II

Properties

8

Genetics of Surface-Active Compounds

JAKOB REISER, ANDREAS K. KOCH, URS A. OCHSNER, and ARMIN FIECHTER* Institute of Biotechnology, Swiss Federal Institute of Technology, Zurich, Switzerland

**Current affiliation*: Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik, Stuttgart, Germany

I. INTRODUCTION

Many microorganisms, both prokaryotic and eukaryotic, are able to grow on water-insoluble substrates like *n*-alkanes, due to alkane-specific oxidation systems, to substrate-induced alterations in their cell surfaces, and to the production of emulsifying agents, some of them acting as biosurfactants. Many of these extracellular and cell-wall-associated compounds have the potential to promote cell attachment to hydrophobic surfaces, to emulsify water-insoluble substances, and to mediate the transport of these unconventional substrates into the cell. Thus, they are important in determining the degree of hydrophobicity that a cell achieves, the wettability of substrates, the adhesion of a cell to the substrate, and the distribution of cells between oil and water. There is an increased interest in developing industrial processes in which highly active biosurfactants with specific properties for specific applications are produced in large amounts. Therefore, the overexpression of genes involved in the biosynthesis of surfactants and emulsifiers and the control of these genes is an important goal to be achieved.

This chapter reviews the current knowledge concerning the genetics of factors affecting the synthesis and/or composition of amphipathic surface-active compounds such as lipopolysaccharides, glycolipids, lipopeptides, and amphiphilic peptides. We also present molecular genetic strategies that have been useful in unraveling the structures of biosurfactants present in the lung.

II. GENETICS OF SURFACE-ACTIVE COMPOUNDS IN GRAM-NEGATIVES

A number of publications have dealt with molecular investigations concerning extracellular or cell-associated compounds of Gram-negative bacteria that, as emulsion-forming agents, enable such microorganisms to grow on water-insoluble substrates [1–8]. Only a few of those reports present results concerning the genetic analysis of biosurfactants and bioemulsifiers, but mutant strains of several Gram-negative species affected in the production of such factors have been described. Such mutant strains may facilitate the isolation of genes involved in the biosynthesis of biosurfactants and bioemulsifiers in the future.

A. *Acinetobacter calcoaceticus*

1. Involvement of Plasmids in Growth on and Dispersion of Crude Oil

A crude-oil-degrading *Acinetobacter* species, *A. calcoaceticus* RA57, was isolated by standard enrichment culture techniques on the basis of its ability to utilize oil sludge [6]. Strain RA57 was found to contain four plasmids. Among those, a 20-kb plasmid called pSR4 was studied in detail. Colonies were isolated at

random after growth in the presence of acridine orange and found to fall into two categories: (1) those that had lost the ability to grow on and disperse crude oil in liquid culture and concurrently were cured of pSR4 and (2) those that retained the ability to both grow on and disperse crude oil and contained pSR4. Strains from the first class continued to grow on hydrocarbon vapors, indicating that the defect associated with the curing of pSR4 was related to the physical interaction of the cells with the hydrocarbon substrate rather than to its metabolism. No differences in either adherence to hydrocarbons or production of extracellular emulsifying activity were found between the two classes of mutants. In growth experiments on crude oil in mixed culture with strains which either contained or lacked pSR4, no sparing of the growth defect was observed. The results are consistent with the possibility that pSR4 encodes a factor(s) that is tightly associated with the cell surface.

2. Mutants of *A. calcoaceticus* Affected in Emulsan Production

Emulsan is an extracellular lipoheteropolysaccharide polyanionic bioemulsifier. Pines and Gutnick [4] have demonstrated the capacity of emulsan to allow wild type *A. calcoaceticus* RAG-1 cells to grow on water-insoluble substrates. This heteropolysaccharide is both a cell-associated capsule and a cell-free product. Only the cell-associated form was biologically active and was required for growth on crude oil. A crude oil-containing medium supplemented with emulsan did not stimulate the growth of the emulsan-deficient mutant strain TR3. In addition, only wild-type cells grew well in a mixed culture experiment where wild-type cells and mutant TR3 cells were co-inoculated in equal numbers in a seawater medium supplemented with 2% crude oil. From the TR3 mutant strain, a revertant strain still affected in emulsan production but capable of growth on crude oil could be isolated. This indicates that besides emulsan other extracellular factors may facilitate the growth of *A. calcoaceticus* on crude oil.

