Chapter 2

Molecular Genetics of Biosurfactant Synthesis in Microorganisms

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Abstract

Biosurfactant (BS)/bioemulsifier (BE) produced by varied microorganisms exemplify immense structural/functional diversity and consequently signify the involvement of particular molecular machinery in their biosynthesis. The present chapter aims to compile information on molecular genetics of BS/BE production in microorganisms. Polymer synthesis in *Acinetobacter* species is controlled by an intricate operon system and its further excretion being controlled by enzymes. Quorum sensing system (QSS) plays a fundamental role in rhamnolipid and surfactin synthesis. Depending upon the cell density, signal molecules (autoinducers) of regulatory pathways accomplish the biosynthesis of BS. The regulation of serrawettin production by *Serratia* is believed to be through non ribosomal peptide synthetases (NRPSs) and N-acylhomoserine lactones (AHLs) encoded by QSS located on mobile transposon. This regulation is under positive as well as negative control of QSS operon products. In case of yeast and fungi, glycolipid precursor production is catalyzed by genes that encode enzyme cytochrome P450 monooxygenase. BS/BE production is dictated by genes present on the chromosomes. This chapter also gives a glimpse of recent biotechnological developments which helped to realize molecular genetics of BS/BE production in microorganisms. Hyper-producing recombinants as well as mutant strains have been constructed successfully to improve the yield and quality of BS/BE. Thus promising biotechnological advances have expanded the applicability of BS/BE in therapeutics, cosmetics, agriculture, food, beverages and bioremediation etc. In brief, our knowledge on genetics of BS/BE production in prokaryotes is extensive as compared to yeast and fungi. Meticulous and concerted study will lead to an understanding of the molecular phenomena in unexplored microbes. In addition to this, recent promising advances will facilitate in broadening applications of BS/BE to diverse fields. Over the decades, valuable information on molecular genetics of BS/BE has been generated and this strong foundation would facilitate application oriented output of the surfactant industry and broaden its use in diverse fields. To accomplish our objectives, interaction among experts from diverse fields likes microbiology, physiology, biochemistry, molecular biology and genetics is indispensable.

Introduction

Enormous structural and functional diversity are implicated in biosurfactant (BS)/bioemulsifiers (BE) produced by microorganisms. BS/BE possesses remarkable applications in diverse fields. With the need for green chemicals, their study is becoming imperative. Therefore, BS/BE

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studies have been focused on by large number of researchers. However, commercial production of these compounds is quite expensive. Use of cheaper and renewable substrates is a necessity.[1-3] However, a great deal of monetary input is required in purification processes.[4] Thus, it represents two faces of a coin; so to overcome this dilemma and subsequently economize and commercialize BS production a better understanding at molecular level is mandatory.

Literature survey illustrates that detailed studies of BS/BE production have been carried out in *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Serratia*, *Candida* spp. BS producing microbes from different resources, viz., fresh water, soil, marine, oil wells and industrial effluents have been studied extensively.[5,6] Among these natural resources, marine environment is attracting interest from many researchers due to its vastness and novelty with respect to products that can be obtained.[7-9] However, this survey clearly illustrates that the maximum reports are focused on rhamnolipid and surfactin production from *Pseudomonas* and *Bacillus* spp. respectively. Few researchers have reviewed the enormous data generated on BS/BE production in microorganisms, briefing molecular biological aspects.[5,6,10-18,19] However, it is important to note that, before and after Sullivan's review[20] on molecular genetics of BS not a single review is devoted exclusively to molecular biology of synthesis BS in microorganisms. A gap of ~10 years indicates that a compilation of molecular mechanisms involved in BS/BE production is essential. Enormous molecular and biotechnological developments have taken place in this decade and therefore, our understanding on the present topic has improved greatly. Therefore, present review is focused at compiling valuable developments in this area. To the best of our knowledge, this chapter would give comprehensive information on molecular genetics of BS/BE production in microorganisms.

Important Aspects Pertaining to Biosurfactant Production in Microorganisms

The mystery why microbes produce BS/BE is still unknown. Justifications include survival on various hydrophobic substrates[21] and desorption from the hydrophobic substrates allowing direct contact with cell, thereby increasing the bioavailability of insoluble substrates.[22] However, few microbes produce BS/BE on water soluble substrates.[23,24] Different biosynthetic pathways and specific enzymes are involved.[25] Synthesis takes place by de novo pathway and/or assembly from substrates.[26] Based on the four assumptions proposed by Syldatk and Wagner,[26] diagrammatic representations for biosurfactant synthesis in microorganisms is given in Figure 1. Induction/repression of BS/BE production are dependent on presence of carbon, nitrogen, phosphate, trace elements and multivalent cations.[27,28] BS/BE production is controlled by environmental parameters.[29,30] Literature survey suggests that complex pathways are involved in BS/BE production.[18] BS/BE producing microbes may harbour plasmids.[31,32] However, genes responsible for BS production are located on chromosomal DNA.[32] Interacellular communication and production of enzymes, pigments and BS occurs by QSS which depends on the production of diffusible signal molecules termed autoinducers.[33] The regulatory machinery is different for different BS/BE producers.

Molecular Genetics of Biosurfactant Production in Bacteria

Acinetobacter Species

Acinetobacter spp. are ubiquitous in nature, being isolated from various sources like soil, mud, marine water, fresh water, meat products etc.[34-38] and reported for production of BE.[34-36,39-40] *Acinetobacter* species are the most promising bacteria producing high molecular weight BS/BE. The first description of the best known marine BE, now exploited commercially as 'Emulsan' appeared in 1972. This emulsifier is produced by *A. calcoaceticus* RAG-1, isolated from the Mediterranean Sea. Emulsan produced by RAG-1 has a heteropolysaccharide backbone with a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid and an unidentified *N*-acetyl amino sugar. Fatty acids (FA) are covalently linked to the polysaccharide through *o*-ester linkages.[42-45] Different species of *Acinetobacter* are known to produce protein polysaccharide complexes. Proteoglycan type bioemulsifier is produced by *Acinetobacter junii*

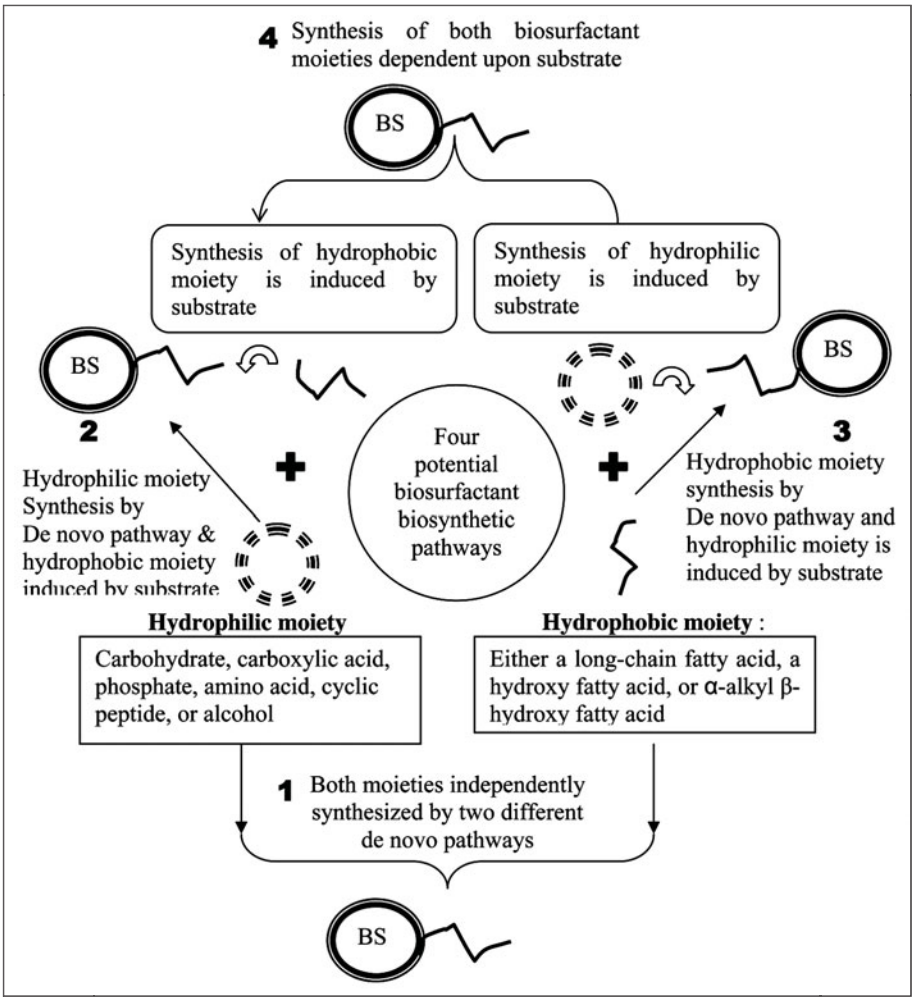

Figure 1. Potential biosurfactant biosynthetic pathways in microorganisms: BS: Biosurfactant molecule. Probable BS biosynthetic pathways operating in different microorganisms. Based on Syldatk and Wagner (1987)[26] four assumptions.

