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Global transcriptome analysis reveals distinct bacterial response towards soluble and surface-immobilized antimicrobial peptide (Lasioglossin-III)

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Anindya Basu,[a]* Biswajit Mishra[a] and Susanna Su Jan Leong[b]*

'Antimicrobial peptide'-immobilized biomaterials are rapidly being developed to tackle concerns related to device-associated bacterial colonization and biofilm formation. This study aims to evaluate the metabolic changes within a bacteria in response to a highly potent antimicrobial peptide lasioglossin-III, in soluble and immobilized forms.

The rising threat from bacterial biofilm colonization on implantable biodevices, which often leads to serious health and financial challenges for patients,[1] has increased the demand for antimicrobial biomaterials. The cationic antimicrobial peptides (AMPs) have shown tremendous potential in this regard due to their membranolytic properties that leads to fast bactericidal activities, and much lower tendencies for pathogen resistance development.[1] Researchers, including our group, have successfully performed proof-of-concept studies demonstrating the effectiveness of AMP-coated biomaterials in eradicating bacteria and biofilms.[2-4] Interestingly, the killing action of immobilized AMPs has been proposed to be different from those in the solubilised form [5] but the exact mechanism of action remains to be well-understood. Considering that the AMPs' antimicrobial action(s) might be influenced by conformational changes due to surface immobilization, deriving a one size fits all mechanism of action is unrealistic given the huge number of varying peptide sequences.[6] Earlier studies aimed at studying the conformational changes of tethered molecules upon encountering bacterial membranes concluded that changes in the surface charge density of molecules can lead to bacterial autolytic responses.[5, 7] Improved understanding of the bacterial response towards the antimicrobial effects of immobilized AMPs will guide optimized AMP immobilization strategies and shed new insights in AMP resistance development. This study aims to investigate the differences, if any, in bacterial response towards soluble and tethered forms of AMPs. The model AMP candidate used in this study is Lasioglossin-III (referred to as Lasio-III throughout the manuscript), which is a promising antimicrobial coating agent for implantable biodevices.[2, 4] To the best of our knowledge, this is also the first systematic study to evaluate bacterial response towards immobilised peptides at the global cellular level where marked difference in the bacterial response towards the different forms of the peptide was observed. We acknowledge that bacterial response could also vary with peptide sequence, concentration and immobilization mode.

In this study, bacteria response was studied by incubating the bacteria population with sub-lethal concentrations of the peptide.[8] The induction of selective and consistent alterations in the bacterial genes was determined by transcript profiling using microarray analysis. Transcript profiling was used to evaluate the regulatory mechanisms that might be involved directly or indirectly in the peptide-induced bacterial susceptibility.[8, 9] leading to new insights into AMPs mechanisms of action.

To study bacterial response to immobilized Lasio-III, a simple immobilization strategy, wherein a peptide monolayer tethered via polyethylene glycol (PEG) spacers onto silicon wafers,[2] was employed. Silicon wafers of 2.54 cm radius were immobilized with a total of 3.4 µg of peptide, as determined by ellipsometry, to achieve a surface peptide concentration equivalent to 0.17 µg/cm^2. The coated wafers were then incubated with an *E.coli* culture in exponential growth phase (OD_{600} = 0.5). To compare the bacterial response to soluble AMPs, the same bacteria culture was incubated with equivalent mass of the peptide in solution, such that the final peptide concentration remained the same as the immobilized peptide concentration.[2] To obtain a global picture of the bacterial response at its dividing stages, the exponentially growing bacterial cultures were incubated with the peptides

