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Abstract

A new family of lipopeptides produced by *Bacillus thuringiensis*, the kurstakins, was discovered in 2000 and considered as a biomarker of this species. Kurstakins are lipoheptapeptides displaying antifungal activities against *Stachybotrys charatum*. Recently, the biosynthesis mechanism, the regulation of this biosynthesis and the potential new properties of kurstakins were described in the literature. In addition, kurstakins were also detected in other species belonging to *Bacillus* genus such as *Bacillus cereus*. This mini-review gathers all the information about these promising bioactive molecules.

Introduction

Between 1949 and 1986, three different families of nonribosomal lipopeptides were identified in *Bacillus* spp.: surfactins, iturins and fengycins (Jacques, 2011). A new family produced by *Bacillus thuringiensis* and named the kurstakins, was discovered in 2000. The recent characterization of the biosynthesis mechanism of these compounds (in 2009 and 2011), their main properties (in 2011 and 2012) and the regulation of their biosynthesis in 2012, open new perspectives for these lipopeptidic compounds. This mini-review is the first one dedicated to them.

Discovery, structure and mass spectrometry detection

Among the spores from six ATCC *Bacillus thuringiensis* strains, the presence of a lipophilic biomarker, named kurstakin, was detected for the first time from the *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Hathout et al. 2000). After a spore washing, the authors identified using LC-MS four $[M+H]^+$ molecular ions of *m/z* 879, 893, 893 and 907 (with a common fragment ion at *m/z* of 609). The molecular masses of these four compounds differed by 14 Da ($-CH_2-$), suggesting that these molecules were homologous lipopeptides. Further acid hydrolysis led to the identification of the corresponding free fatty acids from the four compounds which are 9-methyldecanoic, dodecanoic, 10-methylundecanoic and 11-methyldodecanoic acids, generating kurstakins C_{11} (*iso*-), C_{12} (n- and *iso*-) and C_{13} (*iso*-), respectively. Amino acid analyses revealed the same residues for the four molecules: Thr, His, Ala, Gly, Ser and Glx (Gln or Glu) with molar ratios of 1:1:1:1:1:2. The ascertainment of the chemical structures of the kurstakins was completed by the determinations of (i) the sequence of the heptapeptide: Thr-Gly-Ala-Ser-His-Gln-Gln; (ii) the presence of an amide bond between the fatty acid chain and the first threonine residue and (iii) the presence of a lactone linkage between the serine at position 4 and the C terminus of glutamine at position 7

60(Fig. 1). Such a peculiar lactone ring between the fourth and seventh amino acids of the 61peptidic part was recently reported for a new biosurfactant (licheniformin) produced by 62*Bacillus licheniformis* MS3 (Biria et al. 2010). However, neither the nonribosomal peptide 63synthesis statute of kurstakins in *B. thuringiensis* subsp. *kurstaki* strain HD-1 nor the possible 64occurrence of D- forms among the seven amino acids of their peptidic moiety (compared to 65other known nonribosomal lipopeptides from *Bacillus* spp.) were demonstrated. Three years 66later, a homologous series of three ions at *m*/*z* 892, 906, and 920 similar to those of kurstakins 67was detected by Madonna et al. (2003) in *Bacillus subtilis* ATCC 6051 but these results were 68not confirmed by genetic analyses (see below). Another study dealing with numerous *Bacillus* 69sp. strains isolated worldwide further revealed the presence of kurstakins in 20 from 54 strains 70tested, using MALDI-TOF-MS fingerprinting of whole bacterial cells (Price et al. 2007). 71These were typically identified by the molecular ions of *m/z* 889, 905, 917, and 933 but their 72primary structures or those of other putative kurstakins of *m/z* about 942 and 958 were not 73elucidated. The authors confirmed that these secondary metabolites were retained by the 74spores or cells and not secreted because they predominantly found them in the bacterial 75colonies on agar plates.

