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Surfactin – Novel Solutions for Global Issues

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1. Introduction

The constant demand for new, effective therapeutic agents has triggered intensive research in the field of diverse antimicrobials of natural origin. These compounds are synthesized by all forms of life and have important biomedical and biotechnological properties, and are thus widely considered a potential solution to the growing problem of resistance to conventional antibiotics, fungal infection and life-threatening diseases.

Among these molecules, lipopeptides represent a unique class of bioactive secondary metabolites with increasing scientific, therapeutic and biotechnological interest. The principal representative of the anionic lipopeptide family is surfactin, which is produced by bacterium *Bacillus subtilis*. This most potent known biosurfactant (i.e. surface-active compound of microbial origin), was named surfactin due to its exceptional surface activity. Since its discovery (Arima et al., 1968) and the identification of its molecular structure as a macrolide lipopeptide (Kakinuma et al., 1969) it has been best recognized for its high amhiphilicity and strong tendency for self-aggregation (Ishigami et al., 1995). Due to these characteristics it shows remarkable surface-, interface- and membrane-active properties, resulting in a number of promising biological activities, which are of great relevance in health care and biotechnology. These properties make surfactin a candidate drug for the resolution of a number of global issues in medicine (Banat et al., 2010; Cao et al., 2010), industry (Nitschke & Costa, 2007; Abdel-Mawgoud et al., 2008) and environmental protection (Mulligan, 2009).

2. Structure and physicochemical properties

Surfactin (M.W. 1036 Da), an amphipathic cyclic lipopeptide, is constituted by a heptapeptide (ELLVDLL) with the chiral sequence LLDLLDL interlinked with β-hydroxy fatty acid of the chain lengths 12 to 16 carbon atoms to form a cyclic lactone ring structure (Fig. 1). Hydrophobic amino acid residues are located at positions 2, 3, 4, 6 and 7, while the glutamyl and aspartyl residues, located at positions 1 and 5 respectively, introduce two negative charges to the molecule. Several surfactin isoforms usually coexist in the cell as a mixture of several peptidic variants with a different aliphatic chain length (Hue et al., 2001; Bonmatin et al., 2003; Tang et al., 2007). The pattern of amino acids and β-hydroxy fatty acids in the surfactin molecule depends not only on the producing bacterial strain involved, but also on the type of culture conditions.

Fig. 1. Primary structure of surfactin

The molecular assembly of surfactin in an aqueous solution and the conformation of the molecules in aggregates condition its physicochemical activities and biological properties. Surfactin adopts a β-turn, forming a β-sheet with a characteristic horse-saddle conformation, which is probably responsible for its broad spectrum of biological activities, even at such low concentrations. The β-turn may be formed by an intramolecular hydrogen bond, whereas the β-sheet may depend on an intermolecular hydrogen bond (Bonmatin et al., 1994; Han et al., 2008; Zou et al., 2010). The two charged side-chains are gathered on the same side and form a "claw", providing a polar head opposite to the hydrophobic domain (Tsan et al., 2007).

The three-dimensional structure of surfactin (Fig. 2) was determined via a high-resolution ^{1}H NMR combined with molecular imaging techniques. On one side of the molecule, residues 2 and 6 face each other in the vicinity of the acidic Glu-1 and Asp-5 side chains, which define a minor polar domain (Bonmatin et al., 1995). On the opposite side, residue 4 faces the connection of the lipidic chain constituting a major hydrophobic domain, which includes the side-chains of residues 3 and 7 to a lesser extent, accounting for its amphiphilic nature and its strong surfactant properties (Tsan et al., 2007). Below the critical micelle concentration (CMC), the lipidic chain should extend freely in solution but it strongly participates in hydrophobic interactions in supramolecular structures such as lipid micelles or oligomers at the air/water interface (Peypoux et al., 1999).

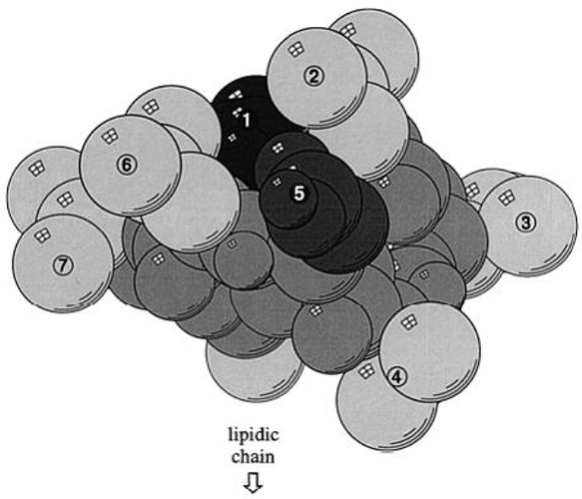

Fig. 2. Three-dimensional structure of surfactin peptide moiety. Backbone atoms are shown in grey. The heavy atoms of amino acid residues (1 to 7) are shown. Pale grey represents hydrophobic residues 2, 3, 4, 6, 7 and the attachment of the lipidic chain. Acidic residues 1 and 5 are in black and dark grey respectively (Peypoux et al., 1999).

Surfactin lowers the surface tension of water from 72 $mN.m^{-1}$ to 27 $mN.m^{-1}$ at a concentration as low as 10 µmol/l (Heerklotz & Seelig, 2001). The critical micelle concentration varies depending on the methods and experimental conditions used, but reaches values of 7.5 – 20 µmol/l (Morikawa et al., 2000; Shen et al., 2010b; Zou et al., 2010), which is about two orders of magnitude smaller than those of most other detergents. Consequently, the consumption of surfactin in practical applications can be lower by several orders when compared with chemical surfactants.

Surfactin has a strong self-assembly ability to form micelles, and these micelles tend to form larger aggregates. Surfactin micelles are inhomogeneous with regard to size distribution with different configurations. The aggregation number of sphere-like micelles is much smaller than that of conventional surfactants, i.e. 11 (Zou et al., 2010) or 20 (Shen et al., 2009). The structure of the micelle is of the core-shell type, with the hydrocarbon chain and the hydrophobic residues forming the core of the micelle (Shen et al., 2009). However, different types of micelles with up to 170 surfactin molecules forming spherical, ellipsoidal and/or cylindrical structures were also found (Heerklotz & Seelig, 2001; Zou et al., 2010).

