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Research paper

Altered EPS Protein Secretion in *Salmonella Typhimurium* Biofilm Following Treatment with DMSO

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Abstract

Dimethyl sulfoxide (DMSO) is known to inhibit biofilm formation by *Salmonella typhimurium* and cause infrared spectral variations of extracellular polymeric substance (EPS) matrix. However, the impact of DMSO treatment on EPS protein secretion in *S. typhimurium* biofilm remains not well investigated. The present study was performed to investigate the EPS protein profile of *S. typhimurium* biofilm following treatment with DMSO. Biofilm was developed in 6-well microplate at 37 ^{O}C for 24 hours. Quantitative resazurin assay and infrared spectroscopy were performed to determine biofilm viability and cell surface chemistry respectively. Subtractive EPS protein profiling was carried out using a combination of polyacrylamide gel electrophoresis and tandem mass spectrometry. Treatment with 32% DMSO caused significant reduction in biofilm viability and alteration of cell surface chemistry. Subtractive comparison of EPS protein profiles highlighted one unique protein band, 52.1 kDa, which was secreted only in 32% DMSO treated EPS matrix and not in control EPS matrix. In turn, three EPS proteins were successfully identified from the 51.2 kDa protein band. In conclusion, altered EPS protein secretion following treatment with 32% DMSO may mediate inhibition of *S. typhimurium* biofilm.

Keywords: *Biofilm; Salmonella typhimurium; dimethyl sulfoxide; extracellular polymeric substances; protein secretion*

1. Introduction

A biofilm is defined as a complex and structured microbial community that tend to attach to inert and living surfaces in the presence of extracellular polymeric substances (EPS) layer. This microbial community differs from its planktonic counterpart in the protein expression pattern that typically results in distinct metabolic profile [1]. On the other hand, the mixture of monolayer biofilm and three dimensional biofilm often produces highly heterogeneous mature biofilm [2, 3]. Across many studies, the spatial heterogeneity in the biofilm structure has made the antimicrobial treatment ineffective. For many decades, the biofilm has been shown to cause a wide range of serious problems to public health and increase the resistance to antimicrobial agents.

Salmonella typhimurium is an important biofilm producer that causes severe gastroenteritis. Its resistance towards a wide range of antibiotics has previously been reported [4]. Recently, EPS matrix has been shown to be crucial for viable biofilm of *S. typhimurium* [5]. In that study, a non-destructive analysis using attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy revealed the correlation between chemical modification of EPS matrix by 32% dimethyl sulfoxide (DMSO) and inhibition of *S. typhimurium* biofilm. Another work demonstrated that chemically modified EPS matrix associated with changes in whole-cell protein expression in *S. typhimurium* biofilm [6].

DMSO is a common organic solvent used to dissolve a wide spectrum of pharmaceutical compounds due to its high polarity. It has been used to treat dermatitis, arthritis, allergic aczema and tissue damage. It can be cytotoxic above 2%, however, an *in vivo* study reveals the low systemic toxicity and safe oral and topical applications of high DMSO concentration (above 70%) [7]. In line with that, Yahya et al. [5] suggested that the application of 32% DMSO for the biofilm control may not result in adverse effects on human. The impact of DMSO treatment on EPS protein secretion in *S. typhimurium* biofilm remains not well investigated. Therefore, the present work was carried out to analyze EPS protein profile of *S. typhimurium* biofilm following treatment with DMSO.

2. Materials and Methods

Microorganism and compound

Microorganism tested herein was *S. typhimurium* ATCC 14028. Gram-staining and assessment of colony morphology were performed to regularly assess culture purity. Bacterial inoculum was adjusted to 12×10^8 CFU mL^{-1} for all biofilm assays, The compound tested in this study was dimethyl sulfoxide (Merck, Germany) [5, 6].

Biofilm formation assay

Cultivation and measurement of biofilms (24 hours) were performed as previously described [5, 6].

Statistical analysis

Data from biofilm formation assay were expressed as mean ± standard deviation with n=3. Paired T-test was performed to determine the significant difference between control biofilm and biofilm treated with DMSO whereby $p < 0.05$ was considered significant.

