Engineering biofilm matrix for efficient biofilm-mediated contaminant removal

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Engineering Biofilm Matrix for Efficient Biofilm-Mediated Contaminant Removal

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Singapore Centre for Environmental Life Sciences Engineering,
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2021

Engineering Biofilm Matrix for Efficient Biofilm-Mediated Contaminant Removal

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Singapore Centre for Environmental Life Sciences Engineering

A thesis submitted to the Nanyang Technological University in

partial fulfilment of the requirements for the degree of

Doctor of Philosophy

2021

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Norazeen Zaiden

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Abstract

The naturally sorptive nature of biofilm matrix is an attractive feature to be exploited for environmental application. The objective of this thesis work is to engineer biofilm matrix into an enhanced biosorbent for efficient biofilm-mediated contaminant removal. *Shewanella oneidensis* was used as an environmentally relevant model organism for biofilm matrix modification through manipulating an abundant matrix-associated protein, BpfA. Arsenic (As) was the contaminant in focus to demonstrate the aimed enhanced sorption by the engineered biofilm.

To understand the role of BpfA in the interaction between *S. oneidensis* and As, comparisons were performed on the global transcriptomics from the RNA-sequencing of the wild-type (WT) and the BpfA-lacking mutant $\Delta bpfA$. When treated with As, both the WT and the $\Delta bpfA$ were shown to upregulate the ars-detoxification system for As tolerance, and the master regulator of metabolic processes, TyrR. Energy-consuming metabolic enzymes and low energy efficient metabolic pathways were downregulated in both. The lack of BpfA resulted in expression loss of genes that are primarily important for managing oxidative stress, protein synthesis and their quality assessment, amino acid and phospholipid biosynthesis, uptake of sulfate and exports of xenobiotics. These losses were observed to be compensated with the expression of megaplasmid-borne genes promoting cellular preservation by regulating the cellular metal content and defense, genetic information, and flux of exogenous genetic materials.

With the aim to display As-binding domains in the *S. oneidensis* biofilm matrix, the chromosomal *bpfA* was genetically fused to *arsR* from *E. coli*, which encodes for a well-characterized As binding protein. The recombinant exhibited poor aggregation under rapid agitation but showed competency in biofilm formation under hydrodynamic conditions. Sorption of As by the engineered biofilms was quantified using packed-bed biofilm reactors and the results showed ~2-fold higher sorption by the recombinant biofilms than the WT biofilms. To overcome the limitation of expressing BpfA-ArsR through the only 1 copy chromosomal *bpfA* in *S. oneidensis*, an expression of a higher copy number of *bpfA* was attempted using vector systems. Due to its high repeats content and to accommodate its insertion into a vector system, the *bpfA* gene was truncated to encode domains deemed necessary for biofilm formation. Biofilm formation assay revealed the necessity of the RTX domain. Constitutive and high induction of BpfA truncates expression resulted in low

amounts of biofilm formation. In contrast, substantial biofilm formed when the BpfA truncates were expressed in an IPTG-inducible vector system. This then allowed a further engineering of it to co-express ArsR, As-specific binding module, at its C-terminal end to be displayed as a component in the biofilm matrix for As sorption. When compared to WT, the As sorption by the biofilms of the vector-expressed BpfA-ArsR recombinants exhibited 3.6-fold more, while the biofilms of the recombinant expressing BpfA-ArsR in the chromosome exhibited 2.4-fold more. Therefore, implicating that vector-expressed BpfA-ArsR can increase As sorption through higher expression of BpfA-ArsR. Taken together, this thesis work reports the practicality of engineering biofilm matrix into a sustainable biological tool for environmental applications. Modifying an extracellular polymeric substance in biofilms can enhance its sorptive nature as shown in this thesis work and may also serve as a strategy to create other functional biofilms.

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Chapter 1 . Introduction

1.1 Research background and problem statement

The sources and diversity of environmental contaminants have emerged beyond geogenic processes since technology advancements contribute to rapid industrial activities [1, 2]. These contaminants include organic and inorganic pollutants that seep into the environment even as trace contaminants, posing as chemical hazards affecting the ecosystems and inhabitants. Prolong exposure to chemical toxicants are closely linked to chronic health effects such as cancer, reproductive, immunological and neurological diseases [3]. Several of these contaminants are inorganics of metals and metalloids such as Zn, Pb, Hg, Cu, Cr, Cd, Ni, As, and Se that cannot be further degraded. Therefore, there is a need to develop removal methods for these ions to mitigate the contamination issue. Membrane technologies and chemical precipitation for metal(loid)s removal can be very efficient, especially for handling high concentrations of contaminants, but they usually incur high operation costs, potentially increase the environmental burden, and may have poor sensitivity to low concentrations of contaminants [4]. In contrast, biotic methods offer sustainable, cost-efficient solutions through harnessing microbial biosorption for sequestering metal(loid)s at low concentrations from wastewater [5]. Microbial biosorption can be improved with genetic engineering technologies, but most of which are limited to cell surface display while the relevance of biofilm matrix as biosorbents are often overlooked. The matrix of self-produced extracellular polymeric substances (EPS) in biofilms [6] has yet to be engineered for efficient biosorption with a high affinity. In this thesis study, it is hypothesized that biofilm-mediated biosorption can be tailored for targeted contaminants by modifying biofilm matrix to display specific binding sites.

The global crisis of As contamination in groundwater is taken as an example for As removal using biofilms that are engineered to display As-binding sites in the biofilm matrix. As is widely distributed in the natural environments, where they minimally exist as native element but frequently in minerals. Epidemiology studies has linked the incidence of exposure to As concentrations of more than the World Health Organization (WHO) guide limit of 10 ppb to prevalence of health complications including skin lesions, cancer, neurological effects, and cardiovascular disease [7]. Weathering of As-containing ores contributes to the elevation of the As local concentrations to more than the natural background concentrations [8]. For instance, a massive As contamination in consumable water affects over 100 million people in

developing areas like China, India, Pakistan, Bangladesh and Vietnam, which needs to be addressed urgently [9]. In the effort to improve the sanitary standards to supply pathogen-free water for the rural inhabitants, the water sources were shifted from surface waters and shallow dug wells to groundwaters through tubewells [10]. Installation of tubewells has led to the leaching of As from the surrounding ores into groundwater, causing widespread of chronic As poisoning. According to WHO, As contamination levels in these As-rich areas could reach up to 5000 ppb, far exceeding their guide limit of 10 ppb [11]. Therefore, there is an imperative need to develop efficient remediation strategies for mitigating As contamination.

Generally, As exists in oxidation states of As(-III), As(0), As(III), and As(V) [12], but in naturally occurring waters, the predominant species present are the toxic inorganics trivalent arsenite [As(III), H_3AsO_3] and pentavalent arsenate [As(V), H_2AsO_4^- , HAsO_4^{2-}] [13]. Redox transformations of As can be influenced abiotically and/or biotically. The interconversion and mobility of As(III) and As(V) species are strongly dependent on the environment redox potential (Eh) and pH states [14]. At pH 7, a reducing condition of anaerobic environment holds the dominant species of As(III), while As(III) gets oxidized into the dominant species of As(V) when an oxidizing condition is introduced [13]. For example, As is frequently associated with Fe and S in pyrites, which are generally formed under reducing microenvironments [13]. Under oxic conditions, weathering of pyrites releases As [15]. Mobilization and entrapment of As in ores can be driven by microbial redox metabolisms that usually couple As transformations with biogeochemical cycles of Fe, C, N and S [16]. Dissimilatory Fe-reducing bacteria may cause the release of As from sorption sites as they either reduce Fe(III) to Fe(II) [17], while dissimilatory sulfate reduction by sulfate-reducing bacteria releases As when generating hydrogen sulfide [18]. Conversely, As entrapment can occur via redox activities of nitrate-reducing Fe(II)-oxidizing bacteria [19], and S-oxidizing bacteria [20]. As biotransformation in bacteria is often mediated by the As detoxification system involving the *ars* (arsenical resistance) operon. This operon contains at least 3 genes: (i) *arsR*, the As-sensing regulatory element, (ii) *arsB*, the efflux pump gene for As(III) extrusion, and (iii) *arsC*, the cytoplasmic As(V) reductase gene [21, 22]. The key component of interest for this study is the As(III)-binding repressor, ArsR. The high specificity, high affinity, and sensitivity of ArsR to As(III) makes it an ideal model contaminant-binding protein for display in the biofilm matrix.

