Review Article

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SURFACTIN: A REVIEW ON NOVEL MICROBIAL SURFACTANT

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Abstract: Surfactin, being a peptide antibiotic; acts as a pharmacologically and commercially important surfactant to the Gram positive bacteria and endospores. They have been recently purified from the microbial strains as lipopeptides in their secretion. Bacillus spp. is one of the common producers to this biosurfactant. They are synthesized non-ribosomally via large multi-enzymes (non-ribosomal peptide synthetases, NRPSs). The surfactin biosynthetic assembly line (inducible operon) consists of three large NRPSs: SrfA-A (402 kDa), SrfA-B (401 kDa) and SrfA-C (144 kDa), comprising of a total of seven modules. These modules are arranged into core and elongation domains which are responsible for lipopeptide chain formation. Current researchers have studied only about the Bacillus subtilis genome and its molecular biological aspects for surfactin biosynthesis and to some extent in Bacillus licheniformis. Due to structurally homology of the NRPSs synthetase complex and Srf gene, there is a plenty of room at the base to be exploited through the tools of molecular and structural biology with the integration of recombinant DNA technology.

Keywords: Surfactin, Lipopeptide, Synthetase, NRPS, Domain

INTRODUCTION

Bacillus subtilis has been used for genetic and biochemical studies for several decades, and is regarded as paradigm of Gram-positive endospore-forming bacteria [1]. Several hundred wild-type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. All of the genes specifying antibiotic biosynthesis combined amount to 350 kb; however, as no strain possesses them all, an average of about 4–5% of a *B. subtilis* genome is devoted to antibiotic production. Peptide antibiotics, also named lipopeptides, represent the predominant class. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases [2]. Furthermore, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages, and consequently the peptide antibiotics are insensitive to oxidation [3]. *Bacillus* lipopepdides are synthesized non-ribosomally *via* large multi-enzymes (non-ribosomal peptide synthetases, NRPSs) [2, 3, 4, and 5]. These biosynthetic systems lead to a remarkable heterogeneity among the lipopeptides products generated by *Bacillus* with regards to the type and sequence of amino acid residues, the nature of the peptide cyclization and the nature, length and branching of the fatty acid chain [6]. Lipopeptides are classified into three families depending on their amino acid sequence: iturins, fengycins and surfactins [5, 6].

An alternative approach to produce novel natural antibiotics and pharmacologically important products is the manipulation of biosynthetic pathways. Several successes were already obtained within the groups of nonribosomal peptides (NRPs) and polyketides (PKs). Nonribosomal peptides (NRPs) represent a diverse group of pharmacologically important natural products synthesized by numerous microorganisms, including filamentous fungi, Gram positive and to minor extent Gram negative bacteria. Among the Gram positive bacteria, the groups of Actinomycetes, as well as members of the genus Bacillus are the primary producers of NRPs [6]

Members of this heterogeneous class of natural compounds comprise the antibiotics Bacitracin A [6], Tyrocidine A [7], Vancomycin [8] and Daptomycin [9] of non-ribosomal origin are also the immuno-suppressant Cyclosporine A [10], the anti-tumor drugs Bleomycin [11] and Epothilone [12], as well as the biotenside Surfactin [13].

Structural Biology of Surfactin:

Despite their structural heterogeneity, NRPs share a common mode of synthesis, namely a stepwise assembly of the peptide backbone directed by large, multi-modular assembly lines, termed nonribosomal peptide synthetases (NRPSs). These enzymes contemporary act as template and biosynthetic machinery [4], and possess a modular organization.

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Each module is responsible for recognition, activation and incorporation of one specific substrate in the growing peptide chain, and can be subdivided in single catalytic units called domains.