3. Surfactin-membrane interactions

Recognition of the interaction between surfactin and membrane is an essential prerequisite to understanding its biological activity. Amphiphilic surfactin molecules destabilize the membrane and disturb its integrity (Bernheim & Avigad, 1970). Since this early observation was made, many excellent studies have focused on the description of the surfactin-membrane bilayer system, documenting its exceptional complexity (see parts 3.1., 3.2.). The hypothetical mechanisms of the interactions of surfactin with membrane structures exhibit a complex pattern of effects, such as insertion into lipid bilayers, chelating of mono- and divalent cations, modification of membrane permeability by channel formation or membrane solubilisation by a detergent-like mechanism.

Surfactin penetrates into the lipid membrane through hydrophobic interactions, thus influencing both the order of hydrocarbon chains and the membrane thickness. Upon this primary collision, the peptide cycle then shows conformational changes, which further facilitate the process of interaction (Maget-Dana & Ptak, 1995). Following the incorporation of surfactin into the membrane, the dehydration of the phospholipid polar head groups occurs, perturbing the local lipid packing and strongly compromising bilayer stability, i.e. its barrier properties. A key step for membrane destabilization and leakage is the dimerisation of surfactin into the bilayer. These structural fluctuations may well explain the primary mode of the antibiotic action and the other important biological effects of this lipopeptide (Carrillo et al., 2003).

3.1 Impact of surfactin nature

The extent of perturbation of the phospholipid bilayer correlates with the concentration of surfactin. In a model dimyristoylphosphatidylcholine (DMPC) bilayer system disintegration occurs in three stages. At low concentrations (up to 4 mol%), surfactin penetrates readily into the outer leaflet of the membrane within the head group and part of the adjacent hydrophobic chain region (Shen et al., 2010b). Here it is miscible with the phospholipids, forming mixed micelles. After it reaches a threshold level, the lipopeptide forms domains segregated within the phospholipid bilayer, i.e. pores in the membrane, and the lipid bilayer

is progressively disrupted into sheet-like lamellar membrane fragments due to increasing strains in the membrane caused by further uptake of surfactin molecules (up to 10 mol%). Finally, at a surfactin concentration higher than 10 mol%, thread-like micelles of 6.5 nm in diameter were detected which tended to organize into loops of various sizes (Kell et al., 2007; Boettcher et al., 2010; Liu et al., 2010; Shen et al., 2010a).

Surfactin-membrane interactions can also be described via the quantification of the local surfactin-to-lipid mole ratio within the membrane R_b. This parameter determines the concentration of membrane-bound detergent and the lipid concentration. Membrane leakage starts at $R_b \sim 0.05$ with an aqueous surfactin concentration of 2 µmol/l. The permeabilising activity of surfactin is thus stronger by one order of magnitude than that of detergents such as Triton X-100, which solubilises the membrane at about $R_b \sim 0.6$. At higher concentrations, i.e. $R_b \sim 0.15$, surfactin-rich clusters are formed in the membrane, inducing leaks. Membrane lyses or solubilisation to micelles begin at $R_b \sim 0.22$ and a concentration of 9 µmol/l (Heerklotz and Seelig, 2007). Periodic variations of fluid, surfactin-rich regions and gel lipid-rich domains within the bilayer membrane result in the formation of stable nanoripple structures with intriguing potential in biomedical and biotechnological applications (Brasseur et al., 2007; Kell et al., 2007; Banat et al., 2010).

Membrane penetration by surfactin is facilitated by the presence of cations (Maget-Dana & Ptak, 1995). The Glu-1 and Asp-5 acidic residues form a tailored "claw", which can easily stabilize a surfactin-Ca^{2+} 1:1 complex via an intramolecular bridge (Maget-Dana & Ptak, 1992). This effect of Ca^{2+} ions on the surfactin conformation promotes the deeper insertion of lipopeptide into the membrane (Grau et al., 1999). Surfactin can also drive mono- and divalent cations through an organic barrier, divalent cations being transported with greater efficiency (Thimon et al., 1992). The selective affinity can be correlated with the partial neutralization of the two acidic residues at the air/water interface in the presence of Na^+ or K^+, whereas Ca^{2+} induces a complete neutralization (Maget-Dana & Ptak, 1992). One physiological result of surfactin cation chelation is the inhibition of the cyclic AMP phosphodiesterase activity (Hosono & Suzuki, 1983). The presence of counterions such as Na^+, Li^+, K^+, Mg^{2+} and Ca^{2+} increases the surface activity of surfactin and reduces its CMC. These ions decrease electrostatic repulsions between the surfactin head groups and enhance the formation of micelles (Li et al., 2009a; Li et al., 2009b).

The cyclic nature of the peptide moiety and the number of negative charges, as well as the fatty acid chain length, play a significant role in lipopeptide activity. Variations both in the peptide and lipid moiety of the surfactin molecule can profoundly modulate the structure-function relationship. Within the large hydrophobic domain, position 4 showed a high contribution as the L-Val4/L-Ile4 substitution induced a 2-fold decrease in CMC and a substantial gain in monolayer stability at the air/water interface (Bonmatin et al., 1995). The loss of cyclic nature weakens the degree of surfactin binding. With regards to the effect of the surfactin acyl chain length, the longer the acyl chain, the better its insertion into the lipid bilayer (Razafindralambo et al., 2009). Higher surface activity was observed with a C14 acyl chain, while antiviral properties were stronger when the C15 chain prevailed in surfactin (Bonmatin et al., 2003; Eeman et al., 2006). The membrane activity of surfactin has also been shown to increase with the number of ionic charges of the polar head (Francius et al., 2008).