The model organism used in this study is *Shewanella oneidensis*, an environmental bacterium of no known pathogenicity. *S. oneidensis* is a facultative anaerobe capable of respiring on a wide range of terminal electron acceptors including metal(loid)s [23] and has great potential in bioremediation applications. In *Shewanella* biofilms, a high molecular weight multidomain protein, BpfA (biofilm-promoting factor A) (285 kDa), that is crucial of its biofilm formation, is one of the most abundant extracellular proteins in the biofilm matrix [24, 25]. In this study, BpfA is used as the vehicle to display ArsR [26] in the biofilm matrix for biofilm-mediated As removal.

1.2 Objective and specific tasks

The objective of this thesis work is to develop a biotechnological platform for the engineering of biofilm matrix to convert biofilms into a sorptive material for sustainable, efficient contaminant removal. As is selected as the contaminant target for testing the engineered biofilm matrix of *S. oneidensis*. Specifically, the tasks to achieve this objective are:

- (1) To investigate the role of BpfA in the responses of *S. oneidensis* to As.
- (2) To engineer *S. oneidensis* biofilm matrix through genetically fusing As-binding domains to BpfA for As removal from water.
- (3) To develop a controllable synthetic biology toolkit for biofilm matrix modification.

Chapter 2 . Literature Review

2.1 Introduction

Biofilm is a form of life characterized as three-dimensional structures developed between air, liquid, or solid interfaces [27]. One feature of a biofilm attracting lots of interest is the self-generated extracellular polymeric substances (EPS), a complex of biopolymers crucial as the binding factor that holds the biological structure sturdiness [28]. Much of these biopolymers are currently explored for their valuable properties as they have potential to serve as an alternative to the chemical polymers in remediation efforts. Their potential in remediation applications is a strategy considered to mitigate environmental deterioration arising from the increasing anthropogenic activities in industrial advancement since the 20th century.

Redistribution of natural resources such as freshwater and minerals used to be generally dictated by geogenic processes such as the climate, weathering, and natural disasters. The escalated demand for precious elements as essential components in industries, or application of engineered systems has resulted in drastic detrimental alterations onto ecosystems health and contributes to resources depletion. According to the United Nations Environment Programme (UNEP) Global Resources Outlook for 2019 report [29], resources extraction has tripled from 27 billion tons in 1970 to 92 billion tons in 2017. This is mainly driven by the doubled world population from 3.7 billion to 7.5 billion people, leading to higher activities of agriculture and minerals extraction to meet food supply and living trends. Inevitably, increasing anthropogenic activities require more consumption of water resources, thereby increasing global water stress by 30 percent. Besides supplying the daily human population domestic needs, redirecting freshwater resources for agriculture not only deplete usable water, but also suffering the freshwater ecosystems biodiversity. For example, analyses of freshwater ecosystems megafauna (defined as animals ≥ 30 kg adult body weight) population between 1970 to 2012 indicated their decline by 88% [30], of which 94% accounted for mega-fishes [31]. Water scarcity is challenged further with groundwater pollution resulting from agriculture runoff that contain nutrients (phosphorus, nitrogen, potassium) found in fertilizers, or pesticides (including herbicides) [32]. Excess nutrients leached into water bodies poses eutrophication, creating damaging hypoxic condition to the aquatic life [33]. Toxicity of organochlorine and organophosphate components in pesticides designed to disrupt the physiology of targeted organisms can extend to the health of the co-existing living communities [34, 35]. Dissolution of metals and metalloids in water bodies resulted also as a

part of the agriculture runoff complex, geogenic processes, the increasing mining activities to meet the growing industrial demand, or electronic wastes serve as the inorganic contaminant sources [36]. Prolonged consumption of metal(loid)s above mandatory regulatory limits were documented to cause a wide variety of clinical complications ranging from respiratory damage, cancer, to neurotoxicity [37]. Ultimately, contamination of water bodies with nutrients or metal(loid)s presents two issues: (1) the water becomes hazardous for consumption, (2) finite supply of global resources such as clean water, and economically significant metals or non-metals like phosphorus become wasted used resources. Negatively impacted environment would not only distort an ecosystem biodiversity, but it can extend to affect population health, and consequently, economic losses. These intricate dynamics between perturbed environments, population health, and economy raises the need to rethink on improving the practice or developing more sustainable approaches to ameliorate the negative influences impacted onto the environment. Ideally, new approaches could offer concurrent removal of pollutant to generate cleaner water, and retrieval of precious resources for their industrial recycling.

Current mitigation approaches to improve water quality for safe consumption includes physicochemical methods such as membrane filtration technologies [38, 39], electro- or chemo- coagulation/precipitation [40, 41]. The general remediation concept applied involves either physically retain pollutants through filter membranes or changing the redox state of a pollutant for precipitation onto chemically binding minerals or membrane, which then facilitate the pollutant removal from water. While these methods are proven to be effective in resulting water quality of lowered pollutant content, setting up these engineered systems are expensive, energy and cost ineffective for maintenance, generates secondary wastes, increases the chemical burden in the already stressed ecosystem, and demonstrates low pollutant removal efficiency at high volume and low concentrations [42, 43]. To counter these disadvantages, there are growing interests in applying biological systems in remediation efforts. Bioremediation exploits the naturally occurring characteristics of living systems, one of which in focus of this review is the bacterial system. Bioremediation presents a sustainable solution that is self-generating, and degradable, making it cost effective and eco-friendly. This chapter reviews the relevance of engaging the bacterial system as a bioremediation strategy, particularly on how their living form as biofilms offer advantageous properties for environmental applications as adsorbents. In addition, factors influencing the efficacy of

biofilm-related adsorbents, current efforts in bioengineering biofilm matrix, and the future perspectives are included.

2.2 Bacteria-mediated bioremediation

Bacteria benefit bioremediation due to their abundance, wide distribution, robust growth rate, which is faster than plants, and them possessing well-evolved resilience for adaptation in changing environment through acquired versatility in conducting biochemical processes. In addition, the high surface area to volume ratio of bacterial cells contributes to the efficiency of bioremediation processes. Bacteria-mediated bioremediation approaches include biotransformation, biomineralization, bioaccumulation, and biosorption (Table 2.1).

2.2.1 Biotransformation

Biotransformation, a metabolically dependent process in living organisms, modify exo- or endo-genous compounds or inorganics, altering their bioactivity or toxicity. It is deemed necessary for a living organism's selective utilization, resistance, or tolerance to chemicals it encounters. In phosphorus-limiting environment, phosphate-solubilizing microorganisms (PSMs) obtain phosphorus by secreting organic acids, enzymes, or siderophores to its surrounding to convert phosphates in solid minerals into a soluble form [44, 45]. PSMs applied in agriculture reduces phosphorus fertilizer input by biologically mobilizing insoluble phosphates and trace elements like iron and zinc to promote crop growth [46]. When exposed to a broad-spectrum antibiotic, tetracycline, bacteria such as *Stenotrophomonas maltophilia*, resist by reducing the potency through removing the antibiotic functional groups enzymically via monooxygenases and peroxidases [47]. Alternative to absolute bacterial resistance, biotransformation facilitates tolerance by detoxifying inorganics that are harmful to cellular physiology. Arsenic tolerant bacteria like *Escherichia coli* expresses arsenic detoxification system [48], comprising of at least 3 essential components, of which intracellular binding of As to ArsR (As-sensitive transcriptional repressor) permits the expression of ArsC (arsenate reductase) to reduce As(V) to As(III) for extrusion through ArsB (As-specific efflux pump). Therefore, bacterial tolerance is achieved by changing As speciation to enable its expulsion via specific membrane transporter. Conversely, As(III)-oxidizing bacteria could express As(III) oxidases to oxidize As(III) into As(V) [48], which found to be less mobile due to its more precipitative nature than As(III), hence, benefiting efforts in arsenic removal from aquatic systems [49-51]. The plethora and efficiency of biotransformation processes in bacteria are useful for remediation exploitation by influencing inorganics mobility, toxic

compounds degradation into inactivity, or converting them into reduced toxicity. Activated sludge for wastewater treatment utilizes such concept to remove polluting nutrients, pharmaceuticals, personal care products, and metals [52-54].