3. Overproduction of Emulsan by Mutant Strains of *A. calcoaceticus*

Nitrosoguanidine-induced, cetyltrimethylammonium bromide (CTAB) tolerant mutant derivatives of the *A. calcoaceticus* RAG-1 strain were shown to grow faster and to produce emulsan at an earlier growth phase as compared to the wild-type strain yielding threefold higher product concentrations [5]. Experiments with resting cells induced upon the addition of chloramphenicol to exponentially growing cultures showed that the CTR-10-49 mutant strain released emulsan at a rate of 30 U/mg h^{-1} and produced almost double the amount of the wild-type within the same time and by the same number of cells, indicating that the effect seen was not simply the result of faster growth. Similar results were obtained with the emulsan overproducing lysine auxotrophic mutant

CTRL-100-1, a CTAB-resistant derivative of RAG-92, which in turn is a lysine auxotrophic derivative of RAG-1. Upon lysine starvation, the method applied to induce resting cells, the CTRL-100-1 mutant strain synthesized emulsan at a rate of 70 U/mg h^{-1} whereas RAG-92 synthesized the product of interest at 20 U/mg h^{-1}. These results indicate that the genetic modification caused an alteration in an emulsifier synthesis-specific step leading to an enhanced capsule production. The fact that the emulsan-overproducing mutants showed an enhanced resistance against CTAB points to a protecting function of emulsan toward the toxicity of this cationic detergent.

B. Pseudomonads

1. Assimilation of Alkanes by *Pseudomonas oleovorans*: The *alkBAC* Operon

The *P. oleovornas* alkane-utilizing system is encoded by the *alkBAC* genes. This operon is located on the so-called OCT plasmid and contains the essential genes for alkane terminal hydroxylation and alkanol dehydrogenation, as well as alkanal dehydrogenation [9]. The OCT plasmid enables *P. oleovorans* to use C_6–C_{12} *n*-alkanes [10]. The question remains whether host-encoded factors are involved in alkane uptake as well. Witholt et al. [11] have proposed that cell wall lipopolysaccharides may be needed to emulsify the hydrophobic substrate prior to its uptake by the cell. Together with the regulatory *alkR* locus, the enzymes of this operon could be expressed successfully in *Pseudomonas putida* and in *Escherichia coli fadR* mutant strains. In those mutants, the fatty acid degradation enzymes are expressed constitutively thus allowing them to grow on *n*-octane as the sole source of carbon and energy [12].

2. Mutational Changes in Physicochemical Cell Surface Properties of Plant-Growth-Stimulating *Pseudomonas* spp.

In an attempt to reach a better understanding of the factors involved in colonizing plant surfaces by bacteria, Weger et al. [13] have investigated bacteriophage-resistant mutant strains of the root-colonizing *Pseudomonas* strains WCS358 and WCS374 lacking the *O*-antigenic side chain of the lipopolysaccharide. These strains were found to differ from their parent strains in cell surface hydrophobicity and in cell surface charge. The observed variations in these physicochemical characteristics were explained by the differences in sugar composition. However, the mutant strains had no altered properties of adherence to sterile potato roots compared with their parental strains, nor were differences observed in the firm adhesion to hydrophilic, lipophilic, negatively charged, or positively charged artificial surfaces. These results showed that neither physicochemical cell surface

properties nor the presence of the *O*-antigenic side chain play major roles in the firm adhesion of these bacterial cells to solid surfaces, including potato roots.

3. Effect of Surface-Active *Pseudomonas* spp. on Leaf Wettability

Leaf surfaces represent a biological interface called the phyllosphere, which is colonized by bacteria, yeasts, and filamentous fungi. These surfaces are covered by a hydrophobic cuticular wax layer, so that water distributes as discrete droplets. It has been proposed that the wettability of the leaf surface may be increased by surface-active compounds produced by bacteria. To more directly assess the role(s) of biosurfactants in leaf wettability, Bunster et al. [14] have isolated *Pseudomonas fluorescens* and *P. putida* strains from the rhizosphere and phyllosphere and tested them for surface activity in droplet cultures on polystyrene. Some of the strains spread over the surface during incubation, and these strains were considered surface-active; strains not showing this reaction were considered nonsurface-active. Similar reactions were observed on pieces of wheat leaves. Supernatants from centrifuged broth cultures behaved like droplets of suspensions in broth, and exposure to 100°C destroyed the activity, indicating that biosurfactants were released into the medium. The average contact angles of the supernatants of surface-active and nonsurface-active strains were 24 deg and 72 deg, respectively, and the minimal surface tensions were 46 mN/m and 6 mN/m, respectively, as estimated from Zinsman plots. After 6 d incubation, wheat flag leaves sprayed with a dilute suspension of a surface-active strain of *P. putida* (WCS 358 RR) showed a significant increase in leaf wettability, which was determined by contact angle measurements. Interestingly, however, a closely related strain (WCS 358 U), with no surface activity on polystyrene did not affect leaf wettability, although it was present in densities similar to those of the surface-active strain. Evidently leaf wettability was caused by *Pseudomonas* biosurfactants. Leaf wettability may affect both the availability of water to microorganisms as well as the redistribution of microorganisms and nutrients on the plant surface. Also, leaf wetness duration, which is an important parameter in the epidemiology of bacterial and fungal diseases may be increased by biosurfactants.