According to the so-called multiple carrier thio-template mechanism, the substrate amino acids and the nascent peptide chain are tethered to the NRPSs via 4´-phosphopantetheine (Ppant) moieties. The prosthetic group represents an integral part of a module's peptidyl carrier (PCP) domain, which has to be converted into its active holo-form by phosphopantetheinylation (Fig. 1A). This priming reaction requires specialized phosphopantetheinyl transferases (PPTase), which catalyze the nucleophilic attack of an invariant serine residue of the PCP domain, onto the -3'-phosphate group of CoA [14]. In analogy to aminoacyl-tRNA synthetases, NRPS adenylation (A) domains select their cognate amino acid from the pool of available substrates and activate it as 2+ aminoacyl adenylate at the expense of ATP and Mg [15]. The activated aminoacyl-O-AMP oxoesters are subsequently transferred to the adjacent holo-PCP and bound as thioester to the 4´-Ppant cofactor [16] (Fig.1B and C).

Substrates are catalyzed by the C domain of the acceptor module. The nucleophilic attack of the amino group of downstream located aminoacyl-S-PCP acceptor onto the up-stream localized peptidyl-S-PCP thioester eventually yields the translocation of the thioester-bound peptide chain to the PCP domain of the acceptor-module [15, 17] (Fig. 1 D). Based on mis-priming experiments, a low selectivity for the upstream donor substrate and a high selectivity for the downstream acceptor residue could be determined for the C domain [17-20]. This property of the C domains probably prevents mis-initiation of the biosynthetic process, since this way, the activated aminoacyl-S-PCP is prevented from directly being transferred to the downstream C domain and accepted as donor acyl-thioester. The iteration of substrate activation, binding as thioester to the PCP domain, peptide chain elongation and translocation to the downstream module (Fig.1E) leads to the formation of the full length peptide chain, bound to the 4'Ppant-cofactor of the last (termination) module. The final product release is then catalyzed by a TE domain, located at the C-terminus of the termination module (Fig. 1E). TE domains, which catalyze product release in NRPS as well as in fatty acid synthase (FS) and PKS assembly lines, belong to the hydrolase family that utilizes the catalytical triade, consisting of acidic residue-His-Ser (or Cys). In a first step, the peptidyl intermediate is transferred from the downstream PCP domain to a highly conserved serine residue, which is part of the core motif GxSxG and the catalytic triade [17, 20].

The peptide-bond formation between aminoacyl or peptidyl-S-PCP donor and an aminoacyl-S-PCP acceptor The subsequent deacylation of the resulting oxoester intermediate occurs by nucleophilic attack of either (i) an internal nucleophile, which depending on its position leads to intramolecolar cyclization and the generation of cyclic or branched-cyclid macrolactones or macrolactames, (ii) water, which causes the release of linear peptides, or (iii) a N-terminal nucleophile of another peptide chain, leading to multi-meric compounds (Fig. 1E). [18, 11]

The described domains are the so-called core domains. A minimal elongation module consists of the three domains: C-A-PCP. An A-PCP di-domain pair forms an initiation module, and in fact - in absence of an up-stream C domain-an amino acid can be activated, tethered to PCP and directly translocated to the downstream module. Finally, the C-terminal termination module (C-A-PCP-TE) has an additional TE for peptide product release.