2.2.2 Biomineralization

Biomineralization is a biologically induced chemical precipitation leading to formation of inorganic composite materials intracellularly or on bacterial surface area [55]. This process may occur through iron mineralization, calcification, or silicification. Magnetotactic bacteria are model microorganisms capable of forming intracellular organelles, magnetosomes, which are accumulation of iron-rich minerals enveloped in lipid bilayer membrane [56]. The magnetosomes were identified as magnetite (Fe_3O_4), and/or greigite (Fe_3S_4) crystals with magnetic properties that are proven useful for Fe and Mn removal from groundwater [57], and other metals in wastewater treatment [58]. Microbial-induced calcium carbonate precipitation are by-products of calcifying bacteria through reaction of their produced metabolites like bicarbonate with Ca^{2+} present in the environment [59]. Carbonate precipitation are applicable as engineering solutions to restore stone or concrete structures [60], and also in immobilizing metals [61]. Biosilicification is the saturation of an essential nutrient, silicic acid that gets incorporated into living organisms as silica, mainly formed on bacterial surface as fossilization strategy to preserve core cellular functions in harsh environments such as hot springs and geothermal plants [62]. While biosilica (silicon dioxide) can form silicate minerals when combined with heavy metals (Mg, Al, Fe, Ca), they are also unstable in biosphere [63], hence, probably a reason for the lack in their application in bioremediation, but feasible as biogenic material in biomimetics [62].

2.2.3 Bioaccumulation

Bioaccumulation often require metabolically active bacteria for molecules uptake, utilization, and intracellular storage [64]. The molecules uptake may engage both passive and active import systems depending on the available sorbate concentration and the cellular need to drive the molecules acquisition. Imported inorganics into the intracellular space are prevented to reach toxic intracellular level by engaging cellular components such as cytosolic polyphosphates, cysteine-rich proteins, and sulfides [65]. Polyphosphate accumulating organisms (PAOs) such as *Candidatus Accumulibacter* are applicable for P removal in wastewater because of its capability in up-taking phosphate and synthesizing intracellular polyphosphates [66]. Cysteine-rich metallothionein proteins facilitate metal(loid)s

accumulation through binding to its sulfhydryl groups [67]. Biogenic hydrogen sulfide (H_2S) generated by sulfate-reducing bacteria can precipitate heavy metals into insoluble sulfides intracellularly or extracellularly [68]. Bioaccumulation is efficient and a sustainable method for pollutant removal, but the requirement of living cells means the need for suitable growth conditions to support cell viability for them to upkeep the biosynthesis functions.

2.2.4 Biosorption

Biosorption is a spontaneous physicochemical process mediated by biological entity in which a substance (sorbate) becomes attached to another (sorbent). Sorbate interaction with a biological matrix serving as sorbent leads to a reduction of sorbate concentration in a solution [69]. The generally applied term “biosorption” encompasses bioabsorption and bioadsorption by living or dead biomass. Bioabsorption and bioadsorption involves only passive processes that are metabolically independent, thus, achievable with dead biomass. Absorption is the uptake of sorbate into a bulk phase, in this case, nutrients or metal(loid)s sorption into cytoplasmic space of live or dead biomass through passive means of molecules importation. Adsorption is a surface energy phenomenon, defined as sorbate adherence onto cellular surfaces physically by electrostatic forces, ionic interactions, molecules complexation, or chelation. The extracellular bacterial surface presents ionic moieties serving as binding sites to metal(loid)s. Cell wall components immobilize metal cations through anionic functional groups of exopolysaccharides, membrane lipids, surface proteins and nucleic acids consisting of amine, carboxyl, hydroxyl, sulfhydryl, and phosphoryl. Being metabolically-independent, adsorption process is more rapid than the energy-dependent bioaccumulation, and shown to demonstrate 70 to 94% adsorption efficiency [70]. Over 70% of the recent findings on metal ion sorption or recovery (Table 2.1) reported bioadsorption as the most common mode of bacterial interaction with inorganics. Adsorption is particularly beneficial for immobilizing inorganics since they are not degradable towards complete inactivation like antibiotics. Furthermore, through manipulation of the aqueous medium, the adsorbed economically valued elements can be desorbed for their industrial recycling. Along with its wide permissible operative parameters (temperature, pH, metabolism independent), self-generated cellular components competent for element-binding, and biodegradability, makes adsorption an efficient, sustainable, environment friendly, and low-cost element removal/recovery method for industrial applications [71].

Table 2.1 Recent (2015 to 2020) studies reporting the various bacteria and their mode of bioremediation for metals.

Metal	Bacteria genus	Biotransformation	Bioaccumulation	Bioabsorption	Bioadsorption	Reference
Mo	<i>Acidithiobacillus</i>		✓		✓	[72]
Cr	<i>Klebsiella</i>	✓	✓		✓	[73, 74]
	<i>Stenotrophomonas</i>		✓			[74]
	<i>Staphylococcus</i>		✓			[74]
	<i>Bacillus</i>	✓				[75]
	<i>Lactobacillus</i>		✓			[76]
	<i>Pseudomonas</i>			✓	✓	[77]
	<i>Microbacterium</i>			✓	✓	[77]
Cd	<i>Escherichia</i>				✓	[78]
	<i>Bacillus</i>				✓	[78, 79]
	<i>Rhodobacter</i>				✓	[80, 81]
	<i>Shewanella</i>				✓	[82]
	<i>Lactobacillus</i>			✓	✓	[83]
	<i>Brevibacterium</i>				✓	[84]
	<i>Pseudoalteromonas</i>				✓	[85]
Hg	<i>Exiguobacterium</i>		✓			[86]
	<i>Rhodobacter</i>				✓	[80]
	<i>Bacillus</i>				✓	[79]

	<i>Vibrio</i>				✓	[87]
Pb	<i>Exiguobacterium</i>		✓			[86]
	<i>Stenotrophomonas</i>		✓			[74]
	<i>Klebsiella</i>		✓			[74]
	<i>Staphylococcus</i>		✓		✓	[74, 88]
	<i>Rhodobacter</i>				✓	[80]
	<i>Lactobacillus</i>			✓	✓	[76, 83, 89]
	<i>Pseudomonas</i>			✓	✓	[77, 90]
	<i>Microbacterium</i>				✓	[77]
	<i>Brevibacterium</i>				✓	[84]
	<i>Pseudoalteromonas</i>				✓	[85]
Ni	<i>Stenotrophomonas</i>		✓			[74]
	<i>Klebsiella</i>		✓			[74]
	<i>Staphylococcus</i>		✓			[74]
	<i>Microbacterium</i>				✓	[77, 91]
	<i>Lactobacillus</i>				✓	[76]
	<i>Pseudomonas</i>				✓	[77]
	<i>Bacillus</i>			✓	✓	[92]
Cu	<i>Microbacterium</i>				✓	[91]
As	<i>Micrococcus</i>				✓	[93, 94]

	<i>Bacillus</i>	✓	✓	✓	✓	[92, 95]
	<i>Rhodococcus</i>	✓	✓	✓	✓	[95]
	<i>Alcaligenes</i>	✓	✓		✓	[95]
	<i>Pseudomonas</i>			✓	✓	[96]
	<i>Acidithiobacillus</i>				✓	[97]
	<i>Yersinia</i>			✓		[98]
	<i>Exiguobacterium</i>	✓		✓	✓	[99]
Fe	Magnetotactic bacteria		✓			[57]
	<i>Bacillus</i>			✓	✓	[92]
Mn	Magnetotactic bacteria		✓			[57]
Au	<i>Lysnibacillus</i>		✓		✓	[100]
	<i>Bacillus</i>				✓	[79]
Al	<i>Bacillus</i>			✓	✓	[92]
Mg	<i>Bacillus</i>			✓	✓	[92]
Pd	<i>Bacillus</i>	✓			✓	[101]
Zn	<i>Klebsiella</i>		✓		✓	[102]
	<i>Pseudoalteromonas</i>				✓	[85]
	<i>Rhodobacter</i>		✓	✓	✓	[81]

✓ reported mode of bioremediation

2.3 EPS in biofilms for bioadsorption

Biofilms develop when free-living cells irreversibly adhered onto biotic or abiotic surfaces, upon which they produce EPS to facilitate their formation into micro-colonies, before they accumulatively divide, differentiate, and matures into a biofilm [27]. In comparison to free-living planktonic single cells, EPS secretion strengthens the bacterial adherence capability, structural stability to a biofilm system, and more importantly, conferring beneficial emergent properties in biofilms for bacteria survival. Bacteria embedded in EPS reduces their vulnerability to starvation and desiccation, increases their resistance against predation, and tolerance to biocidal elements such as antibiotics or heavy metals toxicity [103] by imposing diffusion limitations. The improved bacterial fitness in biofilms contributes to issues in engineered or medical systems such as biofouling [104], biocorrosion [105], and clinical complications [106]. Nonetheless, biofilms are found useful for wastewater treatment [107-109], and production of economically valuable biomaterials [110, 111].

