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Detection of *luxS* gene expression under stressing factors for biofilm formation by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii*

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ABSTRACT

Gene expression constitutes an important role in cellular communication, setting mechanisms for biofilm formation. Genes can be used as molecular markers to monitor viability, stability and maintenance of biofilm eg. in biofilm reactors, bioremediation and biotransformation frequently under stressing conditions to enhance or limit the biofilm formation. In the present study, no pathogenic microorganisms of industrial interest were used. *Propionibacterium freudenreichii subsp. shermanii* DSM 4902^T and *P. acidipropionici* DSM 4900^T strains cannot produce biofilm in culture conditions previously reported. In this regard, chemical culture conditions were modified to stimulate biofilm formation in both strains and determine that under stressing conditions such as 0.6M NaCl, 1.8 M glucose and 10 gL^{-1} yeast extract both Propionibacterium produce biofilm. Finally, *luxS* expression was identified in biofilm of both strains by modified fluorescent in situ hybridization expression (FISH expression).

Indexing terms/Keywords

Biofilm, FISH expression modified, *P. freudenreichii* subsp. *shermanii* DSM 4902^T, *P. acidipropionici* DSM 4900^T, stressing factors and *luxS.*

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INTRODUCTION

Almost all microorganisms are able to induce the production of biofilm as protection layers to assure microbial survival under stressing conditions such as biological, chemical and physical changes at the surrounding environment. Biofilm is a complex assemblage of microbial cells which are irreversibly associated with a surface as well are enclosed in a polysaccharide matrix. It has three components: cell mass that can be constituted by one or more microbial species, the intercellular spaces or water channels and, a matrix of extracellular polymeric substances (EPS). In general, there are five stages for biofilm formation: Initial attachment, irreversible attachment due to EPS, early development, maturation of biofilm architecture and, desorption [1, 2, 3].

Important biotechnological applications of biofilm are processes such as fungal biocontrol, bioremediation, production of chemicals through biosynthesis, fermentation and biotransformation, among others. In addition, reaction rates established in biofilm reactors are usually higher and the operation costs are cheaper than other reactor configuration [3, 4].

Biofilm forming capacity (BFC) and ESP production are closely related to the cellular chemical signaling, allowing the adaptation of microorganisms to environmental changes. A well-known cellular chemical signaling is the Quorum sensing (QS), which is characterized by the production and realizing of chemical signal molecules (auto inducers). Then, microorganism can activate or repress target genes according to population density, environmental dimensions as well as the regulation of biofilm formation.

There are several reports describing specifically two chemical signal molecules, the autoinducer-1 (AI-1) or acylated homoserine lactone (HSL) and theautoinducer-2 (AI-2) that allow bacteria to communicate both within and between different species [5].
The autoinducer-2 (AI-2), a chemical signal molecule that establishes the cell communication called *cross-talk*, was reported in over 40 species of Gram positive and Gram negative bacteria [5, 6] and its production involves the catalytic activity of *LuxS* encoded *by luxS* gene. Database analysis showed that conserved *luxS* homologues genes exist in over 30 species of both Gram-*negative and* Gram-positive bacteria [6, 7, 8, 9, 10, 11*].*Several studies observed a correlation between AI-2 activity and luxS expression during biofilm formation in *Streptococcus bovis, Streptococcus pneumoniae, Edwardsiella tarda, Porphyromonas gingivalis* by molecular tools [12, 13, 14, 15].Furthermore, AbaI protein is highly similar to the members of the family LuxI. It has been shown that this autoinducer synthase was required to produce AI-1 essentially to establish a specific communication within Gram negative bacteria [16]. AbaI protein is encoded by abaI gene and was also identified by molecular tools in *Acinetobacter baumannii* [17, 18].

