Natural transformation in *Streptococcus gordonii* biofilm

By: Fatema Rahman

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Norwegian College of Fishery Science
Faculty of Biosciences, Fisheries and Economics
University of Tromsø
Norway

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Abstract

Natural transformation is one of the three mechanisms of horizontal gene transfer by which bacteria are able to take up free DNA from the surrounding environment. In natural ecosystems, bacteria preferentially attach to surfaces, forming matrix-enclosed communities known as biofilm. Biofilm cells differ phenotypically and physiologically from their free-floating counterparts. **Objective**: To estimate transformation frequency in *Streptococcus gordonii* biofilm and compare that to transformation in a planktonic type of growth. **Methods**: *S. gordonii* challis (ATCC 35105) was grown as biofilm in appropriate liquid media. The plasmid vector pVA838 and chromosomal DNA of *S. gordonii* were used as donor DNA in the transformation assay. Strain-specific synthetic Competence Stimulating Peptide (CSP) was used to induce competence in *S. gordonii*. The biofilm was grown in polystyrene microtiter wells. Cells in the biofilm were harvested to assess the transformation frequency. **Results**: The transformation frequency in biofilm bacteria was found to be approximately 6-fold higher than those of planktonic counterparts. **Conclusion**: This result may either indicate a potential stress-related role of CSP or the fact that CSP participates in the synthesis of bacteriocin-like protein. The finding of enhanced cells in the biofilm in the presence of CSP suggests the involvement of CSP-QS system in the process of biofilm formation. Probably the most confusing finding of this present study was the dramatic increase of the OD value in the liquid culture of *S. gordonii* after addition of CSP in the early growth phase. This study demonstrated that *S. gordonii* cells were able to acquire foreign plasmid DNA much more efficiently than their planktonic counterparts *in vitro*. This finding suggests that biofilm growth mode may provide optimal condition for genetic transformation.

Keywords: Natural transformation; synthetic CSP; quorum sensing; biofilm bacteria.

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Abbreviations:

AHL	Acythomoserine lactone
CFU	Colony Forming Unit
CSLM	Confocal Scanning Laser Microscopy
CO_2	Carbondioxide
CSP	Competence Stimulating Peptide
CV	Crystal Violet
DNA	Deoxyribonucleic acid
EPS	Extracellular Polymeric Substances
$Erythromycin^R$	Erythromycin resistant
HFIR	Homology Facilitated Illegitimate Recombination
$Kanamycin^R$	kanamycin resistant
nM	nanomollar
nm	nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
TAE	Tris-Acetate-EDTA
$Tetracyclin^R$	Tetracyclin resistant
THB	Todd Hewitt Broth
µl	microlitre
µg	microgram

1. Introduction

Bacteria are commonly found in the environment both as free living and as sessile forms. Free living bacteria are termed planktonic, while sessile bacteria are known as biofilm bacteria. Planktonic bacteria are able to adhere to the surfaces and to initiate biofilm formation (Donlan and Costerton 2002). More than 99 % of all bacteria live in biofilm communities (Ashby 1994). In the aquatic environment, the complex biofilm community is composed of other organisms such as diatoms, fungi, unicellular algae and protozoa (Potts 1980; Anderson 1995; Shankar 1998; Nagarkar and Williams 1999; AI 2004). A common general consensus about the biofilm is its high tolerance to antibiotics. However, when bacteria are brought back into planktonic forms, their susceptibility to antimicrobials radically increases (Parsek and Singh 2003; Hall-Stoodley, Costerton et al. 2004). It is estimated that the bacteria within biofilm are 1000 times more resistant to antimicrobial agents than their planktonic counterparts (Gilbert, Das et al. 1997; Brooun, Liu et al. 2000). A biofilm sheds planktonic cells and it seems to be primarily responsible for the manifestation of a disease (Spoering and Lewis 2001).

1.1. Background

1.1.1. Importance of biofilm study: The study of physiological and genetic properties of bacteria within the biofilm has become increasingly important due to increased awareness of the pervasiveness and impact of biofilm on natural and industrial systems. Biofilm is found to play enormous deleterious role in human health and animal production by producing energy losses, equipment damage, product contamination and medical infections (Carpentier and Cerf 1993; Psaltis 2008). However, biofilm also offers huge potential for water treatment, vaccine development for humans and other terrestrial or aquatic animals, larval settlement of several commercially important marine invertebrates and so on (Azad, Shankar et al. 1999; Thompson, Abreu et al. 2002; Dobretsov 2008; Harro, Peters et al. 2010). All these roles have made it crucial to understand what biofilm is, how it is formed, how biofilm bacteria are different from their planktonic counterparts (for example, the exchange of genetic materials by natural transformation within the biofilm).

1.1.2. Definition of biofilm: Anton Van Leeuwenhoek is the first person who observed and described the biofilm bacteria on 17 September, 1683 (Bardell 1983). Although he illustrated bacteria existing either as individual extremely motile organisms or in presumably stationary clusters, the Dutch scientist was paying attention only to planktonic form of bacteria. With the emergence of biofilm associated chronic diseases, the concept of bacteria existing in biofilm was highly accepted in clinical science. Biofilm definition has been evolving constantly with the advance of scientific research and technology. Previously "biofilm" was defined as a bacterial cluster and its encasing matrix (Costerton, Geesey et al. 1978). More recently, a deal of biofilm research has revealed that bacterial biofilm is a dynamic structure and it differs profoundly from its planktonic counterparts both phenotypically and genotypically. The most recent definition of biofilm was presented by Donlan and Costerton (Donlan and Costerton 2002). According to them:

"*Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.*"

1.1.3. Ultrastructure of biofilm: The invention of Confocal Scanning Laser Microscopy (CSLM) represented a great advancement in technology as it helped visualizing the heterogenic structure of biofilm and the biodeterioration of the material supporting bacterial growth (Surman, Walker et al. 1996) (Figure 1). The combination of CSLM and fluorescent molecular staining techniques provided comprehensive information on the three dimensional structure of biofilm and the spatial arrangement of other different microbial species within the biofilm (Lawrence, Korber et al. 1991). It is now widely accepted that the structural unit of biofilm are the microcolonies in an extracellular polymeric substances (EPS) matrix surrounded by interstitial voids and open channels connected to the bulk liquid (Caldwell, Korber et al. 1993; Gjaltema, Arts et al. 1994) (Figure 2). The exchange of nutrients is facilitated by these channels and therefore, biofilm communities become able to develop considerable thickness and complexity.

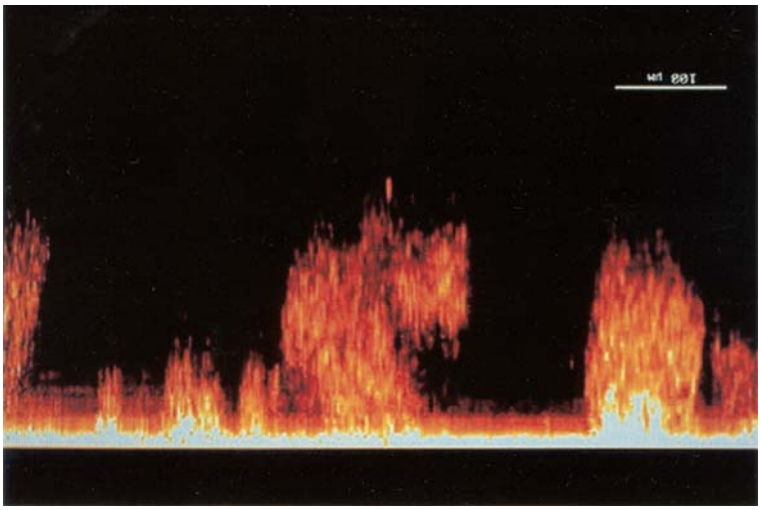

Figure 1: Confocal laser scanning micrograph of a biofilm showing cell clusters and water channels. Adopted from: www.cmr.asm.org (access date: 27.04.2011).

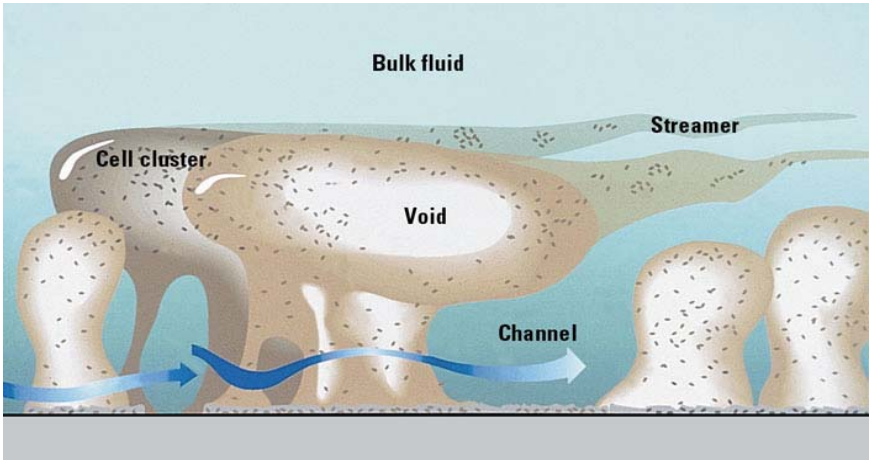

Figure 2: Biofilm of bacteria at microscopic level, showing cell clusters, void spaces, water channels, and slime streamers. Adopted from: http://www.entkent.com/biofilms.html (access date: 27.04.2011).

1.1.4. Biofilm formation: Bacterial attachment to a surface is the first and foremost activity in developing biofilm, because this process allows the microorganisms to anchor in a nutritionally rich environment and to escape to more favorable places when the essential growth factors have been depleted. Bacteria build biofilm in a stepwise manner following three different mechanisms. The first one is the redistribution of attached cells by surface motility (Dalton, Goodman et al. 1996).The second mechanism is the binary division of attached cells (Heydorn,

Nielsen et al. 2000) and the third one is the recruitment of cells from the bulk of fluid to the developing biofilm (Tolker-Nielsen, Brinch et al. 2000). Based on a biofilm study of *Pseudomonas spp*, Stoodley *et al*. have reported a recent diagrammatical representation of the biofilm formation (Heydorn, Nielsen et al. 2000; Stoodley, Sauer et al. 2002). They described five distinct significant episodes (Figure 3) occurring during biofilm formation: a) reversible attachment; b) irreversible attachment; c) aggregation; d) maturation; and e) detachment.