76 New information about the structural diversity of the kurstakins was recently reported 77by MALDI-TOF-MS analyses of the *Bacillus* sp. strain NK2018 (Bumpus et al. 2009) and six 78*B. thuringiensis* strains (Abderrahmani et al. 2011). From culture supernatants of strain 79NK2018 grown in an M9 minimal medium, the first authors showed the presence of six 80kurstakin variants in differing amounts, with molecular masses ranging from 907.4765 to 81953.5192 Da (formulae from $C_{40}H_{65}O_{13}N_{11}$ to $C_{42}H_{71}O_{14}N_{11}$), corresponding to kurstakins with 82a β-hydroxylated fatty acid chain with 12, 13 and 14 atoms of carbon. The occurrence of three 83molecules differing by exactly 18.0103 Da from three other ones was attributed to the 84presence or lack of a lactone ring inside the peptidic part of the molecules, suggesting that

kurstakins might be found currently in culture supernatant as variants with linear peptidic parts. After growth on either AK or LB medium, Abderrahmani et al. (2011) detected the presence of the three C_{11}, C_{12} and C_{13} kurstakin isoforms in six *B. thuringiensis* strains from the 11 tested. Some other molecular ions with m/z of 920, 942 and 958 were also sometimes detected and could correspond to the kurstakin with a C_{14} fatty acid chain (Abderrahmani 2011). To summarize, the kurstakins synthesized by *Bacillus* spp. consist of lipoheptapeptides which (i) are linked to between C_{11} and C_{14} fatty acids, β-hydroxylated or not, with two isoforms (n-, *iso*-); (ii) are partially cyclic (lactone bond between Ser/4 and C-terminal Gln/7) and might even be linear; and (iii) are expected to contain two D-configured amino acids. In MALDI-TOF-MS experiments, the values of the $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ molecular ions detected should range from 878.473 (cyclic C_{11} isoform $[M+H]^+$) to 992.481 Da (linear β-hydroxylated C_{14} isoform $[M+K]^+$), under conditions allowing these biomarkers to be detected (Figs. 1 and 2).

Biosynthesis

The operons potentially encoding kurstakin synthetases in *B. cereus* and *B. thuringiensis* were identified by bioinformatics analyses using two new approaches. In the first one, Bumpus et al. (2009) took advantage of the size of the NRPS enzymes and the presence of unique marker ions derived from the common phosphopantetheinyl cofactor to adapt mass spectrometry-based proteomics to detect selectively NRPS and PKS gene clusters in microbial proteomes without requiring genome sequence information. In these conditions, the authors highlighted in strains *Bacillus* sp. NK2018 and *Bacillus cereus* AH1134 the genes involved in the biosynthesis of the kurstakins. The second approach used PCR with degenerate primers based on the intraoperon DNA sequence alignment of adenylation and thiolation domains of all enzymes implicated in the biosynthesis of the lipopeptide family

(Tapi et al. 2010). Two sets of primers elaborated from first bacillomycin genes, then from kurstakin genes led to the discovery of genes implied in kurstakin biosynthesis (Tapi 2010; Tapi et al. 2010; Abderrahmani et al. 2011). From these two studies the organization of the kurstakin cluster could be predicted. This cluster (Fig. 3) contains three genes (*krsA*, *krsB* and *krsC*) which encode three large multifunctional proteins (KrsA, KrsB and KrsC) constituting the complete synthetase. This latter is organized as follows: KrsA comprises one module (m1), KrsB is constituted of two modules (m2 and m3) and KrsC includes four modules (m4 to m7) and for each module a condensation-adenylation-thiolation motif can be found. In addition, m1 and m6 harbour a supplementary epimerization domain. The module 7 includes a final thioesterase domain enabling the unhooking of the neo-formed peptide from the NRPS and its possible cyclization. The combination of the predictions obtained from different bioinformatics tools (Ansari et al. 2004; Raush et al. 2005; Bachmann and Ravel 2009) led to a peptide with the primary structure: D-Thr_Gly/Ala/Gln/Glu_Ala_Ser_X_D-Gln_Gln.