3.2 Impact of target membrane composition

Several *in vitro* studies have demonstrated the impact of lipid composition on surfactin-membrane interaction and its penetration into the target bilayer; however the mechanism

has so far not been described in detail. Both the polar heads and fatty acid chains play a role in the formation of complexes of surfactin with phospholipids. Surfactin perturbs more strongly membranes containing phospholipids with a shorter chain length (Grau et al., 1999).
These data were also confirmed for the model monolayer system using atomic force microscopy (AFM). This method has become an additional, powerful tool in the investigation of the organization of lipid monolayers and bilayers and the monitoring of their interaction with membrane active peptides, such as surfactin. As the biological activity of surfactin is directly related to its interaction with membranes, understanding the mixing behaviour and domain formation of this molecule within lipid monolayers and bilayers is an important challenge (Deleu et al., 2001; Brasseur et al., 2008).
Polar headgroup composition profoundly affects the interfacial behaviour of surfactin. The miscibility of surfactin with dipalmitoylated (DP) phospholipids decreases in the order phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine (PC>PE>PS). These surfactin-phospholipid interactions are modulated by not only the volume of the phospholipid headgroups (Bouffioux et al., 2007), but also their electrostatic properties and shape (Buchoux et al., 2008). The inverted-cone conformation of surfactin tends to counter-balance the ability of cone-shaped phosphatidylethanolamine molecules to form a hexagonal phase, thereby promoting surfactin stabilization in the membrane. In the case of DPPS, surfactin decreases the electrostatic repulsions between the negative headgroups of DPPS through the large surfactin peptide cycles that result in DPPS-surfactin stability (Grau et al., 1999; Carrillo et al., 2003).
Regarding phospholipid chain lengths, the miscibility between surfactin and phospholipids is higher for shorter chain lengths in the order DMPC (dimyristoyl) > DPPC (dipalmitoyl) > DSPC (distearoylphosphatidylcholine), i.e. 14, 16 and 18 carbon atoms (Bouffioux et al., 2007). On the other hand, the fact that lipid chains are in a fluid or gel phase does not appear to be important, in contrast to other antimicrobial peptides, such as melittin (Buchoux et al., 2008).
There is still some uncertainty regarding the role played by the presence of a negative charge on the phospholipid polar head, which, according to Maget-Dana and Ptak (Maget-Dana & Ptak, 1995) gives rise to electrostatic shielding, preventing the peptide cycle of surfactin from coming close to the phospholipid headgroups. The presence of a net negative charge in the phospholipid monolayer promotes the immiscibility of surfactin into the lipid matrix, therefore favouring surfactin self-assembly. By contrast, this phenomenon is the basis of the pore-forming activity of surfactin in membranes with a significantly high amount of anionic lipids, such as bacterial membranes and some cancer cells (Eeman et al., 2006). A hypothetical model has been proposed for membrane lyses based on charge repulsions between surfactin negative charges and the lipid head group negative charges. This leads to a local increase in membrane curvature and the complete destabilization of the planar membrane, i.e. its direct lyses (Buchoux et al., 2008).
All of the above findings were obtained *in vitro* on model monolayers or bilayers. By contrast, no research deals with the interaction of surfactin with real membrane phospholipids or even intact membranes, where proteins play a crucial role. Our results show subsequent accumulation of cardiolipin (CL) in the *B. subtilis* cytoplasmic membrane during the stationary phase of growth, when surfactin is synthesized. Such an increase in CL, which is regarded as a stress phospholipid stabilizing the membrane bilayer, may therefore support membrane integrity. Additionally, CL bearing two negative charges could prevent the anionic surfactin from coming close to the surface of the membrane bilayer.

However, the putative relation between the surfactin production and the extensive membrane reconstruction would require further analysis (Seydlova & Svobodova, 2008a).

4. Biological and physiological relevance of surfactin

B. subtilis initiates the synthesis of secondary metabolite surfactin through the onset of the stationary growth phase when the culture is becoming short of nutrients and oxygen. Under these famine conditions the cells also activate other survival strategies, such as antibiotic production, sporulation, genetic competence development and the production of extracellular degradative enzymes. Therefore it is reasonable that surfactin or antibiotic synthesis in general provide at least some benefits for the producer, otherwise it would not retain in nature (Stein, 2005).

Lipopeptides are amongst the most frequently produced *B. subtilis* antibiotics. Several possible roles have been proposed for these compounds, such as participation in the acquisition of hydrophobic water-insoluble nutrients and influencing the attachment or detachment of bacteria to and from surfaces (Rosenberg & Ron, 1999). Surfactin is required for raising the fruiting-body-like aerial structures on the surface of *B. subtilis* colonies, where the spores are preferentially developed (Branda et al., 2001). On the other hand, it inhibits the aerial hyphal growth of *Streptomyces coelicolor*, suggesting a possible ecological role (Straight et al., 2006). These properties probably contribute to the survival of *B. subtilis* in its natural habitat.

Surfactin plays a key role in the induction and development of biofilms, i.e. highly structured multicellular communities that adhere to surfaces and constitute the majority of bacteria in most natural ecosystems and are also responsible for many health and industrial problems (Stanley & Lazazzera, 2004). Cells within biofilms are more resistant to biocides and antibiotics; part of this resistance is attributed to the protection provided to the self-produced extracellular matrix, which encases the cells (Lopez et al., 2009b). Swarming, motility in colonies of *B. subtilis* cells, is conditioned by proteins encoded by swrA, swrB, swrC and efp genes (Kearns et al., 2004) and is strictly dependent on the production of surfactin, which reduces surface tension and allows spreading (Kinsinger et al., 2005). Its secretion is stimulated by potassium ions (Kinsinger et al., 2003). Recent improvements in time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging have enabled the demonstration of surfactin distribution and its precise localization within a swarming colony. Secreted surfactin diffuses freely from the mother colony to the periphery of the swarm and forms a gradient (Debois et al., 2008). This gradient generates surfactant waves, i.e. surface-tension gradients on which the colony spreads outward (Angelini et al., 2009). Laboratory strains such as *B. subtilis* 168, which fail to produce surfactin, do not exhibit swarming motility (Julkowska et al., 2005; Patrick & Kearns, 2009).