[a] *School of Chemical and Biomedical Engineering; Nanyang Technological University, 62 Nanyang Drive, Singapore 637459;E-mail: anin0005@ntu.edu.sg*
[b] *Singapore Institute of Technology, 10 Dover Drive, Singapore 138683.*
[c] *Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 117597 Singapore, E-mail: Susanna.Leong@SingaporeTech.edu.sg*
*Correspondences should be addressed to A Basu and SSJ Leong.
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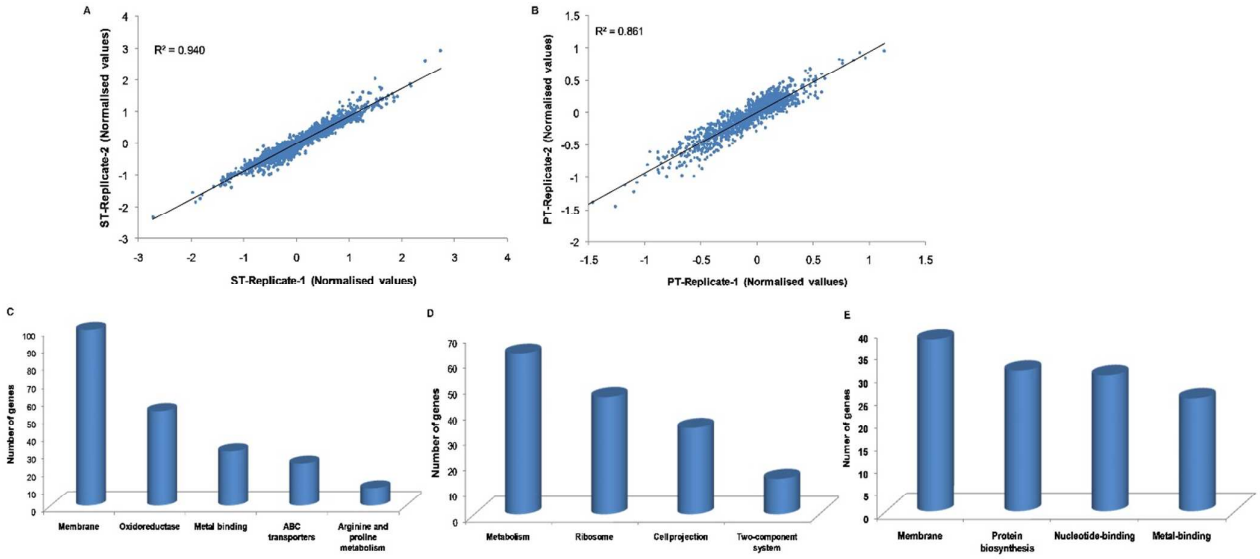
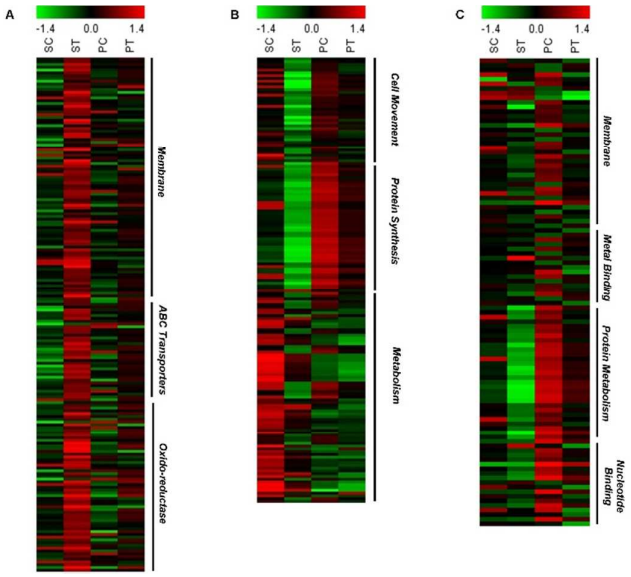


Fig. 1 Correlation graphs for the normalized signals from the mRNA samples from the bacterial samples treated with soluble peptide (A) and immobilized peptide (B). 'ST' stands for bacterial samples treated with peptide in solution form; 'PT' stands for bacterial samples treated with peptide in the immobilized form. Functional classification of the (C) up-regulated and (D) down-regulated genes (as obtained using DAVID analysis) in bacterial samples when incubated with soluble Lasio-III. (E) Major classes of genes regulated in *E. coli* in response to the immobilized Lasio-III. The complete list of the genes involved is presented in supplementary Notes-II.