Infrared spectral analysis

Biofilms in the absence and presence of test compound were cultivated in 6-well microplate. Following incubation at 37 °C for 24 h, nutrient medium was discarded whilst the biofilm fractions were rinsed with distilled water twice, suspended in 0.9% NaCl, 1 mM PMSF, 1% SDS and centrifuged at 4,000 g for 15 min at 4 °C. The resulting cell pellet was used for infrared spectral analysis using a Thermo Scientific NICOLET 6700 ATR-FTIR spectrometer (Thermo Fisher Scientific Inc., USA).

EPS Protein Extraction and Determination

Biofilms with and without test compound were grown in 6-well microplate. Following incubation at 37 °C for 24 h, nutrient medium was discarded whilst the biofilm fractions were rinsed with distilled water twice, suspended in 0.9% NaCl, 1 mM PMSF, 1% SDS and centrifuged at 4,000 g for 15 min at 4 °C. The resulting cell pellet was used for infrared spectral analysis whilst supernatant was incubated with absolute ethanol (Sigma, USA) overnight at −20 °C. Then, the mixture was centrifuged at 4000g for 15 min at 4 °C and pellet containing EPS matrix was suspended in 25 mM Tris, 150 mM NaCl, 0.5% SDS. Protein determination was performed using standard Bradford assay.

Polyacrylamide gel electrophoresis

EPS protein fractions were dissolved in Laemmli sample buffer at the ratio of 1:1 and heated at 95 °C for 5 min. In turn, the fractions were resolved on polyacrylamide gels (12% separating gel) in 25 mM Tris, 192 mM glycine, 0.1% SDS. Electrophoresis, Coomassie staining and subtractive analysis were performed as previosly described [6].

In-gel digestion and protein identification.

The protein band of interest was manually excised, destained, vacuum-dried and trypsin digested according to the previous protocol [6]. Meanwhile, protein identification was performed using 5600 TripleTOF mass spectrometer (AB Sciex, USA) and Mascot database with the previously reported parameter settings [6].

Bioinformatic analysis

Analysis of secreted proteins was conducted using four online tools namely SignalP 4.0, SecretomeP 2.0, EffectiveELD and TMHMM. Analysis of protein interaction network was performed using STRING database v9.0. Identification of significant ($p < 0.05$) biological processes and pathways associated with the protein interaction network were carried out using Gene ontology (GO) enrichment analyses.

3. Results and Discussion

Impaired biofilm viability and altered cell surface chemistry

Biofilm viability in the presence and absence of 32% DMSO is shown in Figure 1. Treatment with 32% DMSO significantly ($p<0.05$) reduced viability of *S. typhimurium* biofilm. Figure 2 shows chemical composition of biofilm cells. Treatment with 32% DMSO caused alteration of infrared (IR) spectra of cell surface in the range between 1700 and 1500 cm^{-1}. Functional groups to be affected were C=O stretch (amide I, 1652cm^{-1}), N–H stretch (amide II, 1558cm^{-1}), C=C stretch (1538cm^{-1}) and N–O asymmetric stretch (1506 cm^{-1}). Impaired biofilm viability and altered cell surface chemistry following treatment with 32% DMSO observed herein are consistent with the previous work [5].

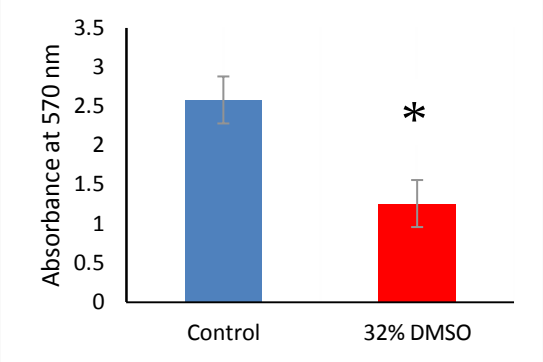

Figure 1: Viability of *S. typhimurium* biofilm. Asterisk indicates significant difference ($p < 0.05$) in comparison with control. DMSO: dimethylsulfoxide.