4. Biosynthesis of Rhamnose-Containing Exolipids in *Pseudomonas aeruginosa*

Rhamnolipids are produced by *P. aeruginosa* cells during the late growth phase [15]. The synthetic pathway was elucidated [16–19] and the optimal conditions for rhamnolipid production by this organism from various radioactive precursors, such as acetate, glycerol, glucose, and fructose were established. Burger et al. [18, 19] described the complete enzymatic synthesis of a rhamnolipid by extracts of *P. aeruginosa*. They were able to show that the synthesis of rhamnolipids

proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyl transferase and that TDP-rhamnose is an efficient rhamnosyl donor in the synthesis of the rhamnolipid according to the following reactions:

TDP-L-rhamnose + β-hydroxydecanoyl-β-hydroxydecanoate
↓Transferase 1
TDP + L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (1)

TDP-L-rhamnose + L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate
↓Transferase 2
TDP + L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (2)

L-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate has been designated as rhamnolipid 1 (RL1) and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate as rhamnolipid 2 (RL2), respectively. Whereas RL1 and RL2 are the principle rhamnolipids produced in liquid cultures, RL3 and RL4, containing two sugar moieties and one fatty acid moiety and one sugar and one fatty acid moiety, respectively, appear to be produced exclusively by resting cells [20].

Previously, it has been shown that rhamnolipid formation by *P. aeruginosa* in a mineral salt medium with 2% alkanes as a carbon source was increased after NO_3^- limitation during the stationary growth phase [21]. Evidently, the control of rhamnolipid production is linked, in some way, to the control of nitrogen metabolism. A direct relationship between increased glutamine synthetase activity and enhanced biosurfactant production was recently found in *P. aeruginosa* cells grown in nitrate and proteose peptone media and increased ammonium and glutamine concentrations repressed both phenomena [22]. It is conceivable that a screen for mutants of *P. aeruginosa* that have increased levels of glutamine synthetase could lead to an enhanced production of biosurfactants. Alternatively, the transfer to and expression in *P. aeruginosa* of a cloned glutamine synthetase gene under the control of a strong promoter may yield the same effect.

A chloramphenicol tolerant strain of *P. aeruginosa* was investigated in terms of its capacity to produce biosurfactants in an inorganic phosphate-limited medium supplemented with chloramphenicol [23]. Several intracellular processes were monitored to correlate biosurfactant production with metabolic changes. In particular, biosurfactant production was preceded by phosphate depletion, followed by increased secretion of alkaline phosphatase and glutamate, and induction of transhydrogenase and glucose-6-phosphate dehydrogenase activity. Cosecretion of alkaline phosphatase and biosurfactant occurred to a greater extent in the chloramphenicol-tolerant strain as compared to the wild type, and extracellular alkaline phosphatase activity increased fourfold while intracellular activity decreased by 50%. Evidently, phosphate metabolism also plays an important role in surfactant production, alkaline phosphatase induction, and glucose metabolism.

5. Genetic Manipulation in the Production of *P. aeruginosa* Biosurfactants and Bioemulsifiers

Rhamnolipids were shown to act as emulsifiers and to stimulate gr wth of *P. aeruginosa* S_7B_1 on hexadecane [24, 25]. *Pseudomonas aeruginosa* U-1, a chemically induced mutant derivative of strain KY-4025, is affected in gr wth on *n*-paraffin and in surfactant production [25]. This strain produces more han 10 times less rhamnolipid than the wild type when grown on *n*-paraffin and is stimulated for growth on the water-insoluble substrate upon exogenou rhamnolipid addition, demonstrating that the mutation must be located in a step involved in rhamnolipid biosynthesis. By taking advantage of the fact that urified rhamnolipids stimulate the growth of *P. aeruginosa* on alkanes, Koch et l. [26] isolated transposon Tn5-GM induced mutants of *P. aeruginosa* PG201 which were unable to grow in minimal media containing hexadecane as the sole carbon source.