Until now*, th*ere is not enough information about *luxS* and *abaI* transcripts used as molecular markers to monitor biofilm formation, both naturally or in different biotechnological processes, for instance in maintenance and cleaning of reactors, biofilters and others [19]. This kind of monitoring could help to determine viability of biofilm forming bacteria and biofilm stability in order to establish different processes in biofilm reactors.

Propionibacterium genus is used in several industrial processes such as probiotic agent production of Swiss-type cheeses, vitamin B12, propionic acid and tetra pyrrolic acids, among others [19, 20, 21, 22, 23]. *P. freudenreichii subsp*. *shermanii* DSM 4902^T and *P. acidipropionici* DSM 4900^T are the most used bacteria at industry level, however they are not forming biofilm in culture conditions previously reported [24, 25, 26, 27].

For stable production of fermentative metabolites, fermentations with immobilized cells are favored over those with free cells. In this regard, cell immobilized reactors constitute the best choice to establish different mode of operation conditions, using biomass carriers of diverse types [20]. Several immobilization techniques have been reported; however, adsorption on a solid support and entrapment inside a polymer matrix are the most studied. Adsorption can be established by chemical, physical and biological interactions, where covalent bond formation offer the advantage to achieve high cell concentration and high productivity, but disadvantages such as a) cell growth inside matrix may be restricted, b) cells leach out of the matrix and c) chemical may affect the cells, can affect the fermentative process. On the other hand, in biofilm reactors, high cell concentrations are also achieved but are economic to operate [5].

The present study pretend to stimulate biofilm production by *P. acidipropionici* DSM 4900^T and *P. freundereinchii* DSM 4902^T under culture stressing conditions, to identify *luxS* and *abaI* expression, that can be used as a molecular markers to monitor biofilm formation, stability and bacterial viability. Considering that biofilm is a biodegradable matrix, it is crucial to identify these transcripts using a novel molecular process such as FISH RNA, detecting *in situ* gene expression.

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MATERIAL AND METHODS

Strains, culture conditions and kinetic growth determination

P. acidipropionici DSM 4900^T *and P. freudenreichii subsp. shermanii* DSM 4902^T strains were grown under anaerobic conditions. The culture medium used according to Dishisha et al. [20] had the following composition per liter: 10 g yeast extract, 20 g glucose, 2.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 0.25 g of L-cysteine HCl and pH was adjusted to 7 adding NH_4OH at 57.6 %. Serum bottles of 100 mL capacity containing the described culture medium (80 mL) were treated using the Hungate technique [28]. They were flushed with oxygen-free nitrogen and subsequently autoclaved at 121 ºC for 20 min. Five mL of each stock culture were inoculated to sterile medium and incubated at 30 °C for five days.

One mL sample of each culture was collected daily to determine the absorbance at 620 nm in a spectrophotometer (Spectro Master). These determinations were used to establish the kinetic growth, doubling time and specific growth rate.

The culture medium composition described here was also modified in following experiments in this study in order to stimulate the biofilm production by the mentioned strains.

Determination of biofilm forming capacity (BFC)

Among a number of described methods to determine the Biofilm forming capacity (BFC) of microorganism [29, 30, 31], the method described by Faleiro was used in this study [32]. The BFC of both strains *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T were evaluated using Falcon 50 mL conical centrifuge tubes containing a glass coverslip (22 x 22 mm) per tube. To keep the anaerobic conditions, the conical tubes were flushed with oxygen-free nitrogen and autoclaved. A volume of 7.5 mL of inoculated culture medium were taken at stationary phase and then, added to each conical centrifuge tube. They were incubated for 24, 72 and 196 h independently *at 30* °C. Subsequently, the coverslips were transferred to another conical tube containing 7.5 mL of 0.1% crystal violet to stain them for 45 min, while the planktonic cells (supernatant) were measured at 630 nm. After that the coverslips were rinsed carefully with deionized water and dried, then transferred to another conical centrifuge tube containing 7.5 mL of absolute ethanol for 10 min. Finally, the absorbance was determined at 570 nm with a spectrophotometer (Spectro Master). The BFC was determined according to the formula described in Table 1 and also the classification in four categories according to the biofilm attachment to a glass surface.