While the precise mechanism of biosynthesis was not yet experimentally analysed, it could easily be deduced from the bioinformatics analysis and the high quantity of available information on the biosynthesis of other lipopeptides from *Bacillus* or *Pseudomonas* species (Sieber and Marahiel, 2005; Raaijmakers et al. 2010; Roongsawang et al. 2010) (Fig. 3). The first synthetase, KrsA, contains one module with a starting condensation domain (Rausch et al. 2007; Kraas et al. 2010) which catalyses the link between a fatty acid chain of different length and isomery and a threonine residue activated by the adenylation domain and covalently fixed to the thiolation domain of this first module. The last domain of this first module is an epimerization domain that will transform the L-Thr to D-Thr. The second synthetase, KrsB, contains two modules responsible for the activation and the incorporation of two other amino acid residues, Gly and Ala. In the third synthetase, KrsC, four modules are involved in the incorporation of the four other amino acid residues: Ser, His, Gln and Gln.

The presence of an epimerization domain in the third module probably modifies the incorporated L-Gln to form the D-configuration. The last module contains a thioesterase which will catalyse the liberation of the peptide and probably its partial cyclization (Kopp and Marahiel 2007). A gene encoding a phosphopantetheinyl transferase was identified downstream from the NRPS complex. This enzyme could be involved in the transformation of the apo-form of the enzymes to the holo-form by the addition of a phosphopantetheinyl group from the coenzymeA to the different thiolation domains (Mofid et al. 2004). A second thioesterase (TEII) is encoded by the next gene (*krsD*). The results obtained by Schwarzer et al. (2002) on the biochemical characterization of two second similar thioesterases named TEII and involved in the surfactin and bacitracin peptide antibiotics biosynthesis showed that these enzymes play a role in the regeneration of the misacylated Peptidyl Carrier Proteins (Thiolation domains). The presence of linear peptide in strain NK2018 could result from the action of this second thioesterase or an insufficient expression or efficacy of the first one.

A sixth gene (*krsE*) situated upstream from the *krsA-C* genes and belonging to the kurstakin cluster was identified by Dubois et al. (2012) in *Bacillus thuringiensis* Bt407. The product of this gene, the protein KrsE, is a presumed efflux protein and could be involved in the secretion of the lipopeptide.

Overview on kurstakin potentially producing microorganisms

In order to identify the genetic potential for kurstakin production among microorganisms, we performed a BLASTp method using KrsA, KrsB, KrsC, KrsD, KrsE and Ppant sequences from *B. thuringiensis* serovar pondicheriensis BGSC 4BA1 as queries. The same cluster leading to kurstakin synthesis was retrieved in the genome of 32 strains for which genomes are sequenced, assembled and either finished or still as drafts (V. Leclère, M. Pupin, W. Hussein, P. Jacques, unpublished data). Without exception, all the strains pointed

out belong to the *Bacillus* genus, more especially to the *B. cereus* species group, indicating that kurstakin production could be considered as the marker for this group. However, no sequences of kurstakin synthetases were present in *Bacillus anthracis* and *Bacillus cytotoxicus* although they belong to the same *B. cereus* group. However, only one genome is available for *B. cytotoxicus* and five genomes are completely sequenced and assembled for *B. anthracis*. So the question of lack of kurstakin synthetase in the *B. anthracis* species remains open and should be related later to the high virulence of the strains. In addition, kurstakins were also detected in *B. subtilis* ATCC 6051 strain (Madonna et al. 2003). This strain was an ancestor of the reference strain 168 (Zeigler et al. 2008), the genome of which was completely sequenced and, surprisingly, no traces of kurstakin genes were found in this genome. The kurstakin cluster is present in most of the genomes of *B. cereus* and *B. thuringiensis* for which genomic data are available (Table 1). Kurstakin genes were also detected in the partially sequenced genome of *B. cereus* BDRD-Cer4 and *B. cereus* AH1134. When the sequences are present in the genomes, they are highly conserved and the organization of the cluster (KrsE-KrsA-KrsB-KrsC-Ppant-TE) is also conserved. However, the strains *B. cereus* AH603, BDRD-ST196, *B. mycoides* DSM 2048 and *B. weihenstephanensis* KBAB4 might produce a variant form with a Glu or Asp instead of Gln at the last position as predicted by NRPSpredictor2 (Röttig et al. 2011). As no amino acid residue can be predicted for module 6, the strain *B. cereus* Rock4-2 can be supposed to produce another member of the kurstakin family varying by the residue at this position..