One emergent property of biofilms beneficial for bioremediation is the increased capacity for sorption of resources such as nutrients and essential metal ions [103]. Removal of nutrients from an anaerobic aquifer was reported to be more efficient by biofilms than their planktonic counterparts [112]. EPS of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella* sp., and *Parapedobacter* sp. were proven competent for binding of Pb(II) [113], Au(III) [114], Hg(III) [115], and Cr(VI) [116]. The competency in elements uptake is attributed to the highly hydrated biopolymer complex, EPS, offering water bulk phase for extracellular sorption and binding sites that are predominantly presented by the exopolysaccharides (carbohydrates), lipids, embedded proteins, and extracellular nucleic acids [6]. Understanding the physicochemical nature of biofilm EPS could provide the avenue on designing a biofilm matrix into a bioadsorbent for efficient pollutant removal or resource recovery.

2.3.1 Extracellular polysaccharides

Extracellular polysaccharides are carbohydrates component of microbial secreted EPS. It sets the EPS nature as neutral, anionic, or cationic, depending on its varying content of neutral monosaccharides, and ionic organic acids. Extracellular polysaccharides have 3 functional importance: aggregative, protective, and architectural [117]. They primarily influence the overall

biofilm scaffolding for stable protective structure with shear-resistance and promote intercellular or surfaces adhesion. The hydrophilicity of extracellular polysaccharides provides hydration to avoid desiccation, and consequently, aids resource sorption to serve as a nutrient sink. Carbohydrates in EPS include homopolysaccharides and/or heteropolysaccharides [118]. Homopolysaccharides are generally linear repeating monosaccharides of D-glucose and L-fructose forming examples like hydrophilic dextran, cellulose, and hydrophobic curdlan. Examples of heteropolysaccharides are alginate, xanthan gum, gellan, colonic acid, and hyaluronic acid, which compose of assembled non-repeating monosaccharides. The abundant hydroxyl groups of polysaccharides set an overall negative charge to the biofilm [119], thus, making the biological structure suitable for cationic inorganics immobilization, and sorption of organic compounds. *Pseudomonas* sp. were shown to produce at least 3 heteropolysaccharides [120] : Pel (cationic glucose-rich polysaccharide, positively responsive to intercellular c-di-GMP levels), Psl (mannose- and galactose-rich polysaccharide), and alginate (uronic acids-rich polysaccharide). Pel and Psl are aggregative polysaccharides, but Psl is noted for its protective function against wide range of antibiotics [121]. Similarly, although alginate has roles in defining biofilm architecture, it exhibits protective function against cationic antimicrobial peptides [122]. While their protective mechanisms remain unclear, a sorptive role by acting as a barrier of diffusion hindrance against biocidal elements has been suggested.

2.3.2 Extracellular proteins

Extracellular proteins form another bulk EPS component in biofilms. Proteins can be released into the biofilm matrix by cell lysis [123], exported through secretion systems [124, 125] and outer membrane vesicles (OMVs) [126, 127], or facilitated by membrane-embedding hydrophobic protein domain [128]. Extracellular proteins can facilitate surface attachment, provide structural stability, or exhibit enzymatic activities. Proteases such as LapG secreted by *P. putida* were shown to modulate the biofilm structure by digesting exopolysaccharides-binding protein (LapA) for regulating bacteria dispersal [129]. Exoenzymes also function to increase biofilm resistance by degrading biocidal compounds like antibiotics [130], or serve as extracellular digestive system by catalyzing reactions on substrates captured in the biofilm into intermediates as nutrient supplies for the consumption by the embedded bacterial cells [103]. Extracellular proteins such as adhesins and components of pili and flagella mediate cell-cell

adhesion and biofilm attachment onto surfaces. Deletion of genes encoding for biofilm matrix proteins (RbmA, Bap1, RbmC) in *V. cholerae* showed losses in rugosity, confer inability to stay adhered onto surfaces, and exhibited drastic sensitivity to shear forces [131]. These indicate their roles in strengthening the biofilm attachment and structure stability. Similarly, lectins (LecA, LecB) produced by *P. aeruginosa* are exopolysaccharide-binding proteins that mediate the biofilm structure stability [132, 133]. Another extracellular protein commonly found in biofilms is a type of amyloid fibre, curli. Curli has become an attractive synthetic biology target [134] due to its self-assembly, structure resilience, tunable length, and capacity to immobilize inorganic ions. The ability of biofilm matrix-associated proteins to bind and retain compounds and inorganics are largely attributed to the presence of the amino acids functional groups that shapes their conformation specificities to compatible substrates, and chemistry bonding. The cysteine-rich proteins such as metallothionein [135] has many thiol side chains presenting sulfhydryl binding sites, which are frequently found competent for binding metal ions with high affinity. The hydroxyl, carboxyl, carbonyl, methyl, amine, and phosphate groups in extracellular proteins serve as binding sites for metal ions. Proteins in the EPS of *P. aeruginosa* exhibited higher Pb(II) sorption capability than polysaccharides, biosurfactants, and DNA [113]. FTIR revealed that Pb(II) was bound mainly to carboxyl, hydroxyl, amine, methyl and phosphate groups.

2.3.3 Lipids

Lipids are biomolecules that can be hydrophobic or amphiphilic. Most common example, phospholipids, are essential component for cellular membranes to maintain bacterial integrity. Despite the advancement of lipidomic technologies, studies on biofilm-related lipids are still lacking. The lipid bilayer vehicles, OMVs, form a part of the lipid content in the biofilm matrix [136] since they relay the exportation of intercellular and membrane proteins, signaling molecules for interspecies communication, toxins, and nucleic acids. Lipid secretion is also important in bacterial attachment onto abiotic or biotic surfaces such as the colonization on copper sulfide minerals by *Acidithiobacillus ferrooxidans* [137], or intestinal substratum by *Listeria monocytogenes* [138]. Deletion of a gene encoding a TetR-like transcriptional regulator, MmbR, for fatty acid beta-oxidation and biofilm formation in *Mycobacterium smegmatis* significantly decreased the intracellular fatty acids and the total lipid content in the biofilm [139]. This led to a deformed and weakened biofilm structure and increased sensitivity to anti-

tuberculosis drugs. Lipid content is frequently reported to be higher in biofilms than in planktonic cultures [140-142]. The common observation in biofilm EPS is the higher abundance of C16-C18 long-chain fatty acids. Over 70% of the total fatty acid in EPS isolated from a thermophilic corrosive consortium belonged to the long-chain C16 species [142]. Long-chain fatty acids are speculated to mediate biofilm structure stability through cross-linking with exoproteins and exopolysaccharides during bacterial adaptation to their microenvironment [140], and surface adhesion [142]. Furthermore, higher lipid accumulation in EPS provides more carboxylic functional groups for sorption [141].

2.3.4 Extracellular nucleic acids (eDNA/RNA)

Extracellular DNA or RNA (eDNA/RNA) are fragments of intracellular nucleic acids released into the EPS. *V. cholerae* extracellular vesicles contained DNA fragments associated with transmembrane DNA-binding protein (ToxR), DNA-binding protein genes, mRNAs for outer membrane genes, and non-coding RNAs [143]. This suggests OMVs as a mode of transport for eDNA/RNA secretion. Nucleic acid secretions were shown to be moderated by quorum sensing (QS) [144] in *Pseudomonas* and *Burkholderia* species, modulation of second messenger c-di-AMP and a transcription factor responsible for cell wall homeostasis in *S. aureus* [145], and transcriptional regulators (H-NS and NlpI) in *E. coli* [146]. Cell autolysis induced by non-coding RNA (RsaE) in *S. epidermidis* [147], and self-produced chemicals like phenazine in *P. chlororaphis* [148] and peroxide in *S. sanguinis* and *S. gordonii* [149] also demonstrated role in eDNA/RNA release. Similarly, antibiotics treatment at subinhibitory levels induces DNA release, which correlates with increased biofilm formation [150, 151]. These suggest nucleic acids release through programmed cell lysis acts as an adaptive mechanism to strengthen the protective biofilm structure when encountering biocidal compounds, as reported in the increased expression of genes responsible for acidic tolerance, and antibiotic resistance [152]. It is speculated that eDNA/RNA stabilizes the biofilm structure through binding interaction with exopolysaccharides since biofilms of PIA-dependent isolates subjected to DNase or RNase [153], or nuclease (NucM) gene deletion [154] resulted in weakened biofilm structure, and displayed higher dispersal. Due to the eDNA/RNA richness in C, N, P, they can also serve as nutrients source [147, 155]. Their phosphate backbone presents anionic binding sites competent for metal capture as reported by

the expression of eDNA-induced genes probable for roles in metal homeostasis [152], and the increased Mg^{2+} sequestration in *P. aeruginosa* biofilms when under Mg^{2+} limitation [156].