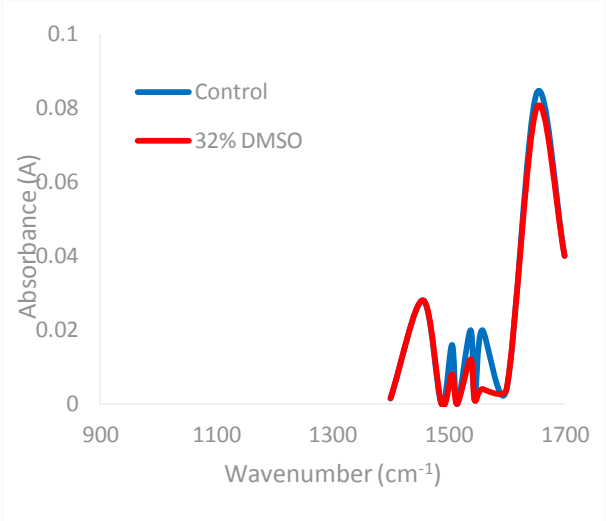

Figure 2: ATR-FTIR spectra of *S. typhimurium* biofilm. DMSO: dimethylsulfoxide.

Subtractive EPS protein profile

To optimize the EPS protein extraction, ethanol precipitation of 24 h EPS fraction was carried out at four different temperatures and incubation times as follows: protocol 1: 4°C for 2 h; protocol 2: 4°C overnight; protocol 3: -20°C for 2 h and protocol 4: -20°C overnight. Only protocol 4 consistently produced at least nine visible protein bands in the range between 17 kDa and 78 kDa. Thus, protocol 4 was used for the subsequent protein analyses. Protein profiles of whole-cell lysate and EPS matrix at 24 h is shown in Figure 3. One dimensional PAGE protein banding pattern of control EPS matrix was 78% similar to that of control whole-cell biofilm while 32% DMSO-treated EPS matrix was 75% similar to that of 32% DMSO-treated whole-cell biofilm. Treatment with 32% DMSO caused changes in the EPS protein secretion at 24 h. Subtractive comparison of protein profiles highlighted one unique protein band, 51.2 kDa, that was secreted only in 32% DMSO-treated EPS matrix and not in control EPS matrix. The absence of 51.2 kDa protein bands in control EPS matrix was confirmed by densitometric analysis. Efficiency of ethanol precipitation in isolation of EPS matrix from microbial aggregates has previously been reported [8]. The present study used gel-based subtractive profiling to investigate the effect of 32% DMSO on EPS protein of *S. typhimurium* biofilm. This approach has previously been taken to analyze whole-cell protein expression in heterogeneous *S. typhimurium* biofilm [6]. According to An and Parsek [9], this approach accounts for physical and chemical heterogeneities in the biofilms. On the other hand, changes in EPS protein profile following treatment with 32% DMSO corroborate the differential protein banding pattern in microbial cells as a result of treatment with antimicrobial agent [10].

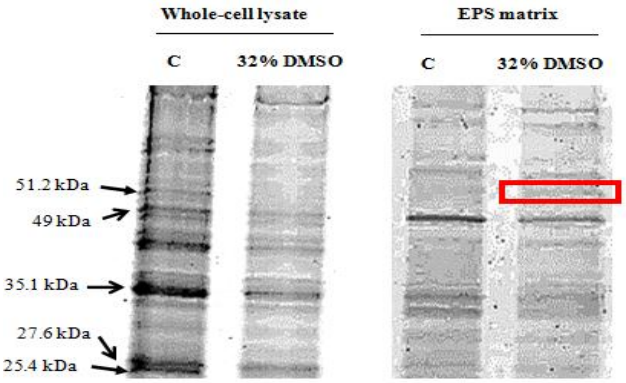

Figure 3: Subtractive EPS protein profile of *S. typhimurium* biofilm. Left panel: whole-cell protein profile in the absence and presence of 32% DMSO [6]; right panel: EPS protein profile in the absence and presence of 32% DMSO. Molecular masses correspond to sizes of major protein bands in Salmonella serovar [6, 11]. Red box indicates the unique protein band which is selected for protein identification. C: control. The amount of protein loaded into each lane was 4 µg (right panel).