(soluble and immobilized forms) for 30 min, which is slightly higher than the doubling time of *E.coli*.[8] Following peptide incubation, the mRNAs were isolated from the bacterial samples for microarray analyses. (For further experimental details the reader may refer to Supplementary Notes-I). This experimental platform allows direct comparison of the bacterial response against the peptides in soluble and immobilized forms, while maintaining the same peptide mass to bacteria ratio. All the experiments were carried out in duplicates where consistent reproducible gene expression levels from the samples were observed (Figure 1A / B). In the following sections, bacterial response against the peptide in the solution and immobilized forms are discussed, followed by a comparison of the overall bacterial response based on the microarray analysis. For convenience, the mRNA samples obtained from bacteria incubated with soluble Lasio-III and the corresponding control (containing only the buffer) are referred to as ST and SC respectively, while tethered Lasio-III and the corresponding control (consisting of the PEGylated surface without any peptide) are referred to as PT and PC respectively throughout the manuscript.

Tethered Lasio-III induce regulation of much smaller number of genes compared to the soluble counterparts

Microarray analysis of the bacterial genes presented a marked difference between the bacterial response against the soluble and immobilized forms of the peptide. 841 genes within *E.coli*, representing approximately 20% of the total bacterial genome, were found to be regulated in response to the soluble form of the peptide compared to only 102 genes in response to the immobilized peptide molecules. To identify the bacterial metabolic systems which were largely affected by peptide treatments, the regulated genes were subjected to functional annotation clustering using the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis tool.[10] Interestingly, despite the disparity in the number of regulated genes, the major gene classes affected due to peptide treatments seemed largely similar (Fig-1C-E), where the corresponding heat-maps depicted in Fig-2. Fold change for each of the genes corresponding to Fig-1C to E are presented in Tables S1A, B and S2 (Supplementary Notes-II), respectively. For both forms of the peptide, the primary genes affected were found to be membrane associated, which was not unexpected considering that Lasio-III is a membrane-interacting peptide.[2] The following sections discuss the changes induced within the bacterial metabolic systems by the two forms of the peptide.



Bacteria adopts synergistic strategies to maintain membrane integrity against Lasio-III

Fig. 2 (A) Heat-maps for the major clusters shown in Fig-1C; (B) Heat-maps for the major clusters shown in Fig-1D; (C)Heat-maps for the major clusters shown in Fig-1E. SC: solution control; ST: soluble peptide; PC: Bacteria treated with the control surface (in the absence of peptides); PT: Bacteria treated with peptide-immobilized surface. Red indicates up-regulation while Green indicates down-regulation.

A unique gene known as the RfaY (waaY) was found to be absent within the ST samples unlike SC, thus indicating inhibition upon peptide treatment. The RfaY gene is responsible for phosphorylation of lipopolysaccharides (LPS) at specific sites [11] thereby asserting net negative charge on the membrane. Inhibition of this gene thus helps reduce the overall negative charge on the outer membrane and reduce attraction of the positively charged AMP molecules.[12] For the PT samples, a different LPS modification strategy was observed where, the fepE gene, responsible for very long O-antigen polymerisation in LPS was found to be up-regulated.[13] Such a response is expected to increase the bacterial outer membrane thickness thereby reducing probable peptide membrane interactions.

In addition to LPS modification, soluble Lasio-III seemed to induce more pronounced impact on the overall bacterial membrane system. For instance, two genes *viz.* dacC and ybjG, both strategically situated within the peptidoglycan biosynthesis pathway and involved in antibiotic resistance were found to be up-regulated,[14-16] indicating the efforts of the bacteria towards strengthening the peptidoglycan layer. Furthermore, in contrast to the observations within the PT samples, microarray data revealed up-regulation of several stress response genes within the ST samples. For instance, genes involved in oxidative, temperature, UV and pH stress like priA, hyaA and YhbO were found to be up-regulated.[17-19] The up-regulation of the yehWXY genes, functioning as an ATP dependant osmoprotection transporter,[20] was also observed.