Within biofilm, cells differentiate from a predominantly unicellular motile state to a genetically identical mixture of cell types with distinct phenotypes. Cells exhibit specialized functions such as sporulation, matrix production, genetic competence, production of surfactin, cannibalism toxins or exoproteases (Kolter, 2010; Lopez & Kolter, 2010). The formation of these multicellular communities involves extensive intercellular communication via the recognition of and responding to small, secreted, self-generated molecules, i.e. quorum sensing. This also applies to surfactin, which does not trigger multicellularity acting as a surfactant, but rather as autoinducer or a signalling molecule for quorum sensing. It causes potassium leakage across the cytoplasmic membrane, which leads to the activation of

protein kinase KinC, affecting the expression of genes involved in the synthesis of the extracellular matrix. This represents a previously undescribed quorum-sensing mechanism (Lopez et al., 2009a).

Extracellular surfactin signalling is unidirectional. Surfactin production is triggered in a small subset of cells responding to another signalling molecule ComX, which is synthesized by most cells in the population. Surfactin then acts as a paracrine signal that leads to extracellular matrix production in a different subpopulation of cells, which can then no longer respond to ComX and therefore cannot became surfactin producers (Lopez et al., 2009d). The blockage of signalling molecules caused by the extracellular matrix has been reported in eukaryotes to define the distinct cell fates in morphogenesis. These results indicate that bacteria display attributes of multicellular organisms.

In the same undifferentiated subpopulation of cells, surfactin can trigger not only the production of extracellular matrix but also cannibalism, as a mechanism to delay sporulation. Cannibal cells secrete Skf (sporulation-killing factor) and Sdp (sporulation-delaying factor) toxin systems while at the same time expressing self-resistance to these peptides. The nutrients released from the sensitive siblings promote growths of matrix producers and their DNA can be taken up by competent cells that originate from the fraction of surfactin producers. The coordinated expression of cannibalism and matrix production can result in a fitness advantage in natural habitats by providing both protection and an effective tool to compete for the same resources with neighbouring bacteria (Lopez et al., 2009c).

The developmental pathways controlling sporulation, cannibalism and matrix production are strongly interconnected – they are activated by the same master regulatory protein Spo0A, which can be phosphorylated by the action of different kinases (KinA-E) and presumably therefore different levels of phosphorylation can be reached. Higher levels are necessary to trigger sporulation, whereas lower levels activate matrix production and cannibalism (Fujita et al., 2005).

In our experiments (unpublished data) we determined an interval of sublethal surfactin concentrations that modify the growth of *B. subtilis* 168 that does not produce surfactin. Unexpectedly, two different effects, dependent on surfactin concentration, were discovered that either inhibit or even stimulate the growth of *B. subtilis* 168, the former concentration being higher than the latter. When an exponentially growing *B. subtilis* culture is exposed to exogenously-added surfactin on a nutrient agar plate, the growth stops for a time and is restored with a decreased growth rate in inhibitory concentration, whereas the stimulatory concentration accelerates growth and results in a higher final density of the population. The observations mentioned in the above paragraph led us to speculate that a low concentration of surfactin may induce both matrix production, which protects the cells from the deleterious effect of surfactin, and cannibalism that provides the population with nutrients released from killed siblings. Although this hypothesis has yet to be verified, it is apparent that some optimum surfactin concentration benefits the population as a whole.

5. Potential biomedical applications

The high demand for new chemotherapeutics driven by the increased drug resistance of pathogenes has drawn attention to the use of biosurfactants as new antimicrobial agents (Seydlova & Svobodova, 2008b). Surfactin exhibits a wide range of interactions with target cell membranes and has potential for various medical applications. Besides its antifungal and antibacterial effects (Thimon et al., 1992), surfactin can also inhibit fibrin clot formation

(Arima et al., 1968), inhibits platelet and spleen cytosolic phospholipase A2 (PLA2) (Kim et al., 1998) and exhibits antiviral (Kracht et al., 1999) and antitumor activities (Kameda et al., 1974). Another interesting property of surfactin is that high surfactin concentration affects the aggregation of amyloid β-peptide (Aβ(1-40)) into fibrils, a key pathological process associated with Alzheimer's disease (Han et al., 2008).

Resistance is generally rare against all lipopeptides and the development of a well-defined resistance mechanism has been suggested to be unlikely (Barry et al., 2001). The explanation for this can be found in the complex chemical composition of membranes. The single-component modification of this target structure can hardly cause resistance to surfactin. Therefore, lipopeptide molecules with their unusual structures, which act rapidly on membrane integrity, rather than on other cell targets, are of growing interest in modern medicine and might hold promise for the development of a new generation of antibiotics (Goldberg, 2001).

This is of particular importance at a time when multi-resistant pathogens overcoming the last-resort drugs, including methicillin and vancomycin, pose a growing threat (Singh & Cameotra, 2004). These antibiotics are used not only in the therapy of nosocomial infections caused by enterococci and *Staphylococcus aureus* (Yoneyama & Katsumata, 2006) but also in the therapy of community-acquired methicillin resistant *S. aureus* (caMRSA), which is much more aggressive than its hospital relatives due to having a particular preference for the young and healthy (Hadley, 2004). The recent detection of Enterobacteriaceae with the New Delhi Metallo-β-lactamase (NDM-1) enzyme, which makes bacteria resistant to the main classes of antibiotics used in the treatment of Gram-negative infections, is alarming (Yong et al., 2009). Furthermore, most isolates carried the bla_{NDM-1} gene on plasmids, which are readily transferable (Kumarasamy et al., 2010).

5.1 Antibacterial, anti-inflammatory and antifungal effects

It has long been asserted that the antibacterial properties of anionic antimicrobial peptides are limited due to the repulsive forces between their negative charge and the negatively charged surface of the bacterial surface. Nevertheless, a number of recent studies show inhibitory effects against different bacteria of high medical, environmental or agricultural importance.

Lipopeptide biosurfactants produced by *B. subtilis* R14 (Fernandes et al., 2007) and the marine *Bacillus circulans* (Das et al., 2008) share a lot of surfactin characteristics and were found to be active against multidrug-resistant bacteria such as *Proteus vulgaris*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*. The minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations used were much lower than those of the conventional antibiotics tested in the same time (Das et al., 2008).

