X- L- Leu	<i>safensis</i> FO-36b

**Table 5**  
***Bacillus* strains with no NRLPs synthetases genes.**

<i>Bacillus azotoformans</i> MEV2011	<i>Bacillus megaterium</i> DSM 319
<i>Bacillus cellulosilyticus</i> DSM 2522	<i>Bacillus megaterium</i> QM B1551
<i>Bacillus coahuilensis</i> p1.1.43	<i>Bacillus methanolicus</i> MGA3
<i>Bacillus coagulans</i> DSM 1 = ATCC 7050	<i>Bacillus pseudofirmus</i> OF4
<i>Bacillus clausii</i> KSM-K16	<i>Bacillus selenitireducens</i> MLS10
<i>Bacillus flexus</i>	<i>Bacillus smithii</i>
<i>Bacillus halodurans</i> C-125	<i>Bacillus tusciae</i> DSM 2912

**Table 6**  
**Nucleotide sequence alignment for partial synthetases genes of NRLPs detected in three *Bacillus* strains by degenerated primers.**

Sequence Origins	Srf primers	Pps primers	Fen primers	Myc primers	Krs primers	Similarity with	Similarity %
<i>B. amyloliquefaciens</i> FZB42	+	+	+	+	-	Surfactin, fengycin and iturin synthetases genes from <i>B. amyloliquefaciens</i> FZB42	100
<i>B. amyloliquefaciens</i> S499	+	+	+	+	-	Surfactin, fengycin and iturin synthetases genes from <i>B. amyloliquefaciens</i> S499	99
<i>B. licheniformis</i> ATCC 14580	+	+	+	-	-	Lichenysin synthetases genes from <i>B. licheniformis</i> ATCC 14580	100
<i>B. pumilus</i>	+	-	-	-	-	Pumilacidin synthetases genes from <i>B. pumilus</i>	100
<i>B. thuringiensis</i> kurstaki	+	-	-	-	+	Kurstakin synthetases genes from <i>B. thuringiensis</i> kurstaki	100
<i>B. thuringiensis</i> isra NRRL HD-522	+	-	-	-	+	Non-ribosomal peptide synthetase from <i>B. thuringiensis</i> serovar <i>israelensis</i> str. AM65-52	99
<i>B. subtilis</i> ATCC 21332	+	+	+	-	-	Surfactin and plipastatin synthetases genes from <i>B. subtilis</i> 168	98 ; 96
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	+	-	+	-	-	Surfactin and plipastatin synthetases genes from <i>B. subtilis</i> 168	100
<i>B. sphaericus</i> 23268T. ( <i>Lysinibacillus sphaericus</i> )	+	-	-	+	-	Surfactin and mycosubtilin synthetases genes from <i>B. subtilis</i> subsp. <i>spizizenii</i> str. W23	93 ; 89

Primers pairs were first tested with *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499, *B. licheniformis* ATCC 14580, *B. pumilus*, *B. thuringiensis kurstaki* and *B. subtilis* 168, which are completely sequenced and are known to harbor NRPS synthetases genes involved in the biosynthesis of the four families of lipopeptides. Srf primers amplified fragments for surfactin, lichenisin and pumilacidin in *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499, *B. licheniformis* ATCC 14580, *B. pumilus*, and *B. subtilis* 168 with similarity percentage of 99-100%, while Fen and Pps primers were amplified fragments for fengycin and or plipastatin family in *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499 and *B. subtilis* 168. Myc primers amplified fragments for iturin family in *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499. Primers pairs were tested with the unsequenced strains; *B. thuringiensis israelienne* NRRL HD-522, *B. subtilis* ATCC 21332 and (*Lysinibacillus*) *B. sphaericus* 23268T. Srf and Krs primers amplified fragments for kurstakin family which were 450 and 1152 bp of length as reported<sup>19</sup> and was showed a 99% similarity with kurstakin synthetase gene from *B. thuringiensis israelienne* AM65-52 on Blast alignment (Fig. 3). In *B. subtilis* ATCC 21332, Srf, Fen and Pps primers amplified three fragments of (431, 449 and 893 bp) length, similar to the surfactin and