Tethered Lasio-III can influence several inner membrane / cytoplasmic proteins

Apart from the regulation of the membrane-lipid components, both forms of Lasio-III molecules showed its potential to interact with membrane-proteins. For the ST samples, our microarray data showed up-regulation of several transport systems including the fepDG and fecBDC systems for iron uptake [21, 22] and / or oxidative-stress response [23] along with the tauABC system for sulphate absorption.[24] Furthermore, genes associated with peptidoglycan-associated porins like the ompF, involved in the uptake of water, ions, glucose and other nutrients were also up-regulated along with transporters involved in cell wall synthesis like dppABCDF.[25, 26]

Compared to their soluble counterparts, surface-immobilized peptides possess significant constraints with respect to flexibility which may impede peptide activity. Consequently, many AMPs reportedly showed reduced antimicrobial activities following immobilization.[27, 28] Our microarray data however, indicated that apart from the membrane associated genes, several genes related to protein and nucleotide metabolism were also regulated in response to the tethered-Lasio-III molecules (Fig-1E). This particular observation was interesting considering that the total length of the immobilized peptide and spacer within this study was designed not to exceed 10 nm.[2] It was therefore presumed that the peptide would be unable to traverse the cell membrane which is typically 20-80 nm thick,[29] and hence exert any direct impact on internal targets. Such indirect intracellular targeting is however, known to be mediated through interactions of anionic membrane lipids and non-penetrating peptides through formation of lipid-domains capable of exerting a global impact on the bacteria[30-33] *via* signal-transduction pathways. More specifically, non-penetrating cationic peptides are often known to induce anionic lipid domain formation within bacterial membranes which disturb the natural distribution of the lipid molecules.[31] For the case of peptide-immobilized surfaces, a high local concentration of positively charged amino acids are presented to the bacteria in its vicinity, thereby inducing clustering of the anionic lipids. Such a situation can result in phase boundary defects formation that influence and or perturb the existing domains in the bacterial membrane. Such domains on the other hand are known to influence a variety of regulatory functions including solute transportation,[34] cell division and sporulation,[35] DNA replication, Z-ring positioning,[36] thus leading to global metabolic dysregulation within the bacteria.[37] Accordingly, global transcription-factors like the ArcA and CRP genes were found to be down-regulated within PT samples, indicating the impact on diverse regulatory systems in the bacteria including majority of its Dense Overlapping Regulons.[38] The regulation pattern of the "immobilized Lasio-III"-treated bacterial genes is shown in the heat-map in Fig-2C and detailed in Table-S2 (Supplementary Notes-II). Interestingly, as shown in Fig-2, the regulated membrane-associated genes were found to be different for the soluble and immobilized peptides. Several inner membrane-protein expression *viz.* yahC, ygiZ and yjfY (a

