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Proteome Analysis of *Pseudomonas putida* F1 Biofilms on Two Substrata

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To understand the importance of substratum in terms of biofilm formation, this study studied differences in the proteins produced by *Pseudomonas putida* strain F1 grown under different conditions. Specifically, this asked what proteins were made when biofilms of *P. putida* were formed on steel mesh balls and glass wool. Two-dimensional gel electrophoresis analysis of total soluble proteins allowed both sets of biofilm-grown cells to be compared with those of planktonically-grown cells. After analysis by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry, fifteen differentially expressed proteins were identified and grouped into several classes: proteins involved in metabolism, transportation, adaptation or protection, biosynthesis and other functions. Five proteins showed differential expression only on one substratum. Enolase, a protein with multiple functions in prokaryotic cells, was up-regulated in mature biofilms both at protein level and mRNA level, indicating the protein expression was regulated in transcription level. These findings will help to understand the development of biofilms and the role of the substratum in this process.

Key words: Proteomics, Biofilm, Substratum, *Pseudomonas putida* F1.

Understanding the proteomics of bacteria growing on different surfaces has many important practical benefits (Kropfl *et al*., 2006; Di *et al*., 2011; Seneviratne *et al*., 2012).These range from the clinical e.g. bacterial growth on implanted medical devices through to more environmental applications. This study focused on the growth of an environmentally important bacterial species, *Pseudomonas putida* and specifically on defining the extent to which the proteome was influenced by growth on different substrata. Like many other strains of *P. putida*, strain F1 can survive in the presence of organic pollutants such as benzene, toluene, ethylbenzene and other aromatic compounds. The aromatic compound degradation pathway (TOD pathway) has been well studied *in vitro* (Zylstra & Gibson, 1989; Parales *et al*., 2008). It is clearly important to be able to make predictions on these processes in terms of the bioremediation of polluted environments. The next step is to develop our understanding of this species when growing as a biofilm, the natural form of growth in environments.

P. putida is not the only species to grow as a biofilm. In natural environments, bacteria typically adopt a biofilm growth mode, with cells sensing the change in environmental factors and transiting from planktonic growth to a multi-cellular aggregation form of growth (Davey & O2 Toole, 2000). The expression of a number of genes or proteins is changed in this process. This makes the cells within the biofilm different from the planktonic cells in phenotype, metabolism and

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other characteristics such as increased resistance to antibiotics, a great problem in the clinic (Manuel et al., 2010). Efforts have been made to try to explain the differential cellular activities of biofilm grown cells compared to planktonic cells. Through applying proteomics methodologies in recent years, proteins involved in a range of cellular processes such as motility, quorum sensing, metabolism, lipid biosynthesis, transport, stress response and protein synthesis have also been found to be involved in biofilm formation (Tremoulet *et al*., 2002 ; Arevalo-Ferro *et al*., 2005 ; Martínez-Gil *et al*., 2010; Kim *et al*., 2012).

Many chemical and physical factors including hydrodynamics, cell surface structures and characteristics of the aqueous medium such as pH, ionic strength and temperature will affect the growth of biofilms (O'Toole *et al*., 2000; Donlan, 2002; Amini *et al*., 2011). Amongst these factors affecting biofilm development, the substratum is of great importance because the substratum affects the composition of 'conditioning film' formed on their surfaces. This consequently affects both microbial attachment and cellular activities in the developing biofilm structure (Donlan, 2002; Anozie *et al*., 2010).

Glass wool (GW) and steel mesh balls (SMB), which have homogeneous characteristics for easy sampling and have relative bigger surface area than other commonly used substrata such as glass slides and silicon tubes, were used in this study (Welin *et al*., 2004; Martínez-Gil *et al*., 2010). The aim of this study was to develop a better understanding of protein expression in biofilm formations in specific relation to the influence of substratum selection on biofilm formation. In order to address this aim, a proteomics-based method was applied to study the differential expression of bacterial proteins during biofilm formation.