SC14. This bioemulsifier is made up of protein (50.5%), polysaccharide (43%) and lipid in a minor fraction (3.8%). 88.7% of the polysaccharide consisted of reducing sugars.[36,41] About 16% of patents on BS have been reported from *Acinetobacter* spp. alone,[38] which indicates the tremendous market potential of exopolysaccharide (EPS).

Emulsan

It is a complex polysaccharide (9.9×10^5) produced by *A. calcoaceticus* RAG-1 and stabilizes oil-water emulsions efficiently.[43,44] In spite of structural complexity, researchers have succeeded in identifying genes implicated in emulsan synthesis and emulsification phenomena. Polymer biosynthesis is accomplished by a single gene cluster of 27 kbp with 20 open reading frames (ORFs) called as *wee* regulon which contains *weeA* to *weeK* genes that accomplish polymer biosynthesis.[46,47] Putative proteins encoded by the *wee* cluster have been tabulated by Nakar and Gutnick[48] in detail.

These genes lead to the formation of polysaccharide containing amino sugars, with *O*-acyl- and *N*-acyl-bound side chain of FA. Further addition of intermediates takes place as follows: WeeA converts UDP-*N*-acetyl-D-glucosamine into UDP-*N*-acetylmannosamine. Consequently, WeeB oxidizes the UDP-*N*-acetylmannosamine into UDP-*N*-acetylmannosaminuronic acid. This regulon possess *wzb* and *wzc* genes which are responsible for biosynthesis of emulsan. Gene products Wzc and Wzb were over expressed, purified and a bulk of polysaccharide was produced successfully.[48,49] The *Wee*E or *Wee*F are possibly involved in formation of UDP-*N*-acetyl-L-galactosaminuronic acid. The gene *WeeJ* further catalyses the formation of diamino 2, 4-diamino-6-deoxy-D-glucosamine, a component of the repeating unit, from UDP-4-keto-6-deoxy-D-glucosamine. The sequence of *Wee*K is similar to dTDP-glucose 4, 6-dehydratase and therefore could possibly be responsible for conversion of UDP-D-glucosamine into UDP-4-keto-6-deoxy-D-glucosamine. The overall process is summarized in detail by Nesper, et al.[50] The monomers gather on a lipid carrier on the cytoplasmic face of the inner membrane. Subsequently, they are transferred by Wzx protein to the periplasmic face of the membrane. Wzy polymerase further catalyzes the polymerization process. Finally, lipid intermediates lead to the formation of a protein-polysaccharide complex which is transported across the periplasm to the outer membrane. This assembly gets accumulated on cell surface and is further excreted as polymer complex in the exterior.[50]

Due to complex nature of exopolymers, genetic studies remained at a nascent level for a long period. However, with the advent of recent technologies and innovations, bioengineering of BE producing microorganisms has become possible. Complex polysaccharide backbone of emulsan was altered by modifying the culture conditions for *A. venetianus* RAG-1.[45,51-53] The emulsan structure was modified by transposon mutagenesis of FA moiety. Analysis of various factors viz., yield, FA content, molecular weight and emulsification behavior demonstrated that parent strain yielded high emulsan as compared to mutant strain. The factors are dependent on the type of FA supplemented during the production process. However, cloning and sequencing of mutants with enhanced emulsifying activity indicated that they were involved in biosynthesis of emulsan. The presence and composition of long chain FAs on the polysaccharide backbone influenced emulsification behaviour. Such studies are highly significant and open newer avenues for applications of amphiphiles in diverse fields.[54] Based on similar kind of studies, an interesting U.S. patent (20040265340) on "Emulsan adjuvant immunization formulations" was filed by Kaplan, et al.[55] The emulsan analog and mutants of *A. calcoaceticus* RAG-1 were produced in presence of different FA sources. Different molecular tools have been employed to modify and improve quality of emulsan produced by *Acinetobacter* spp. (Table 1).

Apoemulsan

It is an extracellular, polymeric lipoheteropolysaccharide produced by *A. venetianus* RAG-1. Purified deproteinized emulsan (apoemulsan, 103 kDa) consists of D-galactosamine, L-galactosamine uronic acid (pKa, 3.05) and a diamino, 2-desoxy *n*-acetylglucosamine.[44] It retained emulsifying activity towards certain hydrocarbon substrates but was unable to emulsify relatively nonpolar, hydrophobic, aliphatic materials.[63,64] It is now known that polymers are synthesized from Wzy pathway. However, there also appears a differing report which claims that the process is based on presence of polysaccharide-copolymerase (PCP).[65,66] However, recently Dams-Kozlowska and Kaplan[58] proved that synthesis of this polymer was dependant on Wzy pathway where, PCP protein controlled the length of the polymer. This was proved by inducing defined point mutations in the proline-glycine-rich region of apoemulsan PCP protein (Wzc). Five of the eight mutants produced higher weight BE than the wild type while four had modified biological properties. This study demonstrated the functional effect of Wzc modification on molecular weight of polymer and the genetic system controlling apoemulsan polymerization. It has been suggested that emulsifying activity and release of polymer is mediated via esterase gene *est* (34.5 kD). A study carried out by Leahy in 1993,[67] proved that lipase is responsible for enhanced emulsification properties. Lipase negative mutants exhibited less emulsification activity. The gene *est* has been cloned and over expressed in *E. coli* BL21 (DE3) behind the phage T7 promoter with His tag system.[68] Further Alon and Gutnick,[57] also showed that *est* gene encodes protein that is located on the outer membrane.

Table 1. Employment of molecular tools for construction of recombinant/mutant strains of* Acinetobacter *and* Pseudomonas *spp.

Organism	Mutation	Objective	Significant Feature	Reference
A. calcoaceticus RAG-1 (Emulsan)	CTAB (Cetyl trimethyl ammonium bromide)	Enhance the yield	High yield achieved	56,57
A. venetianus RAG–1 (Emulsan)	Recombinant point mutations in proline—glycine-rich region of the apoemulsan PCP protein (Wzc)	Alteration in Wzc gene	Molecular weight of polysaccharide was modified	58
A. calcoaceticus A2 (Biodispersan)	N-methyl-N-nitrosoguanidine (NTG)	Alteration of wild type strain	Mutant produced equal/higher polysaccharide; rich protein also secreted along with it.	59
A. calcoaceticus RAG-1	Transposon	Disruption of genes involved in biosynthetic pathways of biotin, histidine, cysteine or purines	Modification in fatty acid (FA) metabolism influenced level and types of FA incorporated into emulsan	54
A. venetianus RAG-1 (Emulsan)	M13 and Primers for parent esterase gene	The pET system for over expression of esterase in *E. coli* BL21 carrying pESTAL-14b/pESTAL-11c and plasmid constructed by ligation of the K*pn*I-*Nde*I-*Bam*H1 fragment form parent *est* gene	Overproduction of His0 tagged recombinant esterase and affected protein confirmation that enhanced apoemulsan-mediated emulsifying activity.	
A. lwoffii RAG-1 (Emulsan)	Mini Tn10Km	One region, transcribed in the upstream direction, appears to encode for 3 complete ORFs, while the second region is transcribed in the opposite direction and encodes 17 complete ORFs	Identification of *wee* gene (27 kbp) containing 20-ORFs for polysaccharide synthesis; Defect in emulsan production with specific activities of 5-14% of parental emulsifying activity	48
P. aeruginosa (Rhamnolipid)	N-methyl-N-nitrosoguanidine (NTG)	Enhance yield of BS	Similar type of biosurfactant with 10 times more BS production	61
P. fluorescens	Tn5	Transposon integration into condensation domains of peptide synthetases	Rhamnolipid reduced surface tension effectively	62

The same gene was sequenced and expressed in *E. coli*. High amount of esterase was found to be associated when cell was grown in presence of nitrogen. Variants resistant to cetyl trimethyl ammonium bromide (CTAB) showed enhanced emulsan production.[56] Site directed mutagenesis revealed that esterase-defective mutants could not release emulsan. Mutant proteins defective were capable of enhancing apoemulsan-mediated emulsifying activity. Bach, et al[60] carried out studies on emulsan from *A. venetianus* RAG-1. It was seen that apoemulsan and esterase are essential for the formation of stable oil-water emulsions.[56,64]