Regulation

The regulation system of kurstakin production has been partially described in *Bacillus thuringiensis* Bt407 (Fig. 4). A transcriptomic analysis indicates that the four genes *krsEABC* form a cluster whose transcription is activated by the NprR-NprX quorum-sensing system during late stationary phase (Dubois et al. 2012). NprR is a quorum sensor activated by its

cognate signaling peptide, NprX. NprR-NprX functions as a typical Gram-positive quorum-sensing system: the pro-signaling peptide NprX is exported from the bacterial cell and after being processed to its active form (presumably a heptapeptide), the peptide is reimported into the bacteria, where it binds to NprR allowing the recognition of its DNA target (Perchat et al. 2011).

The NrpX-NrpR system regulates 41 different genes, divided into four different groups. The first group is composed of genes coding for stress resistance proteins, including cytochrome P450, cysteine dioxygenase and several metabolite exporters. The second group is composed of four genes encoding the Opp permease system, involved in the import of small peptides into the cells. The third group is composed of the NRPS *krs* genes. The last group codes for degradative enzymes and proteins able to bind organic material (Dubois et al. 2012).

Properties

Kurstakins are not recovered in the culture supernatant of producing strains but are found in association with the bacterial cells and particularly on spores (Hathout et al. 2000; Price et al. 2007; Abderrahmani et al. 2011). However, co-infection experiments carried out with a producing strain and a non-producing one in the insect larvae *Galleria mellonella*, suggest that this molecule is secreted (Dubois et al. 2012). This apparent discrepancy between these results suggests that kurstakin is a secreted molecule with a high affinity for membranes. This high affinity could be due to the presence of the basic amino acid histidine which confers a cationic charge to the lipopeptide and thus facilitates its electrostatic interaction with phospholipids of the cell membrane.

Purified kurstakins displayed an antifungal activity against *Stachybotrys charatum*, showing a halo of inhibition identical to the one obtained with polymyxin B used as positive

control (Hathout et al. 2000). Nevertheless, Abderrahmani et al. (2011) showed that no correlation exists between the antifungal activities of the strains and the presence of kurstakins. Indeed, some producing strains did not show any antifungal activity whereas some other ones did not produce kurstakin and showed antifungal properties. However, their evaluation was made with the fungi *Mucor rouxii* DSM 1191, *Rhizopus orizae* DSM 907, *Penicillium roqueforti* DSM 1080, *Aspergillus niger* DSM 737, and *Fusarium oxysporum* DSM 62297, different from those used by Hathout et al. These data indicate that kurstakin might be a pore-forming molecule with a limited spectrum of activity.

The fact that significant colonization of solid media was detected neither for non-producing kurstakin strains nor for a kurstakin-deficient mutant indicates that kurstakins are responsible for the invasive growth (Abderrahmani et al. 2011). Moreover, a strain where the genes *krs*A, *krs*B and *krs*C (Δ*krs*ABC) were deleted was unable both to swarm and to form a biofilm at the air/liquid interface (Dubois et al. 2012). A very interesting property of kurstakin is its ability to enhance the survival of *B. thuringiensis* in the insect cadaver (Dubois et al. 2012). In view of these various properties, kurstakin might allow *B. thuringiensis* to spread across the cadaver, thereby facilitating access to new substrates and increasing its ability to disseminate in the environment.

Perspectives

The research on this fourth family of lipopeptides produced by *Bacillus* spp. has yet to be developed, and several perspectives are worth considering. The precise structure of the different variants should be confirmed by chemical analysis: the presence of the D-amino acid residues should be validated by analysis of amino acid residues after acid hydrolysis and derivatization, e.g., by GC using chirasyl-L-Val column. Confirmation of the presence of linear structure or C14 fatty acid chain should be done by LC-MS-MS analysis and NMR. The predicted biosynthetic pathway proposed in this review should be confirmed by

biochemical analysis of the different domains of the synthetase. Particular attention will have to be paid to the thioesterase domains and their role in the concomitant presence of cyclized and linear forms of the lipopeptides.

Hathout et al. (2000) have evaluated the amount of kurstakin produced at about 15-20 µg/mg of spore. Overproducing mutant cells could be constructed using similar strategies developed by Leclère et al. (2005), Fickers et al. (2009) and Coutte et al. (2010), for the overproduction of other families of lipopeptides from *B. subtilis*. Purification techniques need to be developed to extract the lipopeptides or collect them in the supernatant.