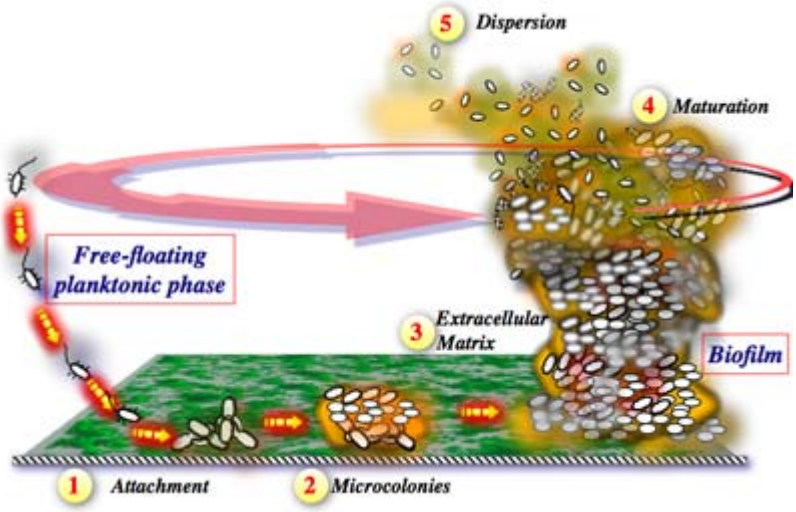

Figure 3: Different steps of bacterial biofilm development.
Adopted from: http://www.pasteur.fr/recherche/RAR/RAR2006/Ggb.html (access date: 27.04.2011).

a) Reversible attachment: The initiation of this stage depends on the availability of nutrients, random collision, active movement of the cell and electrostatic forces. Bacteria posses only a small amount of exopolymeric material around them and they are not yet committed to the differentiation process that leads to biofilm formation at this stage. They can leave the surface anytime to resume the planktonic lifestyle. At this stage, bacteria exhibit a number of species-specific behaviors, including creeping, rolling, aggregate formation and also windrow formation (Marshall, Stout et al. 1971), before they increase the exopolysaccharide secretion for the next attachment.

b) Irreversible attachment: Bacterial transition from reversible to irreversible attachment involves molecular interaction (Dunne 2002; Sauer 2003). The presence of extracellular polymers, physiological changes of bacteria, target material surface and environmental factors may influence the attachment of the bacteria to the surface. In some bacteria, flagellar and twitching motility also play a role in attachment during this stage (O'Toole and Kolter 1998). At the end of this stage the attachment is too difficult to remove from the surface without chemical or mechanical interference.

c) Aggregation: During this stage, the attached organisms start replication actively in order to increase the density and complexity within the biofilm. Water flow containing sufficient nutrients facilitates the replication of the cells.

d) Maturation: The maturation stage results in the generation of multifaceted architecture, pores, channels and a redistribution of organisms away from the substratum. The genomic and proteomics study of the biofilm has revealed that biofilm bacteria are fundamentally different from its counterpart in terms of genetic level and gene expression(Sauer, Camper et al. 2002).

e) Detachment: This stage is a physiologically regulated phenomenon where bacterial cells leave the biofilm either individually or in groups to resume the planktonic form. It has been speculated that nutrients deficiency may lead to detachment of the cells by an unknown mechanisms that allows them to search for nutrient-rich habitats (O'Toole, Kaplan et al. 2000).

1.1.5. Quorum sensing: As biofilm is an assembly of organisms, individual cells may hold responsibility of different activities. The biofilm organisms have a special monitoring and regulatory system which is known as quorum sensing (QS). This system controls the overall behavior of the cells through intercellular signalling within the biofilm. Several studies have revealed that quorum sensing is not only involved in biofilm formation, but it can also influence its development (Parsek and Greenberg 2005). The QS relies on the production and release of tiny signal molecules by the bacterium into its "biofilm environment". These signal molecules have been termed "autoinducers" (Daniels, Vanderleyden et al. 2004). The gram-

positive and gram-negative bacteria use different quorum sensing systems to measure their population density. The acylhomoserine lactone (AHL) system and the peptide-based signaling system are found in several gram-negative bacteria (Parsek, Val et al. 1999; Bassler 2002; Sturme, Kleerebezem et al. 2002), whereas the widespread autoinducer 2 (AI2) system has been found in gram- positive bacteria (Bassler 2002).

a) Mechanism of QS: The quorum sensing or cell to cell communication process is conducted through releasing the QS cells or autoinducers by LuxI protein (Stevens, Dolan et al. 1994). The autoinducers can then diffuse freely outside. The concentration of external autoinducer is a measure of the size of the population (Kjelleberg and Molin 2002) (Figure 4).

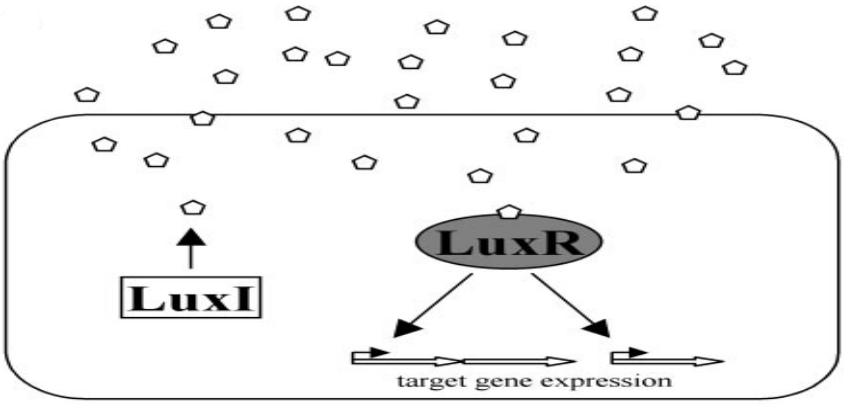

Figure 4: A basic view of quorum sensing mechanism in gram-negative bacteria. (Taga and Bassler 2003).

When a single bacterium releases autoinducers into the environment, their concentration is too low to be detected by the receptor LuxR of gram-negative bacteria. When sufficient bacteria are present, the signaling molecules reach a threshold level that allows bacteria to sense a critical cell mass. As a consequence, a population of cells quickly induces the appropriate phenotypes and genotypes required for a particular environmental condition or for differentiation of the population. The receptor in bacteria not only detects the signaling molecules but also binds autoinducers. There is a low probability of bacteria to detect its own secreted inducers (Taga and Bassler 2003).

b) Role of quorum sensing in biofilm formation : Quorum sensing of bacterial cells plays a vital regulatory role in the development of mature and differentiated biofilm structure (de Kievit and Iglewski 2000). The autoinducers affect several aspects of biofilm dynamics as for instance, the heterogeneity of biofilm, architecture, stress resistance and maintenance (Davies, Parsek et al. 1998; Kjelleberg and Molin 2002; Anguiano-Beltran and Searcy-Bernal 2007; You, Xue et al. 2007). It has also been reported that *Vibrio spp*. uses QS that coordinates the expression of virulence in response to the density of the surrounding bacterial population (Hammer and Bassler 2003; Zhu and Mekalanos 2003; You, Xue et al. 2007).

1.1.6. Natural genetic transformation of bacteria: Natural genetic transformation is one of the horizontal gene transfer mechanisms by which bacteria are able to take up and integrate exogenous free DNA (both plasmid and chromosomal) from their environment. Evidence suggested that the growth of bacteria in biofilm can facilitate the transfer of genetic information between species through natural transformation (Williams, Day et al. 1996; Christensen, Sternberg et al. 1998). It has been also reported that bacteria in biofilm are able to incorporate foreign DNA much more efficiently than their free living counterparts (Li, Lau et al. 2001). Since bacteria can acquire resistance to aquatic environments by taking up extracellular DNA, the ability to transfer genetic material among bacteria is considered a risk indicator for infectious diseases (Wang, Chi et al. 2002).

1.1.7. Mechanism of natural transformation: Bacteria are the only microorganisms capable of natural genetic transformation (Lorenz and Wackernagel 1994). Gene transfer by this mechanism does not require a close genetic relationship or physical contact between donor and recipient cells and even a living donor cell is not necessary for natural transformation to take place either. The natural transformation comprises four interconnected steps: a) competence development; b) DNA binding; c) processing and uptake of DNA; and d) integration of DNA into the chromosome.

a) Competence development in bacteria: The microorganisms must be genetically competent in order to take up free DNA from the environment. Genetic competence is the ability of a cell to take up free DNA from the surrounding medium. Approximately 20 to 50 proteins are found to regulate this physiological state (Thomas and Nielsen 2005). Some bacteria are naturally competent while some others require accumulation of a competence stimulating peptide (CSP) in the surrounding medium . Most naturally transferable bacteria, except *Neisseria gonorrhoeae,* develop time limited competence under specific environmental condition, often in response to growth phase, nutrient access, quorum sensing and stress conditions (Thomas and Nielsen 2005).

b) DNA release and binding: The release and persistence of extracellular DNA in the environment are the prerequisites for the natural transformation. Active release of DNA has been reported in many genera of bacteria including *Streptococcus, Acinetobacter, Bacillus, Alcaligenes, Azotobacter, Flavobacterium* and *Micrococcus* (Lorenz and Wackernagel 1994). Passive release of DNA from dead bacteria occurs after self-induced lysis by breaking down cell walls and membranes. Competent bacteria have specific binding sites on the cell surface where extracellular DNA binds non-covalently. About 50 binding sites in each competent cell of *B. subtilis* and 30 to 80 in *S. pneumoniae* have been reported (Smith, Danner et al. 1981; Dubnau 1991).

c) Uptake of DNA into cytoplasm: Transportation of extracellular DNA into the cytosolic compartment is a complex process (Chen and Dubnau 2004). In gram-positive bacteria, DNA must pass through the cell wall and the cytoplasmic membrane, while in gram-negative bacteria DNA must cross the outer membrane, the cell wall and the cytoplasmic membrane before reaching the cytoplasm. Most other competent species, such as *N. gonorrhoeae* and *H. influenzae,* are able to uptake DNA independently of this sequence and can translocate both plasmids and chromosomal DNA across their membrane (Thomas and Nielsen 2005). When exogenous free DNA binds to the binding sites on cell surface, it is converted into single-stranded DNA (Chen and Dubnau 2004). While the other DNA strand is degraded during uptake.

d) Integration of DNA into host chromosome: After the single-stranded donor DNA has entered the cytoplasm, it can be integrated in homologous regions of the recipient's chromosome. These regions initiate homologous base pairing to form a heteroduplex molecule. Efficiency of integration by homologous recombination depends on the size of the DNA and sequence divergence between donor and recipient DNA regions (Stratz, Mau et al. 1996). The incoming DNA must contain between 25 to 200 base pair in length of high similarity to the recipient genome (Thomas and Nielsen 2005). The integration of foreign DNA into the host genome is accomplished by three different recombination mechanisms, namely homogamic substitutive recombination, heterogamic substitutive recombination and homology- facilitated illegitimate recombination (HFIR) (Figure 5).