The increasing trend to limit the use of chemical food preservatives has generated considerable interest in natural alternatives. It has been observed that a lipopeptide substance containing surfactin is able to damage the surface structure of spores of the recognized food-borne bacterium *B. cereus*, leading to their disruption (Huang et al., 2007). Other results showed that *E. coli* in milk had high sensitivity to a mixture of surfactin with fengycin and can be sterilised by five orders of magnitude even at the temperature of 5.5 °C (Huang et al., 2008). Similar promising observations were made using a combination of surfactin with another lipopeptide iturin to sterilise *Salmonella enteritidis* in meat (Huang et al., 2009). The same antimicrobial peptides were also successful in the antifungal effect

against *Penicillium notatum* (Huang et al., 2010). This is of particular relevance in order to ensure food safety.

A culture broth containing surfactin was used to selectively control bloom-forming cyanobacteria, which cause environmental problems due to the production of malodorous compounds and toxins in eutrophic lakes. The surfactin-containing broth inhibited the growth of *Microcystis aeruginosa* and *Anabaena affinis* at a concentration at which chemical surfactants such as Tween 20, Span 80 and Triton X-100 had no effect (Ahn et al., 2003).

Environmentally-friendly solutions are still needed for application in agriculture. It has been found that surfactin and iturin synergistically exhibit an antifungal effect against the fungal pathogen *Colletotrichum gloeosporioides*, causing damage to crops around the world (Kim et al., 2010). These lipopeptides are less toxic and show better reduction and control of phytopathogens than agrochemicals (Souto et al., 2004; Chen et al., 2008; Kim et al., 2010). In another study a mixture of surfactin and iturin disintegrated the cell wall of the gram-negative phytopathogen *Xanthomonas campestris* (Etchegaray et al., 2008). Surfactin was also shown to display antimicrobial activity against *Paenibacillus larvae*, an extremely contagious and dangerous pathogen of honeybees (Sabate et al., 2009).

Surfactin is known to inhibit phospholipase A2, involved in the pathophysiology of inflammatory bowel disease, which is related to ulcerative colitis and Crohn's disease. Oral administration of a natural probiotic *B. subtilis* PB6 secreting surfactin in a rat model with TNBS-induced (trinitrobenzene sulfonic acid) colitis suppressed the colitis, significantly lowering the plasma levels of pro-inflammatory cytokines and significantly increasing anti-inflammatory cytokine (Selvam et al., 2009). Lipopeptide production by probiotic *Bacillus* strains is one of the main mechanisms by which they inhibit the growth of pathogenic microorganisms in the gastrointestinal tract (Hong et al., 2005).

Several recent studies have revealed the impact of surfactin in silencing the inflammatory effect of lipopolysaccharide (LPS) interaction with eukaryotic cells. Compounds that inactivate LPS activity have potential as new anti-inflammatory agents. Surfactin was shown to suppress the interaction of lipid A with LPS-binding protein (LBP) that mediates the transport of LPS to its receptors. Moreover, surfactin did not influence the viability of the eukaryotic cell lines tested (Takahashi et al., 2006). Surfactin also inhibits the LPS-induced expression of inflammatory mediators (IL-1β and iNOS) (Hwang et al., 2005) and reduces the plasma endotoxin, TNF-α and nitric oxide levels in response to septic shock in rats (Hwang et al., 2007). Surfactin downregulates LPS-induced NO production in macrophages by inhibiting the NF-κB transcription factor (Byeon et al., 2008). The surfactin-induced inhibition of NF-κB, MAPK and Akt pathways also leads to the suppression of the surface expression of MHC-II and costimulatory molecules in macrophages, suggesting the impairment of their antigen-presenting function. These results indicate that surfactin is a potent immunosuppressive agent and suggest an important therapeutic implication for transplantation and autoimmune diseases including arthritis, allergies and diabetes (Park & Kim, 2009).

5.2 Anti-mycoplasma effects

Mycoplasmata are the etiological agents of several diseases and also the most significant contaminants of tissue culture cells. Surfactin is already used commercially for the curing of cell cultures and cleansing of biotechnological products of mycoplasma contamination (Boettcher et al., 2010). The treatment of mammalian cells contaminated by mycoplasmata with surfactin improved proliferation rates and led to changes in cell morphology. In addition, the low cytotoxicity of surfactin to mammalian cells permitted the specific

inactivation of mycoplasmata without having significantly detrimental effects on the metabolism of cells in the culture (Vollenbroich et al., 1997b). A recent study confirmed the potential of surfactin to kill *Mycoplasma pneumoniae* (MIC 25 μM) independently of target cell concentration, which is a significant advantage over the mode of action of conventional antibiotics. Surfactin has exhibited, in combination with enrofloxacin, a synergistic effect resulting in mycoplasma-killing activity at about two orders of magnitude greater than when entire molecules are used separately (Fassi Fehri et al., 2007). More recently, surfactin was described as inhibiting the expression of proinflammatory cytokines and NO production in macrophages induced by *Mycoplasma hyopneumoniae* (Hwang et al., 2008a). In another study, surfactin showed a strong cidal effect (MIC 62 μM) and in combination with other antibacterials exhibited additive interaction, which could be clinically relevant (Hwang et al., 2008b).

5.3 The role of surfactin in surface colonization by pathogens

Swarming motility and biofilm formation are the key actions in the colonization of a surface by bacteria and increase the likelihood of nosocomial infections associated with various medical appliances, such as central venous catheters, urinary catheters, prosthetic heart valves, voice prostheses and orthopaedic devices. These infections share common characteristics even though the microbial causes and host sites vary greatly (Rodrigues et al., 2006). The most important of these features is that bacteria in biofilms are highly resistant to antibiotics, evade host defenses and withstand traditional antimicrobial chemotherapy, making them difficult to treat effectively (Morikawa, 2006). Moreover, in food-processing environments, the control of microorganisms' adherence to material surfaces is an essential step to meet food safety requirements.

Recent studies have suggested that non-antibiotic molecules naturally produced within bacterial communities, such as surface active biosurfactants, could also interfere with biofilm formation by modulating microbial interaction with interfaces (Banat et al., 2010). Biosurfactants, such as surfactin, have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or infection sites. Surfactin decreases the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *Eschericha coli* and *Proteus mirabilis* in polyvinyl chloride wells, as well as vinyl urethral catheters. The precoating of catheters by running the surfactin solution through them prior to inoculation with media was just as effective as the inclusion of surfactin in the growth medium. Given the importance of opportunistic infections with Salmonella species, including the urinary tract of AIDS patients, these results have potential for practical application (Mireles et al., 2001).