Lipopeptides from *Bacillus* spp. are well known for their potential applications in several fields (Jacques, 2011) including biocontrol of plant pathogens (Ongena and Jacques, 2008). Purified compounds could be thus used in different physico-chemical or biological tests in order to characterize their physico-chemical properties and biological activities and their potential applications.

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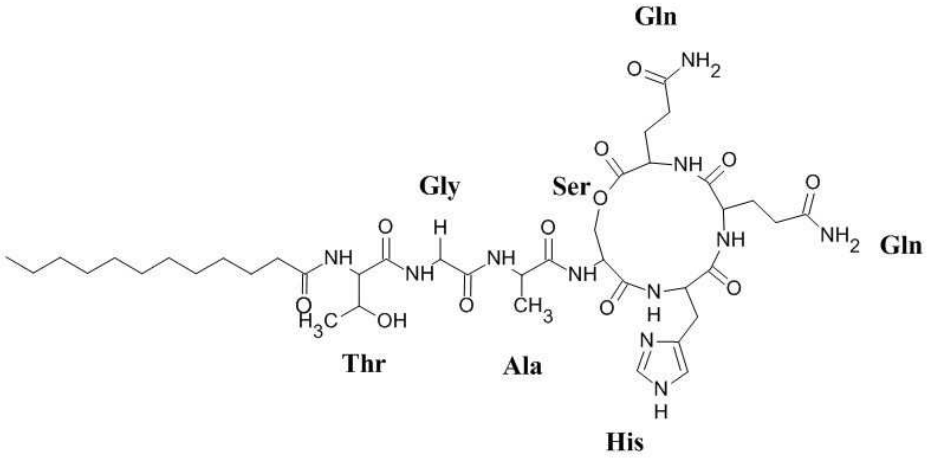
Table 1 Presence of kurstakin genes in sequenced genomes of *B. cereus* and *B. thuringiensis*

Strains	Sequenced genome status[1]	Genes[2]					
		krsE	*krsA*	*krsB*	*krsC*	*sfp*	*krsD*
Reference strain: *Bacillus thuringiensis* serovar pondicheriensis BGSC 4BA1	C	+	+	+	+	+	+
B. thuringiensis							
BMB171; sv chinensis CT-43	C	+	+	+	+	+	+
sv finitimus; sv konkukian str. 97-27; str.Al Hakam	C	-	-	-	-	-	-
Bt407; IBL 200; sv berliner ATCC 10792; sv huazhongensis BGSC 4BD1; sv kurstaki str. T03a001; sv thuringiensis str. T01001; sv pakistani str. T13001; sv pulsiensis BGSC 4CC1; sv sotto str. T04001	U	+	+	+	+	+	+
IBL 4222	U	+	+	+	TCT ?	+	+
B. cereus							
ATCC 14579; B4264; G9842	C	+	+	+	+	+	+
03BB102; AH187; AH820; ATCC10987; E33L; Q1; bv anthracis str.CI	C	-	-	-	-	-	-
172560W; ATCC 10876; BDRD-ST24; BGSC 6E1; F65185; m1550; Rock1-15; Rock1-3	U	+	+	+	+	+	+
BDRD-Cer4	P	+	+	+	+	+	+
AH1134	P	+	+	NF	+	NF	NF
AH603; BDRD-ST196	U	+	+	+	7-D/E	+	+
AH621	U	+	NF	NF	TCT ?	+	NF
AH676	U	+	+	+	+	+	NF
Rock4-2	U	+	+	+	6-X	+	+
Others species							
B. mycoides DSM 2048	C	+	+	+	7-D/E	+	+
B. weihenstephanensis KBAB4	C	+	+	+	7-D/E	+	+

[1] Status of genome sequencing (GS): C complete, U: unfinished, P: partial

[2] NF: Not Found, TCT?: truncated ?, 7-D/E: prediction of amino acid residue incorporated by module 7 is D/E instead of Q, 6-X: no possible prediction for amino acid residue incorporated by module 6

+ : protein present with at least 90% identity with the reference one, - : not present



I.