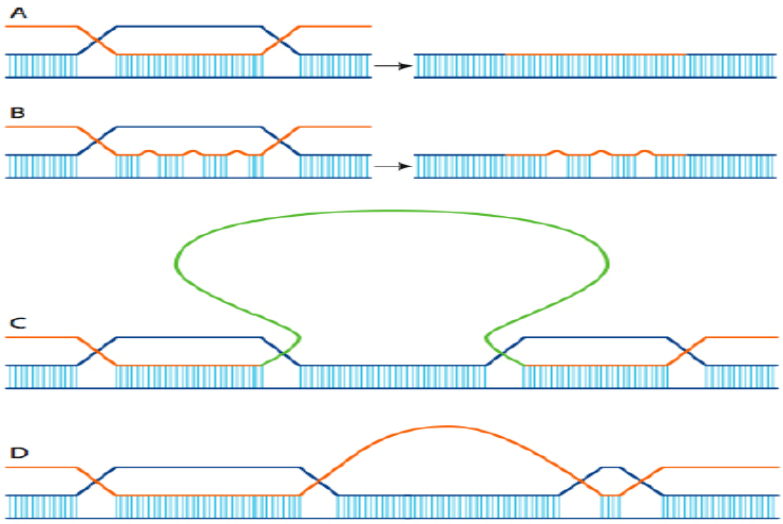

Figure 5: Various recombination types in bacteria. (A) Homogamic substitutive recombination in which donor (orange line) and recipient (blue line) DNA is sequence-identical, leading to no change in the recipient. (B) Heterogamic substitutive recombination occurs when some mismatches exist between donor and recipient. (C) Additive integration occurs when a unique DNA sequence (green line) in the donor is integrated into the recipient through homogamic/heterogamic recombination on each side of the unique DNA sequence. (D) HFIR, integration of donor DNA occurs by a stretch of homology on one side and random microhomology on the other side. (Ray J.L. 2007).

1.1.8. *Streptococcus gordonii* challis as a model host:

Streptococcus gordonii challis (ATCC 35105) (Figure 6), formerly classified as *Streptococcus sanguis* (Kilian, Mikkelsen et al. 1989), belongs to the viridians group. This strain is a gram-positive, facultative anaerobic bacterium. Human oral cavity is the normal habitant of this bacterium.

Figure 6: Colonies of *S. gordonii* in THB agar plate (0.75X, Leica MZ6).

S. gordonii plays an integral role in initiating colonization of dental biofilm by creating surfaces for other colonizers to adhere to. Figure 7 shows a 24- hours *S. gordonii* biofilm grown in the lab during this present study time and stained with fluorescent stain.

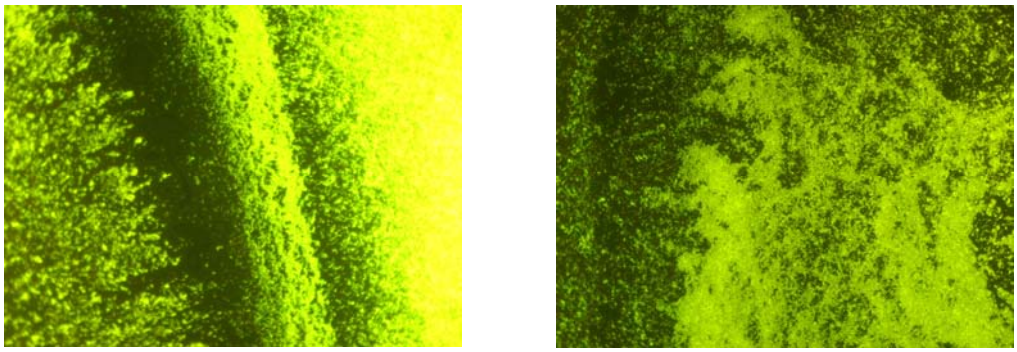

Figure 7: 24 hours old biofilm of *S. gordonii* under laboratory condition during this present study time (biofilm stained with 0.00179mM Syto® 9 for 10 minutes before visualization under fluorescent microscope).

This strain is found to be naturally competent for genetic transformation and this bacterium can efficiently acquire foreign DNA (Macrina, Wood et al. 1980; Pozzi, Musmanno et al. 1987). However, in natural environment *S. gordonii* is mostly found living in a biofilm state, than in a planktonic one. These life styles possess different phenotypic and genotypic characteristics (Ashby 1994; Olson, Ceri et al. 2002). Therefore, it is important to investigate the differences between the frequencies of natural transformation in both growth forms.

1.2. Hypothesis, aims and objectives

Hypothesis: The testable hypothesis was that the frequency **of natural transformation in *S. gordonii* biofilm** is higher than that in planktonic status.

Aim: The aim of this study was two fold: to estimate the natural transformation in biofilm and to compare the transformation frequency in planktonic and biofilm type of growth.

Specific objectives:

- To measure the frequency of natural transformation in *S. gordonii* biofilm and in the planktonic type of the same bacterium using the plasmid pVA838 as a donor DNA.
- To measure the frequency of natural transformation in *S. gordonii* biofilm and in the planktonic type of the same bacterium using *S. gordonii* genomic DNA tagged with kanamycin or tetracycline resistance genes as donor DNA.
- To measure the effect of Competence Stimulating Peptide (CSP) on the growth of *S. gordonii*.

2. Materials and methods

A detailed description of buffers/reagents, laboratory kits, cell culture plates, test tubes, equipments and software used in this study can be found in Annex 1.

2.1. Bacterial strain

Streptococci gordonii challis (ATCC 35105) was used as the recipient bacterium of donor DNA in this study. These bacteria were collected from previously prepared stocks stored at -70°C.

2.2. DNA

Plasmid DNA: The plasmid pVA838 was extracted from *E. coli* following the QIAGEN Midi Kit protocol for plasmid extraction. This plasmid is 9.2 kb in size and contains two markers: Erythromycin and Chloramphenicol. Erythromycin expression occurs in both *E. coli* and *S. gordoni,i* while Chloramphenicol does so only in *E. coli* (Macrina, Tobian et al. 1982).

Genomic DNA: *S.gordonii* DNA isolation was performed using the QIAGEN (QIAGEN Gmbh, Germany, Max-Volumer-StraBe 4) genomic DNA extraction protocol.

2.3. Synthetic Competent Stimulating Peptide (CSP)

19 amino-acid (DVRSNKIRLWWENIFFNKK) synthetic CSP was used to induce competence in *S. gordonii* in the present study.

2.4. Media

Growth media were prepared by dissolving 30gm of Todd Hewitt Broth (Bacto^{TM}, France) in 1L distilled water followed by autoclaving at 121°C for 20 minutes. THB agar media were prepared by adding 30gm of Todd Hewitt Broth (Bacto^{TM}, France) and 15gm agar (Merch-KGaA, Germany) in 1L distilled water followed by autoclaving at 121°C for 20 minutes. The desired concentrations of the antibiotics were added in the media when temperature dropped below 60°C.

2.5. Antibiotics

Erythromycin: working concentration was 400µg/ml (SIGMA-ALDRICH, China).
Kanamycine: working concentration was 50µg/ml (Fluka bioChemika, Sigma-Aldrich, China).
Tetracycline: working concentration was 5µg/ml (SIGMA-ALDRICH, China).

2.6. Growth curve of *S. gordonii*

An overnight culture of approximately16-hrs of *S. gordonii* was diluted 40-fold in THB media and inoculated into a 96-well microtiter plate. The OD_{650nm} of the culture was measured for 23 hours with hourly interval in order to observe the growth pattern of the bacterium.

2.7. Growth curve of *S. gordonii* with CSP

An overnight culture of 16-hrs of *S. gordonii* was diluted 40-fold in THB media. Then CSP of 200 nM was added in the THB media and the culture was inoculated into the wells of 96-well microtiter plate. The OD_{650nm} of that plate was measured for 23 hours with hourly interval in order to observe the growth curve in the presence of CSP.

2.8. Growth patterns of planktonic bacteria before and after CSP exposure

To make *S. gordonii* become competent, the CSP was added into the early log phase and, therefore, bacteria overcome the growth-dependent requirements for spontaneous competence. The overnight culture was diluted 40-fold in THB media and then inoculated into wells of 96-well microtiter plate. The $OD_{650\ nm}$ value was measured every 5 minutes and when it reached a value between 0.06 and 0.08, the CSP with a concentration of 200 nM was added to the culture. After adding CSP, OD value was also recorded every 5 minutes over a 250 minutes time period.

2.9. Plasmid pVA838 extraction

The bacterial culture was prepared by inoculating a single colony of *E. coli* into 20ml of LB media. This was incubated at 37°C for overnight with vigorous shaking. To pellet the bacteria, the suspension was transferred into 50ml falcon tube and

centrifuged at 4°C for 10 minute at 4000 rpm (Heraeus instruments Eppendrof, Centrifuge 5810R). All traces of supernatant were afterwards removed by inverting the centrifuge tube until the entire medium has been drained completely. Plasmid extraction was performed using QIAGEN kit and according to the manufacturer instructions. All the required buffers were prepared according to QIAGEN Plasmid extraction protocol.

2.9.1. Determination of yield: Plasmid concentration was determined by using 0.7% agarose gel according to standard protocol. The result was further confirmed using Nano drop.

2.9.2. Sample loading: The DNA samples were prepared by adding 2µl of 6x loading buffer to 10µl of extracted DNA. The marker of ladder and the samples were then loaded into the wells of the gel. The electrophoresis was run at 90V (BIO-RAD power pac 300, BIO-RAD MINI-SUB® cell GT) for approximately 50 minutes or until the blue was near the bottom of the gel. The plasmid concentration was estimated by comparing the band intensity with the ladder (Figure 8).

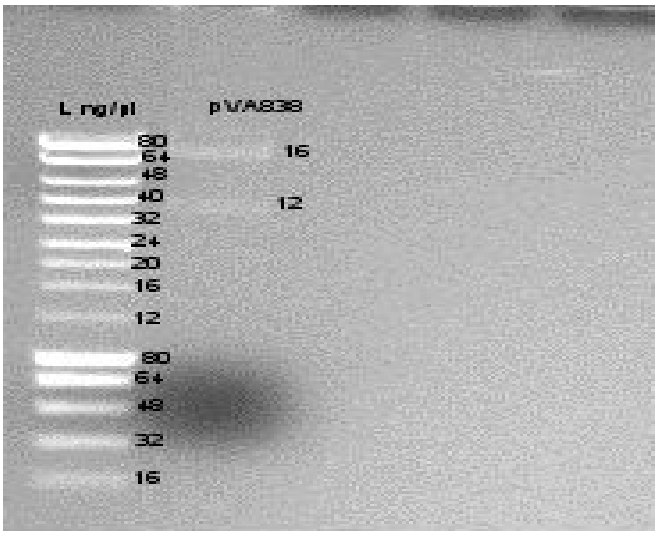

Figure 8: Determining the concentration of Plasmid pVA 838.

2.10. Transformation of *S. gordonii* in liquid media

Different parameters were optimized to measure the transformation frequency of *S. gordonii* in the planktonic mode of growth. The overnight culture of *S. gordonii* challis cells was diluted 40-fold in THB media and then incubated at 37°C until OD_{650nm} value was between 0.06 and 0.08, in which the CSP was added.