MATERIALS AND METHODS

Growth conditions of planktonic cells and biofilms

Bacterial strain *P. putida* F1 (ATCC700007) was inoculated into phosphate and metal reduced Hutner's medium (potassium phosphate buffer 3.8mM and 1/10 of metals "44" solution) (Cohen-Bazire *et al*., 1957) and cultivated at 30°C, with shaking at 220rpm for 16h, 100ml of the culture was inoculated into a 2.5L BioFlow III bioreactor (New Brunswick Scientific, USA) with fresh sterile medium and cultivated at 30°C. Air was pumped into the bioreactor in a flow rate of 1.2L/min and sparged to the medium with the help of the stirrer (250rpm). Fresh sterile medium was pumped into the bioreactor and diluted at a stable dilution rate of 0.027/h to enable biofilm growth. Biofilms grew on SMB (strand diameter 400µm) which were held in mesh baskets in the bioreactor and GW (diameter 15µm) and which were bound surrounding the rods separated evenly in the bioreactor. Bacteria grown under the same conditions but with no substrata were used as controls (planktonic cells). The growth of biofilms was assessed both by total protein quantification and biofilm staining with crystal violet followed by microscopic detection. Biofilms on SMB and GW and planktonic cells were collected after 48h, 28h and 28h of cultivation, respectively.

Total protein quantification for biofilm growth

Triplicate samples were taken at different growth stages and were pulse centrifuged at 5000*g* for 5 seconds to remove the medium and unattached bacteria. The samples were then covered by how much? 0.2M NaOH for 5min. Protein concentration was determined using the RC DC Protein Assay Kit (BioRad, USA) following the manufacturer's recommendations.

Protein sample preparation

Planktonic cells were collected by centrifugation at 5000*g* for 10min at 4°C and washed three times with ice-cold 50mM Tris-HCl buffer (pH7.5). SMB and GW (with adherent biofilms) were washed with fresh 50mM Tris-HCl buffer (pH7.5). The samples were frozen at -70°C and thawed in Sample Buffer (BioRad, USA) in preparation for two-dimensional gel electrophoresis (2-DGE) (urea (8M) added after sonication) with protease inhibitor mixture (PMSF, 1mM; estatin 10mM; pepstatin, 1.45µM and E-64, 40mM) but no Biolyte. After sonication (Soniprep150, Sanyo, Japan) on ice 3 times at 7µA for 20sec with 1min intervals, 20U/mL DNase (Promega, USA) and 10µg/mL RNase (Promega, USA) were added to the samples, left on ice for 30min and centrifuged at 13000*g* at 4°C for 30min. The supernatants were collected and quantified by the RC DC protein assay kit.

2-D gel electrophoresis

Isoelectric focusing, equilibration and

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the manufacturer's recommendations for 17cm, pH4-7 gel strips (BioRad, USA) with the following modifications. 50µg of protein in 300µL sample buffer with 0.2% Biolyte were actively rehydrated for 16h in an IEF cell (BioRad, USA) and focused using a maximum voltage for a total 75000Vh. 12.5% polyacrylamide gel was used for the second dimension. The gels were silver stained using BioRad silver stain kit according to the manufacturer's recommendations and images were recorded by an Image Analyzer (Herolab, Germany).

Image analysis

Gel images were compared using Phoretix (Nonlinear Dynamics, UK) 2-D image comparison software. The same protein spots from each of 2 images of 3 parallel samples were selected to be the representative spots for each condition, protein spots that only appeared in one sample or where protein intensity varied more than 2 times were not selected. After normalized volume comparison, those proteins differentially expressed for more than 3 fold and shown to be significant by Student's t-test ($p<0.05$) were considered to be biologically significant in this study and were chosen for mass spectrometry analysis (Wilkins *et al*., 2003).

In-gel digestion and protein identification

The protein spots of interest from the 2-D gels were in-gel digested using the protocol described by Shevchenko *et al*. (1996) with minor changes. The digested peptide samples were washed with 20mM NH_4HCO_3 for 20min at room temperature and extracted for 3 times using a solution containing 5% formic acid/ 50% acetonitrile for 20min. Supernatants were dried in a speed vac at 20 °C and desalted using Ziptip C18 according to the manufacturer's recommendations (Millipore, USA). After samples were dried with matrix in a vacuum at room temperature, the crystals formed were positively ionized and analyzed by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, USA) (Shevchenko *et al*., 1996; Fountoulakis & Langen, 1997). Peptide fingerprints obtained were used to search in databases using MASCOT software against proteobacteria in order to identify the proteins. Blank gel piece digestion solution was used as an internal standard. The protein was correlated with a function when the best match was significant with a high protein score (a 95% confidence level threshold was used for protein score) and its pI and molecular relative mass to its predicted location on the gels.