Table 1.Semi-quantitative classification of biofilm formation in four categories

Formula	Strong	Moderate	Weak	No forming
$BFC=(ODb-ODc)ODC^{-1}$ (*)	$\geq$1.10	0.70-1.09	0.35-0.69	<0.35

(*) BFC: Biofilm Forming Capacity, ODb: OD 570 nm =bacterial adherence, ODc: OD 570 nm =medium without inoculum ODC: OD 630 nm =bacterial growth [32].

Biofilm microscopic analysis

EPS matrix of biofilm was observed by microscopy. In this sense, another cover slip incubated for 120 h at 30 °C, was stained with crystal violet (0.1%) for 45 min. The excess of staining was removed by consecutive washes with distilled water. Finally, the coverslip was placed onto a slide and observed at 100x using an optical microscope (Olympus).

Biofilm production in stressful growth conditions

Some chemical factors such as sodium chloride, glucose, sodium citrate and yeast extract among others can induce biofilm formation in stressful growth conditions [33, 34, 35]. In this sense modifications of the culture medium described above were tested on the basis of a factorial design. Batch cultures of both strains were established modifying 3 stressing chemical conditions (carbon source: glucose or glycerol, nitrogen source: Yeast extract and, salts: sodium citrate or sodium chloride) at two different concentrations: Sodium chloride 0.6 M and 1.2 M, sodium citrate 15 mM and 35 mM, glucose 1.8 and 3.6 M, glycerol 1.8 and 3.6 M and yeast extract 5 and 10 gL^{-1}.

These experiments with different and combined concentrations could increase or diminish the biofilm forming capacity.

Extracellular Polymeric Substances Extraction (EPS)

Two experiments were carried out to determine EPS composition present in biofilm of both Propionibacteria strains. The first experiment was consisted on centrifugation and membrane filtration, and procedures such as centrifugation, membrane filtration, dialysis and freeze drying were established in the second experiment. In both of them, biofilm was collected in

conical centrifuge tubes of 15 ml and centrifuged at 1000 x g for 20 min at 4°C. Then the supernatant was filtered through a 0.22 µm membrane to be used as the EPS sample. After the later filtration just described, a subsequent dialysis was carried out using 5 KDa cut off, to be finally followed by a freeze-drying treatment during a week. Subsequently, in both cases Chemical Oxygen demand (COD) [37] determination, carbohydrates quantification by anthrone method described by Rodriguez [38] and the determination of total proteins according to Lowry [39] were carried out in order to determine the EPS composition. Culture medium without bacterial cells and biofilm was used as negative culture.

Probes design

Designing Antisense Oligonucleotides program was used to design the probes. This program is proposed to design antisense oligonucleotides, and iRNA in eukaryotes. However, it was also used to design antisense oligonucleotides in prokaryotes in this study, where the antisense oligonucleotide probe binds specifically to target mRNA forming a hybrid [40]. The identification of *abaI* and *luxS* sequences in *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T biofilm, was done using *Lactobacillus plantarum* and *Acinetobacter baumannii* strain M2 as positive controls, with NCBI accession numbers HQ704889.1 and EU334497.1, respectively. Table 2 shows the probe sequences obtained using the program. The syntheses and labeled of the designed probes were done at IDT (In vitro DNA Technology Company, USA).