2.4 Current studies advocating biofilms for environmental applications

Bacterial competency in bioadsorption offer opportunities for synthetic biologists to modify cell surface properties for bioremediation purposes [157]. To date, majority of the designed cell surfaces are limited to transmembrane proteins to serve as the anchoring motifs for extension of passenger protein/peptide modules selected to bind a targeted element. Selection of these transmembrane proteins are based on their ability to be transported across the inner membrane and periplasmic space, for eventual expression on the Gram-negative bacteria outer membrane surface, or for the Gram-positive bacteria, secretion from cytoplasmic space to its thick cell wall. A comprehensive review collated these utilized anchors [158], which include but not limited to outer membrane proteins (OmpA, OmpC, OmpF), ice nucleation protein (INP), Maltose-binding protein (MBP), IgA β -domain, LamB, FimH, and SpA. These anchors have been genetically fused to metal-binding proteins/peptides such as poly-histidine [159], MerR [160], metallothionein [161], synthetic phytochelators [162], or screened peptides [163], for capturing metals from solution. The developed transmembrane recombinant proteins are fixed onto the cell surface. However, the EPS matrix often takes up more than 80% of total biofilm biomass. As such, there is a growing interest in modifying biofilm matrix for improved bioadsorption.

In comparison to exopolysaccharides, lipids, and extracellular nucleic acids, exoproteins present the most feasible EPS component to be synthetically manipulated. There are better-established resources on protein properties and functions, information which are accessible through various proteomic or microorganism databases [164]. Protein expressions are also less complex to achieve, unlike the combining pathways that may be involved for the expression of other EPS components. A frequently utilized exoprotein for biofilm matrix modification is curli. Curli is a functional amyloid fibre that usually forms the dominant proteinaceous EPS constituent. They have importance in attachment for initiation of successful biofilm formation [165]. A canonical curli model system composing of CsgA and CsgB, is often studied in *E. coli* biofilms. The self-assembling CsgA units are not limited to anchorage onto the outer membrane via CsgB, but can also function similarly when detached [165]. CsgA is attractive for synthetic biology attempts

due to its small size (13 kDa) for ease in bioengineering, resistant to high temperature, protease and solvents, and a biopolymer of significant strength [166]. CsgA competency in cell surface and biofilm matrix display was demonstrated in *csgA*-deleted *E. coli* mutant [167] that is transformed with plasmids expressing various fusions of *csgA* to genes encoding functional peptides such as signal tags (hexa-histidine, FLAG, fluorescent proteins), and metal-binding peptides for Au [167], and Hg [168] adsorption. However, attempts in engineering biofilm matrix is still very limited to the curli system in *E. coli*, which may not be a suitable model organism for every environmental conditions. If *E. coli* is not a part of the native microbial community displaying resistance to a given stress condition, chances are their survival rate is low to adequately carry out the designed adsorption function. Another 28 kDa amyloid fibre, TasA, expressed by Gram-positive *B. subtilis*, was similarly designed to carry fluorescent proteins, magnetite biomineralization protein, and hydrolases [169], of which displayed efficient activities of the passenger functional proteins with retained capacity to form biofilm.

Besides amyloid fibres, other studies have attempted on proteins found in two different EPS fractions of biofilm matrix: (1) tightly-bound, (2) loosely-bound. Tightly-bound surface-layer (S-layer) protein of *Lactobacillus acidophilus*, SlpA, was genetically fused to *gfp* (gene encoding fluorescence protein) prior to its expression in *E. coli*, and subsequently harvested for culturing with S-layer stripped *L. acidophilus* [170]. This study evidenced the possibility of exogenous heterologous proteins to decorate *L. acidophilus* surface through anchorage via the SlpA C-terminal domain, thus, omitting the need to genetically modify *L. acidophilus*. Another S-layer protein, RsaA, in *Caulobacter crescentus*, was designed to carry lanthanide-binding modules for adsorption of rare earth elements [171]. Sorption test with Tb^{3+} showed the recombinant adsorbed three-fold change more than the wild-type. Furthermore, desorption was shown possible through citrate elution to free the binding sites for consecutive cycles of sorption/desorption. A protein associated with loosely-bound EPS, LapA, expressed by *P. fluorescens*, was designed to carry cargos up to 500 amino acids in between its N-terminal (retention module) and C-terminal (secretion signal module) [172]. This is a different approach from earlier examples, of which the functional proteins/peptides were fused at their secretion modules. LapA is a large adhesin of 520 kDa, thus, making it inefficient for plasmid expression. LapA was modified to retain only 10% of its full-length protein that still allow its transport to the outer membrane via type-I secretion system (T1SS) and anchored as a transmembrane protein.

This permit decorating the cell surface with functional proteins/peptides it carries within the truncated construct. The recombinant of fused *lapA* and cadmium-binding motif gene (*cadR*) did result an increased Cd^{2+} sorption than empty vector control. However, it was not reported if the exclusion of the 90% LapA portion could have significant loss of its primary function as adhesin molecules in biofilm formation.

2.5 Biofilms as adsorbent

2.5.1 EPS production

EPS production was well-reviewed by More et al [118], which covered influences of the bacterial growth phases, nutrient (C, N, trace elements, vitamins) ratios, and growth conditions (pH, temperature, oxygen availability, presence of metal ions, mono- or multi-species). EPS production is metabolically costly, and so it is often correlated with cell growth. EPS synthesis by *B. subtilis* was reported to be high during the exponential growth phase [173]. Under low carbon/nitrogen conditions, biofilm formation was poor, while when glucose was supplied, increased production of EPS was reported. Similarly, in soil bacterium, *R. eutropha*, EPS production positively correlated with cell growth rate [174]. In contrast, cyanobacterium, *Arthrospira platensis*, displayed no correlation between EPS synthesis and cell growth [175], but showed the dependency of EPS synthesis on nitrate availability and photon flux density. EPS synthesis by thermophilic bacterium, *Chelatococcus daeguensis*, increased with increasing C/N ratio of 3 to 15 [176].

EPS production in activated sludge was found to be process-dependent [177]. Recirculation resulted in a lower EPS content. When supplied with increasing total inorganic nitrogen concentration, the overall EPS content increased, in which tightly-bound EPS (TB-EPS) and exoprotein formed the predominant fractions. In anoxic-aerobic sequencing batch reactor treating saline wastewater, C/N ratios did not affect exoprotein and exopolysaccharide content in the TB-EPS fraction C/N ratios [178]. However, the exoprotein in the loosely-bound EPS (LB-EPS) fraction increased, and the exopolysaccharide decreased when at low C/N ratio, thus, indicating LB-EPS having higher sensitivity to C/N ratios than TB-EPS. Perhaps, this is due to its nature as the peripheral EPS fraction that would have immediate response to its surrounding conditions. Sand column experiments to correlate biofilm formation and porosity [179] revealed

C and P as the main drivers for viscous EPS synthesis. The varying effects of environmental factors suggest that EPS production is closely associated with bacterial species and their adaptive mechanisms in any given conditions. Therefore, there is a need to assess and relate EPS production to the environmental conditions at which a biofilm adsorbent is to be applied.

2.5.2 Sorption process

Sorption efficiency depends on pH, temperature, ionic strength, sorbate and sorbent concentrations, and the contact time between them [180]. The solution pH is considered an important factor since it influences the speciation of inorganics, and the chemistry of the functional groups present in the sorbent [181]. Changes in pH results in protonation and deprotonation of a sorbent's binding sites. At low pH, sorbate competes with excess H^+ ions to associate with binding sites in a sorbent. This competition is reduced with increasing pH, at which exposing the binding sites, thus, creating an overall negative charge that consequently attracts the free cations. High pH (above 7) stimulates precipitation and hydroxide complex formation, leading to reduction in sorption efficiency.

Temperature influences the kinetics energy of sorbate and the thermodynamics of the sorption process [182]. Sorption processes are generally performed at temperature range of 20°C to 35°C. Sorption efficiency improves at higher temperature [183] due to increased kinetic energies of sorbent surface and sorbate, leading to enhancement of their collision frequency. However, higher temperature can impede the sorption process by disrupting the physical properties of the sorbent's binding sites [184].

