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Research Article

Identification of Non-Ribosomal Peptide Synthetase Modifications Involved in Surfactin Production and Quorum-sensing Operon of *Bacillus subtilis* MJ01 Isolated from Oil-Contaminated Soil

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Abstract
The isolation of native microorganism that produced biosurfactants in order to oil pollutants bioremediation and hydrophobic oil hydrocarbons availability inter soil texture has become important issues in bioremediation technology. Surfactin is one of the biosurfactants with more application that produced by *Bacillus subtilis* strains could overcome these problems. Thus in this study, we investigated operon which involved in surfactin biosynthesis and its regulator comQXPA operon due to a high level of surfactin biosynthesis by *B. subtilis* MJ01 isolated from oil contaminated soil based on comparative genomics approaches. Surfactin operon localized and compared among six genomes of close relative strains and MJ01 indicated that missense point mutations on genes of surfactin operon were existence. These mutations affected NPRS protein AMP-binding domain that responsible to bind amino acid to correct the situation on surfactin peptide ring. It seems that lack of hemolytic and anti-microbial function of MJ01 surfactin was due to the creation of missense mutation and modifications in the surfactin biosynthesis NPRS enzyme structure. Moreover, *srf* genes expression regulated by comQXPA quorum sensing operon. MJ01 Quorum sensing operon rearrangement showed that part of the *comQ* gene was extended into *comX* gene and these genes had overlap region. Results suggested that in MJ01 genome has been occurred specific combination of *QS* genes organization. Despite high similarity of three genes *comQXP* among MJ01 with BEST7613 and other subtilis strains group, *comA* gene showed high identity with spizizenii strains group.

Keywords: *Bacillus subtilis*, Srf operon, Quorum sensing, *ComQXPA*, Surfactin

Introduction

Success in technologies of bio remediation is due to the ability of native microbial populations; so that, application of non-native micro-organisms in the alien environment will lead to failure of bio remediation project. Native adapted microbial populations show higher biodegradation potential in a shorter time in the presence of hydro-carbonated pollution. Therefore, in several studies, separation of native microorganisms with bio remediation power from oil and hydrocarbon polluted environment has been used for elimination of pollution (Patowary et al., 2016). Several reports have been offered about separation of various bacteria variants such as *Pseudomonas* and *Bacillus subtilis* from oil reserves or oil polluted soil; which show that these alive specious can be used for bio remediation or production of bio surfactant for reducing or eliminating reducing or eliminating oil pollutions (Gao et al., 2016; Pereira et al., 2013).

One of the main challenges in bio remediation is hydrophobic nature of oil hydrocarbon compounds; this challenge makes these compounds out of the reach of degrading bacteria. Some of the bacteria have gained the ability of secretion of enzymes and secondary metabolites, which aid in the decomposition process during their mutation. These microbes have the potential of producing bio surfactant that is helpful in Solution strengthening of hydrophobic hydrocarbons and increase the accessibility of microbes to the hydrocarbons (Ghazali et al., 2004). Some of the most crucial and applicable bio surfactants, which have been produced by bacteria, are lipo-peptide compounds. The cyclic peptide compounds attached to an

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amphiphilic fatty acid chain have bio environmental and industrial applications (Shaligram et al., 2016). These important biological and industrial compounds include a wide range of materials such as terpenes, bacteriocin, lipo-peptide and many other compounds (Shaligram et al., 2016).

Surfactin is one of the most effective bio surfactants, which has different pharmacological activities such as anti-microbe, anti-virus, anti-cancer, anti-fibrinolytic (hemolytic) and surface tension reduction properties. Surfactin lipo-peptide is a lipo heptapeptide containing beta fatty acid-hydroxy with chain length of 13 to 15 (or 16) carbon atoms. So far, a various variant of surfactin have been reported such as lichenysin from *Bacillus licheniformis* or lacidin from *Bacillus pumilus* Some of the surfactin variants have changes in positions amino acids 2, 4 and 7 (Roongsawang et al., 2010). Final lipo peptide can be formed linear, spinal or as a backbone of branched peptide (Jiang et al., 2016). This anti-biotic should synthesis as an ordered linear chain and finally, it should form 7 amino acids and a chain of beta fatty acid-hydroxy and become cyclic. Therefore, extinguishing one of the modules in NPRS gene cluster will result in the production of a lipo peptide with a missing amino acid (Jiang et al., 2016).