Table 2.Sequences of designed probes to determine *luxS* and *abaI* expression used in this study

Microorganism	N° of Acces	Probe	Sequence	Fluorophores
Lactobacillus plant arum	HQ704889.1	*luxS*	**5'** TGGGAAGACGTACAAGGGAC **3'**	Cy3 (*)
Acinetobacter baumannii	EU334497.1	*abaI*	**5'** AGGGTTGTGTGGTGGGTAGT **3'**	6 Fam (**)

(*) Cy3: Cianina 3, (**) 6 Fam: 6-Carboxifluoresceina

Fluorescent *in situ* hybridization expression (FISH expression)

FISH RNA or FISH expression is a molecular method used to identify mRNA in situ through DNA – RNA hybridization which is detected via fluorescence [41]. In the present study, some modifications to this method were established using RNA protect^{TM} Bacteria Reagent (QIAGEN) to protect bacterial RNA, and also the probe design was done through the bioinformatics program (Designing Antisense Oligonucleotides).

Samples containing only biomass or biofilm were obtained from the growing cultures, correspondingly. A pretreatment was established consisting of 800 µL sample mixed with 500 µL of ARN protect ^{TM} Reagent (QIAGEN) and incubated for 15 min at room temperature. Then, the mixture was placed on ice taking into account the precaution to keep the following experimental procedures also established once. The mixture was centrifuged at 4800 x g for 8 min and washed with PBS three times. Once it was washed, 850 µL of absolute ethanol were added and incubated at 4 °C for 16 h [42]. Subsequently, 8 µL of the sample were fixed on to a slide and dehydrated with ethanol at 50, 80 and 96%. After that, 8 µL of hybridization buffer (0.9 M Na Cl, 20 m M Tris-HCl, 0.01% SDS, pH 7.2) and 8 µL of probe were added over the treated sample. The slide was incubated in a humid chamber at 45 °C for two hours. Then, the slide was treated using the Washing Buffer (5 M Na Cl, 0.5 m M EDTA, 10% SDS, 1 M Tris-HCl pH 7.0) and incubated at 45 °C for 10 min. Finally, the slides were observed at 10x and 100x using a fluorescence microscope (Olympus BX-40) [43, 44].

Statistical analysis

All assays were carried out in triplicates. BFC indexes calculated from experiments were analyzed using ANOVA ($p < 0.05$) with the statistical program R Core Team (2013).

RESULTS AND DISCUSION

Kinetic growth determinations

Some bacterial species can form biofilm able to adhere to certain surfaces under different environmental conditions [1, 4, 45], and it is well known that a crucial key to evaluate the BFC is to determine the period of time when the stationary phase is established [45, 46, 47]. In this regards, the kinetic growth parameters of both strains were determined. The maximum specific growth rate, doubling time, and the start–time of stationary phase were 0.116 h^{-1}, 6 h and 48 h for *P. acidipropionici* DSM 4900^T and 0.05h^{-1}, 14 h and 72 h for *P. freudenreichii* subsp. *shermanii* DSM 4902^T, respectively.

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Biofilm production by *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^{T}under stressing conditions

The BFC indexes for *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T were less than 0.35 (data not show) when they were grown in the culture medium described by Dishisha et al, 2012 [20], being considered by this way as non-bio film forming bacteria.
Among all studies of BFC referring Propionibacterium genus, only P. acnes was reported as a strong biofilm-producing strain [48]. In this regards, in order to stimulate the bio film formation by the former strains, a factorial design was established to determine the effect of chemical stressing factors such as sodium chloride, sodium citrate, glucose, glycerol and yeast extract at different concentrations. The BFC indexes over 1 are shown in Table 3 as a result of the combinations of sodium chloride and glucose as stressing factors for bio film formation by *P. acidipropionici* DSM 4900^T and *P. freudenreichii* DSM 4902^T. In spite of there is not a significative difference of BFC indexes between both strains ($p \leq 0.05$, data not shown), they were found to be strong biofilm-formers with the addition of 0.6 M NaCl as salt, 1.8 M glucose as carbon source and 10 gL^{-1} yeast extract as nitrogen source in culture media. Then, the mentioned combined chemical conditions were established for all the subsequent tests.