Alasan

The polymer produced by *A. radioresistens* KA53 is designated as 'Alasan' and finds significant application in bioremediation.[69] Alasan is an alanine containing complex heteropolysaccharide and protein polymer that stabilizes oil in water emulsions in n-alkanes with chain length 10 or higher and alkyl aromatics, liquid paraffin, soyabean, coconut oil and crude oils.[70] The proteins of alasan have been identified as AlnA, AlnB and AlnC. One of the alasan protein (AlnA) of 45 kDa exhibiting highest emulsification activity was purified[71-74] and denoted high sequence homology to an OmpA-like protein from *Acinetobacter* spp.[75] Four hydrophobic regions in AlnA forming specific structure on the surface of hydrocarbon are responsible for surface activity.[73,74] The AlnB protein exhibited strong homology to perioxiredoxins (family of thiol—specific antioxidant enzymes). It was proposed that all three proteins may be released as a complex with AlnA entering the oil phase and Alnb forming a compact shell around the hydrocarbon, thereby forming stable emulsions.[71] *A. calcoaceticus* RA57 grown on crude oil sludge possesses three plasmids, one of which pSR4, a 20 kb fragment was found to be essential for growth and emulsification of crude oil in liquid culture.[76]

Biodispersan

It is an extracellular, anionic polysaccharide produced by *A. calcoaceticus* A2 which acts as a dispersing agent for water-insoluble solids.[77-79] It is nondialyzable, with an average molecular weight of 51,400 and contains four reducing sugars, namely, glucosamine, 6-methylaminohexose, galactosamine uronic acid and an unidentified amino sugar.[78] Rich protein was also secreted along with the extracellular polysaccharide. Protein defective mutants produced equal/enhanced biodispersion as compared to the parent strain.[59]

Exopolysaccharide (EPS)

A. calcoaceticus BD4, BD413 produces EPS with rhamnose and glucose.[80] EPS production is mediated by proteins like Ptk (protein tyrosine kinases) and was also found in *A. johnsonii*. These proteins encode for virulence factors and may serve as a target for the development of new antibiotics.[81]

Pseudomonas Species

Glycolipid BS production was first discovered by Jarvis and Johnson in 1949.[82] They reported production of an acidic, crystalline glycolipid L-rhamnose and *l*- β-hydroxydecanoic acid from *P. aeruginosa*. This compound was found to be quite similar to a compound of polymer and higher rhamnose-hydroxyacid ratio which was isolated previously by Bergstrom, et al.[83] Later, Hauser and Karnovsky[84] demonstrated the biosynthetic pathway for rhamnolipid production in *Pseudomonas* spp. Burger, et al[85] and Lang and Wagner,[86] demonstrated that *P. aeruginosa* synthesizes mono as well as di-rhamnolipid. Similarly, *P. aeruginosa* synthesizes different rhamnolipid derivatives which include 3-(3-hydroxyalkanoyloxy-) alcanoic acid (HAA), mono-rhamnolipid (L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate)[87-90] and di-rhamnolipid (L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate).[91] Details of different intermediates have been accounted by Pamp and Tolker-Nielsen.[92]

However, studies on regulatory mechanisms came very late with the work of Ochnser, et al[88,89] and Latifi, et al[93] who proposed the involvement of quorum sensing system (QSS) for rhamnolipid biosynthesis in *Pseudomonas* spp. Various components involved in rhamnolipid biosynthesis are

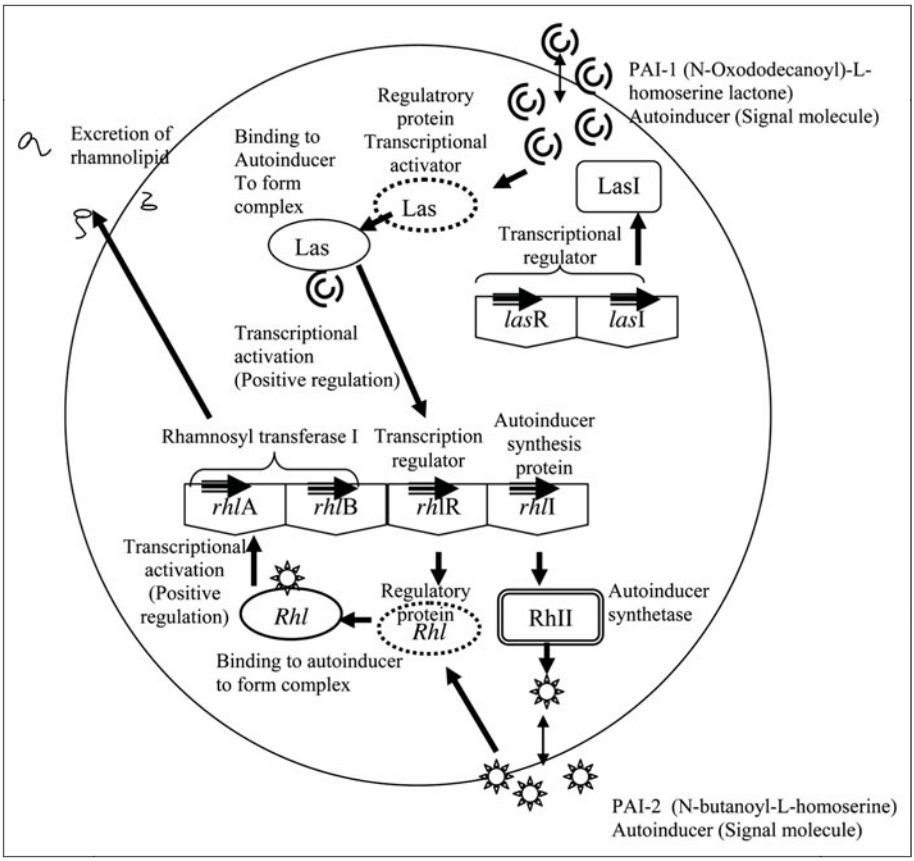

Figure 2. Rhamnolipid synthesis in *Pseudomonas* spp. by two quorum sensing system: Pictorial representation of two quorum sensing system (QSS) present at different regions of *Pseudomonas* spp. chromosome. Thick black bold arrows: Genes on chromosome of *Pseudomonas*; Black arrows: Protein synthesis from gene; Dotted oval indicates inactive regulatory protein; Continuous oval: Active complex of regulatory protein and autoinducer.[14,17,20]

represented diagrammatically in Figure 2. Two QSS regulating rhamnolipid synthesis are present on two different regions of chromosome.[94] Formation of mono and di-rhamnolipids is mediated through two different transferases viz., rhamnosyltransferase I and II. Rhamnolipid synthesis is coupled with nitrogen limitations to the cell.[95] Phosphate limiting conditions are found to enhance BS biosynthesis.[96] Detailed studies have been reported on rhamnosyltransferase I, which contains four genes viz., *rhl*A, *rhl*B, *rhl*R, *rhl*I. Plasmids encoding four genes are sufficient to produce rhamnolipid in heterologous hosts.[97] Genes *rhl*A, *rhl*B are located upstream while *rhl*R, *rhl*I are located downstream of the structural genes (Fig. 2). The *rhl*A and *rhl*B genes code for active rhamnosyltransferase I and are transcribed together as a bicistronic RNA.[88-89,97] Structural proteins are encoded by *rhl*B and present in the periplasm. Inner membrane proteins required for synthesis, transport or solubilization of rhamnosyltransferase are encoded on *rhl*A.[97] In first QSS, genes *rhl*A, *rhl*B are positively regulated by rhlR. Transcriptional activator and autoinducer are encoded by *rhl*R and *rhl*I respectively. Two signal molecules viz., N-butanoyl-Lhomoserine (PAI-2) and hexanoyl-L-homoserine lactone are produced by *rhl*I. Transcriptional activator produced by *rhl*R binds to autoinducer PAI-2 and this active complex causes transcriptional activation of *rhl*A and *rhl*B that encode

rhamnosyltransferase I. The second QSS contains two genes namely *las*R and *las*I.[98,99] In this system autoinducer is encoded by *las*I namely N-(3-oxododecanoyl)-l-homoserine-Lactone (PAI-1) RhlR regulatory protein requires autoinducers N-butyryl-HSL and N-(3-oxohexanoyl)-HSL autoinducer for its activity.[100] Induction of second QSS occurs by cyclic AMP levels as indicated by the presence of *las*R promoter region of both lux-box and binding consensus sequence for cyclic AMP receptor protein.[101] The transcription of *rhl*R system is positively regulated by *las* system.[98,102] The *rhl* system is posttranslationally controlled by *las* system by hindrance of PAI-2 by PAI-1 from binding to RhlR. This situation is created till enough PAI-2 and/or PAI-1 are produced to create blockage effect.[98] Figure 2 illustrates the regulation of rhamnolipid synthesis in *Pseudomonas* spp. It is proved that rhlR expression is strongly influenced by environmental factors and is partially LasR-independent under certain culture conditions. Different regulatory proteins viz. Vfr sigma factor σ54 and RhlR itself regulates expression of rhlR.[103]