Total RNA extraction, cDNA synthesis and real-time PCR

Total RNA was isolated using the TRI_{ZOL} LS Reagent (Invitrogen, USA) according to manufacturer's instructions. Traces of contaminating DNA in the RNA samples were removed using DNA-free™ DNase Treatment and Removal Reagents also according to the manufacturer's recommendations (Ambion, USA).

Each RNA template (1µg in 5µL ddH_2O) was mixed with 10µg of 10 base random primers (Invitrogen, USA), denatured at 70°C for 5min and immediately cooled on ice. A master mix containing all other reagents including 10U of ImProm-II™ Reverse Transcriptase (Promega, USA), reaction buffer and dNTPs (reaction concentration 1mM) was added to the tube to a volume of 15µL. The master mix was mixed with RNA and primers mixture and cDNA was synthesized in all samples at the same time in a thermal cycler (PHC-3, Techne, UK) at 25°C for 5min followed by 42°C for 60min.

Primers for real-time PCR were designed using Primer Express software (Applied Biosystems, USA) in the conserved regions of the respective genes. Primers for internal standard RNA polymerase sigma factor (*rpoD*) were designed as: forward 5'-CGCAACCGCCGTCTCGTATC-3' and reverse, 5'-CCTCCGCGTAAGTCAGGTAG-3' with a predicted product size of 73bp. Primers used for enolase were designed as: forward, 5'-CGAAATCTTCCACCACCTCAA-3' and reverse, 5'-CTTCGTTGGATGCCAGGTTAG-3' with a predicted product size of 101bp.

A 10x dilution of cDNA products (2µL) was mixed with 10µL of the SYBR Green PCR Master Mix (Applied Biosystems, USA), 4µL of forward and reverse primers each in a final concentration of 0.4µM. Real-time PCR was carried out in an ABI 7000 system (Applied Biosystems, USA) in a series of reactions start from hold at 50°C for 2min, 94°C for 10min for denaturation; 40 cycles of denaturation (95°C for 15sec) followed by annealing (60°C for 30sec) and extension (72°C for 30sec). By dissociation curve analysis, only those reactions that were primer dimmer and other

artifact free were considered to be valid. A standard curve was generated by the software according to the dilution rate of the *rpoD* gene in a cDNA sample. The mRNA relative concentration of the *eno* to *rpoD* was calculated with the help of the standard curve (Savli *et al*., 2003).

RESULTS AND DISCUSSION

Growth of biofilms on SMB and GW substrata

The growth of *P. putida* F1 biofilms achieved under the cultivation conditions described was assessed microscopically and also by calculating the total protein yield. Bacterial cells started to attach to the strands of the SMB and GW after cultivation started with a few bacteria attaching to the glass wool after 6h cultivation and attachment getting faster after 18h. After 28h (on GW) or 48h (on SMB), the protein yield reached a stable stage with tower shaped biofilms formed, and remained in a similar amount in the following 8h (on GW) or 24h (on SMB) (Figure 1). This indicated the formation of mature biofilms, as exemplified by the images of biofilms on GW shown in Figure 2, tower shaped mature biofilms (pointed by an arrow) formed on the glass wool with large bare spaces. The time points of 28h (GW) and 48h (SMB) were then selected to study the protein expression of biofilms. Since the cultivation was in a chemostat and the growth of planktonic cells was steady, the sampling time of planktonic cells at 28h time point would not affect the final comparison.

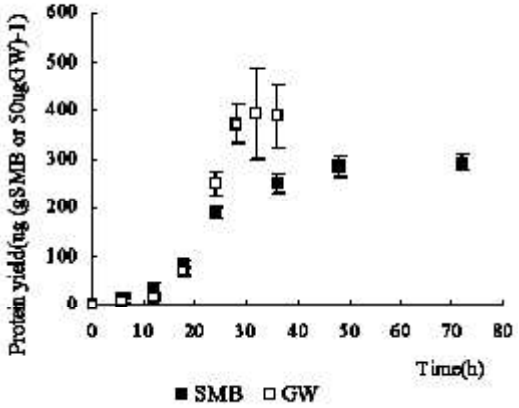

Fig. 1. Growth of *P. putida* F1 biofilms on SMB or GW. Error bars show the average protein yield from triplicate cultivation. SMB, steel mesh balls; GW, glass wool