Some of these mutants turned out to be affected in rhamnolipid produ tion in the following way. The mutant derivative 65E12 was found to be un ble to produce extracellular rhamnolipids under any of the conditions tested and lacked the capacity to take up ^{14}C-labeled hexadecane nor did it grow in media con aining individual alkanes with chain lengths ranging from C_{12} to C_{19}. However, growth on these alkanes and uptake of ^{14}C-hexadecane could be restored provid d that small amounts of purified rhamnolipids were added to the cultures. Mutan 59C7 did not produce rhamnolipids and was unable to grow in media con aining hexadecane, nor was it able to take up ^{14}C-hexadecane in significant amou ts, but the addition of small quantities of rhamnolipids restored growth on alka es and ^{14}C-hexadecane uptake. Interestingly, the rhamnolipid production capa ity of mutant 59C7 on hexadecane was restored by this rhamnolipid boost. In g ucose-containing media, however, mutant 59C7 produced rhamnolipids at level about twice as high as those of the wild-type strain. Using rhamnosyl transferase ssays, mutant 65E12 turned out to be affected in the production of this key e zyme, whereas strain 59C7 showed transferase activities indistinguishable fr m the wild-type cell extracts. It is thus possible that this strain carries a m tation affecting a gene whose product is involved in the control of rhamnolipid b iosynthesis. These mutants will be instrumental in isolating the correspondin wild-type genes. The isolation and characterization of such genes will allow us to unravel rhamnolipid biosynthesis at the molecular level and may, in the lo g run, yield strains with increased production capacities.

By chemostatic selection with paraffins isolated from high-paraffin oil, a strain of *P. aeruginosa*, SB1, that can use even-numbered straight-chain alkane from C_{10} to C_{36} and beyond was isolated [3]. This strain was reported to grow r pidly with oil-well paraffin or candle wax as a sole source of carbon and e ergy, producing large amounts of surface-active agents. The production of emul fying

agents was particularly apparent when liquid hydrocarbons such as decane, dodecane, tetradecane, and hexadecane were used as growth substrates. A mutant derivative, SB3, that could not produce the emulsifier did not grow on any of the liquid hydrocarbons, unless the emulsifier was added to the culture medium. Interestingly, however, mutant SB3 could grow on solid hydrocarbons as rapidly as the wild type. During growth on solid hydrocarbons, such as tetracosane ($C_{24}H_{50}$) or candle wax, both SB1 and SB3 cells were reported to produce surface-active agents that wet these hydrophobic substrates so as to disperse them throughout the media. Thus, different surface-active compounds were produced when the cells were grown on different hydrocarbon substrates. The *P. aeruginosa* SB30 strain, which is a derepressed mutant derivative of the original SB1 strain was shown to produce large amounts of an emulsifier while growing on either hexadecane, glucose, or a cheap substrate such as chicken fat [3]. Normally, the wild-type strain produces large amounts of emulsifier only during growth on hexadecane but not on glucose or chicken fat. SB30, however, produced five to six times more emulsifier while growing on such substrates. This mutant, therefore, allows rapid production of high amounts of emulsifier, with considerable savings in time and expense.

6. Genetic Construction of Lactose-Utilizing Strains of *P. aeruginosa* and Their Application in Biosurfactant Production

With a view toward using whey as a cheap substrate for the production of biosurfactants, Koch et al. [27] have constructed lactose-utilizing strains of *P. aeruginosa*. For this purpose the *E. coli lac*ZY genes were inserted into the chromosomes of *P. aeruginosa* strains PAO-1 and PG-201 using a bicomponent transposition system, yielding transconjugant strains with one set of *lac*ZY genes integrated into the chromosomes at unique locations. The transconjugants grew well in lactose-based media (minimal medium and whey), albeit with reduced initial rates as compared to growth in glucose-based minimal media. *Pseudomonas* rhamnolipids were produced during stationary growth in lactose-based minimal media and whey, showing that waste products can be effectively used for important biotechnological processes.