plipastatin synthetases genes of *B. subtilis* 168 (98 and 97%), respectively (Fig. 4). This was confirmed before<sup>17</sup> and this strain was characterised before as surfactin and fengycin producer strain<sup>9, 25, 26</sup>. Srf and Fen primers amplified two fragments (416 and 425 bp) from (*Lysinibacillus*) *B. sphaericus*, which have a partial similarity with surfactin and mycosubtilin synthetases genes, respectively from *B. subtilis* subsp. *Spezizenii*. Str W23. This was confirmed before<sup>17,19</sup>. These fragments may due to unknown polyketide-non ribosomal peptide molecule encoding by four synthetases genes, which were detected by bioinformatics in *Bacillus* (*Lysinibacillus*) *sphaericus* genome sequence, these four synthetases genes two annotated as polyketide synthase WP\_036221991.1, WP\_036222373.1, one annotated as non ribosomal peptide synthase WP\_036221997.1, and the last annotated as hypothetical protein WP\_036222000.1, respectively (Fig. 5). It is interesting to note that the four genes are adjacent, spanning a locus of about 22718 nt. Fen primers was described before that are more degenerated than the other primers pairs, and this could explain their lower specificity compared to the others. Also, when these genes are not present, other genes containing adenylation and thiolation domains are detected as observed by primers from other species.

Bacillus thuringiensis serovar israelensis strain AM65-52, complete genome  
Sequence ID: [CP013275.1](#) Length: 5499731 Number of Matches: 1

Range 1: 2338169 to 2339298		<a href="#">GenBank</a>	<a href="#">Graphics</a>		
Score	Expect	Identities	Gaps	Strand	
2015 bits(1091)	0.0	1117/1130(99%)	0/1130(0%)	Plus/Minus	
<b>Features:</b> <a href="#">non-ribosomal peptide synthetase</a>					
Query	1	CGAATTTAGGTATAACCATTTTTTGTGTTGTAACCTTTTCCTGTGTCACCAGTACCTTTATTT			60
Sbjct	2339298	CGAATTTAGGTATAACCATTTTTTGTGTTGTAACCTTTTCCTGCCTCACCAGTACCTTTATTT			2339239
Query	61	GAGCATCTTCTAAAATGTACTGCAGTCGACTTTCCGGATACGCTGGATCAATTGGGACAT			120
Sbjct	2339230	GAGCATCTTCTAAAATGTACTGCAGTCGACTTTCCGGATACGCTGGATCAATTGGGACAT			2339179
Query	121	ATGCTCCTCCCGCCTTTATAAATTCCTAAGAGACCGACAATCATCTCGGATGAACGTGTAA			180
Sbjct	2339178	ATGCTCCTCCCGCCTTCATAATTCCTAAAAGACCGACAATCATCTCGGATGAACGTGTAA			2339119
Query	181	CGCAAATGCCAACTAATGATTGCGCATGTTACACCAAttttttGTAAATAATGTGCCAACT			240
Sbjct	2339118	CGCAAACCCCAACTAATGATTGCGCATGTTACACCAATTTTTTGTAAATAATGTGCCAACT			2339059
Query	241	GATTCGAGCGTTCATCCAACCTCTCGATATGTTAGTTCTTCATCTTCGCACACTACTGCAA			300
Sbjct	2339058	GATTCGAGCGTTCGTTCCAACCTCTCGATATGTTAGTTCTTCATCTTCGCACACTACTGCAA			2338999
Query	301	TTGCTTCTGGTGTttttttCACCTGTTTTTCAAACATTGTATGAATCGTACTTTCTTCCA			360
Sbjct	2338998	TTGCTTCTGGTGTTTTTTTCACCTGTTCTTCAAACATTGTATGAATCGTACTTTCTTCCA			2338939
Query	361	TATCTATAACACGCGATTTATTCCATTCTAGTAGTTGCTTATATTCTACTTTTCGATA			420
Sbjct	2338938	TATCTATAACACGCGATTTATTCCATTCTAGTAGTTGCTTATATTCTACTTTTCGATA			2338879