2.10.1. Optimizing the incubation time after adding CSP: To optimize the incubation time, *S. gordonii* were cultivated as described previously. After OD_{650nm} reached a value between 0.06 and 0.08, the CSP at a concentration of 200 nM was added to the culture, in order to induce competence, and then incubated at 37°C for 0 minutes, 20 minutes, 40 minutes, 60 minutes and 80 minutes respectively, before adding donor plasmid DNA pVA838 at a concentration of 1.2µg/ml). The culture was then incubated at 37°C for additional 30 minutes and afterwards the bacteria were diluted if necessary and plated in THB plates with or without the proper antibiotic to measure the transformation frequency. The transformants were selected on THB agar plates supplemented with erythromycin (400µg/ml). For each experiment, three different parallel plates were used with the proper controls. In brief, the culture was plated on three different plates: one for recipient titer (THB agar plates), another for transformant titer (THB agar plates supplemented with erythromycin), and a third one for negative control (THB agar plates supplemented with erythromycin). Transformation frequencies were determined after 48 hours of incubation in a 5% - CO_2 (Anaerocult® C mini) environment at 37 °C.

2.10.2. Optimizing of the CSP concentration: A 40-fold diluted culture was incubated at 37°C until OD reached a value between 0.06 and 0.08. At this point, two different concentration of CSP were added (200 nM and 400 nM, respectively) in the media and incubated at 37°C for 60 minutes and 80 minutes, before adding the donor DNA, i.e., plasmid pVA838 (1.2µg/ml). Competent recipient cells were afterwards incubated at 37°C for additional 60 minutes. Transformants were then

serially diluted in the normal saline and selected on THB agar plates supplemented with erythromycin (400µg/ml). Transformation frequencies were determined after 48 hours of incubation in anaerobic jar (5% - CO_2)..

2.10.3. Optimizing the concentration of donor DNA: To optimize the plasmid DNA concentration, an overnight culture was diluted 40-fold with THB media and incubated at 37°C until OD_{650} reached a value between 0.06 and 0.08. The CSP at a concentration of 200 nM was added to the culture and then incubated at 37°C for 80 minutes. Three different concentrations of donor plasmid DNA pVA838 were added to the culture (1.2µg/ml, 2.4µg/ml and 3.6µg/ml, respectively). The competent recipient cells were incubated at 37°C for additional 60 minutes after adding the donor DNA. Transformants were plated using proper dilution and then selected on THB agar plates supplemented with erythromycin (400µg/ml). Transformants were calculated to determine the transformation frequency after 48 hrs of incubation in anaerobic jar containing 5% -CO_2.

2.10.4. Measuring the transformation frequency in liquid media with optimized parameters: With all parameters optimized for natural transformation, another experiment was performed to determine the actual transformation frequency of S. gordonii. The 16- hours overnight culture was diluted 40-fold in THB media and then incubated at 37°C until OD reached a value between 0.06 and 0.08 at 600nm. The CSP with the proper concentration was added to the culture and incubated again at 37°C for 80 minutes in order to induce competence to the cells. Plasmid DNA pVA838 (1.2µg/ml) was added and the culture was incubated for additional 60 minutes. Transformants were then serially diluted in the normal saline and selected on THB agar plates supplemented with erythromycin (400µg/ml). Transformation frequencies were determined after 48 hours of incubation in anaerobic (5% CO_2) environment.

2.11. The effect of the CSP on the biofilm

To examine whether the CSP had any effect or not on the growth rate of biofilm bacteria, an overnight culture of *S. gordonii* was diluted 60-fold in THB media. Afterwards, two experiments were conducted at the same time: one using CSP with THB media, and one without adding CSP. The cells were inoculated into different 6-wells of 12 -well polystyrene microtiter plates (1ml/ well) and incubated at 37°C for 24 hours in order to form biofilm. Planktonic bacteria were removed from each well and the remaining cells were stained with 150 µl per well of crystal violet (CV) (2.3% w/v, Accustain Crystal Violet Solution, Sigma Diagnostics) for 15 minutes. The wells were washed twice with milli-Q water and air dried. Biofilm formation was quantified by solubilization of CV staining in 200 µl of 95% ethanol per well. The OD was measured at 650nm.

2.12. Optimizing the CSP concentration and incubation time following DNA addition to the biofilm

Biofilm assays were carried out with two different parameters in order to determine the optimum experimental conditions. To optimize the CSP concentration and incubation time before adding donor DNA, overnight culture of *S. gordonii* was diluted 60-fold in THB media. This culture was afterwards inoculated into 6-well of 12 well polystyrene microtiter plates (1ml/ well) and incubated at 37°C for 24 hours, in order to form biofilm. Planktonic cells were removed by discarding the supernatant liquid and the wells were washed once with normal saline (0.9% NaCl). Fresh THB media with CSP (400 nM and 800 nM respectively) was added into wells of 12-microtiter plates and incubated for 80 minutes, in order to induce competence of the biofilm cells. For natural transformation, the plasmid pVA838 (1.2µg/ml) was added to the biofilm and incubated for an additional 1 hour and 2 hours. The biofilm bacterial cells were afterwards scraped off with a scraper and vortexed vigorously for a few seconds in order to disperse the cells in the biofilm. Transformants were serially diluted in normal saline and selected for on THB agar plates supplemented with erythromycin (400µg/ml). Transformation frequencies were determined after 48 hours of incubation in anaerobic jar containing 5% CO_2.

2.12.1. Measuring transformation frequency with optimized parameter in the biofilm:

The 60-fold diluted overnight culture in THB media was inoculated into wells of 12 well polystyrene microtiter plates (1ml/ well), and incubated at 37°C for 24 hours, in order to allow biofilm formation. Planktonic cells present in the well were removed by discarding the supernatant liquid, then wells were washed once with normal saline (0.9% NaCl). Fresh THB media with CSP 800 nM was added into wells of 12-microtiter plates and incubated for 80 minutes, in order to induce competence of the biofilm bacteria. The plasmid pVA838 (1.2µg/ml) was then added to the biofilm and incubated for an additional 2 hours. The biofilm bacterial cells were scraped off with a scraper and vortexed vigorously for a few seconds in order to disperse the cells uniformly. Transformants were serially diluted in the normal saline and a proper dilution is used to plate the cells on THB agar plates supplemented with erythromycin (400µg/ml). Transformation frequencies were determined after 48 hours of incubation in anaerobic atmosphere containing 5% CO_2.

2.13. Calculation of transformation frequency

Transformation frequencies were calculated as the number of transformants per recipient per ml.

Transformation frequency = CFU (Colony Forming Unit) transformants / Total CFU.

2.14. Statistical analysis

When required, data were logarithmically or square root transformed and analyzed by t-test or one-way Anova using Excel (Microsoft®Office 2003) after verification of variance homogeneity and normality of distributions. Data are presented as mean ± standard deviation of means (SD) and statistical differences accepted as significant when $P<0.05$.

3. Results

3.1. Growth curve of *S. gordonii*

The growth curve of *S. gordonii* is illustrated in Figure 9. In this study, *S. gordonii* was monitored photometrically and the growth curve was plotted by reading the optical density at 650 nm over time, with the starting value normalized to zero. The average value of five wells was calculated to plot the growth curve (Figure 9) (see Annex 2 for details). The lag phase lasted around 1 hour, and it was followed by the log phase that ended after around 6 additional hours. The stationary phase completed after less than one hour, when the death phase set in. The doubling time was calculated to be 0.8283 hours.

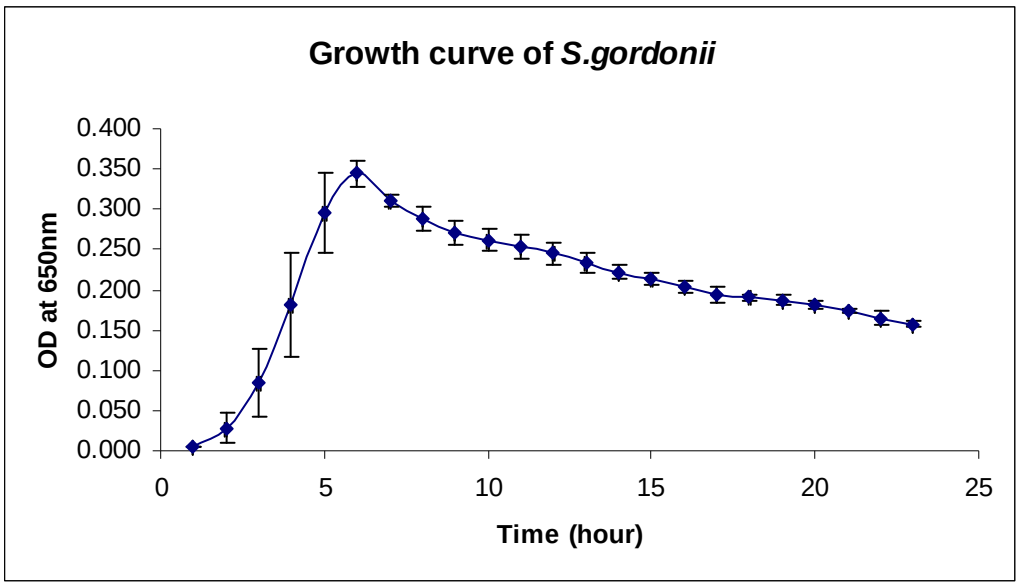

Figure 9: Growth curve of *S. gordonii*. The 23 hrs measurement is the average of 5 samples. Data are shown as mean ± SD of n=5 replicates.

3.2. Comparison between growth curve with and without CSP

To examine the response of S. *gordonii* to CSP exposure, parallel experiments were performed, using two cultures: one in which the CSP (200 nM) was added, and a second one without the addition of CSP. The OD_{650nm} value was recorded every hour, over a 23 hours time period to monitor the growth curve. The graph (Figure 10) (see Annex 3 for raw data) indicates that CSP may have affected the growth rate of *S. gordonii*. *S. gordonii* reached its growth peak in about 6 hour. However, the bacterium reached its peak in about 7 hour in the presence of CSP.

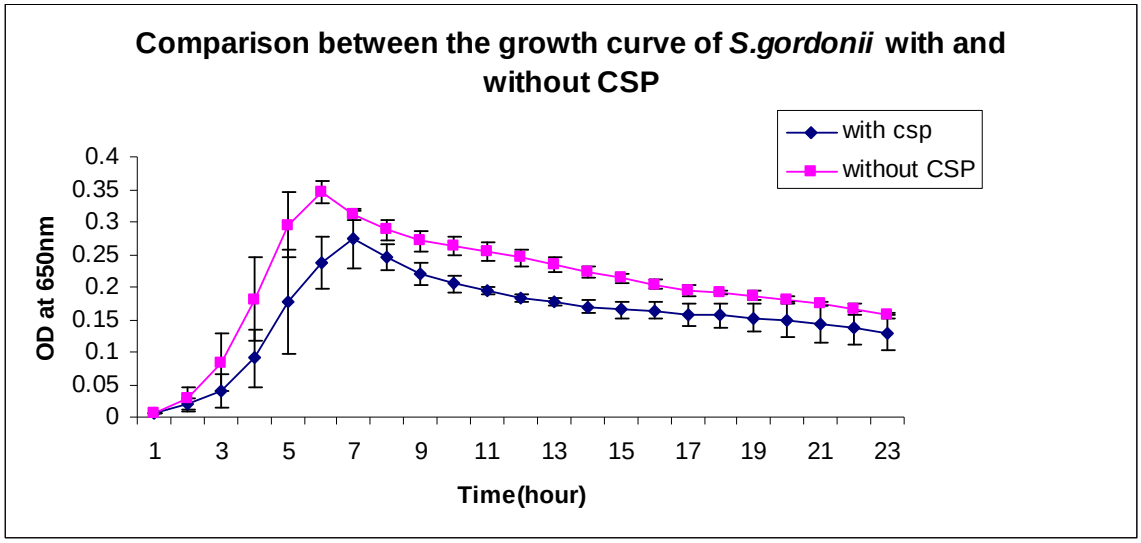

Figure 10: Effect of CSP on the growth rate of *S. gordonii* over 23 hours time period. Data are shown as mean ± SD of n= 5 replicates.