C. *Serratia marcescens*

1. Mutants of *S. marcescens* Affected in Cell-Surface Hydrophobicity

Distinct extracellular and cell-bound factors were shown to affect the cell-surface hydrophobicity of certain *Serratia* strains [2]. Enrichment for spontaneously occurring nonyhydrophobic mutants of *S. marcescens* yielded two types: (1) a

hydrophobic mutant that exhibited partial hydrophobic characteristics compared to the wild type, as determined by adherence to hexadecane and polystyrene, and (2) a pigmented, nonhydrophobic mutant whose colonies were translucent with respect to those of the wild type. These data suggest that the pronounced cell-surface hydrophobicity of the wild type is mediated by a combination of several surface factors.

2. Serraphobin and Serratamolide as Modulators of *Serratia* Cell-Surface Hydrophobicity

Serraphobin, a 70 kDa protein recovered from the cell surface and from the culture supernatants of wild-type *S. marcescens* cells has been demonstrated to be capable of binding to hexadecane droplets [8]. Serraphobin was either totally absent or present only in minor amounts in hydrophobicity-deficient mutants and in wild-type cultures grown at elevated temperatures (39°C instead of 30°C). Besides this hydrophobin, *S. marcescens* produces a hydrophilin called serratamolide, an aminolipid [28] that plays an important role as a wetting agent [7]. Mutant NS38-9, a derivative of the *S. marcescens* NS38 strain, is deficient in the production of this compound. This was shown by direct colony thin-layer chromatography [29] and by spreading small droplets of washed cell suspensions over a polystyrene surface. In contrast to the wild type, mutant cells did not show spreading activity. The production of this spreading activity was shown to be temperature dependent in that wild-type colonies lacked the capacity to produce serratamolide at 38°C. The data suggest that the presence of serratamolide on *S. marcescens* cells results in a reduction in hydrophobicity possibly by blocking hydrophobic sites on the cell surface and, together with serraphobin, serratamolide seems to act as a modulator of cell-surface hydrophobicity. On the one hand, the production of amphiphilic wetting agents such as serratamolide or the rubiwettins [30] lower the surface tensions of aqueous media and may, therefore, act as emulsion-forming molecules. On the other hand, increased cell surface hydrophobicity results in a better adherence to hydrophobic substrates and consequently, in faster growth rates on carbon sources like hexadecane.

III. GENETICS OF BIOSURFACTANT PRODUCTION IN *Bacillus subtilis*

A. Biosurfactants Produced by *Bacillus* spp.

Bacillus subtilis produces surfactin, which is a cyclic lipopeptide with exceptional surface activity [31, 32]. Surfactin lowers the surface tension of water from 72 mN/m to 27 mN/m, inhibits fibrin clotting, and lyses erythrocytes. The lipopeptide contains a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and seven amino acids (Glu-Leu-Leu-Val-Asp-Leu-Leu) [33]. The biosynthesis of surfactin

has previously been studied in intact *B. subtilis* cells by incorporating ^{14}C-labeled precursor amino acids directly into the product [34]. [^{14}C]-Acetate appeared in the fatty acid portion of surfactin and was also partially converted into leucine. An enzyme was subsequently isolated and partially purified from a cell-free extract, which catalyzed ATP-P_i-exchange reactions mediated by the amino acid components of surfactin. This activation pattern is consistent with a peptide-synthesizing multienzyme complex that activates its substrate amino acids simultaneously as reactive aminoacyl phosphates. The large-scale production of this surfactant has been investigated [35] and a yield of surfactin of up to 0.8 g/L was obtained in a batch cultivation by continuously removing the product by foam fractionation. A similar aminolipid has been isolated from *B. licheniformis*, and its properties are described in a separate chapter in this volume [36].

B. Identification of Genetic Loci Responsible for Surfactin Production

A study of peptide antibiotic synthesis in *B. subtilis* focusing on the isolation of genes required for the production of surfactin has recently been initiated [37]. Three genes, *sfp*, *srfA*, and *comA* (previously called *srfB*) have been identified and subsequently isolated [38, 39]. The first, *sfp*, is found in surfactin-producing strains of *B. subtilis* and all of the surfactin-nonproducing strains of *B. subtilis* examined so far carry the genes required for surfactin production, with the exception of a functional *sfp* gene. When transferred by genetic transformation, *sfp* is necessary and sufficient to render cells of a nonproducing strain surfactin positive [39]. Mutations of *comA*, in addition to blocking competence development (a stationary phase-induced phenomenon) at an early stage, also render *sfp*-bearing cells surfactin negative [39], and the *comA* gene product has been shown to be required for the expression of a *srfA*::Tn*917lac* fusion as well as other *com* genes that function in later stages of competence development [39]. The *comA* product is homologous to the response regulator class of two-component regulatory proteins and is likely to be a DNA-binding protein [40]. This observation suggests that surfactin production and competence development are regulated by a common signal transduction pathway. The *srfA* gene has been shown to be a large operon of over 25 kb encoding functions responsible for surfactin production, competence development, and sporulation [41].