Identified EPS proteins

A list of EPS proteins identified by QTOF analysis is shown in Table 1. Three EPS proteins from 51.2 kDa protein band were identified as elongation factor Tu (KNU68004), enolase (KNB35421) and phosphoglycerate kinase (KNB39376). It should be noted that these identified EPS proteins were secreted only in 32% DMSO-treated EPS matrix and not in control EPS matrix. Representative QTOF mass spectra are shown in Figure 4. Table 2 presents the results of analysis of secreted proteins. All the QTOF-identified EPS proteins were categorized as secreted proteins. Functional interactions among QTOF-identified EPS proteins are depicted in Figure 5. Enolase was predicted to be functionally linked to phosphoglycerate kinase (by five STRING criteria) and elongation factor Tu (by two STRING criteria) while no functional linkage between phosphoglycerate kinase and elongation factor Tu was predicted. GO enrichment analysis showed that glycolysis was significant ($p<0.05$) for this protein association. Identification of three EPS protein (elongation factor Tu KNU68004, enolase KNB35421 and phosphoglycerate kinase KNB39376) in the present study corroborates Yahya et al. [6]. They demonstrated the presence of these proteins in whole-cell of *S. typhimurium* biofilm using the same experimental approach. Elongation factor Tu is the protein required for protein synthesis and is considered as one of the most abundant and highly conserved proteins in prokaryotes. It mediates the binding of the aminoacyl tRNA into a free site of the ribosome. Expression of elongation factor Tu in *S. typhimurium* has previously been reported [12]. Enolase is an enzyme essential for the degradation of carbohydrates via glycolysis. It catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Expression of enolase in *S. typhimurium* has previously been reported [13]. Phosphoglycerate kinase is a major enzyme in glycolysis. It is involved in the second step of the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate. Expression of phosphoglycerate kinase in *S. typhimurium* has previously been reported [14]. Meanwhile, secretion of glycolytic enzymes (enolase KNB35421 and phosphoglycerate kinase KNB39376) was only present in 32% DMSO treated EPS matrix and not in control EPS matrix is in agreement with [15]. They suggested that a combination of glycolytic enzymes with proteases and deoxyribonucleases in extracellular matrices is effective in inhibiting biofilm formation and promoting biofilm dispersal. This implies that secretion of glycolytic enzymes (enolase KNB35421 and phosphoglycerate kinase KNB39376) into EPS matrix following treatment with 32% DMSO may promote dispersal of *S. typhimurium* biofilm. In the functional interaction networks, hubs are nodes with high connectivity or molecules that have the high number of functional interactions. The hub molecules are expected to play crucial roles in the cellular processes and hence possess biological significance [16]. In the present study, enolase KNB35421 was identified as the hub protein in *S. typhimurium* biofilm because it was found to have seven functional linkages. This finding is in accordance with a large-scale study on protein-protein interaction in *E. coli*. [17]. According to [13], enolase also showed functional linkages with ATP synthase subunit alpha, heat shock protein 60 kDa, flagellin, ATP synthase subunit beta, outer membrane protein A and outer membrane TolC in *S. typhimurium*. Trigger factor, glyceraldehyde-3-phosphate dehydrogenase, elongation factor Tu and ATP synthase subunit alpha have also been suggested as the hub proteins in *S. typhimurium* biofilm[6]. The biological pathway identified in the present study namely glycolysis has previously been shown to be involved in the biofilm formation [18].

Table 1: Identified EPS proteins in *S. typhimurium* biofilm.