The *rhl*I negative mutant is unable to produce rhamnolipid on its own. However, addition of synthetic N-acylhomoserine lactone (signal molecule) initiates BS production by mutant. Holden, et al[104] carried out studies to find out whether the BS genes are expressed in unsaturated porous media contaminated with hexadecane and play role in biodegradation process. For this purpose, the *gfp* reporter gene was integrated with either the promoter region of *pra*, which encodes for the emulsifying PA protein and/or to the promoter of the transcriptional activator *rhlR*. It was found that GFP was produced in culture, which indicated that the *rhlR* and *pra* genes are both transcribed in unsaturated porous media. The *gfp* expression was localized at the hexadecane-water interface. Other interesting studies carried out by Pamp and Tolker-Nielsen[92] demonstrated the BS produced by *P. aeruginosa* has additional role in structural biofilm development. Genetic evidence showed that mutant deficient in *rhlA* lack the ability to synthesize BS and could not form microcolonies. This indicates significant role of *rhlA* in BS biosynthesis and biofilm development. The protein AlgR2 responsible for regulation of nucleoside diphosphate kinase also down regulates rhamnolipid production in *P. aeruginosa*.[105] Lequette and Greenberg[106] in 2005, worked on identifying the role of QSS responsible for rhamnolipid biosynthesis on biofilm architecture. They introduced a *rhlA-gfp* fusion into a neutral site in the *P. aeruginosa* genome and highlightened the activity of *rhlAB* promoter in rhamnolipid-producing biofilms. Campos-Garci´A, et al[107] identified a new *gene rhl*G which is a homologue of the *fabG* gene encoding NADPH-dependent β-ketoacyl acyl carrier protein (ACP) reductase. This is necessary for synthesis of FA. This gene *rhl*C is obligatory for synthesis of b-hydroxy acid moiety of rhamnolipids and partly contributes to production of poly-β-hydroxyalkanoate (PHA). This study proved that different pathways are involved in synthesis of FA moiety of rhamnolipids than those for general FA synthetic pathways.

Till the year 2001, it was obvious that, rhamnosyltransferase 1 (RhlAB) catalyses the synthesis of mono-rhamnolipid from dTDP-l-rhamnose and β-hydroxydecanoyl-β-hydroxydecanoate, whereas di-rhamnolipid is produced from mono-rhamnolipid and dTDP-l-rhamnose. For the first time, Rahim, et al[91] in 2001, reported dependance of di-rhamnolipid synthesis on rhamnosyltransferase gene. Gene *rhlC* encode for rhamnosyltransferase which catalyses di-rhamnolipid (l-rhamnose-l-rhamnose-β-hydroxydecanoyl-β-hydroxydecanoate) production in *P. aeruginosa*. RhlC is a protein consisting of 325 amino acids (35.9 kDa). The *rhlC* gene is located in an operon with an upstream gene (PA1131) of unknown function. A σ^{54}-type promoter for the PA1131-*rhlC* operon was identified and a single transcriptional start site was mapped. Biological role of RhlC was confirmed by insertional mutagenesis studies and allelic replacement. Inhibition of QSS was demonstrated by work with mutants. Deletion mutants, complementation studies and northern blot analysis on *P. aeruginosa* strain PR1-E4: a *lasR* deletion mutant revealed that overproduction of the *P. aeruginosa* DksA homologue down regulated transcription of the autoinducer synthase gene *rhlI* thereby inhibiting QSS.[108]

Pseudomonas species are known to produce different types of BS viz., rhamnolipids, cyclic lipopeptides- putisolvins, lipopolysaccharide. Two types of cyclic lipopeptides (putisolvins I and II) are produced by *P. putida* PCL1445, which possess surfactant activity and also plays

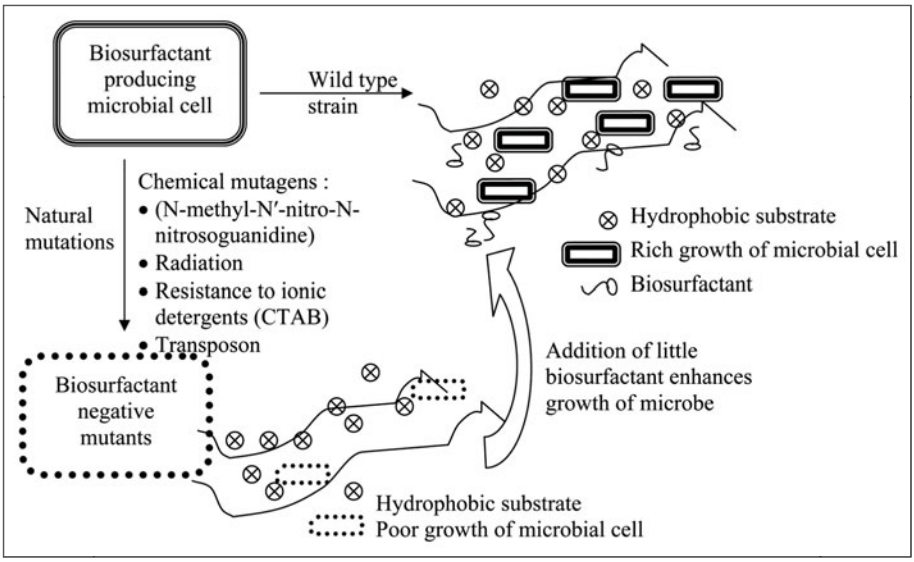

Figure 3. Effect of biosurfactant production on growth in presence of hydrophobic substrates.

significant role in biofilm formation and degradation. Mutants from Tn5*luxAB* library of strain PCL1627 defective in BS production contained transposon inserted in a *dnaK* homologue located downstream of *grpE* and upstream of *dnaJ* indicating positive regulation of these genes in BS synthesis. Two-component signaling system GacA/GacS was involved in BS synthesis.[109] Studies on co-existence of *Burkholderia cepacia* and *P. aeruginosa* in lungs of cystic fibrosis (CF) patients as mixed biofilms correlated the formation of biofilms to *cep*-regulated BS production.[110]

Generally hydrocarbon utilizing microbes produce BS. *P. aeruginosa* degrades hexadecane only if it can produce rhamnolipid.[10,111-113] Mutated *Pseudomonas* spp. produce low rhamnolipid BS.[114-116] Whereas, rhamnolipid defective mutants grow very poorly on hydrocarbons.[117] Pictorial representation is given in Figure 3. Ability of hydrocarbon uptake can be improved by addition of BS in the growth medium. This concept was proved by various studies viz., Koch, et al[118] constructed a transposon TN5-GM induced mutant of *P. aeruginosa* PG201 which could not grow on minimal medium with hexadecane. It was found that the same culture grew well with rhamnolipid supplementation. Al-Tahhan, et al[119] showed that emulsifier makes the cell surface more hydrophobic through release of lipopolysaccharide (LPS). *P. aeruginosa* grew well on paraffin in presence of emulsifier in the production medium. All these observations clearly suggest role of BS/BE in survival of microbes on hydrophobic substrates. Natural or chemical mutations are employed to improve quality and yield of BS/BE from microorganisms.[61] In the year 1995, Iqbal, et al[120] demonstrated hyper—production of BS, high biodegradation and emulsification of crude oil by an EBN-8 a gamma ray induced mutant of *P. aeruginosa*. The same mutant produced 4.1 and 6.3 of rhamnolipids (g/L) when grown on hexadecane and paraffin oil respectively.[113] Another gamma ray induced *P. putida* 300-B mutant gave high yield of rhamnolipid (4.1 g l^{-1}) on soybean waste frying oil as carbon source and glucose as growth initiator over the wild type strain.[121] A research team of Koch, et al[122] constructed a lactose utilizing strain of *P. aeruginosa* by insertion of *E. coli* lac Y genes. Two reporter systems, lacZY and lux4B, were incorporated into chromosome of *P. aeruginosa* UG2. This recombinant strain could utilize lactose and produced BS efficiently. Similar studies were also carried out by Flemming, et al.[123] Their work proved to be efficient in sensitive detection and quantitative enumeration of *P. aeruginosa* UG2Lr (spontaneous rifampin-resistant derivative) using supportive data from antibiotic resistance, bioluminescence and PCR analyses. Ochsner, et al[97] constructed recombinant strains of

P. putida and *P. fluorescence* by knocking down genes responsible for pathogenicity thereby produce harmless BS producing stains. This is the best example of application of molecular knowledge in producing biotechnologically improved stains.