C. Overproduction of Surfactin by Stable Mutants of *B. subtilis*

Ultraviolet (UV) mutagenesis of *B. subtilis* ATCC 21332 cells yielded a stable mutant strain that produced over three times more surfactin than the parent strain [42]. Approximately 1000 colonies of UV-treated cells were examined for

enhanced hemolytic activity on blood agar plates. One mutant (*Suf-1*) produced a significantly larger hemolytic zone and was not an auxotroph. The mutation was mapped by protoplast fusion. This information provides a target site for future genetic manipulation.

IV. MOLECULAR GENETICS OF LUNG SURFACTANTS

A. Physiological Roles and Composition of Lung Surfactant

Lung surfactant is a complex mixture containing primarily phospholipids (phosphatidylcholine and phosphatidylglycerol) with small amounts of proteins, carbohydrates, and neutral lipids. It is found at the air–liquid interface of the alveoli and is essential for normal respiration. In premature infants, an insufficiency of surfactant can cause alveolar collapse, leading to hyaline membrane disease. One promising treatment for the disease is the use of bovine-based surfactant replacement therapy using preparations derived from organic solvent extracts of bovine surfactant. The biochemistry and physiology of the pulmonary surfactant system has been reviewed [see, e.g., Refs. 43 and 44].

B. Structure of Surfactant-Associated Proteins

Hydrophobic proteins with molecular weights between 5 and 18 kD have been identified as the only protein components in the bovine-based preparations now under investigation in treating hyaline membrane disease. An additional protein with a molecular weight of approximately 35 kDa (termed SP-A or SAP-35) is also present in natural surfactant [45]. It is a more hydrophilic glycoprotein, which has been shown to be unrelated to the small hydrophobic proteins and is not found in appreciable amounts in organic extracts from mammalian surfactants being investigated for clinical use [46, 47]. SP-A is insoluble in ether/ethanol or chloroform/methanol and contains an approximately 70-amino-acid collagenlike amino-terminal domain which is rich in glycine and hydroxyproline. The proteins SP-A and Clq, a subunit of the first component (C1) of the classical complement pathway, are structurally homologous molecules, each having an extended collagenlike domain. Evidence has been presented indicating that SP-A can substitute for Clq in enhancing FcR-mediated phagocytosis by monocytes and macrophages and CR1-mediated phagocytosis by macrophages *in vitro* [48].

Two classes of small, organic solvent-soluble proteins have been identified as important components of lung surfactant. These proteins are distinct gene products with unique amino acid sequences. In humans, one of the surfactant-associated proteins with an N-terminal phenylalanine, SP-B [SPL(Phe)] has a molecular weight of approximately 6 to 8 kDa (reduced) or 18 kDa (unreduced).

The other, with an N-terminal glycine, contains a stretch of polyvaline residues and is now termed SP-C [also referred to as SPL (pVal) or SP5]. It has a molecular weight of 4.5 kDa. Both of these proteins have been identified in human surfactant and their primary amino acid sequences have been deduced from cDNAs derived from the mRNAs encoding them [49–51]. The amino acid sequences of bovine and porcine SP-B were recently reported [52, 53]. The porcine peptide was shown to be 79 amino acids long, but the precise C-terminal end of the human protein is not known.