3.3. Effect of the synthetic CSP on the *S. gordonii* growth

To examine the response of S. *gordonii* to CSP exposure, parallel experiments were performed, using four cultures, two for each growth mode: one in which the CSP (200 nM) was added, and another one left without the addition of CSP. In the case of the planktonic culture, the OD_{650nm} value was recorded over a 7 hours time period. In the case of the biofilm mode of growth, the OD_{650nm} value was registered after 24 hours

(Figure 11) (see raw data in Annex 4). The CSP had negative effect on the growth rate of planktonic *S. gordonii* (t-test, $P < 0.0001$). The CSP positively influenced the growth rate of *S. gordonii* (t-test, $P < 0.0001$) in the biofilm state

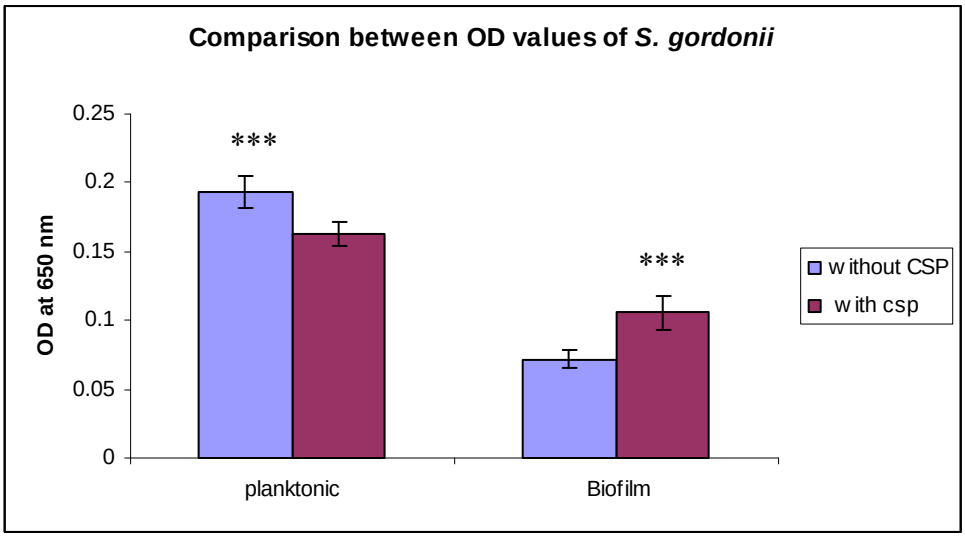

Figure 11: Effect of CSP on OD value of *S. gordonii* over 7 hours time period (planktonic) and 24 hours (biofilm). Data are shown as mean ± SD of n=7 replicates.

3.4. The effect of the addition of the CSP on the log phase of *S. gordonii*

The change in growth curve at the log stage was recorded in following the addition of the CSP to the culture (Figure 12) (see Annex 5 for raw data). It was noticed that after adding the CSP (200 nm) during the log phase, the OD_{650nm} increased and reached its maximum in 5 minutes and then dropped to the average density after another 5 minutes. Afterwards, the cells continued to grow exponentially, as expected.

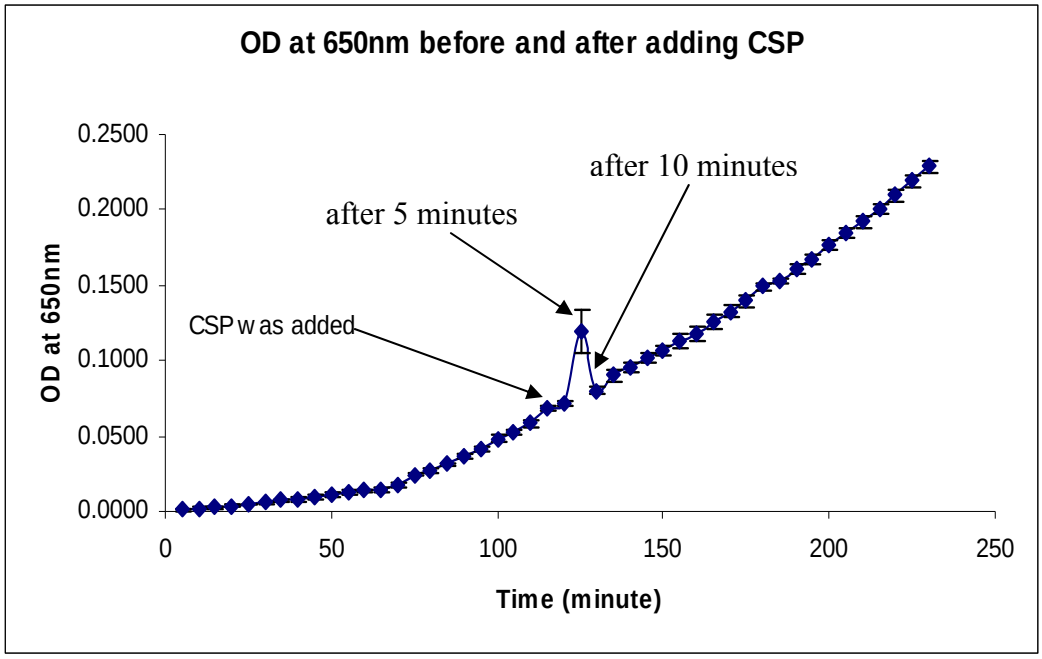

Figure 12: Effect of CSP addition on the log phase of growth curve of *S. gordonii*. The results correspond to mean values and ± SD of n=5 replicates.

3.5. Optimization of incubation time after the exposure to the CSP for maximum competence

The transformation frequencies after five different incubation time periods were calculated (see raw data in Annex 6). These frequencies were 6.24E-07, 1.59E-05, 5.70E-05, 6.20E-05, and 4.23E-04 if the donor DNA was added immediately after the addition of the CSP, at 20 minutes, 40 minutes, 60 minutes and 80 minutes, respectively. Figure 13 shows that the transformation frequency was seemingly enhanced after 80 minutes of incubation.

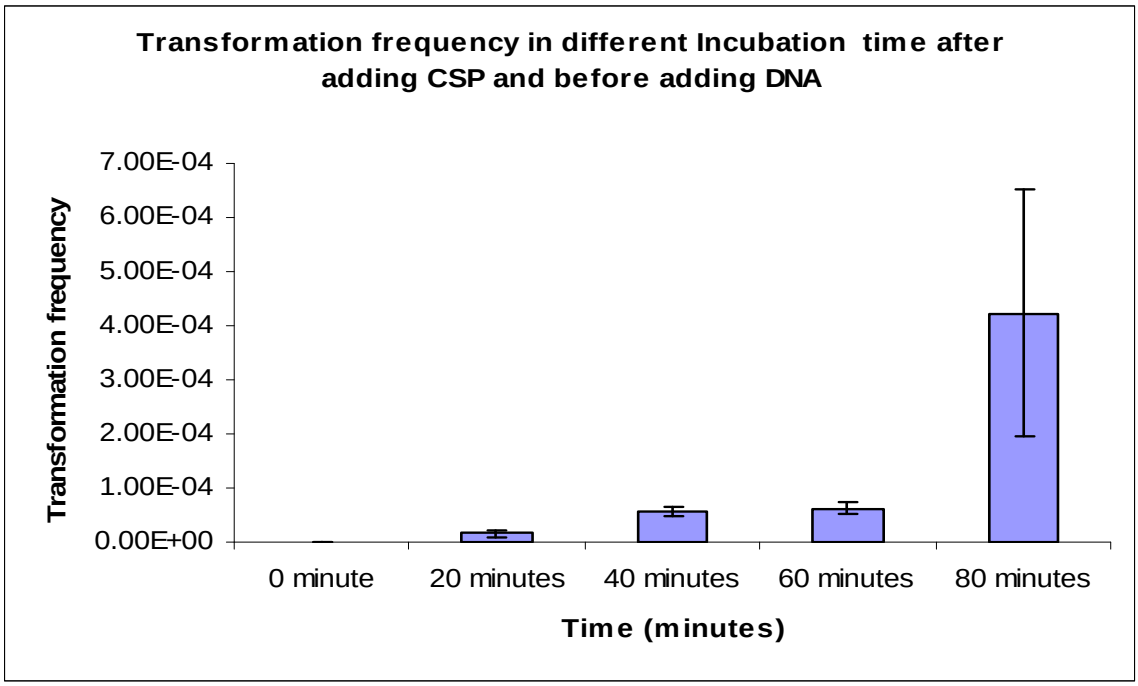

Figure 13: Transformation frequency in liquid media with different incubation time points after adding CSP and before adding 1.2µg/ml pVA838. Mean values ± SD of n=3 independent experiments.

3.6. Optimization of the CSP concentration for maximum competence and transformability

The CSP was used to induce competence at two different concentrations. In brief, the *S. gordonii* culture was incubated for 60 minutes and 80 minutes, after exposure to 200nM and 400 nM of the CSP before adding the donor DNA, i.e., pVA838. The transformation frequency was: 6.24E-05 (200 nM CSP, 60 minutes incubation), 1.77E-04 (400nM CSP, 60 minutes incubation), 1.57E-04 (200 nM CSP, 80 minutes incubation), 4.49E-05 (400nM CSP, 80 minutes incubation) (see Annex 7 for raw data). Figure 14 indicates that the transformation frequency was seemingly higher when 200 nM of the CSP was used and the culture incubated for 80 minutes before addition of the donor DNA.