The use of different culture media and or stressing factors could affect cell adhesion and biofilm formation in different microorganisms. Then, the chemical, physical and biological conditions can be modified to favor the biofilm formation. In this sense the manipulation of nutrient availability for bio film formation is an interesting application in industry. Several studies determined that cations including sodium, calcium and iron increase adhesion by reducing electrostatic repulsion and stabilization of the interactions between the negatively charged bacterial surface of *Pseudomonas fluorescens, Lacto bacillus casei, Sphingomonas paucimobilis* and glass surfaces [50, 51]. In addition, NaCl and glucose are also associated to enhance biofilm formation in *Lactobacillus casei* CG11, *Sphingomonas paucimobilis* and *Staphylococcus aureus*, besides it was described that biofilm formation in *S. aureus* involve *rbf* gene expression when NaCl or glucose concentrations are increased in the culture medium [52, 53, 54]. However, Martinez, 2011 [33] described that $FeCl_3$ and NaCl could inhibit the production of bio film in e.g. *Pseudomonas aeruginosa* and *Stentrophomonas maltophilia.*
Furthermore, other studies also described that increased concentrations of carbon and nitrogen sources and low concentration of potassium and phosphate enhance EPS synthesis [34, 55].

On the other hand, modifications at physical culture conditions such as aireation could determine biofilm formation. It was demonstrated that *B. subtilis* grown in nutrient broth with aerated conditions was unable to produce biofilm, while in minimal medium without aeration, biofilm formation was evident [45, 49].

Table 3. Indexes of Biofilm forming capacity (BFC) for *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T grown in culture conditions containing different concentrations of chemical stressing factors (salts, carbon sources and nitrogen source)

	Variables			Outcomes	
Strain	[Salts]	[Carbon source]	[Yeast extract]	BFC index	BFC Classification
P. acidipropionici DSM 4900^T	Sodium citrate 35 Mm	GlyOH 1.8 M	5 gL^{-1}	**0.8**	Moderate
	Sodium citrate 35 mM	Glc 3.6 M	10 gL^{-1}	**0.7**	Moderate
	NaCl 0.6M	GlyOH3.6 M	10 gL^{-1}	**0.9**	Moderate
	NaCl 0.6M	Glc 1.8 M	5 gL^{-1}	**1**	Strong
	NaCl 0.6M	Glc 1.8 M	10 gL^{-1}	**1.2**	Strong
P. freudenreichii subsp. shermanii DSM 4902^T	Sodium citrate 35 mM	Glc 3.6 M	5 gL^{-1}	**0.7**	Moderate
	NaCl 1.2 M	Glc 1.8 M	10 gL^{-1}	**0.7**	Moderate
	NaCl 0.6M	Glc 1.8 M	10 gL^{-1}	**1.7**	Strong

Glc: Glucose, GlyOH: Glycerol.

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EPS composition

The EPS were extracted from the experiment described above and COD, total proteins and carbohydrates. When the first experiment was conducted based on only centrifugation and membrane filtration, the COD and carbohydrates determinations gave higher values than the second experiment including dialysis and freeze drying. However, the protein contents showed to be lower in the former experiment compared to second one (Table 4). Moreover the EPS matrix obtained from *P. freudenreichii subsp. shermanii* DSM 4902^T strain showed higher amounts of organic matter and protein with the exception of carbohydrates concentration (11 mg mL^{-1}) obtained by second assay of EPS extraction. To any further extent, this behavior would be related to the increased production of biofilm by this strain.