Substances containing surfactin have also been shown to possess specific anti-adhesive activity that selectively inhibits the biofilm formation of two pathogenic strains of *S. aureus* and *E. coli* on polystyrene by 97% and 90%, respectively (Rivardo et al., 2009). In another study, Rivardo et al. observed a synergistic interaction between surfactin and silver, acting as effective antibiofilm agents. Negatively charged surfactin increases metal solubility and may therefore facilitate the penetration through the exopolymeric substance that encapsulates biofilm and provides its protection (Rivardo et al., 2010). Moreover it was demonstrated that surfactin increases the efficiency of eradication of different antibiotics against a urinary tract-infective *E. coli* strain (Banat et al., 2010).

The preconditioning of stainless steel and polypropylene surfaces with 0.1% (w/v) surfactin reduces the number of adhered cells of food pathogens *Listeria monocytogenes* and

Enterobacter sakazakii. The absorption of surfactin on polystyrene also reduced the colonization of *Salmonella enteritidis* (Nitschke et al., 2009). Considering that surfactin has an anionic nature, the observed anti-adhesive effect can be due to the electrostatic repulsion between bacteria and the molecules of surfactin adsorbed onto the polystyrene surface (Zeraik & Nitschke, 2010). All in all, these results outline a new potential of surfactin as an anti-adhesive compound that can be explored in the protection of surfaces from microbial contamination.

5.4 Anti-viral activity

Surfactin is active against several viruses, including the Semliki Forest virus, herpes simplex virus (HSV-1 and HSV-2), vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and the murine encephalomyocarditis virus. The inactivation of enveloped viruses, especially herpes viruses and retroviruses, is significantly more efficient than that of non-enveloped viruses. This suggests that the antiviral action of surfactin is primarily due to the physicochemical interaction between the membrane active surfactant and the virus lipid membrane (Vollenbroich et al., 1997a). One important factor for virus inactivation is the number of carbon atoms in the acyl chain of surfactin. The capacity for virus inactivation increases with rising fatty acid hydrophobicity. During the inactivation process, surfactin permeates into the lipid bilayer, inducing complete disintegration of the envelope containing the viral proteins involved in virus adsorption, and penetration to the target cells. Its absence accounts for the loss of viral infectivity (Kracht et al., 1999).

Recently, it has also been observed that antimicrobial lipopetides containing surfactin inactivate cell-free viruses of the porcine parvovirus, pseudorabies virus, Newcastle disease virus and bursal disease virus (Huang et al., 2006).

5.5 Antitumor activity

Surfactin has been reported to show antitumor activity against Ehrlich's ascite carcinoma cells (Kameda et al., 1974). A recent study on the effect of surfactin on the proliferation of a human colon carcinoma cell line showed that surfactin strongly blocked cell proliferation. The inhibition of growth by surfactin was due to the induction of apoptosis and cell cycle arrest via the suppression of cell survival regulating signals such as ERK and PI3K/Akt (Kim et al., 2007).

Another study revealed that surfactin inhibits proliferation and induces apoptosis of MCF-7 human breast cancer cells trough a ROS/JNK-mediated mitochondrial/caspase pathway. Surfactin causes the generation of reactive oxygen species (ROS), which induce the sustained activation of survival mediator ERK1/2 and JNK, which are key regulators of stress-induced apoptosis. These results suggest that the action of surfactin is realized via two independent signalling mechanisms (Cao et al., 2010). The induction of apoptotic cell death is a promising emerging strategy for the prevention and treatment of cancer.

5.6 Thrombolytic activity

The plasminogen-plasmin system involved in the dissolution of blood clots forms part of a variety of physiological and pathological processes requiring localized proteolysis. Plasminogen is activated proteolytically using a urokinase-type plasminogen activator (u-PA), which is initially secreted as a zymogen prourokinase (pro-u-PA). Along with activation by u-PA, the plasminogen itself has an activation mechanism involving conformational change. The reciprocal activation of plasminogen and prourokinase is an

important mechanism in the initiation and propagation of local fibrinolytic activity. Surfactin at concentrations of 3 – 20 μmol/l enhances the activation of prourokinase as well as the conformational change in the plasminogen, leading to increased fibrinolysis *in vitro* and *in vivo* (Kikuchi & Hasumi, 2002). In a rat pulmonary embolism model, surfactin increased plasma clot lysis when injected in combination with prourokinase (Kikuchi & Hasumi, 2003). Surfactin is also able to prevent platelet aggregation, leading to the inhibition of additional fibrin clot formation, and to enhance fibrinolysis with the facilitated diffusion of fibrinolytic agents (Lim et al., 2005). The anti-platelet activity of surfactin is due not to its detergent effect, but to its action on downstream signalling pathways (Kim et al., 2006). These results suggest a possible use for surfactin in urgent thrombolytic therapy related to pulmonary, myocardial and cerebral disorders. Moreover, surfactin has advantages over other available thrombolytic agents because it has fewer side effects and therefore has potential for long-term use.

5.7 Antiparasitic activity

Vector control is a key point of various strategies aiming at interrupting the transmission of mosquito-borne diseases. The culture supernatant of a surfactin producing *B. subtilis* strain was found to kill the larval and pupal stages of mosquito species *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. As few biocontrol agents or insecticides are effective against mosquito pupae, this could be a promising tool for application in control programmes (Geetha et al., 2010).

Surfactin was also reported to act as a Sir2 inhibitor (silent information inhibitor 2). Sir2 belongs to the NAD^+ dependent histone deacetylases, which modulate the acetylation status of histones, regulate transcription, DNA replication and repair and have been implicated in pathogenesis of *Plasmodium falciparum*, causing cerebral malaria. Surfactin functions as a competitive inhibitor of NAD^+ and an uncompetitive inhibitor of acetylated peptide. Surfactin was also found to be a potent inhibitor of intra-erythrocytic growth of *P. falciparum in vitro*, with an IC_{50} value in the low micromolar range (Chakrabarty et al., 2008).