Ionic strength is another factor influencing sorption process. Sorption of Hg(II) by *B. subtilis* decreased with increased ionic strength [183]. This study also reported higher Hg(II) sorption with increasing initial metal concentration. This observation is based on another factor of sorbent concentration. Given a sorbent concentration, increasing sorption will be achieved with higher supply of sorbate, until it reaches a saturation point. Similarly, allowing longer contact time increases the chance of sorbate binding to the sorbent, but again, a saturation point is reached when the binding sites are occupied [183, 185].

2.6 Engineering biofilm matrix into a bioadsorbent with high specificity for a contaminant

Bacteria-mediated bioadsorption serves as an eco-friendly, cost-efficient method for pollutant removal or resource recovery. It is based on exploiting binding properties present on bacterial surfaces. It is a metabolism-independent approach, allowing the use of both living and dead biomass. Synthetic biologists have engineered bacterial cells to convert them into a bioadsorbent with desirable binding properties. However, most of these efforts are limited to surface modifications for bacterial cells, although in a biofilm the biomass of EPS is often substantially higher than that of bacterial cells. To date, the effort of engineering EPS to convert the biofilm matrix into sorptive materials is still at its infancy.

While the biofilm matrix is found to be naturally sorptive, it is not specific. This thesis hypothesizes the potential for biofilm matrix to be engineered into a bioadsorbent with high specificity for a contaminant removal through a modification of an EPS component. The most feasible EPS component for modification would be of proteinaceous nature because the amount of available data will allow precision in devising a strategy when engineering the biofilm matrix. The biofilm matrix protein would also need to be of significant abundance to serve as the anchoring motif to present high number of functional displays to capture the targeted contaminant. The functional display to be carried by the biofilm matrix protein needs to have high specificity and affinity for binding the contaminant. For this thesis, instead of using the classical study model, *E. coli*, there is a need to showcase a different environmental model organism, preferably with high adaptability to presence of contaminants such as metal(loid)s in both freshwater and marine systems. Taken together, the listed criteria aim to demonstrate how the biofilm matrix can be optimized into a beneficial biotechnology tool for contaminant removal.

Chapter 3 . New insights on bacterial responses to arsenic: How does biofilm-promoting factor influence detoxifying mechanism of *Shewanella oneidensis*?

3.1 Introduction

Arsenic (As)-contaminated natural waters affect ~220 millions of people globally, of which, ~60% are in Asia due to their heavy reliance on natural water resources containing As with a concentration above the WHO guideline limit level (10 ppb) [186-188]. Prolonged consumption of As-contaminated water poses chronic health risks, including the development of cancer [189]. As predominantly exists as As(III) and As(V), depending on the pH and redox potential of the environment, consequently influencing As solubility and adsorption [188]. As(III) species are more soluble in water and deemed to be more toxic than As(V) species, therefore As(III) oxidation is an effective approach for As remediation through immobilization [188]. Some environmental bacteria are capable of mobilizing As from As-bearing solid minerals to water, which complicates the As contamination problem in groundwater. Under anoxic conditions, reductive dissolution of ferric (hydr)oxide minerals and/or reduction of As(V) to As(III) mediated by microorganisms such as *Shewanella oneidensis* could liberate As from solid minerals into the water [190]. Exposure to As induces oxidative stresses that perturbs cellular physiological processes of environmental bacteria [191]. To cope with As-induced oxidative stresses, *Shewanella* spp. employ As-reductase, ArsC, to reduce As(V) to As(III), which is subsequently extruded through specific transporters such as ArsB [192]. *S. oneidensis* has also been shown to be capable of biomethylating As into the lesser toxic organic forms, monomethylarsonate acid (MMA) and dimethylarsinic acid (DMA) when provided S-adenosyl methionine or methylcobalamin as methyl donors [193].

In addition to microbial transformation, biofilm formation is also involved in detoxification of environmental contaminants such as As [194]. A key mediator of biofilm formation in *S. oneidensis* is a large transmembrane protein, BpfA (biofilm-promoting factor A), serving as an adhesin [24]. BpfA predominated in extracellular polymeric substances (EPS) of *Shewanella* sp. HRCR-1 [25]. Deletion of *bpfA* in *S. oneidensis* reduces cell aggregation and biofilm formation [24]. The role of BpfA in mediating *S. oneidensis* response to As has yet to be explored.

The objective of this study was to determine the influence of BpfA on the responses of *S. oneidensis* to As. Specifically, we compared transcriptional profiles of *S. oneidensis* MR-1 wild-type (WT) and the *bpfA*-deleted mutant ($\Delta bpfA$) under As(III) stresses. Our results suggest that BpfA influences a network of genes involved in stress responses to the oxidative damages. Identifying the role of BpfA on responses of *S. oneidensis* to As provides new insights on As-tolerance of environmental bacteria.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

S. oneidensis MR-1 WT and $\Delta bpfA$ were cultured overnight aerobically at 30°C in Luria-Bertani (LB) medium prior to subsequent experiments in modified M1 medium (MM1). The MM1 medium (pH ~7.0) [195] contained 30 mM HEPES, 7.5 mM NaOH, 28.04 mM NH₄Cl, 1.34 mM KCl, 1.09 mM NaH₂PO₄, 0.68 mM CaCl₂, trace amounts of mineral, vitamins, and amino acids, and supplemented with 10 mM sodium lactate as the sole carbon source [196]. The in-frame deletion of *bpfA* in WT was previously prepared by Liu Weijie through homologous recombination using suicide vector, pDS3.0 [197] ligated to a crossover PCR fused product of the upstream and downstream fragments of *bpfA* (SO_4317) open reading frame. The crossover PCR fused product were generated using 2 primer pairs (bpfA-5O & bpfA-5I; bpfA-3O & bpfA-3I) listed in Table 3.1 to give 2 PCR products (upstream and downstream genomic segments flanking *bpfA*) with overhangs that were designed to anneal in a subsequent PCR to fused them, hence, termed as a crossover PCR product. Potential mutants with integrated crossover PCR fused product were initially cultured in LB without NaCl before sequentially selected on LB without NaCl supplemented with 10% sucrose, and then, in LB with 10% sucrose and 15 µg/ml gentamycin. The gene deletion was confirmed by PCR using primers (bpfA-FO & bpfA-RO) flanking the recombination region, and sequencing.

Table 3.1 Primers for crossover PCR fused product for *bpfA* deletion in *S. oneidensis*

Primers	5' → 3'
bpfA-FO	AGAGCCGAGCTCGATAAGTTAG
bpfA-5O	TAGCACGAGCTCCTACCAGCAAGAGTAAAGCACC
bpfA-5I	CGTTAGCTGCAGACCTAGGAAAATTAGCTCCCAACAGCCAAAG

bpfA-3I	TTCCTAGGTCTGCAGCTAACGGTCATTAATCTGACATCAATTATTC
bpfA-3O	TAAAGCGCATGCCAATGATGATCATAACCGCCAACC
bpfA-RO	GGGTTGGGTATAGAGTGTAAG

3.2.2 Growth curve of *S. oneidensis* WT and $\Delta bpfA$

To characterize the growth curve of $\Delta bpfA$ in comparison to its WT control, overnight LB cultures of both organisms were washed in MM1 twice through centrifugation of 12000 rcf for 5 minutes each. Washed pellets were standardized to OD₆₀₀ of 0.1 in MM1 supplemented with 10 mM sodium lactate (L7022, Sigma Aldrich, Germany). Cultures were incubated at 30°C, 200 rpm, and sampled over 24 h for OD₆₀₀ reads using spectrophotometer UVmini-1240 (Shimadzu, Japan). Experiment was performed in three biological replicates.

3.2.3 Assessment of *S. oneidensis* WT and $\Delta bpfA$ tolerance to arsenic

To determine BpfA significance for *S. oneidensis* in tolerance to As, the WT and $\Delta bpfA$ cultures were blotted onto MM1 agar and solution supplemented with 10 mM sodium lactate and concentrations of As ranging from 5 μ M to 500 μ M (375 ppb to 37500 ppb). Overnight cultures of WT and $\Delta bpfA$ were washed in MM1 twice through centrifugation of 12000 rcf for 5 minutes each. Washed pellets were initially standardized to OD₆₀₀ of 1.0 in MM1 before serially diluted 10 times up to 10⁻⁶. Diluted cultures of 10 μ l from dilution 10⁻² to 10⁻⁶ were blotted onto MM1 1.5% agar supplemented with NaAsO₂ (S7400, Sigma-Aldrich, Germany) concentrations of 5 μ M, 10 μ M, 100 μ M, 200 μ M, 300 μ M, 400 μ M and 500 μ M. Culture blots were allowed to dry within class II biological safety cabinet (Nuaire, USA) before incubated at 30°C incubator until sufficient colony formed for comparison. For the assessment in MM1 solution, cultures standardized to OD₆₀₀ of 0.1 were seeded into MM1 solution supplemented with similar As concentrations, but instead up to 400 μ M. Cultures were incubated at 30°C incubator for 48 h. Experiments were performed in at least three technical replicates.