According to application of *B. subtilis* bacteria as a plant growth stimulus, surfactin is necessary for formation of biofilm in producer cells and its accumulation around plant root (Aleti et al., 2015), but it prevents formation of biofilm by other bacteria via attachment to cells' surface and their created interference (Roongsawang et al., 2010). It seems that surfactin acts through permeation to the membrane and degrading it; in fact, this action is the reason of its hemolytic, anti-microbial and anti-virus properties (Aleti et al., 2015).

Surfactin has strong surface properties that lead to a reduction of water surface tension from 72 to 27 mN/m in Critical Micelle Concentration (CMC) 25-220 mg/L due to its variants and determined conditions. Substitution effect of Glu1 in the lichenysin variant of surfactin increases its efficiency up to 2 times more than surfactin (Roongsawang et al., 2010).

Biosynthesis gene cluster of surfactin named *srfA* is more than 25 Kb. The operon has an open reading frame (ORF), *srfA*-A, *srfA*-B, *srfA*-C, and *srfA*-Te. The amino acid sequence of first three ORF is homolog with other NRPS; while the last ORF encodes a thioester from putative II type. *SrfA*-A/B/C include 3 moduli and each ORF can be classified to the functional domain type lately (Roongsawang et al., 2010).

Genetic studies show that production of surfactin is under direct effect of 3 chromosome genes of *comA*, *srf* and *sfp*. *Sfp* gene only can be found in surfactin producer races and its transference to surfactin producer races will enhance production of this effective material (Desai and Banat, 1997). *Srf* Operon is required for producing lipo peptide, surfactin anti-biotic, competitive development and efficient sporulation; it activates by Com QXPA Quorum Systems in *B. subtilis* (Guan et al., 2016). Operon operation mechanism of the quorum is through secreting ComX molecules signaling and their accumulation outside the bacteria cells. These ComX molecules attach to ComP receivers on the bacteria membrane and activate them. Activation of ComP leads to phosphorylation of ComA response regulator, which finally can lead duplication from most of the genes. Microarray studies indicate that srfA A-D is under the direct effect of ComQXPA regulon (Oslizlo et al., 2015).

ComX has the interaction effect with histidine kinase attaching to ComP membrane and stimulates the process of auto phosphorylation; it transfers its phosphor to serine residues to respond ComA regulator. Phosphorylated ComA attaches to ComA box (T/GcGG-N4-CCGCA) on the upper side of the srfA promoter as a tetramer, and begins the duplication from srfA. In addition, the mutation in 3 amino acids except aspartate at the end of N- of ComA reduces production of surfactin. These three amino acids may interfere with phosphorylation mechanism. Glucose can stimulate duplication from Com A and consequently increase *srfA* (Roongsawang et al., 2010).

In previous studies that were carried out by Jahanbani et al., (2015) on MJ01 bacteria, it was determined that the bacteria can produce effective material of surfactin at relatively preferable level (1.1 g/lit). Therefore, as operon *srf* is regulated by bacteria quorum system (comQXPA), recognition and understanding the difference between quorum operon of these bacteria with their similar homologous among their close relatives have gained attention.

Moreover, the properties of produced surfactin by MJ01 bacteria such as lack of hemolytic power, anti-bacterial and anti-fungal properties have strengthened the hypothesis that NRPS coding genes, which biosynthesize surfactin, have mutants. Therefore, this study has been carried out to study *srf* operons and quorum through comparing genomics analysis, in order to answer above questions and recognize surfactin biosynthesis mechanism and also the competition among these bacteria.

Materials and Methods

Bacteria genome of *B. subtilis* has been interpreted and sequenced in the laboratory of biotechnology Institute of Shiraz University. This genome with accessibility Number CP018173.1 is available in NCBI genome data base. Alignment, visibility, and manipulation of sequences have been evaluated by CLC genomic Workbench Software V.09. Genome interpretation data have been used in an online platform of MicroScope (Vallenet et al., 2012; Vallenet et al., 2009).