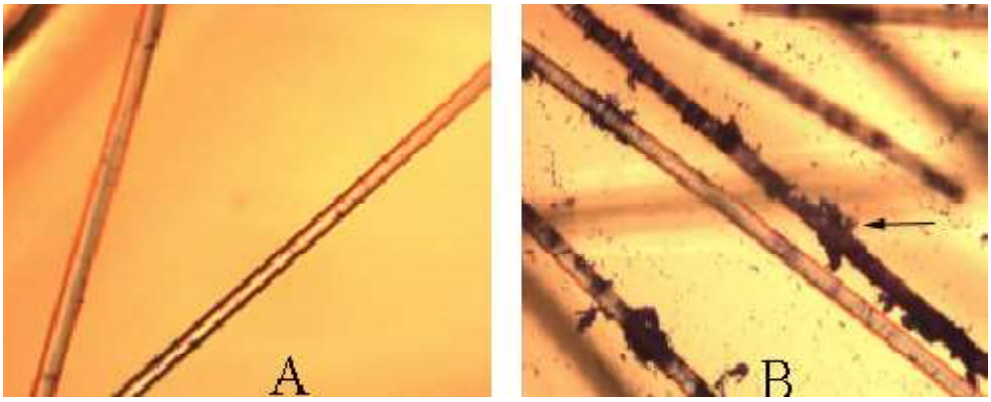

Fig. 2. Growth of *P. putida* F1 biofilms on GW. The biofilms were crystal violet stained after cultivated for 6h (A) and 28h (B) in a chemostat. GW, glass wool. Arrow shows a representative mature biofilm

Protein differential expression in the two growth modes

Whole cell proteins were extracted from triplicate samples and subjected to 2-DGE. In other experiments, it was found that the majority of the proteins expressed were located in the pH4-7.5 region of 2-D gels (data not shown). As such, the most closely matching commercially available strips, those gel strips operating over the region from pH4 to pH7, were thus used. According to image comparison software, 435 out of total 528 protein spots (GW biofilm) and 454 out of 512 spots (SMB biofilm) were reproducibly detected in the triple 2-D images, the reproducibility of the 2-D images was above 82%. The protein expression in biofilms cells significantly differed from their counterpart planktonic cells. Selected major up-regulated proteins are indicated by arrows in Fig. 3.

Table 1. Differentially expressed proteins identified by MALDI-TOF analysis in biofilm cells grown on SMB and GW

Spot No. Protein function identified	pI[a]	Mr (kDa)[a]	Peptide coverage (%)	Protein expression[b] (up-regulated)	Accession No.
Proteins involved in metabolism					
1. Ketol-acid reductoisomerase	5.5	36.6	45	SMB, GW	NP_746787
2 Arginine deiminase	5.6	46.8	34	SMB	NP_743162
3 Ornithine carbomoyltransferase	5.9	38	40	SMB	CAD10424
4 Glutamine synthetase, type I	5.2	52.1	45	SMB, GW	NP_747147
5 Uroprophyrin-IIIC- methyltransferase, putative	5.0	40.5	43	SMB, GW	NP_742357
Transport or membrane proteins					
6 Outer membrane protein OprF	4.5	37.2	40	SMB, GW	NP_747154
7 General amino acid ABC transporter, periplasmic binding protein	5.8	36.7	30	GW	NP_744239
8 Putrescine ABC transporter, periplasmic putrescine-binding protein	6.0	40.2	41	SMB, GW	NP_747282
9 ABC transporter, periplasmic binding protein	5.8	38.6	54	GW	NP_743883
10 Tricarboxylate transport protein TctC, putative	6.2	35.1	25	SMB, GW	NP_743576
Adaptation or protection related proteins					
11 Tld protein	5.1	50.4	35	SMB, GW	NP_743101
12 Phosphoglycerate kinase	5.2	40.2	24	SMB, GW	NP_747066
13 Enolase	4.9	45.6	30	SMB, GW	NP_743769
Protein biosynthesis					
14 Elongation factor Tu-A	5.2	43.8	54	SMB	AAY29576
Proteins involved in other basic biofilm activities					
15 Periplasmic glucan biosynthesis protein	6.1	62.3	28	SMB, GW	NP_747127

[a] Obtained from databases.
[b] Proteins were up-regulated in the biofilms formed on the substrata shown below. SMB, steel mesh balls; GW, glass wool.