Surfactin can also be used as alternative treatment for nosemosis. When exposed to surfactin, the spores of *Nosema ceranae*, the causative agent of the most frequently parasitic infection in *Apis mellifera*, reveal a significant reduction in infectivity. Moreover, when surfactin is administered *ad libitum* and is introduced into the digestive tract of a bee, it also leads to a substantial reduction in parasitosis development (Porrini et al., 2010).

6. Obstacles and perspectives

In general, biosurfactants produced from microorganisms possess more advantages over their chemical counterparts, such as diversity, biodegradability, lower toxicity, biocompatibility and stability over wide range of pH. Nevertheless, they have not been widely used so far due to their high production costs, caused primarily due to low yields and high recovery expenses that cannot meet the economic needs of industrial production. Similar limitations hinder the exploitation of surfactin potential applications in medicine and industry, as well as environmental protection. Numerous studies have been made on the optimization of surfactin yields at the level of production conditions, hyperproducing mutant construction and downstream processing of the crude product or in seeking surfactin producers in extreme habitats (Das et al., 2008) and the development of novel methods for the rapid screening of producers (das Neves et al., 2009). On the other hand, the

relatively low (μmol/l) effective concentration in biological systems could facilitate its use in biomedicine.
However, surfactin also needs to conform to some additional requirements, such as detailed knowledge of the mechanism of interaction with the target cells and possible cytotoxicity effects to the treated macroorganism. Genetic and biochemical engineering approaches to create a tailor-made molecules (Symmank et al., 2002), or surfactin analogues with modified properties represent a possible solution for the future. Surprisingly, almost no research has been focused on the principle of surfactin resistance of the producer, which can not only bring a valuable piece of information for improving yields, but is also crucial for possible medical applications.

6.1 Toxicity

One of the plausible drawbacks of the potential use of surfactin in medical applications is its haemolytic activity, as observed in *in vitro* experiments, which results from surfactin's ability to disturb the integrity of the target cell membranes. The concentration-dependent haemolytic effect of surfactin was described as the concentration of surfactin that bursts 50% of red blood cells (HC_{50}), which is equal to 300 μmol/l (Dufour et al., 2005). On the other hand, surfactin concentrations used in various biomedical studies were far below the threshold, i.e. 30 μmol/l. The lowest surfactin concentration that completely inhibited the growth of mycoplasmata after 48 h (MIC) was 25 μmol/l (Fassi Fehri et al., 2007); 30 μmol/l surfactin treatment displayed significant anti-proliferative activity in human colon cancer cells (Kim et al., 2007) and was able to induce apoptosis in human breast cancer cells (Cao et al., 2010). The same surfactin concentration is also capable of inhibiting the immunostimulatory function of macrophages (Park & Kim, 2009).
The LD_{50} (Lethal Dose, 50%; the dose required to kill half the members of a tested population) of surfactin is at > 100 mg/kg, *i.v.* in mice (Kikuchi & Hasumi, 2002). An oral intake of up to 500 mg/kg per day of the lipopeptide did not show apparent toxicities. Surfactin demonstrated no maternal toxicity, fetotoxicity, and teratogenicity in ICR mice (Hwang et al., 2008c). Surfactin did not show any toxicological effects at dose 2500 mg/kg after a single oral administration in rats. The no-observed-adverse-effect level (NOAEL) of surfactin was established to be 500 mg/kg following repeat (4 weeks) oral administration. No surfactin-related toxicities in survival, clinical signs, haematological parameters and histopathological observations of haematopoietic organs were found (Hwang et al., 2009).
Surfactin did not influence the viability of HUVEC (human umbilical vein endothelial cells) up to 30 μg/ml after 24 h. Surfactin was also regarded as being less toxic than other surfactants, as judged from the results of an acute toxicity study in mice (Takahashi et al., 2006) and also as a safer anti-endotoxin agent in comparison with polymyxin B (Hwang et al., 2007).
Another option for reducing surfactin toxicity is to design a tailor-made molecule. Minor alterations in the chemical structure of the molecule may lead to a dramatic adjustment in the toxicity profile of any compound. Genetic engineering of the surfactin synthetase resulted in the production of a novel antimicrobial agent. Reduced toxicity against erythrocytes concomitant with an increased inhibitory effect on bacterial growth was observed (Symmank et al., 2002). Similarly, linear forms of surfactin have lower surface and haemolytic activities and can even protect red blood cells against the action of other detergents. Linear surfactin analogues could be incorporated into cyclic surfactin in order to

take advantage of its protective effect (Dufour et al., 2005). An alternative approach is to deliver cyclic surfactin in a liposome of a specific phospholipid constitution into different kinds of target cells (Bouffioux et al., 2007). Thus, similar surfactin derivatives may exhibit reduced toxicity against eukaryotic cells, which could improve their therapeutic applications. These synthetic analogues appear as an interesting research tool to investigate the subtle structure-function variations on the membrane activity of surfactin. In the future, it is expected that potential applications will be found in the biomedical and biotechnological fields, enabling the design of new surfactants with tuneable, well-defined properties (Francius et al., 2008).

Surfactin can be also regarded as a toxic agent that can insult the producing microorganism membrane. All antibiotic-producing bacteria ensure their self-resistance by coding for various means of self-defense mechanisms that are activated in parallel with antibiotic biosynthetic pathways; their expression subsequently increases in time in order to avoid suicide. The cytoplasmic membrane can be reasonably supposed to be the site of self-resistance against surfactin. The major advantage of drugs targeting the integrity of the membrane constitutes the multistep modification of this structure, necessary to bring about cell resistance. On the other hand, any use of antibiotics could lead to the selection of resistant variants of pathogens at some level. Nowadays, only limited information is available concerning the molecular background of surfactin tolerance in producing bacteria. However, as the ultimate source of resistance genes are almost certainly the producers (Hopwood, 2007), the elucidation of the self-protective resistance mechanism in the producer *B. subtilis* at the level of surfactin target site – the cytoplasmic membrane – is inevitably important.