To identify producer operons of secondary metabolites such as surfactin an online tool named AntiSMASH with the address of http://antismash.secondarymetabolites.org/ was used. AntiSMASH is a predictor and comparing tool, which is used for coding the zones of secondary metabolites, bacteriocin and non-ribosomal lipo-peptide antibiotics by bacteria (Weber et al., 2015).

Results

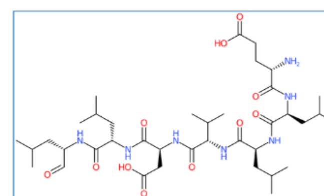
Surfactin Biosynthesis Operon

Surfactin coding operon in the position of 1484194 to 1458804 locates on MJ01 genome. This operon is similar to gene cluster existing in the data base of 86% in terms of their gene sequences (Fig. 1). Consideration of homologs gene cluster of this operon in relative bacteria indicates that this operon has the most similarity (97%) with its homologs bacteria in variants of *B. subtilis* subsp.spizizenii W23and ATCC 6633. The homologs similarities between these operons are 91 and 78% at variants of 168 and spizizenii TU-B-10 (the closest relative (fig2). Based on the data that achieved from AntiSMASH tool and interpretation of MJ01 genome, the positions of SrfA operon and *sfp* gene were determined on the genome. This operon has 4 genes *srfA-ABCD* at positions of 1458804 to 1484950. *Sfp* gene locates in 5017 open pair which is in the lower side of surfactin biosynthesis operon (Table 1).

Table 1. The position of surfactin NRPS biosynthesis operon and sfp gene on MJ01 genome

Gene name	Start	Final	Branch	Length	Fs
***srfA*-A**	1458804	1469567	+	10764	F1
***srfA*-B**	1469580	1480331	+	10752	F2
***srfA*-C**	1480367	1484194	+	3828	F3
***srfA*-D**	1484222	1484950	+	729	F4
sfp	1489293	1489967	-	675	F5

Fs: Functions
F1 = Surfactin synthetase
F2 = Surfactin synthetase
F3 = Surfactin synthetase
F4 = Surfactin synthetase
F5 = 4-phosphopantetheinyl transferase



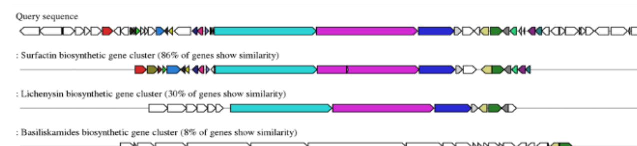


Figure 1. Gene cluster biosynthesizing surfactin lipo-peptide and its predicted structure at variant MJ01 and its comparison with gene cluster of other bacterial variants existing in the data base

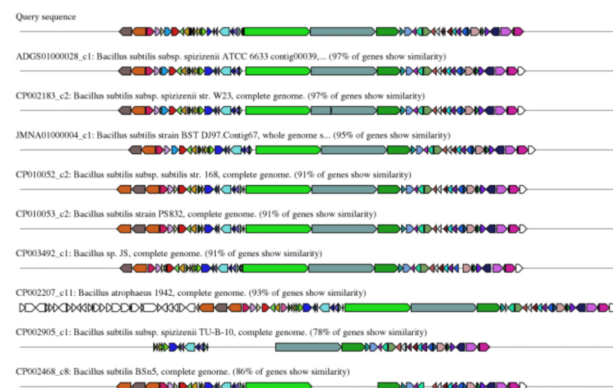


Figure 2. Gene cluster comparison between MJ01 and other bacterial variants which biosynthesize surfactin lipo-peptide. Preserved synteny zones are observable in comparison with aimed and considered sequences. Different genes are indicated by different colors and homologs genes are shown by similar colors.

The nucleotide comparison between surfactin operon genes of MJ01 with other relative variants of *B. subtilis* showed that point mutation in genes of this operon caused codon change of missense substitutions. Mutation in NRPS enzyme protein caused changes at the functional domain protein and as a result, the structure of amino acids of surfactin lipo-peptide and their efficiency have been changed (Jiang et al., 2016).