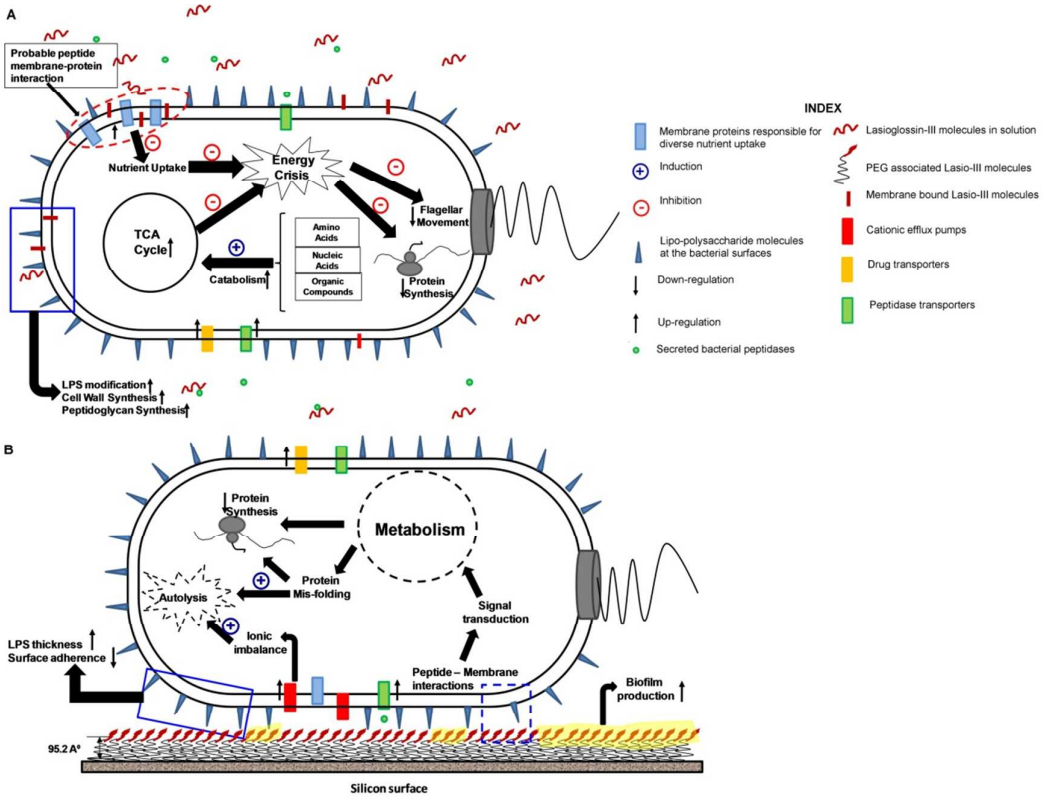


Fig.3 (A) Bacterial response towards soluble Lasio-III molecules: Apart from interacting with the membrane lipids, Lasio-III molecules also appeared to interact with or affect other membrane protein components, particularly those involved in trans-membrane nutrient uptake, leading to an energy crisis within the bacterial cell. To alleviate this phenomenon, the bacteria instigates several responses through modification of its membrane components as well as its energy metabolism. The bacteria tries to reduce the negative charges on its membrane and produces several peptidases to neutralize the toxic peptide, with simultaneous reinforcement of its membrane components. The bacteria stops all energy demanding processes including protein synthesis and flagellar movement, and channel most of its cellular resources towards energy production. **(B) Bacterial response towards Lasio-III-immobilized surface:** Due to the constraints associated with the PEG spacers, it is rather unlikely that the immobilized peptides can completely penetrate the bacterial membrane. Nonetheless, the peptides seem to possess the capability to induce membrane-protein mediated interference to signal transduction mechanisms affecting some of the major metabolic pathways that can ultimately lead to an autolytic response from the bacteria. To address these challenges, the bacteria responds by up-regulating several defence mechanisms mainly involving efflux pumps and preparing thicker O-antigen layer on the bacterial surface. Furthermore, to protect itself from the toxic surface, the bacteria enhances biofilm production and reduces its adhesive tendencies towards the peptide-coated surface.

periplasmic protein), were also found to be regulated indicating the far-reaching effects of the constrained Lasio-III molecules. Of particular interest was the observed regulation of the cationic efflux pumps like ECs0610 which can lead to ionic imbalances within the bacteria that can ultimately trigger autolytic responses.[39]

Soluble Lasio-III strongly impacts bacterial metabolism while tethered Lasio-III induces apoptotic response