II.

FA_Thr-Gly-Ala-Ser-His-Gln-Gln		**FA**_Thr-Gly-Ala-Ser-His-Gln-Gln
	O————	
iC$_{11}$	877.465	n.r.
C$_{12}$	891.481	n.r.
C$_{12}$[-OH(3)]	907.476	925.486
iC$_{12}$	891.481	n.r.
iC$_{13}$	905.496	n.r.
iC$_{13}$[-OH(3)]	921.491	939.502
C$_{14}$[-OH(3)]	935.507	953.518
	A	**B**

Fig. 1 I. Chemical structure of cyclic kurstakin with a C12 fatty acid chain. **II.** Structures and calculated molecular masses (Da) of the different isoforms of kurstakins characterized until now by MALDI-TOF-MS. **A**. Partially cyclic molecules (Hathout et al. 2000; Bumpus et al. 2009); the square brackets **[-OH(3)]** in bold correspond to the presence of a β-hydroxylated fatty acid; **B**. Linear molecules (Bumpus et al. 2009). The amino acids Thr and Gln (in position 6) are expected to be under the D- form (Abderrahmani et al. 2011) (It is worthy of

note that L- and D- forms have never been chemically determined). The first kurstakin homologues, chemically characterized by Hathout et al. (2000), did not contain a β-hydroxylated fatty acid, and comprised the sole *i*C_{11}-, *i*C_{12}- and *i*C_{13} isoforms isolated so far. **FA** = fatty acid; n.r. = not reported to date.

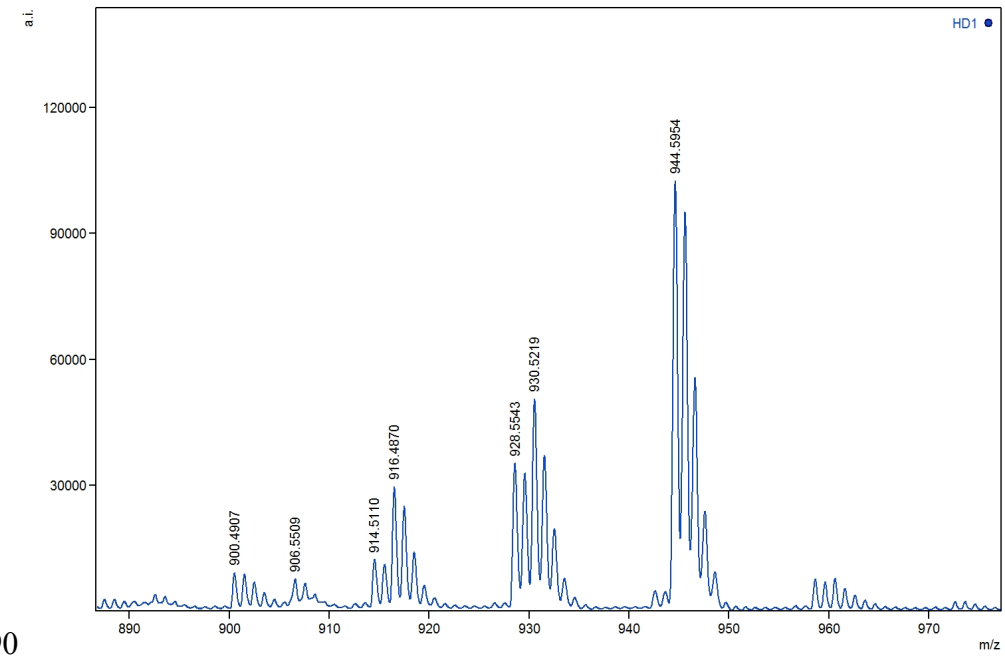


Fig. 2 Typical pattern of whole cell MALDI-TOF MS analysis of the kurstakin producing strain *B. thuringiensis* subsp. *kurstaki* HD-1 (Caradec et al., unpublished data).