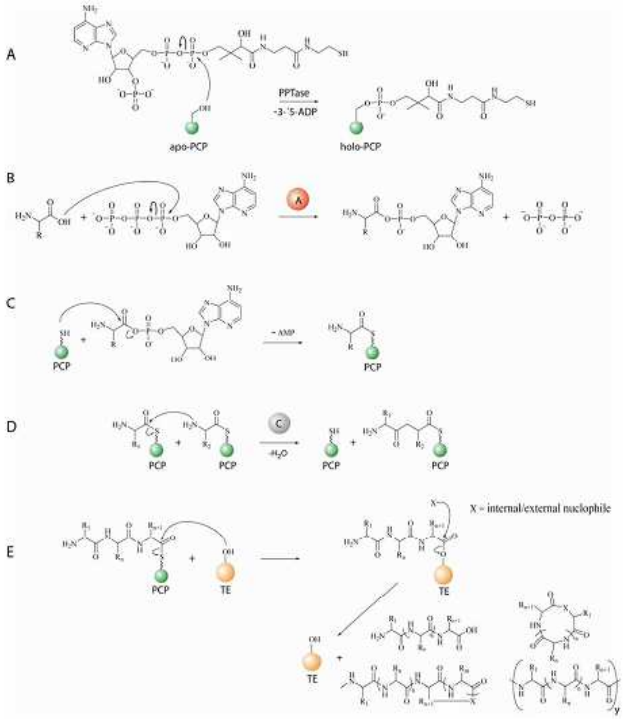
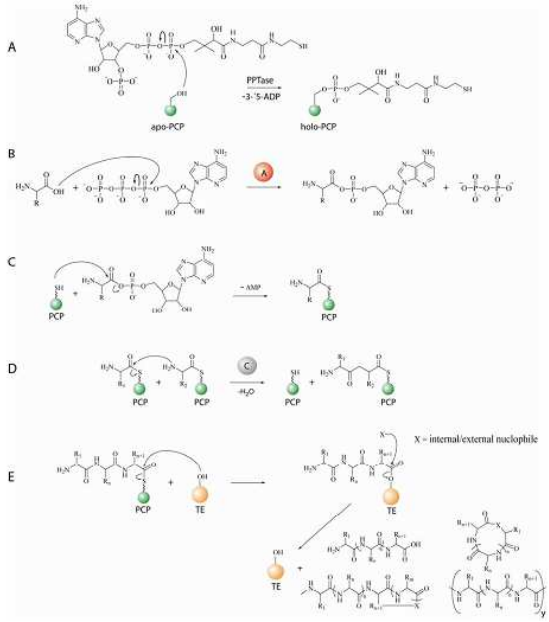


Fig 1: The five essential reactions of NRPs biosynthesis

(A) The post-translational modification of the PCP domain (in green) is carried out by a specific PPTase, which catalyze the nucleophilic attack of the hydroxyl-group of an invariant serine residue of the PCP domain onto the 3'-phosphate group of CoA, resulting in the covalent loading of the Ppant cofactor too PCP. **(B)** An adenylation (A) domain (in red) selectively recognizes a substrate amino acid and activates it as aminoacyl adenylate at the expense of ATP and Mg^{2+} **(C)** The activated amino acid is bound to the Ppant cofactor of the associated PCP as aminoacyl thioester. **(D)** The C domain catalyzes the peptide bond formation between two intermediates covalently bound to neighbouring PCP domains. **(E)** The thioesterase domain, localized at the C-terminus of the last module, forms an oxoester intermediate with the peptide chain and finally catalyzes the product release either in linear, circular, branced circular or multi-meric form.



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Many NRPS modules contain additional domains, which modify the activated aminoacyl or peptidyl-thioester intermediates. Examples for those optional domains are the epimerization (E) domain, N-methylation, (M) domain, oxidation (Ox) domain, formylation (F) domain, cyclization (Cy) domain. Many pharmacologically important non-ribosomal peptides undergo one more of the mentioned modifications, which were shown to be fundamental for their activity.

Surfactin Biosynthesis:

The surfactin biosynthetic assembly line (inducible operon) consists of three large NRPSs: SrfA-A (402 kDa), SrfA-B (401 kDa) and SrfA-C (144 kDa), comprising of a total of seven modules. The corresponding NRPS genes are organized in the srfA operon. The first step of surfactin biosynthesis is the loading of the fatty acid chain on the L-glutamate residue, previously activated by the first module. In contrast to the majority of NRPSs assembly lines that begin with a initiation module the surfactin protein template starts with an elongation module C-A- PCP (Fig 2) [20]