Regulation of genes related to trans-membrane nutrient transport within the ST samples, as discussed above, could imply plausible peptide membrane-protein interactions leading to reduced nutrient supply within the cell resulting in metabolic disturbances. Consequently, altered transcript levels of several genes involved in TCA cycle, were found to be up-regulated, which is in agreement with *E.coli* response to peptides like lactoferricin B.[40] Accordingly, genes like fbaB, maeA, yihU, poxB, Acs, fadB, metF, talA, tktB, and yahI, directly involved in the TCA cycle, were found to be up-regulated. Furthermore, supporting the energy crisis hypothesis, several genes involved in energy derivation from diverse organic -compounds (oxidoreductase genes, Fig-1C) including different carbon and nitrogenous sources were also found to be up-regulated (Fig-2A). For example, genes like DgoA and ugpABCE, involved in Glyceraldehyde-3-Phosphate and pyruvate production or uptake from carbon sources, were upregulated[41-43] while the rutABCDEFG operon genes, involved in pyruvate production from pyrimidines[44] and other genes like

astABCD[45], gabD, gabT, gadB, fadB, paaI, paaK and adhP (all associated with degradation of various amino acids to produce substrates for the TCA cycle) were also up-regulated. Degradation and diversion of amino acids towards energy yielding processes is further supported by the down-regulation of genes involved in the synthesis of ribosomal sub-units (46 genes), indicating a probable reduction in protein synthesis. In addition to the protein synthesis machinery, the microarray data also suggest bacterial tendency to arrest flagellar movement (another high energy consuming process), as evidenced by the down-regulation of genes like: (i) flhC,D (DNA binding transcriptional dual regulators responsible for flagellar assembly)[46] and (ii) fliA,G,M, (sigma-factor for transcription initiation of a number of genes involved in motility and flagellar synthesis).[47]

In contrast to the above-mentioned findings, intense metabolic regulations were not observed for the PT samples. However, the only similarity in terms of metabolic-regulation, was that the genes associated with the protein synthesis were also found to be down-regulated within the latter samples. However, the associated regulation of genes with chaperonic activities (like yehL, prfH and pphB) in response to the tethered Lasio-III molecule indicated a slightly different mechanism of action; while yehL is associated with the MoxR AAA+ family of ATPase transporters with probable molecular chaperonic activities,[48] prfH is involved in peptide chain releasing steps during translation.[49] Such observations along with simultaneous down-regulation of protein synthesis machinery, suggested that exposure to the tethered peptide might lead to some signal cascades causing protein mis-folding. As a result, the bacteria stops further protein synthesis to reduce stress induced by the accumulation of mis-folded proteins. Of particular interest is the up-regulation of the pphB gene which is often associated with protein mis-folding. The pphB gene is known to exhibit phosphatase activity towards phosphorylated serine, threonine and tyrosine residues within proteins[50] and play vital roles in controlling cell cycle dynamics[51] including triggering apoptotic responses.[52] Such responses are often associated with regulation of DNA and RNA metabolisms, as evidenced by the up-regulation of genes like yjiS, Z3355, rihB, nupX. While the first two genes (yjiS and Z3355) are involved in the DNA replication process, nupX is a nucleoside transporter and rihB is a ribonucleoside-hydrolase that utilises both cytidine and uridine.[53]

Table 1. Difference in the bacterial response towards the Lasio-III in solution and immobilized forms.

	Solution	***Immobilized***
Membrane system		
LPS modification		
	Reduces net negative charge through the RfaY gene	Elongation of the LPS layer through FepE
Peptidoglycan		
	Strengthening the peptidoglycan layer through ybjG and dacC genes along with osmoprotection related genes	Related genes not found to be regulated
EPS production		
	Biofilm or EPS production not observed	EPS and biofilm production observed along with reduction of cell adherence through regulation of omp related genes
Membrane proteins		
	Regulation of several ion and nutrient transport related proteins	Such genes do not seem to be regulated
	Such autolytic response not observed	Regulation of several periplasmic proteins including those related to ionic balance and protein mis-folding can lead to autolytic response within the bacteria
Resistance and Metabolism		
	Amino-peptidases and drug exporters up-regulated	Drug transporters and ion channels up-regulated some of which can also trigger apoptotic response
	TCA cycle genes up-regulated	No such regulations observed
	Protein synthesis machinery down-regulated	Protein synthesis machinery down-regulated
	Flagellar movement down-regulated	Flagellar movement not regulated
	Genes involved in energy derivation from organic compounds up-regulated	Such regulations not observed