Bacillus *Species*

Surfactin is a cyclic lipopeptide BS produced by *Bacillus* spp. The first report on surfactin production dates back almost to 4.5 decades. Arima, et al[124] were the pioneer researchers who reported production of surfactin from *Bacillus* species. Surfactin the most effective BS reducing surface tension efficiently (72-27 dynes/cm)[125,126] has low CMC (critical micelle concentration) value and finds potential applications in biotechnology and medicine. It is important to note that more than 70% of research on BS is accounted for *Bacillus* spp. alone. Surfactin production, structure, enzymes involved in biosynthesis, organization and genetics of production has been reviewed in great detail.[13] Due to great potential of surfactin and its diverse applications, it became necessary to study the underlying genetic mechanisms. However, the advent of these studies was not until 1988. Kluge, et al[127] laid the foundation for molecular studies by proposing a non ribosomal mechanism of surfactin synthetase. A brief summary of genetic machinery involved in surfactin synthesis is tabulated in Table 2.

Surfactin contains β-hydroxyl FA, usually β-hydroxytetradecanoic acid, synthesized by a 27 kb *srfA* operon. It is under regulation of QSS. First QSS involves nonribosomal peptide synthetases with four open reading frames (ORFs) in the *srf*A operon.[139,140] Operon *srf*A catalyses three multi-functional enzymes for surfactin synthesis.[141] (Cosmina, et al 1993). These modular building blocks are called as surfactin synthetases encoded by *srfA*, *srfB* and *srfC*. The *srf*A locus plays a key role in surfactin production; Nakano and coworkers[142] isolated *srf*A locus by cloning the DNA flanking srfA::Tn917 insertions followed by chromosome walking. This region was an operon (>25 kb) and the gene *srfA* codes for template enzymes while; another gene *Sfp* located downstream of the *srfA* operon encodes for 4′- phosphopantotheinyl transferase. This gene product modifies enzymes to their functional forms for their transcription.[143-146] Study on Tn9171ac mutations confirmed that surfactin production required both the intact 5′ as well as 3′ end of *srfA*. The 5′ region was responsible for sporulation and competence for DNA uptake along with surfactin production and contains 20,535 bp. This region contains *srfA* promoter and two ORFs *srfAA* and *srfAB* encoding surfactin synthetase I and II. The *srfAA* contains three amino acid activating domains for Glu, Val and Leu, while *srfAB* peptide synthesizing domain contains domains for activating Val, Asp and D-Leu. Gene *srfC* contains activating regions for Leu[128-130] and encodes thioesterase Type I motif responsible for termination of peptide.[131]

A third locus within *srfA* operon, the *srfB gene* is required for surfactin production. *srfB* is also necessary for expression of *srfA-lacZ* and is identical to an early competence gene *comA*. Surfactin production is under *ComA* (*SrfB*)-dependent regulation operating at the transcriptional level. *srfA* is positively regulated by product of *srfB*.[147,148] Subsequently, SrfD stimulates the initiation process.[149] However, release of surfactin is still unknown. There is an assumption that passive diffusion releases surfactin across the cytoplasm membrane.[150] Once the cell density attains a maximum level, ComX get accumulated in the medium and interacts with membrane bound histidine kinase ComP and the response regulator ComA.[151] Further, after phosphorylation, by ComP; ComA binds to promoter *srf*A and transcription begins. Competence stimulating factor (CSF), a signal peptide influences *srf*A expression.[139,142,152] It is transported across the membrane and interacts with at least two different intracellular receptors depending upon its concentration. Mutation in ComA inhibits development of competence indicating that, *com*A gene is responsible for expression of *srf* and other com genes.[148] In addition to all these proteins, ComR and SinR also influence *srf*A expression.[138] ComA is regulated positively as well as negatively by ComP under the control of the ComX pheromone.[153] The authors also suggested that *srf* expression requires SpoOK and another, as yet unidentified, extracellular factor under variable pH conditions. The gene *spoOK* codes for an oligopeptide permease that functions in cell-density-dependent control of sporulation and competence.[154,155] Thus molecular machinery ensures appropriate surfactin synthesis.

Table 2. Genetic machinery involved in surfactin synthesis from* Bacillus *spp.

Operon/Genes/Operator/ Promoter/Protein	Function	Reference
Quorum sensing system I		
srfAA[†‡]	Amino acid activating domain for Glu, Leu, D-Leu Expression of *com*S gene[#]	128,129
		130
SrfAC[†]	Encodes a thioesterase of a Type I motif responsible for peptide termination	131
sfp	Surfactin production	132
Sfp[†]	Activation of surfactin synthetase by post translational modification	132
Quorum sensing system II		
ComQ	Modification of *com*X to form signal peptide ComX	133,134
ComP (Membrane bound protein)	Gets autophosphorylated upon stimulation and transfers its phosphate group to ComA	
Phosphorylated ComA ComS (located within and out of frame srfA gene)	Binds comA-box and initiate transcription of surfactin peptide synthetase, *srf*AA-AD operon and *com*S Development of competence	135
ComX (Signal peptide)	Controls expression of *srf*A and interaction with	
	• Membrane bound histidine kinase ComP • Response regulator ComA	
*Spo*OK (Oligopeptide permease) RapC	Transfer of Competence stimulating factor (CSF) through the cell membrane; Phosphotransferase activity	136
ComR (Polynucleotide phosphorylase)	Enhances *srf*A expression posttranscriptionally	137
SinR (Transcriptional regulator)	Negatively controls *srf*A possibility by regulating *com*R	138

[‡]: Multifunctional subunit of surfactin synthetase; [†]: Part of peptide synthetase; [#]: Embedded within but out of frame with *srf*B.

The sfp locus plays a significant regulatory role at the transcriptional level. The *sfp* locus from a producer strain *B. subtilis* ATCC 21332 was transferred to a standard *B. subtilis* 168 and further subjected to transposon mutagenesis. Studies suggested that, *B. subtilis with a* sfp^0 genotype contains some genes required for surfactin synthesis; *sfp* locus responsible for surfactin production alters the transcriptional regulation of *srf*.[128] A *gsp* gene with sequence homology to *sfp* gene from Gramicidin operon of *B. brevis* complemented in trans, a defect in the *sfp* gene and was able to initiate surfactin synthesis in a non producer strain *B. subtilis* JH642 with an sfp^0 phenotype.[145] Additionally, *Sfp* gene is also responsible for hydrocarbon degradation.[156] *sfp* gene was successfully integrated in chromosome of *B. subtilis* to enhance bioavailability of hydrophobic liquids.[157] Sequencing of *sfp* gene revealed 100% sequence homology to amino acid sequence reported earlier by Nakano, et al.[132] A research team of Morikawa, et al[158] worked on cloning and nucleotide sequencing of regulator gene in *B. pumilis*. Studies indicated that out of three large

ORFs (ORF1, 2, 3), ORF3 was essential for surfactin synthesis. Additionally, production of antimicrobial substances or other secondary metabolites is associated with resistance to the producing organism. Tsuge, et al[144] proposed function of gene *yerP* as a determinant of self resistance to surfactin in *B. subtilis* 168. YerP was homologous to the resistance, nodulation and cell division (RND) family of proteins, which confers resistance to wide range of noxious compounds to the secreting organism. Mutagenesis with mini-Tn10 transposon indicated that the transposon had inserted itself in the *yerP* gene in surfactin susceptible mutant. The molecular machinery for BS synthesis in *B. licheniformis* is similar to that in surfactin synthesis.[159,160] A recombinant strain of *B. licheniformis* KGL11 was constructed by inserting the surfactin synthetase enzyme. This mutant produced 12 times the BS of parent strain.[161,162] With better understanding of the molecular phenomena, many attempts were aimed to enhance BS/BE production. Mulligan, et al[163] were successful in obtaining a threefold higher BS production over wild type employing recombinant *B. subtilis* with modified peptide synthetase. A plasmid pC112 with lpa-14, a gene was used to construct a recombinant strain of *B. subtilis* MI113. High yield of surfactin was achieved by fermentation technology.[164] Another recombinant strain of *Bacillus subtilis* MI113 (pC115), was constructed from *B. subtilis* RB14C. This recombinant strain had a gene responsible for surfactin, iturin production and produced new surfactin variants along with usual surfactin when cultured in solid-state fermentation employing soybean curd residue (okara) as substrate.[165] Along with large number of research papers published, enormous patents on BS production appear to date.[38] Carrera, et al[166,167] filed U.S. patents (5,264,363; 5,227,294) on *B. subtilis* ATCC 55033 mutant strain which produced 4-6 times better BS over wild type. Another US patent (7,011,969) on *B. subtilis* SD901 strain mutated with N-methyl-N'-nitro-N-nitrosoguanidine resulted in 4-25 times more surfactin production.[168] Such studies are opening arrays for improved BS production technologies. Various mutant/recombinant strains of *Bacillus* spp. have been constructed for better quality and optimum quantity of surfactin production (Table 3).