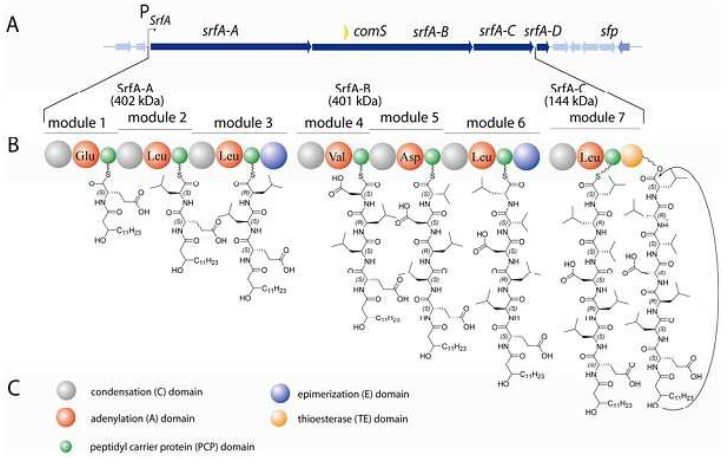
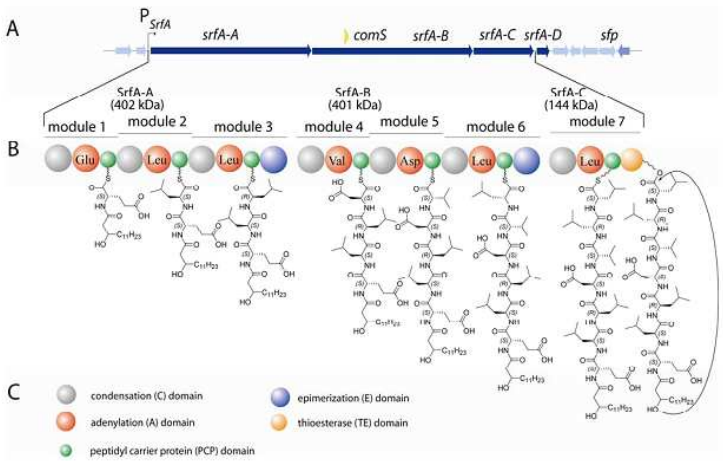


Fig 2: The surfactin biosynthetic assembly line

(A) The surfactin biosynthetic gene cluster of *Bacillus subtilis* encodes for the non-ribosomal protein template for the synthesis of the lipoheptapeptide surfactin. **(B)** This biosynthetic complex consists of three surfactin synthetases: Srf-A,-B,-C, consisting of seven distinct modules, each responsible for recognition, activation and loading of a single amino acid substrate. Two epimerization domains are found in module 3 and 6, converting L-Leu into D-Leu, respectively. The cyclizationn and release of the final heptapeptide as macrolactone is catalyzed by the TE domain.



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The first C domain catalyzes the formation of a covalent peptide-bond between the -amino group of L-glutamate and the -carboxyl group of the fatty acid. The acyl-chain donor could be a CoA-dependent acyl transferase, observed for the first time in the crude cell extract of *B. subtilis* ATCC 21332 [18]. However, this 40 kDa-protein could not be isolated in subsequent studies and the mechanism of surfactin lipo-initiation has not been completely clarified yet. Recently, it was proposed that the protein TE II, encoded by the gene *srfA-D* and involved in the proof-reading of peptide

synthesis [19] may also act as acyl transferase, and possibly be required for in the lipo-initiation of surfactin synthesis [20].

Surfactin biosynthesis proceeds according to the multiple carrier thiotemplate mechanism, by a stepwise activation and loading of the single amino acid substrates. Two epimerization domains, located at the C-terminus of SrfA-A and SrfA-B, respectively, catalyze the conversion of the L-Leu into D-Leu at position 3 and 6. The release of the peptide product from the protein template as macrolactone is catalyzed by the TE domain and is the result of the nucleophilic attack of the-hydroxy moiety of the fatty acid onto the carboxy group of the Leu 7. The resolution of the x-ray structure of Srf-TE provided more insight into the structural basis of the cyclization reaction. [2]