Positioning the missense point mutation on surfactin operon genes and its effect on the functional section of NRPS protein showed that

these mutations affect the AMP-binding domain which is responsible for joining correct amino acid to surfactin peptide chain (Fig. 3).

Jiang et al., (2016) studied mutation on surfactin operon genes and creation of changed surfactin peptide chain; they indicated that mutation in Lucien No. 3 and 6 of produced surfactin by *B. subtilis* bacteria caused it to loss its hemolytic power, but its anti-fungal power has increased. On the other hand, the presence of acid aspartic in surfactin peptide chain will cause losing surfactin anti-microbial power. In the other word, the absence of acid aspartic in surfactin peptide chain increases hemolytic and anti-bacterial powers of produced surfactin (Jiang et al., 2016).

Therefore, it seems that according to surfactine hemolytic, anti-microbial and anti-fungal inactivity, the main reason for changes in surfactin efficiency is missense mutation and changes in structure of NRPS enzyme; therefore, the produced surfactin, which has peptide chain, doesn't have common septet amino acids, and so it doesn't show the common hemolytic and anti-bacterial powers.

Quorum Operon ComQXPA and its Effect on Surfactin Biosynthesis

Quorum operon includes 4 genes which locate on 75902 to 80088 bp on complementary strands of MJ01 genome. *Com A* gene has been interpreted as two coding sequences, which each encodes a segment of the regulated protein of quorum (QS); it has been interpreted and recognized as a false gene (Table 2).

BLAST search of the entire sequence of the operon in local data base, which included 40 complete genomes of *B. subtilis* strains, showed that this operon with BEST7613 strain had 94.84% similarity and consequently it was similar to *B. subtilis* subsp.*subtilis* str.KCTC 3135 and delta6 variant (94.84%).

Consideration of similarity of this operon genes by local data base indicated that comA gene had the most similarity with spizizenii TU-B-10 strain (99.84%), while, its similarity with BEST 7613 was 98.28%. Other com QXP genes had the most similarity with BEST 7613 strain which were equal to 95.34, 94.05 and 93.72% orderly. Alignment of this operon with homologs operon in spizizenii TU-B-10 strain showed that nucleotide Polymorphism exists as 3 genes of comQXP in two strains with high frequency.

QS operon guides its regulatory activities via ComA through the cell. The result of studies in *B. subtilis* indicates that more than 10% of genomes are under control of quorum sensing network (QS) which is done by duplication factor of ComA (Wolf et al., 2015). Comell and Grossman (2005) found that most of the genes, which are under the effect of ComA, are coding genes of the membrane or secretory proteins or even proteins that are extracellular and involve in the production of membrane products. These processes include competitive development, enzyme digest, production of anti-biotic and exopolysaccharides, metabolisms and fatty acid transference. Their results showed that regulation path of ComX, ComP and ComA has been acted by Pheromone and kinases that are only under the effect of ComA (Comella and Grossman, 2005)

Dogsa et al., (2014) considered quorum system of *B. subtilis* and found that bacteria of these two groups can be divided into two groups in terms of quorum operon organization. The first group includes quorum operon genes that have not overlap, and the second group includes *comQ* and *comX* genes that have overlap. Because of the stop codon mutation, this overlap has caused the development of final C from *comQ* 13-18 amino acid to *comX* (Dogsa et al., 2014).

The study of quorum operon organization of MJ01 showed that this bacterium had overlap operon and some parts of the *comQ* gene have extended to the *comX* gene; based on Dogsa et al. classification *B. subtilis* MJ01 locates in the second group. It seems that overlap of reading framework leads to controlling character duplication and reduction of the requirement for complex regulatory paths (Johnson and Chisholm, 2004). In addition, this kind of overlapping organization in involved genes is common in regulating processes such as Loci that regulate comQXPA genes expression in bacteria (Comella and Grossman, 2005; Dogsa et al., 2014).

As a result, it seems that in MJ01, a unique type of shuffle combination of QS operon genes has occurred. Qua despite the high similarity of three genes (*comQXP*) from Qs operon in the MJ01 bacterium with BEST7613 and bacteria of subtilis group, *comA* gene of this operon is similar with strains of the spizizenii group as well.