3.2.4 Sample preparation for RNA-Seq

The overnight *S. oneidensis* MR-1 WT and $\Delta bpfA$ cultures in LB was washed twice in MM1 and resuspended to OD₆₀₀ of 0.1 in MM1. They were grown aerobically at 30°C, 200 rpm. At the late exponential growth phase (~7-9 h), As(III) in the form of NaAsO₂ (S7400, Sigma-Aldrich, Germany) was added to a final concentration of 5 µM. After 1 h exposure to As(III), cells from 500 µl of the cultures were harvested, mixed thoroughly with 1 ml RNeasy Protect Bacteria Reagent (QIAGEN, Germany), and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. DNA was removed through on-column DNase digestion with 27 Kunitz RNase-free DNase (QIAGEN, Germany) for 1 h at room temperature. Total RNA and their DNA remnants were quantified using Qubit™ RNA BR assay kit (Q10211, Thermo Scientific, USA), and Qubit™ dsDNA HS assay kit (Q32851, Thermo Scientific, USA). Their quality was assessed on Nanodrop 2000 (Thermo Scientific, USA), and TapeStation 2200 (Agilent, USA) using RNA ScreenTape (5067-5576, Agilent, USA). Acceptable sample quality consists of RNA integrity of above 8.0, absorbance ratio of 260/280 and 260/230 above 1.8, and contaminating DNA of less than 5%. Three biological replicates were conducted for each condition.

3.2.5 RNA sequencing and data analyses

Transcriptome sequencing was performed on the HiSeq2500 system (Illumina, USA) using sequencing method of paired-end 100 bp length read. The generated RNA-seq raw data were uploaded onto Galaxy web platform at usergalaxy.eu for adapter removal (Cutadapt), quality assessment (FastQC), ribosomal RNA exclusion (SortmeRNA), mapping non-ribosomal RNA reads to reference *S. oneidensis* MR-1 genome assembly, GCF_000146165.2 (Bowtie2), gene expression measurement (featureCounts), and selection of differentially expressed genes (DeSeq2). Significant changes in gene expression were filtered based on at least 2-fold change with p-value <0.001. Common genes between groups of interest were determined using Venn diagram webtool, InteractiVenn [198]. Gene functions were assigned using the following databases in reference to *S. oneidensis*: Gene Ontology Resource (geneontology.org), KEGG genes (genome.jp/kegg/genes), and InterPro (ebi.ac.uk/interpro).

3.3 Results and discussion

3.3.1 Growth of WT and $\Delta bpfA$ in the presence of As

When comparing the end OD₆₀₀ of WT and $\Delta bpfA$, the $\Delta bpfA$ showed an approximate of 50% increase in biomass yield (Figure 3.1A). Given that BpfA is a large surface protein (285 kDa) that relies on ATP-dependent T1SS for its exportation [199], the mutant lacking BpfA may have released this metabolic expense, allowing $\Delta bpfA$ to generate higher biomass yield. When challenged with As(III) on minimal medium agar, $\Delta bpfA$ exhibited comparable growth as WT up to 200 μ M of As(III), but eventually formed more colonies than WT at 300 μ M, after which no growth was observed beyond that (Figure 3.1B). Similarly, in liquid medium, $\Delta bpfA$ grew better than WT at 300 μ M of As(III) (Figure 3.1C). To reveal molecular mechanisms underlying the observed differences in As tolerance, we compared the transcriptomic profiles of WT and $\Delta bpfA$ in the presence of 5 μ M (375 ppb) As(III), a low concentration mimic the As-contaminated waters [186].

Figure 3.1 Growth assessment of WT and $\Delta bpfA$ in presence of As. (A) Growth curves over 24 h in minimal medium without As. (B) Culture blots on minimal medium agar, and (C) minimal medium solution supplemented with concentration range of As.

3.3.2 Differentially expressed genes (DEGs) in both WT and $\Delta bpfA$ in response to As

The As-treated WT and $\Delta bpfA$ were compared with their respective untreated controls. A total of 75 and 56 DEGs (≥ 2 -fold change expression difference with $p\text{-value} \leq 0.001$) were observed in As-treated WT and As-treated $\Delta bpfA$, respectively (Figure 3.2A). Among them, 16 DEGs (9 upregulated and 7 downregulated) are shared by both WT and $\Delta bpfA$ in response to As, while 99 DEGs (37 upregulated and 62 downregulated) are distinct for WT and $\Delta bpfA$.

Upregulated DEGs in both WT and $\Delta bpfA$ under As stress predominantly consists of the *ars* detoxification system (Figure 3.2B, Table 3.1). Eight DEGs form part of a gene cluster in *S. oneidensis* that includes arsenic-resistance permeases (*acr3*, *yraQ*), As reductase (*arsC2*), As-sensitive transcription repressor (*arsR*), a thioredoxin (*arsD*) that serves as antioxidant agent, NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (*gapA-I*) that catalyses the synthesis of 1-arseno-3-phosphoglycerate and functions synergistically with a major facilitator superfamily-type efflux pump (*arsJ*) for its extrusion [200], and toxic anion resistance protein (*telA*) [201]. Similar *ars* detoxification system was shown to be expressed in the closely related *Shewanella* ANA-3 [202] indicating the primary mode of resistance to either As(III) or As(V) in *Shewanella* spp. The *telA* gene is not well-studied, but its expression in *Rhodobacter sphaeroides* is linked to tellurite resistance [203]. TelA protein sequence has similarity to the major vault protein (MVP) in eukaryotes, and MVP exhibits aggregate formation, termed vaultosome in the cell periphery when exposed to oxyanions including As(III) and As(V) [201]. The vaultosome was hypothesised to function as reversible stress granules in mediating resistance to toxic oxyanions but the exact mechanism remains elusive [204]. In addition, a transcriptional regulator gene *tyrR* is also upregulated. TyrR is a transcriptional factor can function either as activator or repressor *E. coli* K12 [205], but it is yet to be well-established in *Shewanella* spp. Through *in silico* analyses of transcriptional networks across *Shewanella* spp., TyrR is predicted as a master regulator of amino acids metabolism, glyoxylate shunt pathway, and biosynthesis of chorismate, an essential precursor for amino acids and metabolites [206]. Our data suggests TyrR acting as a transcriptional repressor on the glyoxylate shunt pathway since genes responsible for the

pathway, *aceA* and *aceB*, are downregulated in both WT and $\Delta bpfA$ (Figure 3.2C, Table 3.1). This metabolic pathway is a variant of the TCA cycle (Krebs cycle) with lower energy efficiency [207], and in agreement with our data, *S. oneidensis* cultured under aerobic condition resulted in 2-fold lower *aceAB* activities [208]. In *Pseudomonas aeruginosa*, disrupted *aceA* and *aceB* expression has been shown to improve survival during oxidative stresses [209]. It is plausible that downregulation of *aceA* and *aceB* observed here might benefit *S. oneidensis* to survive better under the As stress. In support of this finding, the NAD⁺-dependent aldehyde dehydrogenase gene (*aldA*) and Fe/NADH-dependent glutamate synthase genes (*gltD*, *gltB*) are also downregulated in both WT and $\Delta bpfA$. Their downregulation implies the additional need for *S. oneidensis* to repress energy-consuming metabolism.

Figure 3.2 DEGs in response to As in WT and $\Delta bpfA$. (A) Distribution of DEGs from genomic or megaplasmid. (B) Venn diagram of overlapped upregulated (left) or downregulated (right) DEGs from As-treated WT and $\Delta bpfA$ data.