Cyclic isoform with C_{11} fatty acid chain: 900.4907 $[M+Na]^+$; 916.4870 $[M+K]^+$

Cyclic isoform with C_{12} fatty acid chain: 914.5110 $[M+Na]^+$; 930.5219 $[M+K]^+$

Cyclic isoform with C_{13} fatty acid chain: 906.5509 $[M+H]^+$; 928.5543 $[M+Na]^+$; 944.5944 $[M+K]^+$

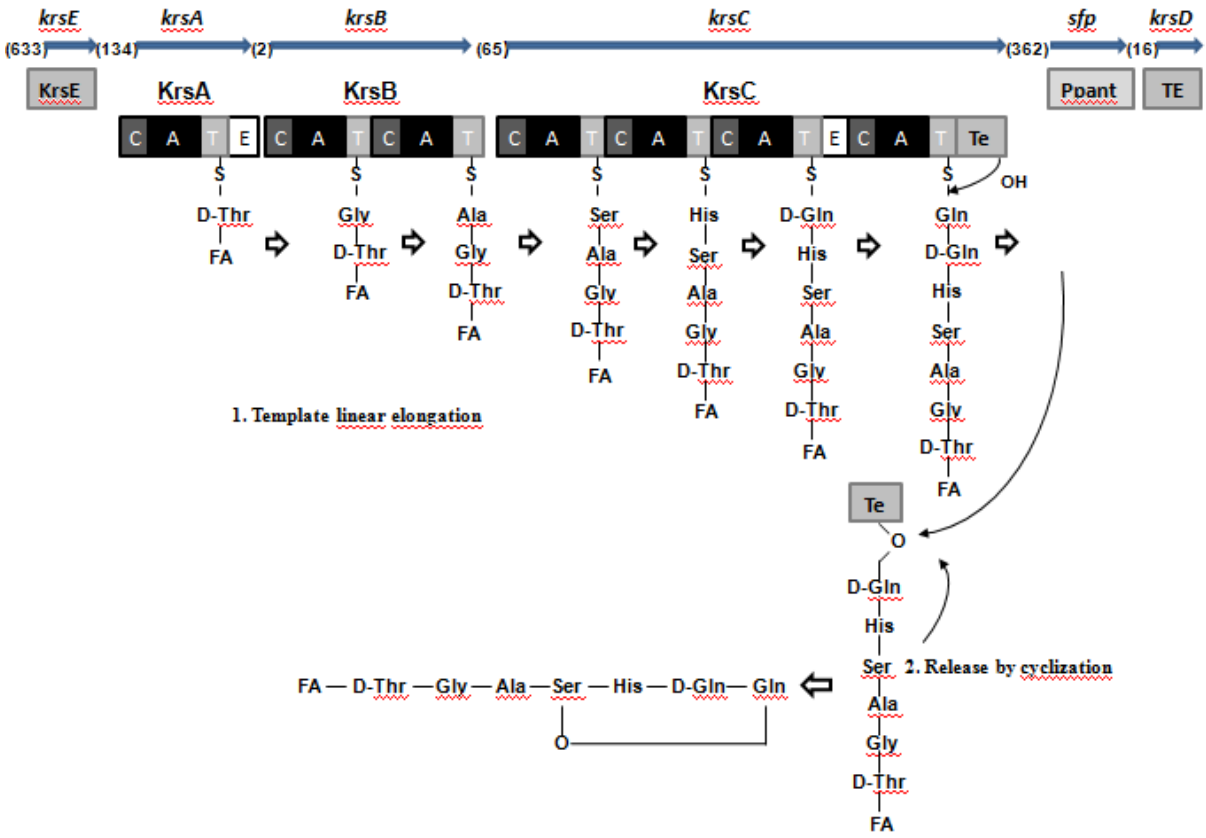


Fig. 3 Hypothetical kurstakin biosynthetic assembly line based on bioinformatics analysis

Situated downstream from the putative *krsE* gene which potentially encodes for an efflux protein which could be involved in the kurstakin secretion (bthur0010_59510), the biosynthetic complex consists of three kurstakin synthetases: KrsA, KrsB and KrsC (encoded by three genes: bthur0010_59520, bthur0010_59530 bthur0010_59540 in *Bacillus thuringiensis* serovar pondicheriensis BSCG 4BA1), divided into seven distinct modules. Each module is responsible for recognition, activation and loading of a single amino acid substrate. In the first module, a starter condensation domain links a fatty acid chain to the amino acid residue (Thr) activated and fixed in this module. Two epimerization domains are found in modules 1 and 6, converting the corresponding amino acid in the D-stereoisomer. The cyclization and release of the final heptapeptide is catalyzed by the first adjacent Te