Table 4. EPS composition with and without dialysis and lyophilization treatment of bacterial biofilm obtained from *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T

Methods of EPS obtention	C+MF	C+MF+DL +FD	C+MF	C+MF+DL+FD	C+MF	C+MF+DL+FD
Microorganism	**COD mg O2L^{-1}**		**Carbohydrates mg mL^{-1}**		**Proteins mg mL^{-1}**	
***P. acidipropionici* DSM 4900^T**	188.2±0.53	41.9±0.36	247.0±0.27	22.1±0.34	0.03±0.25	2.4±0.48
***P. freudenreichii subsp. shermanii* DSM 4902^T**	665.3±0.46	107.5±0.49	358.4±0.23	11.0±0.48	0.04±0.37	3.0±0.43

C= Centrifugation, MF= Membrane filtration, DL= D yalisis, FD=Freeze Drying

The composition of EPS is significantly affected by the extraction method [56, 57]. Several EPS extraction techniques can get different amounts of EPS. Even for similar culture conditions, variations up to 100 times of EPS amount obtained were reported. Pan *et al,* 2010 [35] cannot determined proteins in algal-bacterial bio film using centrifugation as a sole method for EPS extraction. However Tapia *et al* 2009 [55] described that the amount and composition of EPS extracted by centrifugation was similar to that obtained by heating and the addition of EDTA. This could be attributed to the low sedimentation rate of cells and the strong action of shearing forces on individual cells resulting in a high extraction of EPS, even without a pre-treatment, but also in a certain cell lysis. Nevertheless, since EPS showed protein/carbohydrate ratios slightly higher for the centrifugation and heating methods, the extraction with EDTA could be more recommendable because of its combination of a high extraction with a low cellular lysis.

Identification of *luxS* homologue gene expression by FISH expression method

FISH expression method was used in order to determine the involved mRNA in the biofilm samples from both strains subject of this study. Designed probes for *luxS* and *abaI* were used. Figure 1 shows that only luxS was identified in both strains, interestingly, *luxS* transcripts were previously reported in *Proponibacterium acnes* [48].

The fact that abaI was not identified in both strains, might have been due to a non-appropriate design of the probes for this gene or, on the other hand, this gene could be detected in early stages of bio film formation. Besides, *abaI* was only reported in *Acinetobacter baumannii* strains. This gene codified a homologue enzyme to LuxI that is involved in acyl-homo serine lactone (AHL) synthesis, signal molecule in Gram negative bacteria. This kind of communication is specie-specific. In addition, *luxS* and *abaI* expression were not detected in non-bio film-forming bacterial growth in culture medium without addition of stressing factors [20].

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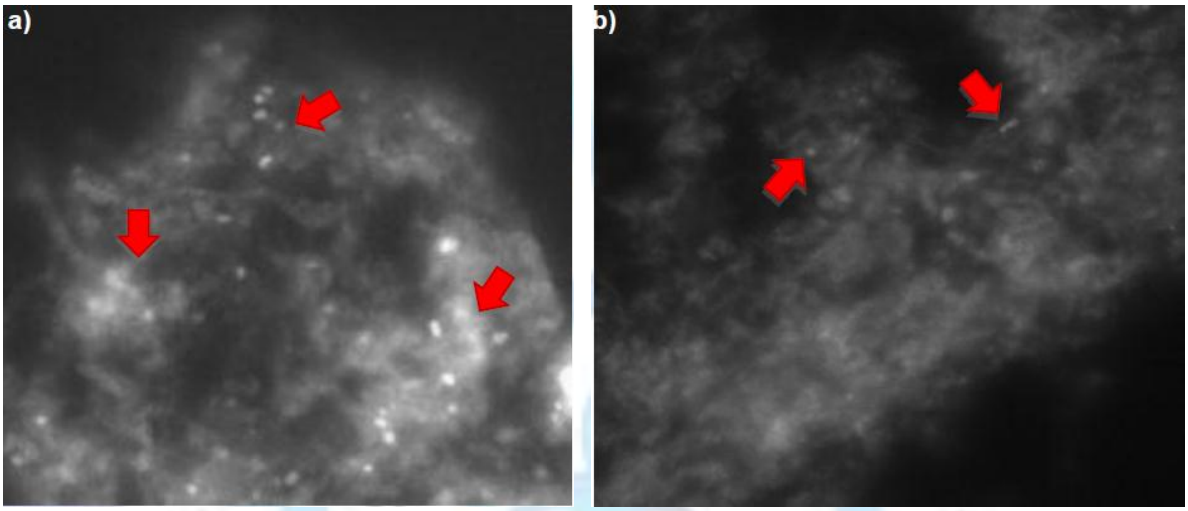

Figure1. Fluorescence microscope images (100x) of a) *P. acidipropionici* DSM 4900^T and b) *P. freudenreichii subsp. shermanii* DSM 4902^T biofilm. The arrows indicate *luxS* gene expression in bacteria.