**Figure 3**  
**Kurstakin fragment BLAST alignment of *B. thuringiensis* isra. NRRL HD-522.**





**Lipopeptides production definition**

The obtained results show clearly that, at the studied conditions, in all cases, strains produced much more surfactin types than iturin and fengycin and low amount of kurstakin. *B. amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type). *B. subtilis* strains produced

two families of lipopeptides surfactin and fengycin (plipastatin type) or no production. While, strains of *B. licheniformis* and *B. pumilus* produced only surfactin family with two types of lipopeptides (lichenysin and pumilacidin, respectively). The two strains belongs to *B. thuringiensis* were found to produce one lipopeptides family (kurstakin) as mentioned (table 7).

**Table 7**  
**Bacillus strains harbor lipopeptides synthetases genes with their production.**

Bacillus strains	Produced Lipopeptides families							
	Surfactin types	Production mg. L <sup>-1</sup> ± SD	Fengycin types	Production mg. L <sup>-1</sup> ± SD	Iturin types	Production mg. L <sup>-1</sup> ± SD	Kurstakin types	Production mg. g <sup>-1</sup> ± SD
<i>B. thuringiensis kurstaki</i>	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	102 ± 28.12
<i>B. thuringiensis isra.</i> NRRL HD-S22	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	62 ± 19.42
<i>B. pumilus</i>	Pumilacidin	642 ± 13.52	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
<i>B. licheniformis</i> ATCC 14580	Lichenysin	358 ± 17.09	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
<i>B. subtilis str.</i> 168	Surfactin	0.0	Plipastatin	0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
<i>B. subtilis</i> ATCC 21332	Surfactin	1060 ± 7.31	Plipastatin	226 ± 9.54	-	0.0 ± 0.0	-	0.0 ± 0.0
<i>B. amyloliquefaciens</i> S499	Surfactin	872 ± 12.23	Fengycin	103 ± 9.46	Bacillomycin	589 ± 14.17	-	0.0 ± 0.0
<i>B. amyloliquefaciens</i> FZB42	Surfactin	294 ± 8.03	Fengycin	62 ± 11.34	Mycosubtilin	210 ± 14.22	-	0.0 ± 0.0

\* Results are means of triplicate experiments ± standard deviation (SD).

The production of *B. amyloliquefaciens* FZB42 were 294 ± 8.03, 62 ± 11.34 and 210 ± 14.22 mg.L<sup>-1</sup> surfactin, fengycin and mycosubtilin respectively. *B. amyloliquefaciens* S499 were 872 ± 12.23, 103 ± 9.46 and 103 ± 9.46 mg.L<sup>-1</sup> surfactin, fengycin and bacillomycin respectively. *B. subtilis* ATCC 21332 were 1060 ± 7.31 and 226 ± 9.54 mg.L<sup>-1</sup> of surfactin and plipastatin respectively. While, *B. subtilis* 168 has no production. The production of *B. licheniformis* ATCC 14580 strain was 358 ± 17.09 mg.L<sup>-1</sup> of lichenysin, and *B. pumilus* was produced 642 ± 13.52 mg.L<sup>-1</sup> of pumilacidin. Also, the strains of *B. thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522 were produce 102 ± 28.12 and 62 ± 19.42 mg.g<sup>-1</sup> cells, respectively of kurstakin. The regulation of surfactin biosynthesis has been well described in the literature<sup>27</sup> but little information is available concerning fengycin

iturin and kurstakin. Our results clearly show that the regulations of the four families of lipopeptides are different; molecular analyses should be performed in further studies to characterize the regulation mechanism of lipopeptides families biosynthesis.

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**CONFLICT OF INTEREST**

Conflict of interest declared none.

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