Resistance mechanisms adopted by *E.coli* against Lasio-III

Apart from reducing surface membrane charges, *E.coli* adopts diverse resistance strategies to alleviate the multi-faceted problems when exposed to the soluble Lasio-III which mainly involve over-expression of peptidases and efflux

pumps.[54, 55] Accordingly genes like (i) ypdF, a metalloenzyme with aminopeptidase activity,[56] and (ii) mdtA and mdtQ which are membrane fusion proteins functioning as RND type drug exporters were found to be up-regulated within the ST samples.[57, 58]

Several resistance mechanisms were also triggered within the PT samples: (i) up-regulation of genes like yjiJ which is a stress toxin releaser, and (ii) regulation of efflux pumps such as Ecs2885, also known as multi-drug efflux system protein (MdtE).[59] These pumps not only help in maintaining ionic balance[60] within the bacteria but also play a pivotal role in establishing resistance against adverse environments through diverse strategies including biofilm development.[61] Accordingly, simultaneous up-regulation of protein phosphatase 2 and biofilm-related genes such as ttdA[62] were also observed. Supporting this, thegjE (ttdT) gene, also located in the same operon as ttdAB genes encoding L-tartaratedehydratase,[63] was found to be up-regulated. Interestingly, the bacteria was found to down-regulate the ompX gene which is known to enhance bacterial tolerance to hydrophobic antibiotics and reduce cell surface contact.[64, 65] Amongst the other outer membrane porins, elevated levels of the ompG gene was observed in bacteria incubated with tethered Lassio-III. This outer membrane porin is not only involved in non-specific channel transportation of solutes less than 600 Da but also involved in co-operative association with the mexXY multi-drug efflux system that provides aminoglycoside resistance in some Gram negative bacteria.[66] Such effects can induce up-regulation of some of the apoptotic genes which is again in conformity to the previously reported hypotheses related to ionic imbalance induced autolysis.[39]

Overall, our microarray data indicated marked distinction in *E. coli*'s response towards the soluble and immobilized peptide molecules and is summarised in Table-1 and Fig-3. For the case of soluble Lasio-III, it seems that alongside membrane disruption, the peptide induces trans-membrane nutrient uptake inhibition leading to an internal energy crisis. To mitigate the multifaceted problems, the bacteria tends to reduce its membrane charge whilst simultaneously reinforcing the membrane. To neutralize the toxic peptide, the bacteria releases several peptidases and initiates an energy conservation process by diverting its resources towards the TCA cycle whilst curtailing on other energy demanding processes like protein synthesis and flagellar movement. However, when exposed to tethered-Lasio-III, the bacteria develops longer LPS layers, where although the peptide cannot penetrate the bacterial membrane, it still induces membrane-protein mediated interference to major metabolic pathways that can ultimately lead to plausible apoptotic responses within the cells. The bacteria responds by up-regulating several defence mechanisms mainly involving up-regulating efflux pumps, enhancing biofilm production and developing strategies to reduce its adhesive tendencies towards the peptide-tethered surfaces.

Hence, from a material design perspective, the use of longer spacers for peptide immobilization purposes can potentially help to maintain peptide flexibility and overcome the limitations induced by the growing LPS layer. Alternatively, coating strategies involving co-immobilization of agents that can degrade the LPS layers, might improve the antimicrobial performance of the implantable device as well. Considering the effectiveness of the soluble peptide, efforts to retain membrane-protein binding capability of the immobilized AMPs can probably increase the potency of the AMP coated surfaces as well. Hence significant efforts need to be directed towards evaluating the peptide secondary structures following immobilization and characterization of binding kinetics of some of the target proteins on the coated surfaces. The tendency to form biofilms suggest another mode of the bacteria's attempt to reduce peptide activity. It can thus be said that immobilization strategies using self-cleaning strategies to reduce biofilm adhesion will ensure complete elimination of bacterial colonization on the implantable biodevices like catheters.

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