Serratia *Species*

Followed by *Acinetobacter*, *Pseudomonas* and *Bacillus* strains, *Serratia* is one of the well-studied bacterium in terms of molecular genetic studies of BS production. *Serratia*, a Gram-negative organism is known to produce extracellular surface active[172] and surface translocating agents.[173] *S. marcescens* produces a cyclic lipopeptide BS 'Serrawettin' which contains 3-hydroxy-C10 FA side chain. BS production is correlated with populational surface migration.[174] The mobility (swarming/sliding motility) and cell density of a population is monitored; depending on this information, regulatory systems control gene expression. This helps the microbial community in interacting with its surrounding.[175,176] The SpnIR QSS is responsible for regulation of flagellum- independent population surface migration and synthesis of BS (prodigiosin) in *S. marcescens* SS-1.[173] Later on, Wei, et al[177,178] confirmed that *spnIR* quorum-sensing genes were located on a Tn*3* family transposon, Tn*TIR*. They also proved that SpnR negatively regulated transposition frequency of Tn*TIR*. This group for the first time reported direct evidence of involvement of a *luxIR*-type QSS in regulation of transposition frequency.

BS production is controlled by auto-induction system which subsequently helps in swarming of cells.[176] *S. marcescens* ATCC 274 produces temperature dependant serrawettin W1[cyclo-(D-3- hydroxydecanoyl –L-seryl)$_2$]. Presence of *swr*W gene encoding serrawettin W1 aminolipid synthetase was identified in *S. marcescens* 274 by transposon mutagenesis. The swrW had all four domains of nonribosomal peptide synthetase (NRP), responsible for condensation, adenylation, thiolation and thioesterisation. The swrW NRP is unimodular and specifies only lysine.[179] The authors also proposed a pathway for serrawettin synthesis based on their findings. Parallel production of serrawettin and pigment production in *S. marcescens* 274 is coded by an ORF namely pswP. Synthesis of serrawettin is believed to be through non ribosomal peptide synthetases (NRPSs) system which is a product of the *pswP* gene. A single mutation in the gene is responsible for parallel disruption of both, pigment as well as BS production in *S. marcescens*.[180] In another study, screening of serrawettin W1 overproducing mini Tn5 insertional mutants

Table3. Employment of molecular tools for construction of recombinant/mutant strains of* Bacillus *spp.

Organism	Mutation	Objective	Significant Feature	Reference
Bacillus	Transformants	Integration of *sfp* gene in chromosome	Improved avaibility of hydrocarbon.	156,157
B. subtilis ATCC21332	Recombinant	Modification of peptide synthetase	3 fold yield than the parent	
B. subtilis MI 113	Recombinant	Insertion of a plasmid pC112 with Ipa-14	High yield	164
B. subtilis	Recombinant	Modification in surfactin synthetase	Surfactin without hemolytic activity of erythrocytes	169
B. pumilus		Nucleotide sequence of regulator gene: 3 large ORFs; ORF3 *psf-1* sequences	Surfactin synthesis Similar with operon of *B. brevis*	158
B. licheniformis (Lichenysin)	Recombinant strain	Enzyme complex with surfactin synthetase	Similar with that of surfactin synthetase (*srf*A)	159,160
B. subtilis	M13	The mutated PsrfA fragments were inserted into pT-Klac for construction of plasmids. Promoter activity, pMMN102 was derived from msf33 and pMMN97-101 and -103 were constructed from msf29. pMMN104 with a 10-bp insertion between two ComA boxes. PsrfA of pMMN103 (5-bp insertion between the ComA boxes).	Found that transcription of srfA operon is dependent on the transcriptional activator ComA.	170
B. subtilis (Surfactin and Iturin)	Recombinant MI113(pC115)	Gene responsible for biosurfactant synthesis cloned in *B. subtilis* RB14C	Production of new surfactin variants on soya-bean curd residue (okara)	165
B. subtilis (Lipopeptide antibiotic surfactin)	Tn917	Campbell-like recombination at pBR322 homology present in both SPpc2del2::Tn9J7::pSK10A6 DNA and pMMN derivatives *srf*B located between *aroG, ald* in genome.	Competence and surfactin production resorted by a single DNA fragment of 1.5 kbp namely *srf*B gene is *com*A	148

continued on next page

Table 3. Continued

Organism	Mutation	Objective	Significant Feature	Reference
B. subtilis (lipoheptapeptide surfactin)	In vivo and in vitro recombination	Peptide synthetase modified by eliminating large internal region of the enzyme with complete amino acid incorporating module. Permissive fusion sites identified for the engineering of peptide synthetase genes	Surfactin with altered antimicrobial activity. The selection of the recombination site is of crucial importance for a successful engineering.	169
B. subtilis	The srfA operon of surfactin synthetase; competence regulatory protein ComS	Construction of *B. subtilis* strain expressing epitope-tagged *srfB*. Plasmid pOH9 with carboxy-terminal end of *srfB* fused to the DNA encoding the influenza virus hemagglutinin 1 (HA1) epitope was constructed.	Found that *Srf* expression alters with changes in culture pH. ComP acts both positively and negatively in the regulation of ComA and that both activities are controlled by the ComX pheromone.	153
B. subtilis	Oligonucleotide directed mutagenesis	Ser-to-Ala substitutions made in the amino-acylation site of each domain	Part of the srfA contains the region required for competence development and is composed of the first four amino acid-activating domains responsible for the incorporation of Glu, Leu, D-Leu and Val into the peptide moiety of the lipopeptide surfactin. Fourth, Val-activating, domain is required for competence, suggesting that some activity, other than amino-acylation and perhaps unrelated to peptide synthesis, possessed by the fourth domain is involved in the role of srfA in regulating competence development.	139
B. subtilis 168 and 801	Transposon mini-Tn*10*	Deletion of internal region of the *yerP* gene of *B. subtilis* strain 168 yielding a *yer*P deficient strain 802	The gene *yerP* determines surfactin self-resistance.	144

continued on next page

Table 3. Continued

Organism	Mutation	Objective	Significant Feature	Reference
B. subtilis		Expression of *sfp* gene in *E. coli* , plasmid-amplified in *B. subtilis*	No effect on surfactin production and repression of a *lacZ* transcriptional fusion of the *srfA* operon, which encodes enzymes that catalyze surfactin synthesis. The *sfp* represents an essential component of peptide synthesis and directly or indirectly affects regulation of surfactin biosynthesis.	132
B. subtilis	Recombinant strain	To create *sinR* and *degU* mutant strains bearing multicopy *comS*, plasmid pMMN284 DNA in combination with DNA from strain LAB2274 (D*sinR*::Phleor) or LAB2275 (*degU*::Neor) DNA To create a *comS*9::9*lacZ* D*sinR* strain, DNA from LAB1874 (*comS*9::9*lacZ* Cmr) and LAB2274 (D*sinR* Phleor) was used to transform JH642 cells with selection for Cmr and screening for Phleor.	It was found that *sinR* is required for optimal *comS* expression but not transcription from the *srf* promoter and that SinR at high concentrations represses *srf* transcription initiation.	138
B. subtilis		The peptidyl carrier protein (PCP) domains of surfactin synthetase by transferring the 4-phosphopantetheinyl moiety of coenzyme A (CoA) to a serine residue conserved in all PCPs.	Several residues of Sfp involved in CoA interactions are not conserved and CoA binding may vary between members of this super family.	171

suggested a down regulating mechanism for BS production. The transposon was inserted between the *hexS* gene. *hexS* is a suppressive gene controlling production, therefore insertion and deactivation resulted in enhanced production of exolipids. Thus, target specific repression of *hexS* gene product in transcription is elucidated.[181] Such abortion of repression can be useful for large scale and economical production of surface active agents. Production of BS and thereby surface migration in *S. marcescens* SS-1 is controlled by N-acylhomoserine lactones (AHLs) of QSS located on a mobile transposon.[173,177] Production of BS is under negative control. *S. marcescens* SS-1 produces four AHLs via spnI. The production is regulated by SpnR in *spnI/spnR* QSS. The SpnR is a homologue of the transcriptional regulator LuxR.[173] Furthermore, deletion of this *spnR* gene to produce an isogenic mutant strain *S. marcescens* SMΔR was found to enhance BS activity.[174] Upstream of *spnI* is a gene *spnT* encoding a 464 amino acid protein.[173] The *spnT* is cotranscribed with *spnI* and also functions as a negative regulator of BS production and sliding motility. Thus mobility and horizontal transfer of these genes was proved by Wei, et al.[178] Similar correlation of genes (*swr*/QS) and enzyme involvement in BS production and swarming motility exists in *S. liquefaciens*.[182,183] This interdependence is obligatory for *S. liquefaciens* MG1 to develop swarming colony. The gene *swr*I encodes a similar putative AHL synthase for synthesis of extracellular signal molecules *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone. Expression of *swrA*, encoding serrawettin synthetase, is a homoserine lactone (HSL) and is dependent on QSS.[176,183] The flagellar master operon (*flhDC*) and AHL are involved in flagellar mobility and cell density regulation.