Despite MJ01 genome has all the biosynthesis genes for antibiotic and peptide bacteriocins and is able to confirm the presence of resistance genes to the antibiotic, it shows a different appearance in the laboratory media. Therefore, it seems that according to logical relationship of antibiotic biosynthesis operons such as *srfA* operon (surfactin producer) with signaling and logical systems of *comQXPA*, this regulatory system and mutations in NRPS enzyme, which is responsible for surfactin biosynthesis, can be factors for changing the behaves of MJ01.

Table 2. Characteristics and positions of quorum operon *comQXPA* on MJ01 bacteria genome

Gene name	Start	Final	Branch	Length	Fs
comQ	79102	80088	-	987	F1
comX	78951	79118	-	168	F2
comP	76627	78936	-	2310	F3
comA-1	76388	76546	-	159	F4
comA-2	75902	76387	-	486	F5

Fs: Functions
F1 = Isoprenyl transferase
F2 = Competence pheromone precursor
F3 = Two-component sensor histidine kinase
F4 = Two-component response quoeume-sensing regulator-part-1
F5 = Two-component response quoeume-sensing regulator-part-2

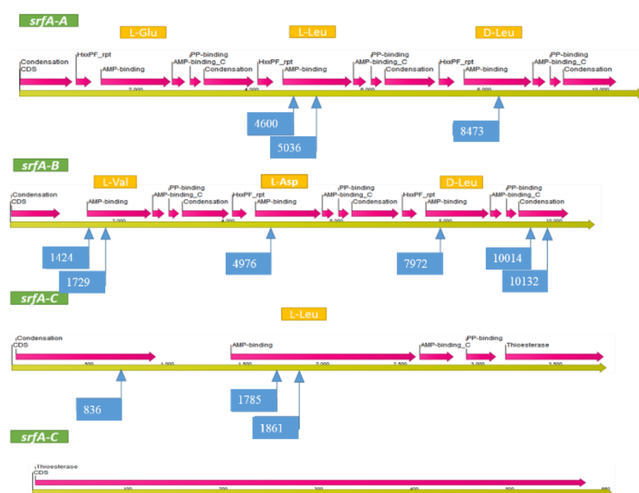


Figure 3. The position of the missense mutation on surfactin operon gene of MJ01 genome. The functional domain has been done based on Pfam (pink). A missense mutation (the blue boxes) located on the AMP-binding domain of surfactin biosynthesis enzyme (NRPS). The digits inside blue boxes show nucleotide number from the beginning part of the gene. Involved amino acids of each domain- binding have been mentioned in yellow boxes.

Discussion and Conclusion

Discovering and annotating coding sequences of PKS and NRPS enzymes in MJ01 genome and its comparison with relative strains and references indicated that although extracted lipo-peptides from this bacterium do not show anti-microbial, anti-fungal and hemolytic activities, there is not any significant difference between that enzymes and bacteria of *B. subtilis* group and their close relatives in terms of operon organizations, the structure of biosynthesis genes of biosurfactants and sequence of nucleoid. Therefore, it seems that enzyme machine that biosynthesis non-ribosomal lipo-peptide in MJ01 bacteria may be the subject to changes or modifications after translation of enzyme protein, as a result it causes their peptide products could not show its effect. Mutation in subunits of enzyme protein domain (NRPS) can substitute amino acids in the structure of lipo-peptide chain (Jiang et al., 2016). This substitution can reduce surfactin activity in *B. subtilis* up to two times. Therefore, the exact study of reasons and effective factors in the appearance of such inefficiency can be a solution for biosynthesis engineering of anti-fungal and anti-microbial lipo-peptides.

Proper growth of this bacterium in culture media showed that it should have high competition and quorum abilities. Although high polymorphism in *comQXPA* genes of *B. subtilis* bacteria which have been separated from soil has been reported previously (Oslizlo et al., 2015), but considering *comQXPA* quorum operon in MJ01 bacteria showed that nucleotide changes caused that *comA* gene has been considered as a false gene in the interpretation of MJ01 genome. Therefore, according to regulating the role of QS operon gene, consideration of gene network relationship under the effect of *comQXPA* and its effect on secretory mechanisms and extracellular lipo-peptides activities of MJ01 bacteria through data of genome expression seems necessary.

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