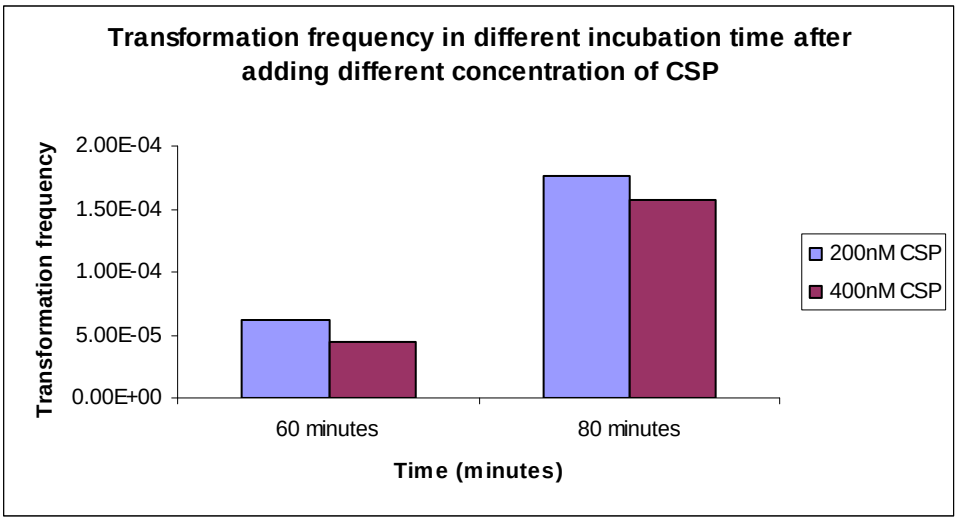

Figure 14: The effect of CSP concentration and incubation hours on transformation frequency.

3.7. Optimization of the plasmid (pVA838) concentrations for maximum transformability

The transformation frequencies were calculated to be 5.46E-04, 5.92E-04 and 7.41E-04 after adding 1200ng, 2400ng and 3600ng of donor DNA, respectively (Figure 15) (see Annex 8 for raw data). The transformation frequency was not significantly different with different concentration of donor plasmid DNA (one-way Anova, $P>0.05$).

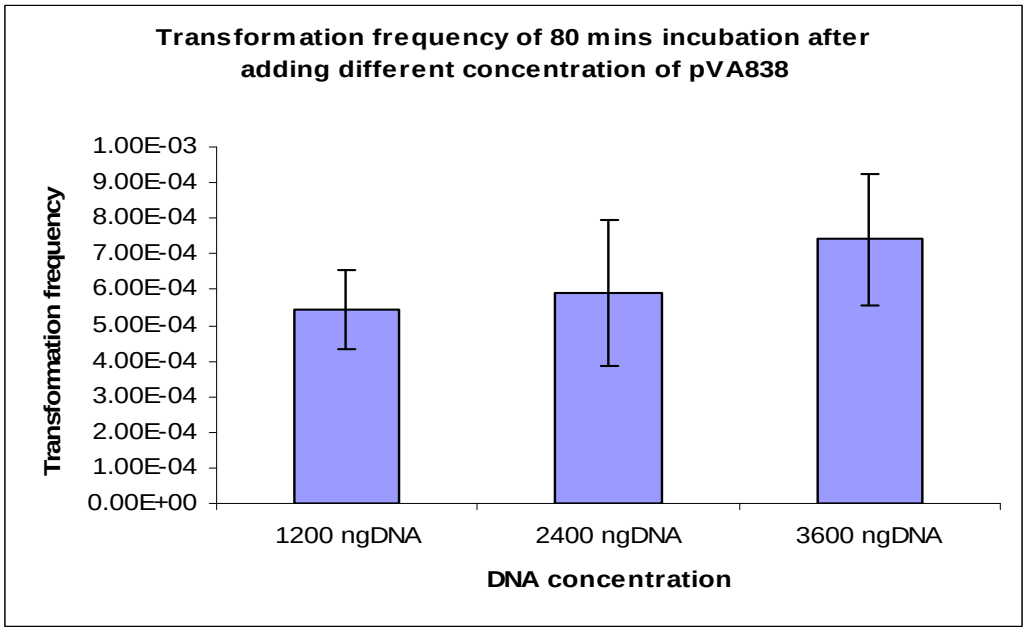

Figure 15: Transformation frequency in liquid media with different concentration of plasmid DNA. The results correspond to mean values mean values ± SD of n=2 replicates.

3.8. Optimizing the CSP concentration and incubation time after adding DNA to the biofilm

The transformation frequencies with two different CSP concentrations and two incubation time were calculated. These frequencies were 1.35E-04 and 1.63E-03 if the donor DNA was added immediately after the addition of the 400 nM CSP,1 hour and 2 hours respectively, and 2.61E-04 and 4.05E-03 03 if the donor DNA was added immediately after the addition of the 800 nM CSP, 1 and 2 hours, respectively (Figure 16) (see Annex 9 for raw data). The transformation frequency was significantly higher after 2 hours incubation than after only 1 hour, at both CSP concentrations (t-test, $P < 0.0001$). The CSP concentration did not influence the transformation frequency in bacteria (t-test, $P>0.05$).

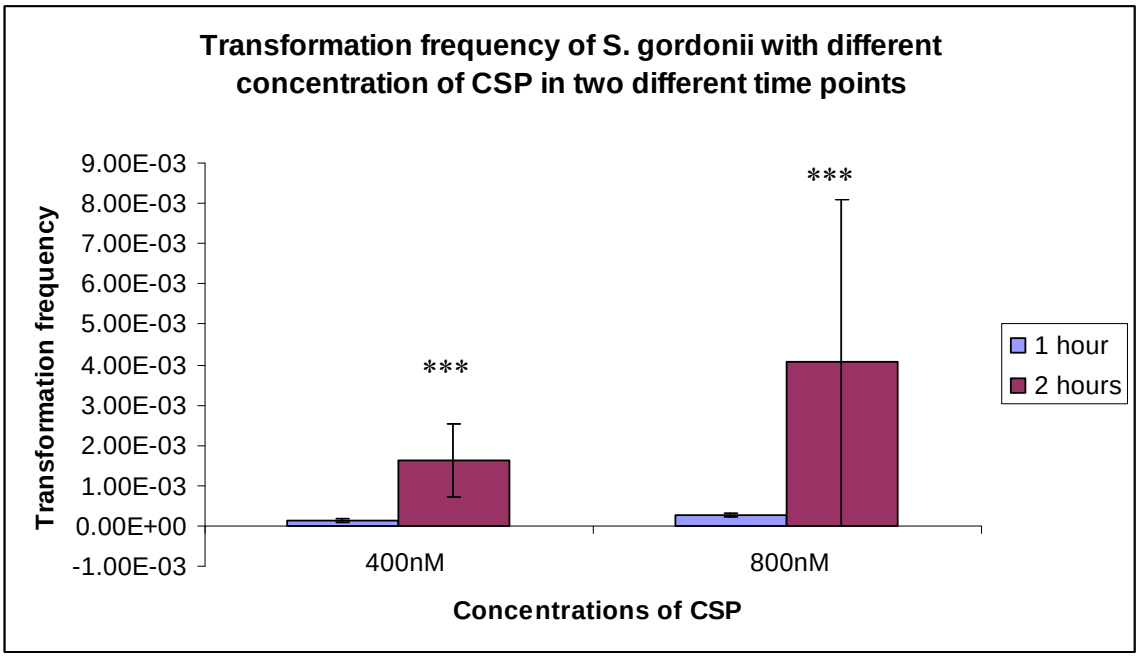

Figure 16: Effect of different CSP concentration and incubation time in biofilm mode of growth. Mean values ± SD of three replicates.

3.9. Comparison between the transformation frequency of planktonic and biofilm bacteria

The transformation frequency was calculated for both biofilm and planktonic bacteria, under optimized parameters. The transformation frequency of plasmid pVA838 was observed to be approximately 6-fold higher than in planktonic bacteria (Figure 17) (see Annex 10 and 11 for details). This transformation frequency was found to be significantly different (t-test, $P < 0.001$).

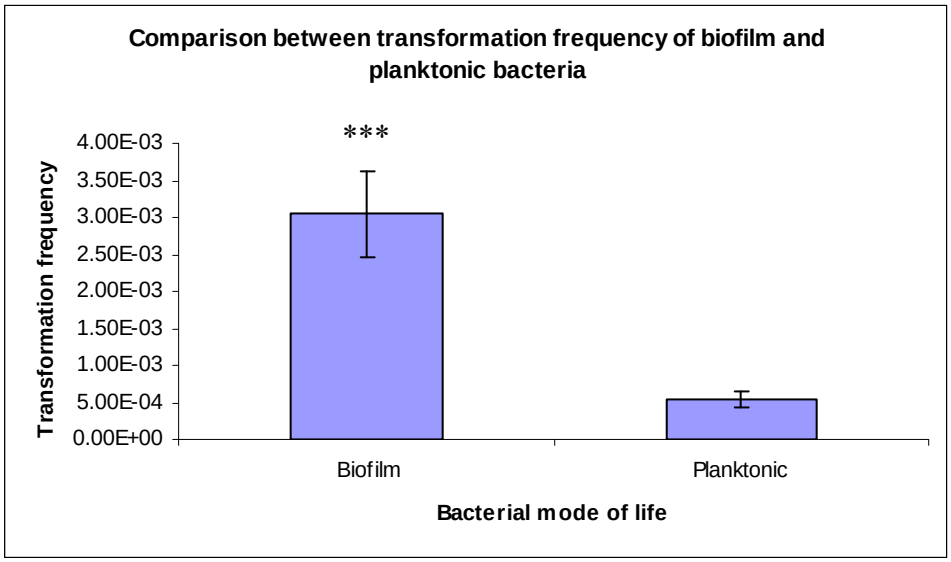

Figure 17: Comparison between transformation frequency of biofilm and planktonic bacteria. Mean values ± SD for n=3 replicates.

3.10. Tagging *S. gordonii* genomic DNA with *tet* and *nptI* genes

The EZ-Tn5^{Tm} <KAN-2> and EZ-Tn5^{Tm} <TET-1> insertion kits were used to tag the *S. gordonii* DNA with kanamycin and tetracycline resistance genes. After the insertion experiment was performed, some colonies were found in the kanamycin resistant agar plate. However, PCR revealed that there was no positive insertion in the tested colonies (Figure 18).

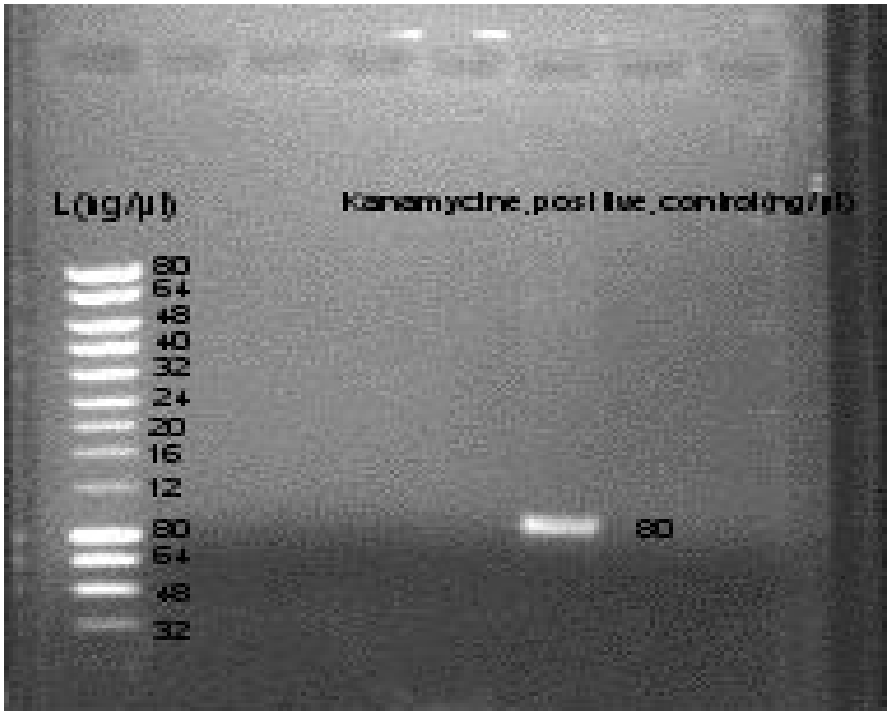

Figure 18: PCR amplification shows only positive control for kanamycin resistant marker.