Mutant strain of *S. liquefaciens* was developed by transposon mutagenesis to construct a non-swarming mutant deficient in serrawettin W2 production. Sequence analysis indicated homology with gene *swrA* that encodes a putative peptide synthetase. Expression of *swrA* is controlled by QSS. Transposon mutagenesis involving the promoter less *lux*AB reporter confirmed action of *swrA* gene via QSS in production of the lipopeptide BS. The gene *swrA* encodes a putative peptide synthetase.[183] Microbes are able to change their cell surface hydrophobicity during different growth phases, morphogenesis and differentiation.[184] Cell surface hydrophobicity is affected by cell bound and extracellular factors viz., serraphobin (capacity to bind with hexadecane) and serratamolide (act as wetting agent). Serratamolide negative mutants revealed that serratamolide increases cell surface hydrophobicity.[185] Various BS producing, mutant/recombinant strains of *Serratia* have been constructed employing molecular approaches (Table 4).

Molecular Genetics of Glycolipid Synthesis in Fungi and Yeast

Candida

Sophorolipids (SLs) are one of the most common glycolipids produced by *Candida* species.[19,186-190] SL is composed of sophorose disaccharide glycosidically linked to a hydroxy FA. Genes involved in biosynthesis of SLs were identified, characterized and cloned by several workers.[188,191,192] Mono-oxygenase enzyme, cytochrome P450 dependant on NADPH (nicotinamide adenine dinucleotide phosphate) is essential for FA conversion. The *CPR* (cytochrome P450 reductase) gene of *Candida bombicola* was isolated using degenerate PCR and genomic walking. The CPR gene is made up of 687 amino acids. Heterologous expression in *Escherichia coli* proved functionality of the gene. The recombinant protein had NADPH-dependent cytochrome *c* reducing activity.[193] The genes of cytochrome P450 are diverse among them and also within the genome of a single organism. The phenomenon responsible for induction and expression of these genes was unknown.[194] Specific glycosyltransferase I leads to the coupling of glycosidic linkage of glucose and FA. Glycosyltranferase II carries out subsequent glycosidic coupling. Both glycosyltransferases have been partially purified.[195-197] Like other microorganisms *C. bombicola* produces glycolipid when grown on alkanes. Cytochrome P450 monooxygenase obtains reducing equivalents from NADPH cytochrome P450 reductase (CPR). The *CPR* gene of *C. bombicola* was isolated, sequenced and expressed in *E. coli*. The recombinant protein shows NADPH-dependent 'cytochrome c' reducing activity.[19,186]

Table 4. Employment of molecular tools for construction of recombinant/mutant strains of* Serratia *spp.

Organism	Mutation	Objective	Significant Feature	Reference
S. marcescens SS-1 SpnR-defective isogenic mutant, SMΔR	Homologous recombination	Isogenic *spnR* insertion deletion mutant of *Serratia marcescens* SS-1 where a 2 kb Sm-resistant DNA	SMΔR strain exhibited better ability for surface tension reduction and diesel emulsification than SS-1 strain did, it is reasonable to assume that the SMΔR strain produced more biosurfactant. Thus, deletion of *spnR* gene may enhance biosurfactant production from the *S. marcescens* strain.	174
S. marcescens	Mini-Tn5	Purified protein encoded in his (6)-hexS bind to DNA fragments of the upstream region of *pigA* and *swrW* genes and not to that of the *pswP* gene.	Over production of exolipids; Plasmid carrying hexS yielded low prodigiosin and serrawettin W1 with reduced activity of exoenzymes (protease, chitinase and DNAse) except phospholipase C.	181
S. liquefaciens MG1	Tn5	Transposon carrying a promoterless *luxAB* reporter the *luxAB* transposon most likely been integrated into a gene, designated *swrA*, is essential for surfactant production.	The gene *swrA*, encodes a putative peptide synthetase. Expression of *swrA* is controlled by quorum sensing.	183

Mycobacterium, Corynebacteria, Rhodococcus

Trehalose lipid (TL) contain carbohydrates and long-chain aliphatic acids/hydroxy aliphatic acids and are most effective BS produced by *Mycobacteria*, *Corynebacteria* and *Rhodococcus* species.[27] Finerty[198] studied genes responsible for glycolipid biosynthesis in *Rhodococcus* sp. H13-A. A Genomic library was generated using *E. coli-Rhodococcus* shuttle vector pMVS301. Tn917 transpositional mutagenesis in *Rhodococcus*, was employed for isolation and analysis of sporulation and developmental genes in strains of *Bacillus*.

Pseudozyma, Ustilago maydis

Mannosylerythritol lipid (MEL) are produced by genus *Pseudozyma*. A yeast strain *P. antarctica* produces MEL. Genetic study was conducted on prospective genes involved in MEL production.[199] Under nitrogen limitation, *Ustilago maydis*, a dimorphic basidiomycete produces

two different classes of glycolipids, ustilagic acids and ustilipids. Ustilagic acids contain cellobiose linked O-glycosidically to 15, 16 dihydroxyhexadecanoic acid, while ustilipids are derived from β-D-mannopyranosyl-D-erythritol and belong to the class of mannosylerythritol lipids.[200] The first report of molecular characterization of glycolipid production using mutants came very recently in 2005 by Hewald, et al.[200] They identified two genes *emt1* and *cyp1* responsible for production of extracellular glycolipids by the fungus. Gene *cyp1* codes for cytochrome P450 monooxygenase and is involved in synthesing 15, 16 dihydroxyhexadecanoic acid. *U. maydis Emt1* codes for a protein which resembles eukaryotic prokaryotic glycosyltransferases and transfers GDP-mannose to form mannosyl –D-erythritol. DNA micro-array analysis revealed that *emt1* is part of a gene cluster which comprises five open reading frames. Three proteins namely Mac1, Mac2 and Mat1, contain short sequence motifs characteristic for acyl- and acetyltransferases. Mac1 and Mac2 are essential for MEL production and are involved in acylation of MEL. Enzyme Mat1 acts as an acetyl coenzyme which is dependent on acetyltransferase. Mat1 displays relaxed regioselectivity and is able to acetylate MEL at both, the C-4 and C-6 hydroxyl groups.[201] Fifth protein is an export protein of the major facilitator family. This is the first report on presence of a gene cluster for production of extracellular glycolipids in a fungus. With these studies, authors introduce the possibility of transfer of genes between species or recent progenitors, for secondary metabolite production in fungal species.