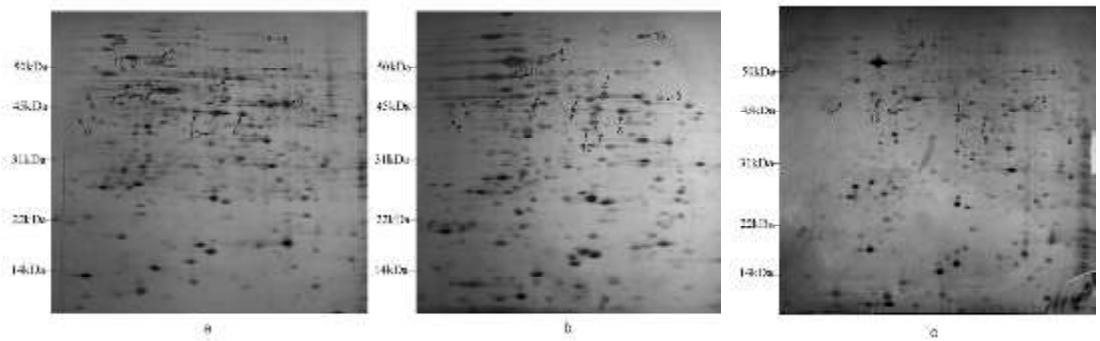

Fig. 3. Two dimensional gel image comparison of proteins from biofilms and planktonic cells. A representative gel from duplicate of two samples under each condition was presented. 50µg proteins were loaded on a 17cm, pH4-7 gel, focused at highest voltage of 10000v for 75000Vh. The gels were silver stained. a, 48h biofilms on SMB; b, 28h biofilms on GW; c, planktonic cells. Arrows point to the main up-regulated proteins analyzed. Numbers indicate the protein spots being identified

It was hypothesized that those proteins that were over expressed in both biofilms were essential for the development of biofilms, whereas those over expressed only in biofilm cells grown on one substratum were reflected the impact of each individual substratum. Twenty seven proteins pointed by arrows in Figure 3 which were detected to be significantly differentially expressed were analyzed by MALDI-TOF, other spots were not analyzed because they were either low abundant or difficult to select without contamination by other proteins. Fifteen proteins were identified with known function (Table 1), others were unable to be identified due to incomplete database information. Five metabolic proteins, five membrane proteins, three adaptation or protection related proteins, one protein biosynthesis related protein and one protein related to other activities were identified. Their identity, pI and molecular relative mass obtained from databases, as well as the matched peptides coverage of total protein and their expression are listed in Table 1.

Among the proteins identified, ketol-acid reductoisomerase, arginine deiminase (ADI), ornithine carbomoyltransferase (OCTase), outer membrane protein OprF, several ABC transporters, phosphoglycerate kinase, enolase and periplasmic glucan biosynthesis protein have been previously identified to be over expressed in biofilms. These play roles in either altering metabolism and transportation activities, adaptation to the biofilm microenvironments or surface adhesion. Periplasmic glucan biosynthesis protein may also have some role in antibiotic resistance (De Mot *et al*., 1994; Beenken *et al*., 2004; Sriramulu *et al*., 2005; Manuel *et al*., 2010; Barczak *et al*., 2012).

Glutamine synthetase (GS), uroprophyrin-IIIC- methyltransferase, tricarboxylate transport protein TctC, elongation factor Tu-A and TldD protein, were found in this study to be up-regulated in biofilms on both substrata, indicating changed metabolism, transportation, protein synthesis and stress response activities when mature biofilm formed.

Arginine deiminase (ADI) and ornithine carbomoyltransferase (OCTase) were up-regulated in biofilms grown on SMB but not significantly on GW. These two enzymes, together with carbamate kinase, catalyze the conversion of arginine to ornithine, ammonia, and CO_2, with ATP produced for cellular activities(Zeng & Burne . 2010). It has been found that in *P. aeruginosa*, these proteins were only expressed under anaerobic conditions (Trunk *et al*., 2010), and in *Bacillus licheniformis, these proteins were induced by* anaerobiosis in the presence of arginine (Maghnouj *et al*., 2000). Although in *Streptococcus gordonii and S. rattus*, the expression of these enzymes are believed to be related to the pH homeostasis in biofilms, they could also be induced by anaerobiosis through a Fnr-like protein (Flp) (Liu *et al*., 2008). Thus, the higher expression of ADI and OCTase in *P. putida* F1 seen in this study could also be a consequence of much reduced oxygen levels or anaerobiosis induced in biofilm microenvironments. What was interesting is that ADI and OTCase were not found