Accession	Mascot score	Peptide matches	Sequence coverage (%)	Description	Functional categories
KNB39376	211	3	14	Phosphoglycerate kinase	Carbohydrate metabolism
KNU68004	131	2	2	Elongation factor Tu	Translation
KNB35421	232	6	14	Enolase	Carbohydrate metabolism

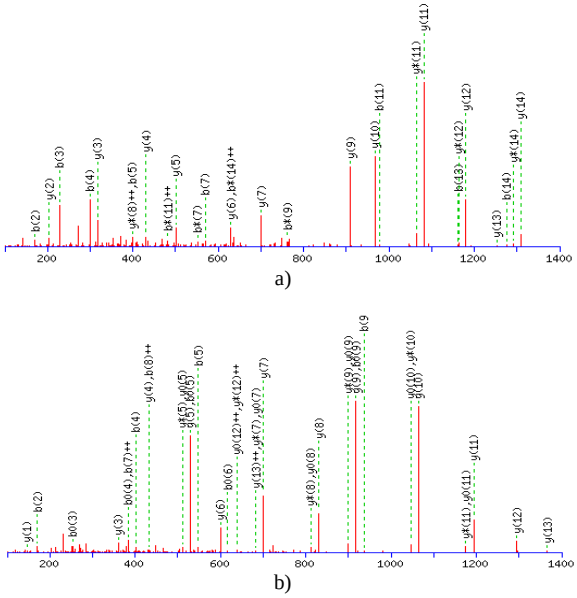

Figure 4: Representative QTOF mass spectra of EPS proteins. a) Fragment of AVGAVNGPIAQAILGK found in enolase. b) Fragment of VATEFSETAPATLK found in phosphoglycerate kinase.

Table 2: EPS proteins which are classified as secreted proteins. Data are expressed as + and – which indicate calculated probability score above threshold level and calculated probability score below threshold level respectively.

Accession	Protein name	Prediction softwares				Secreted proteins
		SignalP 4.0	SecretomeP 2.0	EffectiveELD	TMHMM	

KNB39376	Phosphoglycerate kinase	-	-	+	-	**Yes**
KNU68004	Elongation factor Tu	-	-	+	-	**Yes**
KNB35421	Enolase	-	-	+	-	**Yes**

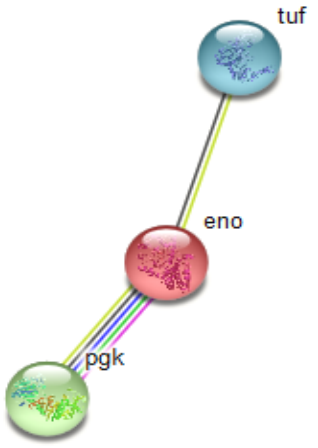

Figure 5: Protein interaction network showing functional associations between QTOF-identified EPS proteins. Tuf: elongation factor Tu; Eno: enolase; Pgk: phosphoglycerate kinase. Functional associations are represented by lines or edges while proteins are represented as nodes.

General discussion

The present study shows a limitation related to ATR-FTIR analysis to obtain IR spectra for biofilm cells. In this case, difficulty in identifying the spectral variations between control biofilm and 32% DMSO treated biofilm may be due to the use of inappropriate biofilm assay system (6-well microplate) prior to ATR-FTIR analysis. Development of biofilm in ATR flow cell system may be useful to achieve the high confidence IR spectra. In 2008, Ojeda et al. [19] demonstrated the application of *in situ* flow cell ATR-FTIR for the study of *Pseudomonas putida* biofilm.

Despite several effective antibiofilm agents being available, the search for effective antibiofilm agents and the investigation of their mode of actions still continue due to the high prevalence of infectious diseases worlwide. These continuous efforts include the microplate screening of various medicinal plant species [20, 21] and existing chemical compounds [5] for their efficacy against biofilm-forming bacteria, omic analysis of biofilms [1, 9, 13, 14] and application of bioinformatic tools to identify the essential and non-homologous proteins in biofilm-forming bacteria [22].

4. Conclusion

This study demonstrates extended analysis of antibiofilm action of DMSO against *S. typhimurium* biofilm. Treatment with 32% DMSO inhibits *S. typhimurium* biofilm by altering the cell surface chemistry and EPS protein secretion. Secretion of glycolytic enzymes in EPS matrix may account for the mode of action of DMSO.

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