410domain. Immediately downstream are situated two genes, coding for a phosphopantetheinyl
411transferase (bthur0010_59550) and a closely adjacent type II thioesterase (bthur0010_59560),
412which are expected to belong to the kurstakin cluster. The numbers in brackets correspond to
413the spaces (in nucleotides) between these different open reading frames borne by strain BSCG
4144BA1. C: Condensation domain; A: Adenylation domain; T: Thiolation domain; E:
415Epimerization domain; Te: Thioesterase domain; Ppant: Phosphopantetheinyl transferase
416(Sfp); TE: Type II thioesterase; FA: Fatty acid

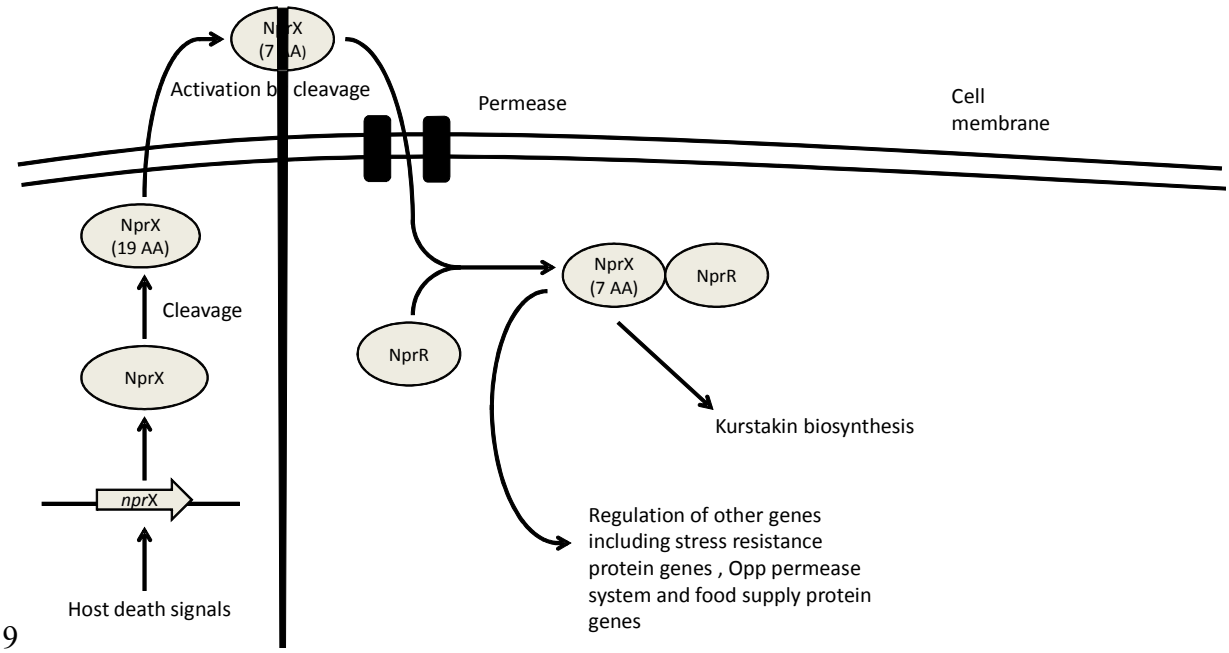


Fig. 4 Regulation of kurstakin biosynthesis

The NprX peptide is produced as an inactive form and is then activated by two successive cleavages. The first cleavage happens in the cell cytoplasm, and leads to a 19 amino acid peptide formation. After this first cleavage, the peptide is exported out of the cell, and is cleaved a second time in a 7 amino acids peptide, leading to its active form NprX. This peptide is then reimported within the bacterial cell through the Opp permease system, and is bound with the NprR regulator. NprR forms a complex with the heptapeptide NprX whose production is regulated by host-death signals. The NprX-NprR complex activates the kurstakin production, by binding to the -35 box of the promoter of the *krs* genes (Perchat et al. 2011).