It is well known that the widespread test to identity AI-2 molecule is the bioassay based on *Vibrio harveyi* BB170. Nevertheless, this method has many drawbacks such as 1) growth and luminescence are strongly influenced by trace elements such as Fe^{3+} vitamins, lactic acid, glucose introduced directly into the bioassay causing inhibitory effects, 2) borate interferes with the detection of AI-2 by giving false positive results and 3) several studies reported the low concentrations and instability of AI-2 in biological samples [58, 59]. Consequently, *luxS* gene homologues identification by molecular methods is more specific and quicker than bioassay based *on V. harveyi*.

The present study describes a novel procedure to identity transcripts by FISH RNA. Antisense probes were designed to determine gene expression involved in biofilm formation by FISH expression method. Therefore, this technique has the potential to provide information in gene expression studies in single cells. It is a very useful tool to analyze different functional aspects of genome expression; however few protocols have used to determine mRNA in microbial cultures by FISH [60, 61]. This method requires a partial target sequence for genetic mapping, the presence of hundreds of copies from these sequences into a single cell to transcripts are necessary to be detected by microscopy. Transcripts (mRNA) of a specific gene in bacterial cells are often less abundant than rRNA, which can be considered as a disadvantage.

On the other hand, another alternative is *in situ* Reverse Transcription (ISRT) method when exist low number of target copies [62, 63]. Transcripts (mRNA) inside of bacterial cells can be detected by ISH (*in situ* Hybridization) when the digoxigen in labeled nucleotides (e.g., 200 bases) are used as a probe. In this case, an increased signal is achieved by using multilabeled nucleotides in a sole probe rather than amplifying the gene target. Therefore, *in situ* reverse transcription (ISRT) with a single primer binding to RNA and extension of the primer by using reverse transcriptase in the presence of labeled nucleotides will also be sufficient to detect low copy number of RNA. ISRT allows multiple incorporation of labeled nucleotides (e.g., DIG-dUTP or CY3- dUTP) into a single copy of transcribed cDNA. It should provide a more intense signal than the standard ISH with a monolabeled probe for in situ detection of RNA sequences. However the IRST method is more time demanding, further treatment of the sample and in addition the cost is higher than FISH expression [64, 65].

Consequently, the use of this method (modified FISH expression) allows monitoring the expression of genes involved in bio-film formation in *Propionibacterium* strains. Techniques using fluorescent molecules are excellent tools for studying the specific gene expression in bio film. Then, the use of these methods in single strains may help to understand the structural dynamics of EPS matrix during bio film formation [66].

Moreover the sensitivity of the method is closely related to the amount of available target (mRNA) in the cells so that the signal is detected in a fluorescence microscope. However, as an internal control of the method, a reverse transcriptase PCR (RT-PCR), real time PCR and proteomic analysis would be carried out during biofilm formation and determine the difference between genes that can be more expressed than other genes, to support this study [67].

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CONCLUSION

It was established that *P. acidipropionici* DSM 4900^T and *P. freudenreichii subsp. shermanii* DSM 4902^T strains could produce biofilm under conditions not reported so far; therefore giving new insights in the study of biofilm formation by non biofilm-formers. Furthermore luxS identification in biofilm formation can also give clues about the molecular genetics behind the biofilm formation. This study can have future impact in the production of biofilm with the aim of acquiring complex biopolymer or matrixes, but also in the study of the eradication of such complexes, especially in health field.

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