Immediately downstream of the coding region of srfA-C, the srfA-D gene is localized, encoding for the type II thioesterase (TE II), which was shown to have an editing activity. According to the so called "cleaning model", TE IIs restore the peptide synthesis after mis-loading of PCP domains by catalyzing the hydrolysis of the unprocessed aminoacyl-S-PCP substrates. [19, 21]

About 4 kb downstream the *srf*A operon, the *sfp* gene is located, encoding for the PPTase Sfp, which is required for the conversion of the PCP domains of the surfactin biosynthetic complex from their inactive apo- to the active holo-form [14]. Due to its high substrate tolerance, *Sfp* is widely exploited in vitro as well as in vivo for modification of different peptidyl and acyl carrier proteins either with their natural cofactors or with cofactor mimicries.

The strategies described for introducing targeted modifications in NRPs can be divided into two categories: the rational manipulation of biosynthetic NRPS template, and the chemoenzymatic approach.

Current Genetic Study on Surfactin Producing (*sfp*) Locus:

The molecular genetics of antibiotic *subtilin* production has cloned the gene. Recently, a genetic locus involved in the biosynthesis of the dipeptide antibiotic bacilysin was mapped in the *B.subtilis* 168 genome [22]. Additionally, powerful strategies can be designed for studying the regulation of gene expression in *B. subtilis* with the recent development of new techniques such as transposon mutagenesis and gene fusion analysis [23]. The *srf::Tn917* mutation was found to be closely linked to *sfp* and both loci mapped by PBS1 phage transduction to the chromosomal region between *aroI* and *mtlB* which enhanced the production of surfactin. Also the deletion of *spoOH* (*encoding* σ^H) caused 50% increase in surfactin production showing that *srf* gene is independent of role from σ^H transcription [24].

The srfA native promoter was replaced by the constitutive promoter P (repU) in *B. subtilis* 168 after integration of a functional *sfp* gene. Moreover, the plipastatin synthesis was further disrupted in the *B. subtilis* 168 derivatives. In liquid media, an earlier and higher expression of P (repU), than that found with P (srfA), led to a specific surfactin production of fivefold higher after 6 h of culture. On solid media, not only the invasive growth and the haemolytic activity but also the antifungal activity of the constitutive strains was improved when compared to the parental strain BBG111. As expected, the disruption of the plipastatin operon strongly reduced in vitro antifungal properties but interestingly, enhanced specific surfactin production (1.47 g) of biomass, spreading behaviour and haemolytic activity of the strains.

Bacillus subtilis YB8 was found to produce the lipopeptide antibiotics surfactin and plipastatin B1. A gene, *lpa-8*, required for the production of both lipopeptides were cloned from strain YB8. When this gene was inactivated in strain YB8, neither surfactin nor plipastatin B1 was produced. However, the defective strain transformed with an intact *lpa-8* gene had restored ability to produce both peptides.[25] Nucleotide sequence analysis of the region essential for the production of the peptides revealed the presence of a large open reading frame.[26] The deduced amino acid sequence of *lpa-8* (224 amino acid residues) showed sequence similarity to that of sfp (from surfactin-producing *B. subtilis*), *lpa-14* (from iturin A- and surfactin-producing B. subtilis), *psf-1* (from surfactin-producing *Bacillus pumilus*), *gsp* (from gramicidin-S-producing *Bacillus brevis*), and *entD* (from siderophore-enterobactin-producing Escherichia coli), which are able to complement a defect in the *sfp* gene and promote production of the lipopeptide antibiotic surfactin. The sequence similarity among these proteins and the product similarity of cyclic peptides suggest that they might be involved in the biosynthesis or secretion of the peptides. [24, 26]

CONCLUSION

In spite of many laboratory based success in biosurfactants production and its immense commercial applications, the production of biosurfactant at a plant scale remains a challenging issue as the composition of final product is affected by the nutrient, micronutrient and environmental factors. Guidelines and regulations should be formulated for use of biosurfactants in different sectors. It is expected that in future, super-active microbial strains will be developed using genetic engineering for production of biosurfactants at industrial level using renewable substrates as raw material.

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