Reconstitution of hydrophobic surfactant peptides with synthetic phospholipids imparts virtually complete surfactantlike properties to synthetic phospholipids, including rapid surface adsorption and lowering of surface tension during dynamic compression. Surfactant lipid extracts containing the two hydrophobic peptides as the sole apoproteins have been used for therapy in hyaline membrane disease in newborn babies. Sarin et al. [54] have synthesized pulmonary surfactant apoprotein SP-B peptides by solid phase chemistry and demonstrated their ability to enhance the surface-active properties of synthetic lipid mixtures. The synthetic peptides were reactive with antiserum generated against the native bovine surfactant peptide. The peptides conferred surfactantlike properties to synthetic lipid mixtures as assessed by a Wilhelmy balance and pulsating bubble surfactometer. Likewise, mixtures of synthetic SP-B peptides and lipids restored compliance of isolated surfactant-deficient rat lungs. This work demonstrates the utility of SP-B as a functional component of pulmonary surfactant mixtures for treatment of respiratory distress syndrome or other disorders characterized by surfactant deficiency. Curstedt et al. [53] have presented unambiguous evidence that native SP-C is a lipopeptide with two palmitoyl groups covalently linked to the polypeptide chain. The deacylation conditions involving treatment with KOH, trimethylamine, or dithioerythrol, the presence of two cysteine residues in the polypeptide, and the absence of other possible attachment sites establish that the palmitoyl groups are thioester-linked to the two adjacent cysteine residues (Fig. 1). In contrast, the major form of porcine SP-B is a dimer without fatty acid components. Long-chain acylation may constitute a means for association of proteins with membranes and could conceivably modulate the stability and biological activity of surfactant films.

C. Molecular Biology of Surfactant Protein Genes

Molecular genetic tools are currently being applied by a number of groups in order to understand the biophysical properties of the surfactant-associated proteins and their mechanism(s) of formation and to provide substrates for reconstitution studies. A review describing the function and regulation of expression of pulmonary surfactant-associated proteins has recently appeared [55].

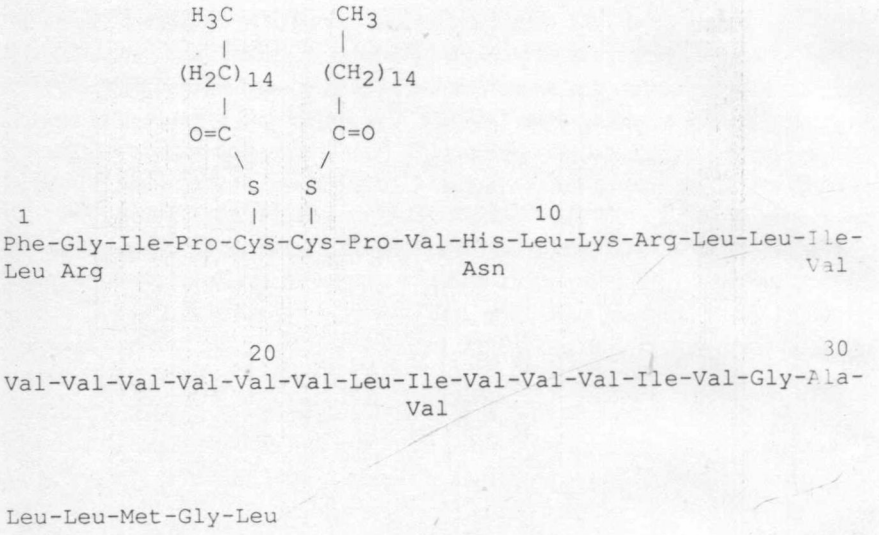

FIG. 1 Structure of intact SP-C. The amino acid sequence of human SP-C is shown with residues that differ in the porcine molecule indicated below the human sequence. The sulfurs implicit in the Cys symbols are shown to emphasize the thioester nature of the linkages. Adapted from Ref. 53, with permission.

1. The Gene Encoding SP-A

The cloning of the human SP-A gene and cDNA was first reported in 1985 [45]. For this purpose, a full-length cDNA encoding the canine 32-kDa pulmonary surfactant apoprotein [56] was used in reduced stringency hybridization conditions to screen bacteriophage lambda-based genomic and adult lung cDNA libraries. The 248-amino-acid sequence deduced by the single open reading frame was also found to be encoded by the genomic isolate. In a parallel study, Floros et al. [57] have prepared and sequenced tryptic fragments of the 35-kDa SP-A protein and oligonucleotide probes were synthesized based on the amino acid sequences. A human lung cDNA library was then screened using the oligonucleotide probes, and clones encoding these proteins were identified and characterized. By *in vitro* transcription–translation experiments individual clones were associated with particular proteins of 29 and 31 kDa. The data obtained suggest that cotranslational modifications of two primary translation products account for many of the isoforms observed.