4. Discussion and conclusion

In natural ecosystem, bacteria exist in two forms: free-floating planktonic and biofilm. Biofilm mode of growth is a big challenge in clinical science. It has been estimated that 60% of all infections in developed countries are related to biofilm bacteria (Costerton, Stewart et al. 1999). Over 99% of bacteria in natural population are found in biofilm, and possess different characteristics and respond differently to the environmental factors (Ehrlich, Hu et al. 2005). In agreement with other studies, this study revealed that there might be some differences in the transformation of *S. gordonii* planktonic and biofilm forms.

In the present study, synthetic CSP was used to overcome the spontaneous competence constraints in bacteria. The experiments carried out in this study showed that planktonic bacteria grew slowly when cells were treated with synthetic CSP from the lag phase compared to untreated cells. When CSP was added in the early log

phase, cells dramatically increased their number, but their growth decreased within a short interval of time. A previous study showed that development of competence in *S. pneumoniae* triggers cell lysis (Steinmoen, Knutsen et al. 2002). Besides this, competence induced-cells of this species lyse competence-deficient cells of the same species during co-cultivation (Steinmoen, Knutsen et al. 2002). Marco R. *et al* also reported that CSP induces a competence-derived stress in *S. pneumoniae* that was nearly undetectable as a phenotype, and that could be detrimental to bacterial cells under some conditions (Oggioni, Iannelli et al. 2004). The detrimental role of CSP in *S. pneumoniae* could be the explanation for slow growth rate of *S. gordonii* planktonic cells in the lag phase, since CSP response of *S. gordonii* was found to be very similar to that of *S. pneumoniae* (Vickerman, Iobst et al. 2007). The sudden change in the log phase after addition of CSP might be due to the competence regulatory system.

However, biofilm formation by *S. gordonii* treated with CSP showed an increased growth compared to untreated bacteria, indicating that CSP may facilitate biofilm formation. Recent studies have demonstrated that biofilm formation, acid tolerance, and the transformation in *S. mutans* are mediated by quorum sensing (Cvitkovitch, Liu et al. 2003). This signaling system depends on a competence-stimulating peptide. Disruption of QS genes resulting in reduced or altered biofilm formation has been reported in *S. gordonii* (Loo, Corliss et al. 2000). Another report described that CSP-treated *S. intermedius* cells formed biofilm at a 100% higher rate than untreated cells after 24 hour incubation (Petersen, Pecharki et al. 2004) suggesting a positive effect of CSP on biofilm formation in that species.

The present study found that the transformation frequency increased with increasing incubation time, after exposure to CSP (200nM) in liquid culture. The 80 minutes incubation was likely to be the best time in order to obtain high transformation frequency. This result was different from the one reported by Vickerman, Iobst *et al* in *S. gordonii* (Vickerman, Iobst et al. 2007). These authors recorded highest transformation frequency at 30 minutes incubation, while after this time the transformation frequency started to decline.

The observations included in this present study also differed from the observation reported by Rodriguez *et. al.* (Rodriguez, Callahan et al. 2011), where competence peaked after approximately 10 minutes of exposure to CSP and returned to near baseline levels after 40 minutes.

Furthermore, the experiments with varying concentration of CSP (200 nM and 400 nM) in liquid media indicated that this concentration had an effect on transformation frequency. In liquid culture, 200 nM of CSP appeared to be the effective concentration for transformation. Similar results were demonstrated by Rodriguez, Callahan *et. al.* in their study with *S. gordonii* (Rodriguez, Callahan et al. 2011). They found the peak response of CSP concentration between 200-240 nM/ ml. However, the findings by Vickerman, Iobst *et al.* with *S. gordonii* showed different results, and the level of synthetic CSP (range from 25 to 200 nM/ml) did not appear to affect the competence response. The transformation frequency with variable concentrations of plasmid DNA was not significantly different in liquid culture as observed in this present study.

The effect of two different concentrations of CSP (400 nM and 800 nM) and two different incubation periods (1 and 2 hours) on transformation frequency suggested that the 2 hours incubation after exposure to CSP was the best incubation time for plasmid transformation in biofilm. This effect of incubation time was also found during a transformation experiment performed on *S. mutans* biofilm (Li, Lau et al. 2001).

In the present work, although the bacteria were grown for 24 hours to form a dense biofilm, the CSP was necessary for bacteria to become competent and no transformants were detected without the use of CSP. The same results were also highlighted in another study (Vickerman, Iobst et al. 2007). Moreover, Yung-hua Li *et al* reported that transformation frequency in biofilm *S. mutans* following addition of synthetic CSP was 100-fold higher than without CSP.

Natural transformation is considered today a driving force in bacterial adaptation and evolution. The biofilm mode of growth is thought to provide a selective advantage in the transformation process (Petersen, Pecharki et al. 2004). Sessile bacteria in biofilm

exhibit characteristics that are distinct from that of planktonic bacteria. Bacterial cells growing in biofilm were found to be able to acquire foreign DNA more efficiently than their free-living counterparts. Yung-hua li *et al* reported that quorum sensing mediated transformation frequency in *S. mutans* cells growing in biofilm was 10- to 600-fold higher than those of planktonic *S. mutans*. The same frequency was also reported by C. Vitkovitch *et .al.* in a study with the same bacterial strain (Cvitkovitch 2004). These results were also recorded by another work that reported 100 times higher transformation frequency in biofilm population than their planktonic fraction (Perumbakkam 2005). The results reported in the present study confirm the conclusions of the previous studies in other Streptococcus (Cvitkovitch 2004), as the transformation frequency in *S. gordonii* was found to be higher in biofilm cells compared to their planktonic counterparts. Approximately 6-fold higher transformation frequency was found in biofilm of *S. gordonii* cells than free-living planktonic cells.

In conclusion, this present study shows that CSP may induce competence and slow the growth of *S. gordonii* when in liquid media. This result may either indicate a stress-related role of CSP or the fact that CSP participates in the synthesis of bacteriocin-like protein as suggested by others (Suntharalingam and Cvitkovitch 2005). The finding of enhanced cells in the biofilm in the presence of CSP suggests the involvement of CSP-QS system in the process of biofilm formation. Probably the most confusing finding of this present study was the dramatic increase of the OD_{650nm} in the liquid culture of *S. gordonii,* after addition of CSP in the early growth phase. The explanation of this finding demands further investigation.

Finally, this study shows that *S. gordonii* cells were able to acquire foreign plasmid DNA much more efficiently than their planktonic counterparts *in vitro*. This finding suggests that the biofilm growth mode may provide optimal condition for genetic transformation in bacteria. This finding may have important implications when assessing the potential of antibiotic resistance gene transfer to pathogens temporarily resident in the biofilm.

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Annex 1: Details on materials and methods

Buffers and reagents

1xTAE buffer containing 10 mM Tris-Cl, PH-7.5 and 1 mM EDTA.

Buffer B1 (Bacterial lysis buffer) 50mM Tris-Cl, pH 8.0; 50mM EDTA, pH 8.0; 0.5% Tween® - 20; 0.5% Triton X-100.

Buffer B2 (Bacterial lysis buffer) 3 M guanidine HCl; 20% Tween® - 20.

Buffer QC (wash buffer) 1.0M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol.

Buffer QF (Elution buffer) 1.25 M NaCl; 50mM Tris-Cl, pH 8.0, 15% isopropanol.

Buffer QBT (Equilibration buffer) 750mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol 0.15% Triton X- 100.

Buffer P1 50mM Tris-Cl, pH 8.0; 10mM EDTA.

Buffer P2 200mM NaOH, 1%SDS.

Buffer P3 3.0 M Potassium Acetate, pH 5.5

6X Loading Buffer.

Smart ladder (Eurogentec, USA).

Proteinase K QIASEN, 10 ml solution.

Lysozyme (Sigma Chemicals, SERVA/Boehringer Ingelheim Bioproducts).

Ethanol 70%, and 95% (Sigma-Aldrich, Germany).

Isopropanol prima (Kemetyl, Norge AS).

Glycerol 20% (Merck KGaA, Germany).

Agarose (SeaKem®LE agarose, USA).

Ethidium bromide (Sigma-Aldrich, Germany).

MilliQ water.

Crystal violet (Fluka® analytical, Sigma- Aldrich chemic GmbH).

Anaerocult® C mini and Anaerocult® C MERK, Germany.

Fluorescent dye Syto® 9 (Invitrogen).

Kits

Ez-Tn5™<KAN-2>Insertion kit (EPICENTRE BIOTECHNOLOGIES Cat. No. EZ1982K).

Ez-Tn5™<TET-1>Insertion kit (EPICENTRE BIOTECHNOLOGIES Cat. No. EZ1921T).

QIAGEN Genomic tip (100/G).

QIAGEN® plasmid Midi Kit (100) cat. No. 12125, QIAGEN GmbH, D-40724 Hilden.

Cell culture plates and test tubes

96-well microtiter plate (FALCON® -MICROTEST™96, Becton Dickinson, France).

12-well microtiter plate (Becton Dickinson, USA).

8-well microtiter plate (NUNC™, USA).

Petridishes (FRAGILE, Norway).

15ml conical tube (NUNC™, USA).

50ml falcon tubes (SASTEDT, Germany).

Appendorf tube 2ml (Plastibrand®, Germany).

Software

Quality one (version 4.3.0).

Soft Max Pro (version 5.4.1).

Microsoft Excel 2003.

Equipments

Cabinets (Thermo, Biological safety cabinets, model 1,2).

Incubator 37°C.

Water incubator (50°C and 37°C) (Grant, England).

Spectrophotometer (Molecular device, VERSA max).

Spectrophotometer (Thermo scientific).

Centrifuges (Biofuge fresco, Heraeus instruments Eppendrof, Centrifuge 5810R).

Freezer Forma Scientific (-75°C and -20°C).

PCR machine (PTC-200) (Peltier Thermal cycler) (Bio-RAD, Mexico).

Shaker – MS1 minishaker.

Electrophoresis machine BIO-RAD power pac 300, BIO-RAD MINI-SUB® cell GT.

NanoDrop® ND-1000 spectrophotometer.

Annex 2: OD values at 650nm used to plot the growth curve of *S. gordonii* (liquid culture).