Exploitation of Biosurfactant Molecular Genetics in Biotechnological Applications

The inherent genetic machinery controls phenotypic expression for any particular organism. Understanding of this molecular machinery and its mechanism will play pivotal role in tailoring efficient microbes for potential, economic products. There has been an ever increasing progress in biotechnology in recent years, which has generated enormous opportunities. Initially biotechnological tools were aimed at hyperproducing mutant/recombinant strains. Mutant of *P. aeruginosa* PTCC 1637 produced 10 times BS to that of wild type. Those of *B. subtilis* MI113 and *B. licheniformis* KGL11 enhanced production by 8 and 12 times respectively. Remarkably *B. subtilis* SD901 mutant produced 4-25 times higher yield.[202] Recombinant and/or mutant strains provide huge impetus for further studies (Tables 1, 3 and 4). Biotechnological applications have been recently extended to initial screening methodology of BS producers. The best example is represented from the work by Hsieh, et al.[143] The *sfp* locus was used for PCR based detection of BS producing *B. amyloliquefaciens* and *B. circulans*. Such methods would authenticate the conventional screening methods enlisted in the brief review of Bodour and Miller-Maier.[203] On similar lines, *P. rugulosa* NBRC 10877 was identified as MEL producer on the basis of rDNA sequence.[204] Direct search for genes involved (Fig. 4) would be faster and less laborious. Newer invention like those of Whiteley, et al[205] could be used to identify modulators and genes of QSS signals in bacteria. Novel indicator strains and vectors have been engineered. Techniques like electroporation are useful in transformation studies and have been used successfully in *Pseudozyma*.[206,207] The cationic liposome bearing MEL (produced by *C. antartica*) has been demonstrated to increase dramatically gene transfection efficiency into mammalian cells. Similar studies have been reported by Inoh, et al[208] in 2004. Thus, molecular tools would help to regulate and modify biosynthetic pathways to improve BS production technologies. Such significant findings can be used to upgrade lab scale studies towards field application. Advent of techniques in identification, isolation and manipulation of structural genes involved in BS biosynthesis has made it easier to improve existing BS production technologies. The first genetically engineered bioluminescent strain *P. fluorescens* HK44, with a plasmid containing pUTK21 (naphthalene degradation), transposon and introduced *lux* gene fused within a promoter for naphthalene catabolic genes was released for bioremediation process. The strain HK44 was capable of generating bioluminescence in response to soil hydrocarbon bioavailability. Authors suggested that *lux*-based bioreporter microorganisms can prove a practical alternative in determination of biodegradation in situ, with the process being well-monitored and controlled.[209]

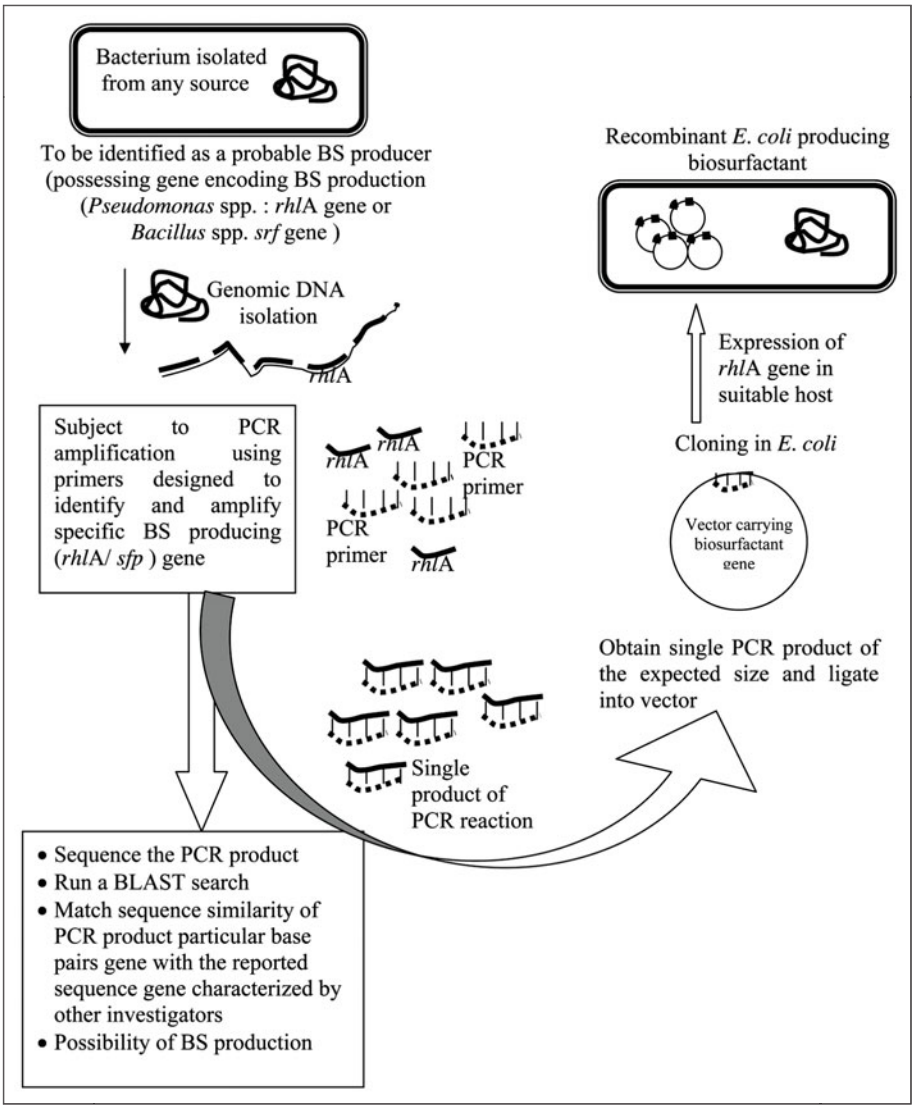

Figure 4. Molecular approach for screening of biosurfactant producers.

It is possible to use naturally occurring molecular tools for investigation purpose. Three cryptic plasmids from both *A. calcoaceticus* BD413, BD4 were isolated, characterized, sequenced and used in the construction of *E. coli* shuttle plasmids. Studies were done to clone and express the alcohol dehydrogenase regulon from *A. lwoffii* RAG-1. Gene expression and transformation in emulsan production and cell surface esterase activity in *A. lwoffii* RAG-1 were also analyzed.[210] The gene (*alnA*) was cloned, sequenced and over expressed in *E. coli*. The recombinant emulsifier protein (AlnA) exhibited 70% emulsifying activity as compared to that of native protein and 2.4 times more than that of the alasan complex. Thus, for the first time Toren, et al[74] in the year 2002, successfully produced a recombinant surface-active

protein using a defined gene. The existing molecular knowledge has opened gateways in drug discovery and manipulations. Protein products from microbes can be used for formulation of newer antibiotics and/or life saving drugs. Dams-Kozlowska and Kaplan,[58] introduced a promising and new approach for bioengineering emulsan analogs which has novel application in the field of medicine as biological adjuvants for vaccine and drug delivery.[211,212] Research team of Symmank, et al[169] genetically tailored peptide synthetase, which produced surfactin with reduced haemolytic activity. Rhamnolipid was synthesized in a heterologous host of *P. putida* by cloning *rhlAB* with *rhlRI* from the pathogenic producer strain *P. aeruginosa*.[213] These discoveries are highly commendable and certainly provide promising approach towards conversion of pathogenic to avirulent strains. It appears that, although there is no dearth to the data accumulated which is constantly building up; its actual filed implementation is in a stage of infancy. Thus, maximum exploitation of molecular mechanisms will not only add to our existing understanding of BS production; but will also help bridge the gap between research and actual application.

Conclusion

Irrespective of structural complexity, molecular mechanisms involved in polymer synthesis have been revealed. Among the low molecular BS, the genetic mechanisms in *Pseudomonas* and *Bacillus* have been clearly elucidated. The BS production in both microbes is under the influence of QSS. Different genes are involved and interplay of these genes ensures efficient BS synthesis. Mere choosing of substrates, optimization of physicochemical parameters are not enough. Understanding the genetic mechanisms will help in accelerating research towards achieving economical production. Continued research is adding to the ever expanding knowledge of this field and will certainly prove to be a boon for surfactant industry. Although the utility of genetically modified organisms seems to be farfetched due to environmental constraints; Nevertheless, an understanding of the genetic mechanisms and molecular biology of production of biosurfactants will help us in better understanding of the production phenomena. This will form the basis for further manipulation of conditions resulting in optimal and faster production of these surface active agents. More concerted efforts are needed for an optimal exploitation of generated information. A strong foundation of molecular mechanisms will help in an application oriented outlook at the surfactant industry.

Future Prospects

Over the decades, valuable information on molecular genetics of BS/BE has been generated and this strong foundation would facilitate application oriented output of the surfactant industry. Promising biotechnological advances have expanded the applicability of BS in therapeutics, cosmetics, agriculture, food, beverages and bioremediation. Interaction among experts from diverse fields like microbiology, physiology, biochemistry, molecular biology and genetics is necessary. With the knowledge at hand, BS with desired qualities can be produced. Mutants and recombinants can be generated to achieve desired yield and properties of BS. Potent but harmless strains can be constructed by employing biotechnological advances. However, meticulous and concerted efforts in unfolding the molecular phenomena of BS production in yeast and fungi are essential. PCR based detection methods can be used to authenticate newer BS producers obtained by conventional screening methodologies. Additionally, switch on/off regulatory mechanisms if involved in BS production need to be discovered and investigated.

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