to be significantly up-regulated in biofilms grown on GW; one explanation is that the different accumulation of biofilms on the two substrata may result in different microenvironments. As shown in Figure 1, at the sampling point, the protein yield was 284μg/g SMB and 7420μg/g GW. However; considering the factors of density and strand diameters of the two substrata, for 1g of each substratum, the surface of GW is about 100 times in size of that of SMB. Thus, the biomass on certain surfaces of SMB was about 4 times of biomass on GW. The differential amount of biofilm accumulated on the surface of different substrata may be due to the difference of the roughness of the substratum surfaces. The higher accumulation of SMB biofilms stimulated the usage of oxygen, generating lower oxygen conditions in the microenvironment of SMB biofilms than in GW biofilms. The different metabolic activities thus resulted in different expression of some membrane and transport proteins, as shown in Table 1.

Enolase, a glycolytic enzyme converting 2-phosphoglycerate to phosphoenolpyruvate, was up-regulated in biofilm cells. It is a protein with multi functions. As well as its catabolic and hypoxic stress response functions, enolase is involved in RNA processing and transcript turnover in Gram negative *Escherichia coli*, (Chandran & Luisi, 2006; Burger *et al*., 2011). In Gram positive Streptococci, enolase was surface expressed and acted as plasminogen-binding receptor, and thus has a role in pathogenesis (Díaz-Ramos *et al*., 2012). As enolase has not been found to be surface expressed in Gram negative bacteria, the up-regulation of enolase in biofilm cells was most probably due to the oxygen or nutrient limitation stress in biofilms. However, it is hard to explain why the expression of enolase in GW biofilm was higher than in SMB biofilm. More work is required to investigate the functions of enolase in biofilm formation.

Due to the important roles of enolase, study on the expression of enolase in *P. putida* F1 biofilm cells may be helpful in elucidating the multiple roles of enolase. The expression of enolase gene (*eno*) in planktonic and biofilm cells was compared using real-time PCR analysis. The mRNA concentration of enolase relative to RpoD in the two biofilms as well as in the planktonic cells was shown in Figure 4. The mRNA level of the *eno* gene in both biofilms was more than 2-fold higher than in planktonic cells. This was compatible to the protein expression level and indicated that the regulation of enolase expression was in the transcriptional level.

It can be concluded that during biofilm formation, the influence of substrata on biofilm formation can in turn influence biofilm structure with different nutrient and oxygen conditions created in their microenvironments. As such, this has a direct impact on cellular activities including metabolism, transport, stress response and biosynthesis. In this study, it was found that the multifunctional enzyme enolase may serve as a stress response protein to limited oxygen in mature biofilms.

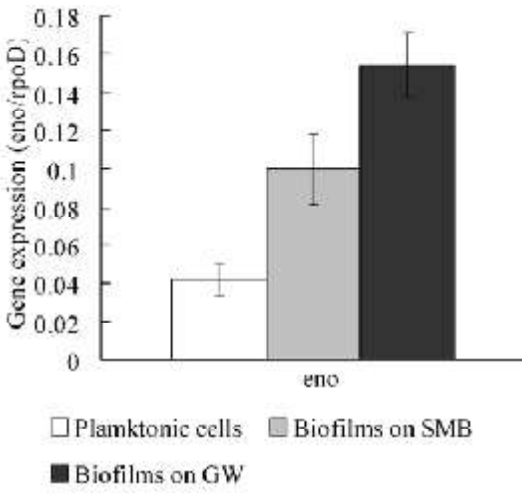

Fig. 4. Relative expression level of *eno* in planktonic and biofilm cells using *rpoD* gene as internal control. The expression level of *rpoD* in each sample was labeled as 1. The mean value was obtained on the basis of duplicate of three samples of each template. Planktonic cells and biofilms on SMB and GW were cultivated in a chemostat for 28h, 48h and 28h, respectively. *eno*, enolase gene; *rpoD*, RNA polymerase sigma factor; SMB, steel mesh ball; GW, glass wool. The results were generated from duplicate of three samples

Overall, this study emphasizes the importance for greater research in this area in order to understand the importance of the role of the substratum in influencing the formation of bacterial biofilms.

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