The extracytoplasmic transcription σ^w factor, controlling genes that provide intrinsic resistance to antimicrobial compounds produced by *Bacilli*, was recently identified (Butcher & Helmann, 2006). Nevertheless, none of these resistance systems were proven to be engaged in surfactin resistance. The only gene plausibly involved in surfactin resistance is swrC (Tsuge et al., 2001; Kearns et al., 2004). It codes for the first published example of an RND family of the proton-dependent multidrug efflux pumps in Gram-positive bacteria and contributes to the secretion of surfactin. However, surfactin production was observed even in a swrC-deficient strains that persistently survived at concentrations higher than 10,000 μg/ml (Tsuge et al., 2001). This finding suggests the existence of other additional mechanisms that participate in the surfactin self-resistance of the producer.

In order to examine the self-protective mechanisms of the cytoplasmic membrane against the deleterious effect of surfactin, we have constructed a mutant derivative with an abolished ability to synthesize surfactin (Fig. 3) complementary to the wild type surfactin producer *B. subtilis* ATCC 21332 (Seydlova et al., 2009). In this mutant, the sfp gene essential to the synthesis of surfactin was replaced with its inactive counterpart from the non-producing strain *B. subtilis* 168, bearing a frame shift mutation (Nakano et al., 1992). This isogenic pair of strains, differing only in surfactin production, represents a key tool for the comparative study of surfactin-induced changes in the cytoplasmic membrane of *B.* subtilis producing surfactin. Our preliminary data show that the synthesis of surfactin coincides with the substantial reconstruction of phospholipid polar headgroups, leading to a more stable bilayer. On the other hand, GC/MS analysis revealed a minor alteration in membrane fatty acids, implying that surfactin operates mainly in the polar region, which is in agreement with recent findings observed *in vitro* (Shen et al., 2010b).

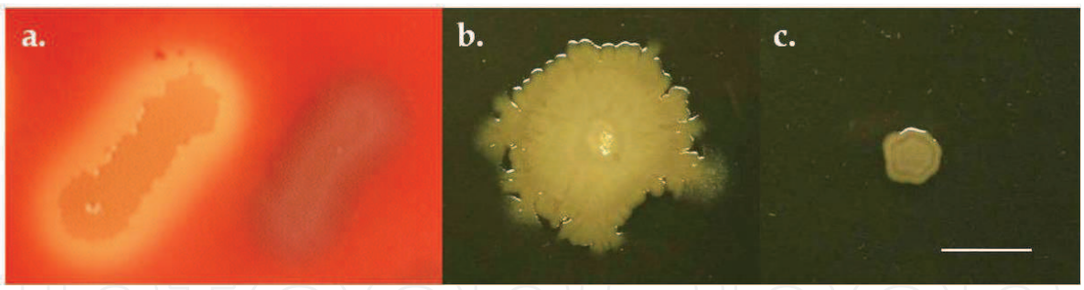

Fig. 3. The *B. subtilis* ATCC 21332 surfactin-producing strain and its mutant derivative minus surfactin production, accompanied by the absence of haemolysis (a - right) and swarming motility (b – wild type, c - mutant; bar 10 mm)

6.2 Economics of surfactin production

The high production cost of biosurfactants, which cannot compete with chemical surfactants, has been a major concern in commercial applications. Different strategies have been proposed to make the process more cost-effective, such as the optimization of fermentative conditions and downstream recovery processes, use of cheap and waste substrates and the development of overproducing strains (Banat et al., 2010).

Several advances in the optimization of culture conditions and downstream processing have been published recently. The amount and type of a raw material can contribute by 10-30% to total production costs in most biotechnological processes (Mukherjee et al., 2006). Interesting, cheap and renewable sources have been described from agroindustrial crops and residues. A promising perspective for large-scale industrial application was shown using the already commercialized, cottonseed-derived Pharmamedia medium (Al-Ajlani et al., 2007) or cashew apple juice for surfactin production (Ponte Rocha et al., 2009) reaching high yields of 2000 mg/l and 3500 mg/l, respectively.

A number of studies also deal with the improvement of culture and environmental parameters, the optimization of medium components and trace elements for the fermentation of surfactin. Carbon source (glucose), nitrogen source (ammonium nitrate), iron and manganese were found to be significant factors. It was reported that the addition of 4 mmol/l Fe^{2+} leads to a 10-fold increase in surfactin yield (Wei et al., 2004) and the addition of Mn^{2+} ions enhances lipopeptide production by a factor of 2.6 (Kim et al., 2010).

Apart from wild-type surfactin-producing strains, a few mutants have been selected and tested for surfactin production. Physical mutagenesis by ion beam implantation was used successfully to prepare a mutant that produced up to 12.2 g/l of crude surfactin (Gong et al., 2009). Another recombinant strain was obtained using random mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, reaching a maximum production level of 50 g/l (Yoneda et al., 2006).

Downstream processing such as recovery, concentration and purification account for the greater part of the total cost of a biotechnological product (Mukherjee et al., 2006). The most common isolation techniques for biosurfactants use precipitation, solvent extraction and chromatographic purification. These techniques are already well established for lab-scale applications, but cost hinders their use in industrial production. Lately, many advances have been reported for the recovery and purification of surfactin, including different combinations of ultrafiltration and nanofiltration through polymeric membranes with molecular weight cut-off. High surfactin recovery and purification were achieved, showing

potential for application (Isa et al., 2007; Chen et al., 2008; Juang et al., 2008; Shaligram & Singhal, 2010).

7. Conclusion

Surfactin, as a natural product with a multitude of auspicious features applicable in biomedicine, has attracted the intense attention of many research entities during the last decade. This systematic effort has resulted in substantial progress in understanding the different aspects of surfactin physicochemical properties, interactions with cell membranes and even its physiological role for the producer itself. A number of activities, such as antimicrobial, immunosuppressive, antitumor and antiparasitic activities, have been described and explored. This is of particular importance, especially at time when drug resistance among causal organisms for many life-threatening diseases is on the rise; other means of therapy are needed, or are entirely absent.

In spite of its immense potential, surfactin use remains restricted thus far. Further research needs to be carried out into the interaction of surfactin with target membranes and its global effect on the macroorganism and natural microbiota in order to validate the use of surfactin in biomedical and health-related areas. Last but not least, the mechanism of surfactin resistance also presents a crucial challenge. Nevertheless, it is only a matter of time before surfactin and its great biomedical potential are harnessed.

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9. References

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