2. The Gene Encoding SP-B

Antiserum generated against small hydrophobic surfactant-associated proteins with molecular weights between 6 and 14 kDa was used to screen a bacteriophage expression library constructed from adult human lung polyA$^+$ RNA [49]. This resulted in the identification of a 1.4 kb cDNA clone that was shown to encode the N-terminus of the surfactant polypeptide SP-B. Hybrid-arrested translation with this cDNA and immunoprecipitation of ^{35}S-methionine-labeled *in vitro* translation products of human polyA$^+$ RNA with a surfactant polyclonal antibody resulted in identification of a 42-kDa precursor protein. Blot hybridization analysis of electrophoretically fractionated RNA from human lung detected a 2.0 kb RNA that was more abundant in adult lung than in fetal lung. The larger RNA and translation product indicates that SP-B is derived by proteolysis of a large polypeptide precursor. In a parallel study, Jacobs et al. [58] have partially sequenced one of the low-molecular-weight proteins and specific oligonucleotides deduced from the sequence were used as probes. The cDNA clone selected by hybridization a human lung mRNA, which, upon *in vitro* translation, yielded a 42-kDa protein. The same protein was observed when the cDNA clone was expressed following transfection into monkey COS cells. The SP-B precursor is not homologous to SP-A and has no collagenlike regions, nor do other parts of SP-A share homology with SP-B. A cDNA sequence encoding a related protein from canine lungs termed SP-18 has recently been described [50].

A complete SP-B cDNA was used to isolate the gene encoding the SP-B precursor from a genomic library of human embryonic kidney DNA [59]. The entire SP-B gene was sequenced and is approximately 9.5 kb long, with 11 exons and 10 introns. Southern blotting of human genomic DNA probed with SP-B cDNA indicated the presence of only one SP-B gene in the human genome, and the gene was localized on chromosome 2.

3. The Gene Encoding SP-C

An oligonucleotide probe based on the valine-rich amino-terminal amino acid sequence of SP-C was utilized to isolate cDNA and genomic DNA encoding the human proteolipid SP-C [51]. The primary structure of a precursor protein of approximately 20 kDa, containing the SP-C peptide, was deduced from the nucleotide sequence of the cDNAs. Hybrid-arrested translation and immunoprecipitation of labeled translation products of human mRNA demonstrated a 22-kDa precursor protein, the active hydrophobic peptide being produced by proteolytic processing to 5 to 6 kDa. The precursor contains an extremely hydrophobic region of 34 amino acids that comprises most of the mature SP-C [60]. This hydrophobicity explains the unusual solubility characteristics of SP-C and the fact that it is lipid-associated when isolated from lung. Two distinct human genes encoding SP-C were identified and sequenced [61]. In both genes, the active

hydrophobic region of the polypeptide was located in the second exon that encodes a peptide of 53 amino acids. The entire nucleotide sequence of the two classes of SP-C genes differed by only 1%. The SP-C gene locus was assigned to chromosome 8.

V. SURFACE ACTIVITY OF PROTEINS AND AMPHIPHILIC α-HELICES

A. Surface Activity of Proteins

The various structural properties of proteins, namely, surface hydrophobicity, net charge, molecular size, and the presence of regions involved in protein–protein interaction, have all been considered as factors influencing their surface properties. In addition, the stability of proteins seems to be an important factor. The conformation of an unstable protein can easily change to become hydrophilic toward an aqueous phase and hydrophobic toward an air or oil boundary, so that a pronounced reduction of surface or interfacial tensions facilitates foaming and emulsification.

To elucidate the role of structural stability in determining the surface properties of proteins, Kato and Yutani [62] have compared the surface properties of wild-type and six mutant α-subunits of tryptophan synthase substituted at position 49. The surface tension, foaming power, emulsifying activity, and foam stability of the seven proteins tested were found to correlate linearly with the free energy of denaturation of the proteins. These results indicated that the surface properties of the α-subunits of tryptophan synthase change in proportion to their conformational stabilities.

The observations of Kato and Yutani [62] on tryptophan synthase mutants are consistent with differences in surface properties of hemoglobin (Hb) mutants in previous studies [63]. The Hb studies were stimulated by the observation of decreased mechanical stability of deoxy sickle cell Hb (HbS). *Mechanical stability* is a term used to describe the tendency of proteins to precipitate from solution upon shaking. Deoxy-HbS precipitates much more readily during shaking in comparison to the normal HbA variant and thus has a lower mechanical stability. When other hemoglobin variants were tested, a relatively large range of mechanical stability was observed. Furthermore, the surface tension of solutions of Hb mutants was also found to vary. Among a variety of Hb mutants studied, there was a generally good correlation between surface properties and mechanical stability. In addition, the mechanical stability of oxy-HbS is much greater than deoxy-HbS. These differences appear to be due to the greater denaturability of deoxy-HbS, which makes it more ready to denature and spread at the air–water interface.