Time (hours)	Sample1	Sample2	Sample3	Sample4	Sample5	Average	Standard deviation
1	0.005	0.005	0.005	0.005	0.005	0.005	0.000
2	0.015	0.06	0.02	0.025	0.02	0.03	0.018
3	0.06	0.161	0.065	0.07	0.065	0.08	0.043
4	0.146	0.296	0.161	0.156	0.146	0.18	0.065
5	0.246	0.377	0.296	0.291	0.266	0.30	0.050
6	0.327	0.332	0.362	0.362	0.342	0.35	0.016
7	0.302	0.312	0.322	0.312	0.307	0.31	0.007
8	0.271	0.291	0.291	0.312	0.276	0.29	0.016
9	0.251	0.271	0.281	0.291	0.261	0.27	0.016
10	0.246	0.266	0.266	0.281	0.251	0.26	0.014
11	0.231	0.261	0.261	0.266	0.246	0.25	0.014
12	0.226	0.251	0.256	0.256	0.236	0.25	0.013
13	0.216	0.246	0.241	0.241	0.226	0.23	0.013
14	0.211	0.226	0.231	0.226	0.216	0.22	0.008
15	0.201	0.216	0.216	0.221	0.211	0.21	0.008
16	0.196	0.201	0.206	0.216	0.201	0.20	0.008
17	0.181	0.196	0.196	0.206	0.191	0.19	0.009
18	0.191	0.191	0.191	0.196	0.186	0.19	0.004
19	0.176	0.186	0.191	0.191	0.191	0.19	0.007
20	0.176	0.176	0.186	0.181	0.186	0.18	0.005
21	0.176	0.171	0.171	0.176	0.176	0.17	0.003
22	0.171	0.171	0.166	0.166	0.151	0.17	0.008
23	0.161	0.161	0.156	0.156	0.151	0.16	0.004

Annex 3: OD value at 650nm used to plot the growth curve of *S. gordonii* (with CSP) (liquid culture).

Time (hour)	Sample1	Sample2	Sample3	Sample4	Sample5	Average	Standard deviation
1	0.005	0.005	0.005	0.005	0.005	0.005	0.000
2	0.015	0.035	0.015	0.015	0.015	0.019	0.009
3	0.025	0.085	0.03	0.025	0.03	0.039	0.026
4	0.07	0.166	0.08	0.08	0.055	0.0902	0.044
5	0.141	0.317	0.161	0.161	0.106	0.1772	0.081
6	0.221	0.261	0.271	0.261	0.176	0.238	0.040
7	0.271	0.261	0.317	0.317	0.206	0.2744	0.046
8	0.251	0.226	0.271	0.256	0.226	0.246	0.020
9	0.211	0.211	0.241	0.236	0.201	0.22	0.017
10	0.196	0.196	0.221	0.216	0.196	0.205	0.012
11	0.191	0.186	0.201	0.201	0.191	0.194	0.007
12	0.181	0.176	0.186	0.186	0.186	0.183	0.004
13	0.176	0.171	0.176	0.176	0.186	0.177	0.005
14	0.171	0.161	0.166	0.161	0.186	0.169	0.010
15	0.166	0.151	0.161	0.161	0.186	0.165	0.013
16	0.166	0.151	0.161	0.156	0.186	0.164	0.014
17	0.161	0.146	0.146	0.151	0.186	0.158	0.017
18	0.161	0.141	0.146	0.146	0.186	0.156	0.018
19	0.161	0.136	0.141	0.136	0.186	0.152	0.022
20	0.161	0.131	0.131	0.131	0.186	0.148	0.025
21	0.161	0.121	0.126	0.121	0.186	0.143	0.029
22	0.156	0.111	0.126	0.121	0.171	0.137	0.025
23	0.156	0.111	0.111	0.111	0.161	0.13	0.026

Annex 4: Effect of the synthetic CSP on the *S. gordonii* growth.

Planktonic *S. gordonii*		Biofilm *S. gordonii*	
With CSP	Without CSP	With CSP	Without CSP
0.177	0.181	0.108	0.075
0.169	0.213	0.122	0.068
0.165	0.204	0.123	0.079
0.164	0.194	0.091	0.079
0.158	0.191	0.101	0.065
0.156	0.187	0.096	0.065
0.152	0.181	0.098	0.071
Average 0.163	0.193	0.105571	0.071714
STD 0.008485	0.011902	0.01266	0.006075

Annex 5: OD value at 650nm used to observe the effect of CSP addition on the log phase of growth curve of *S. gordonii* (liquid culture).

Time (minutes)	Sample1	2	3	4	5	Average	STDEV
5	0.0007	0.0015	0.0009	0.00048	0.0012	0.0010	0.0004
10	0.0017	0.0027	0.0023	0.0011	0.0018	0.0019	0.0006
15	0.0026	0.0031	0.0027	0.002	0.0029	0.0027	0.0004
20	0.0036	0.0046	0.0039	0.0034	0.0037	0.0038	0.0005
25	0.0052	0.0054	0.0052	0.0041	0.0051	0.0050	0.0005
30	0.0055	0.0068	0.0061	0.0053	0.0061	0.0060	0.0006
35	0.0071	0.0084	0.0076	0.0055	0.0079	0.0073	0.0011
40	0.0084	0.0097	0.0084	0.0061	0.0083	0.0082	0.0013
45	0.0096	0.0107	0.0103	0.0078	0.0102	0.0097	0.0011
50	0.0109	0.0126	0.0112	0.0089	0.011	0.0109	0.0013
55	0.0122	0.014	0.0121	0.0099	0.0128	0.0122	0.0015
60	0.0136	0.015	0.0134	0.0112	0.0146	0.0136	0.0015
65	0.014	0.015	0.013	0.015	0.0149	0.0144	0.0009
70	0.018	0.02	0.018	0.018	0.0155	0.0179	0.0016
75	0.024	0.025	0.023	0.025	0.024	0.0242	0.0008

80	0.029	0.027	0.026	0.028	0.027	0.0274	0.0011
85	0.031	0.031	0.032	0.033	0.031	0.0316	0.0009
90	0.035	0.036	0.037	0.038	0.036	0.0364	0.0011
95	0.04	0.041	0.042	0.043	0.041	0.0414	0.0011
100	0.049	0.049	0.047	0.051	0.046	0.0484	0.0019
105	0.053	0.053	0.052	0.055	0.051	0.0528	0.0015
110	0.058	0.059	0.057	0.061	0.056	0.0582	0.0019
115	0.068	0.07	0.067	0.071	0.066	0.0684	0.0021
120	0.072	0.073	0.07	0.074	0.069	0.0716	0.0021
125	0.098	0.123	0.128	0.134	0.115	0.1196	0.0139
130	0.078	0.081	0.08	0.08	0.083	0.0804	0.0018
135	0.089	0.093	0.091	0.094	0.084	0.0902	0.0040
140	0.091	0.095	0.095	0.098	0.098	0.0954	0.0029
145	0.097	0.102	0.102	0.105	0.105	0.1022	0.0033
150	0.101	0.107	0.109	0.109	0.109	0.1070	0.0035
155	0.108	0.113	0.117	0.117	0.108	0.1126	0.0045
160	0.111	0.117	0.122	0.122	0.116	0.1176	0.0046
165	0.118	0.124	0.129	0.129	0.127	0.1254	0.0046
170	0.126	0.131	0.135	0.137	0.134	0.1326	0.0043
175	0.133	0.139	0.143	0.141	0.141	0.1394	0.0038
180	0.145	0.151	0.149	0.151	0.149	0.1490	0.0024
185	0.149	0.154	0.154	0.154	0.154	0.1530	0.0022
190	0.156	0.161	0.164	0.162	0.159	0.1604	0.0030
195	0.165	0.169	0.17	0.169	0.164	0.1674	0.0027
200	0.172	0.178	0.179	0.178	0.175	0.1764	0.0029
205	0.181	0.187	0.186	0.186	0.183	0.1846	0.0025
210	0.188	0.196	0.196	0.193	0.189	0.1924	0.0038
215	0.196	0.204	0.204	0.202	0.197	0.2006	0.0038
220	0.203	0.213	0.214	0.211	0.207	0.2096	0.0046
225	0.213	0.222	0.223	0.219	0.218	0.2190	0.0039
230	0.221	0.231	0.232	0.228	0.231	0.2286	0.0045

Annex 6: Transformation frequency in liquid media with different incubation time points after adding CSP and before adding 1.2µg/ml pVA838 (liquid culture).

Incubation time (minute)	Transformation frequency	Average of transformation frequency	Standard deviation
0 minute	6.67E-07	6.24E-07	3.7753E-08
0 minute	5.95E-07		
0 minute	6.10E-07		
20 minutes	2.40E-05	1.59E-05	7.0694E-06
20 minutes	1.27E-05		
20 minutes	1.10E-05		
40 minutes	6.77E-05	5.70E-05	1.6746E-05
40 minutes	6.57E-05		
40 minutes	3.77E-05		
60 minutes	6.51E-05	6.20E-05	9.8653E-06
60 minutes	5.10E-05		
60 minutes	7.00E-05		
80 minutes	1.87E-04	4.23E-04	2.29E-04
80 minutes	6.45E-04		
80 minutes	4.38E-04		

Annex 7: Transformation frequency in different CSP concentration and incubation hours (liquid culture).

CSP concentration	60 minutes incubation	80 minutes incubation
200nM CSP	6.24E-05	1.77E-04
400nM CSP	4.49E-05	1.57E-04

Annex 8: Transformation frequency in different concentration of plasmid pVA838 (liquid culture).

DNA concentration	Transformation frequency in 80 minutes incubation time	Average transformation frequency	STDEV
1200 ngDNA	6.25E-04	5.46E-04	0.00011135
1200 ngDNA	4.68E-04		
2400 ng DNA	7.36E-04	5.92E-04	0.00020328
2400 ng DNA	4.48E-04		
3600 ng DNA	8.71E-04	7.41E-04	0.00018375
3600 ng DNA	6.11E-04		

Annex 9: Transformation frequency in different concentration of CSP with two incubation time points in biofilm.

Incubation time	CSP of 400 nM	CSP of 800 nM	Average of 400nM	Average of 800 nM	STDEV of 400nM	STDEV of 800nM
1 hour	2.00E-04	1.94E-04	1.62E-04	2.61E-04	3.7528E-05	5.7754E-05
	1.60E-04	2.96E-04				
	1.25E-04	2.92E-04				
2 hour incubation	1.43E-03	1.85E-03	1.65E-03	1.90E-03	1.88E-04	2.74E-04
	1.76E-03	1.65E-03				
	1.76E-03	2.19E-03				

Annex 10: Transformation frequency with optimized parameters in liquid culture.

Incubation time (minutes)	Transformation frequency	Average of transformation frequency	STDEV
80 minutes	6.44E-04	4.40E-04	2.56E-04
80 minutes	4.31E-04		
80 minutes	5.24E-04		

Annex 11: Transformation frequency with optimized parameters in biofilm.

Incubation time (hours)	Transformation frequency	Average of transformation frequency	Standard deviation
2 hours	2.97E-03	3.04E-03	0.00057644
	3.65E-03		
	2.51E-03		