3.3.3 Distinct DEGs in WT and $\Delta bpfA$ in response to As

Bulk of the distinct, upregulated DEGs in WT (Table 3.2) are indicative of protective roles in maintaining cellular homeostasis. The list includes genes encoding antioxidant-like thioredoxins (*trxC*, *trxA*) and oxidative resistance protein (*ohr*), chaperones and folding catalysts (*grpE*, *ibpA*, *secB*, *dnaK*, *dnaJ*, *hspG*, *groES*, *groEL*, *clpB*), proteases against nonessential and misfolded proteins (*hslV*, *hslU*, *lon*), arginine and proline biosynthesis (*argD*, *astA*), phospholipid biosynthesis (*gpsA*), ATP-binding cassette-type transporters for sulfate uptake and xenobiotic exports (*sbp*, *cysP*, *yadG*). Toxic effects of As on organisms are mediated by oxidative stresses resulted from reactive oxygen and nitrogen species that are damaging to the nucleic acids and proteins [210]. The distinct DEGs observed for WT in response to As can be considered as the highly relevant pool of genes forming cellular defense mechanism to cope with As toxicity. In contrast, these genes were not differentially expressed in As-treated $\Delta bpfA$, suggesting their probable association with *bpfA* in inducing adequate cellular stress responses to toxin exposure.

Genes *etfB* and *trpD* are the only distinct, upregulated DEGs observed for $\Delta bpfA$ in response to As (Table 3.2). The *etfB* encodes for electron transfer flavoprotein subunit B (EtfB), which heterodimerizes with EtfA to channel electrons derived from fatty acid and amino acids metabolism to the membrane-bound respiratory chain [211]. Deletion of *etfB*, but not *etfA*, in *Burkholderia cenocepacia* impaired its growth and viability [212], suggesting the importance of *etfB* to cell viability. This could explain the upregulation of *etfB*, but not *etfA*, in As-treated $\Delta bpfA$. The *trpD* encodes for anthranilate phosphoribosyltransferase, an enzyme involved in tryptophan synthesis [213]. The upregulation of *trpD* suggests a higher amount of tryptophan is required for oxidation to sustain the increased EtfB-mediated electron transfer [213].

WT under As stress mostly resulted in downregulation of signal transduction, and transport systems (Table 3.2). Locus tags SO_2124, SO_2123 are putative for signal transducers, one of which is methyltransferase, CheR. Downregulation of CheR, which methylates glutamate residues of methyl-accepting chemoreceptors proteins (MCPs) as part of sensory adaptation [214] may have been impacted by the earlier mentioned downregulated glutamate synthases, *gltDB*. Histidine kinase (SO_0544), response regulator (SO_2366) of two-component signaling systems, and protein kinase, YeaG (SO_2882) are potentially ATP-dependent as the monosaccharide

(SO_3714), and PAAT family-related polar amino acid (SO_4714) uptake systems. SO_1560 was similarly reported to be repressed under Cr(VI) in *S. oneidensis* [215], but not well-documented for the others. Since they are ATP-dependent, their downregulation may be *S. oneidensis* attempt in reducing energy-consuming processes to maintain viability. It could also be resulted due to As toxicity through interference with the sulfhydryl groups of enzymes, or mimicking phosphates that could have siphoned the stable ATP formation [216]. Downregulation of the high-affinity phosphate uptake protein, PstS (SO_1560) is expected since it is usually expressed under low-phosphate condition [217], which is not the case for this study. Opposed to As-treated WT, downregulated DEGs in As-treated $\Delta bpfA$ not only comprised of genes in transport, and signal transduction, but markedly in transcription and energy metabolism (Table 2). Transcriptional regulators for CadC family (SO_0490), and AcrR (SO_1415), speculates As suppressing effect on cadmium response, and energy-consuming multi-drug efflux systems that are not specific for moderating arsenic stress [218, 219]. Notably, downregulated transcriptional regulators for HlyU (SO_3538), Sigma54 specific for Fis family (SO_3059), and LysR family (LTTRs) (SO_0843), suggest a combined effect of *bpfA* loss and As, since they are not observed in the As-treated WT. These transcriptional regulators belong to protein family classes that has diverse modulation on virulence [220], rRNA expression [221], metabolism, quorum sensing and motility [222]. The lack of BpfA expression may have directed *S. oneidensis* to limit its metabolic expenses on those extensive cellular activities to prioritise coping under metal(loid) stress such as arsenic. This notion is further supported by the downregulation of energy metabolism related genes, periplasmic [NiFe] hydrogenases, HyaAB (SO_2098, SO_2099). HyaAB functions in hydrogen cycling during fermentative growth. Our data is derived from aerobic condition, and given the HyaAB sensitivity to oxygen [223], its low expression is expected since its activity can be deemed less crucial for viability.

It is noted that majority of the DEGs in response to As in WT and $\Delta bpfA$ are associated with the chromosome with a few from the megaplasmid (Figure 3.2B). *S. oneidensis* possesses a 16 Mbp megaplasmid comprising of 173 open-reading frames (CDS) [23], of which over 70% of them are unknown, putative functions, or transposases. One upregulated DEG in As-treated WT is of unknown function (SO_A0057), and 4 downregulated DEGs encode for proteins associated with the CzcA efflux system (SO_ SO_A0154, SO_A0155, SO_A0156), which will be discussed further in section 3.5.

Compared with As-treated WT, substantially less upregulated DEGs, but more downregulation observed for $\Delta bpfA$ in response to As. This suggests a narrowed focus of the mutant on expressing only essential genes for survival when incapacitated to form biofilm. To reveal the genes whose expression may have been affected by the lack of BpfA, we compared the transcriptomic profiles of WT and $\Delta bpfA$.

Table 3.2 DEGs observed for both WT and $\Delta bpfA$ in response to As. DEGs derived from comparative analyses between As-treated WT against untreated WT, and As-treated $\Delta bpfA$ against untreated $\Delta bpfA$. DEGs selected based on fold-change expression difference ≥ 2 , with p-value ≤ 0.001 .

Upregulated DEGs in both WT and $\Delta bpfA$ in response to As			Expression (Log₂)	
Gene ID	Gene name	Gene function	WT+As vs WT	$\Delta bpfA$+As vs $\Delta bpfA$
SO_0534	<i>acr3</i>	Arsenical-resistance protein	4.0	4.2
SO_0538	<i>gapA-I</i>	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase for arsenate detoxification	4.0	4.0
SO_0533	<i>arsC2</i>	Arsenate reductase thioredoxin-coupled, LMWP family	3.8	3.7
SO_0532	<i>arsR</i>	Arsenical resistance operon repressor	3.6	3.4
SO_0535		Uncharacterized membrane protein, YraQ family	3.3	3.3
SO_0536	<i>arsD</i>	Redox-active disulfide protein 2	3.2	3.4
SO_0539	<i>arsJ</i>	MFS-type efflux pump specific for 1-arseno-3-phosphoglycerate	3.1	3.6
SO_0540	<i>tela</i>	Tellurite resistance protein	2.1	2.4
SO_1669	<i>tyrR</i>	Transcriptional repressor protein	1.4	1.8
Downregulated DEGs in both WT and $\Delta bpfA$ in response to As				
SO_4480	<i>aldA</i>	Aldehyde dehydrogenase	-2.0	-1.7
SO_1484	<i>aceA</i>	Isocitrate lyase	-1.8	-1.7
SO_1483	<i>aceB</i>	Malate synthase	-1.6	-1.5
SO_1324	<i>gltD</i>	Glutamate synthase [NADPH] small chain	-1.5	-1.1
SO_1325	<i>gltB</i>	Glutamate synthase [NADPH] large chain	-1.5	-1.0
SO_0595		Uncharacterized	-1.3	-1.4
SO_3891		Uncharacterized	-1.1	-1.2

Table 3.3 Distinct, upregulated DEGs in WT and $\Delta bpfA$ in response to As. Data derived from comparative analyses between: As-treated WT against untreated WT, and As-treated $\Delta bpfA$ against untreated $\Delta bpfA$. DEGs selected based on fold-change expression difference ≥ 2 , with p-value ≤ 0.001 .

Distinct, upregulated DEGs in WT in response to As			Expression (Log ₂)
Gene ID	Gene name	Gene function	WT+As vs WT
SO_3802	<i>yadG</i>	Uncharacterized efflux ABC transporter, ATP-binding protein	2.0
SO_1524	<i>grpE</i>	Heat shock protein	1.9
SO_2863		Uncharacterized	1.7
SO_0907	<i>nqrF-I</i>	Na(+)-translocating NADH-quinone reductase subunit F	1.7
SO_2277	<i>ibpA</i>	16 kDa heat shock protein A	1.7
SO_4162	<i>hslV</i>	ATP-dependent protease subunit	1.7
SO_0052	<i>secB</i>	Protein-export protein SecB (maintains pre-export unfolded state)	1.7
SO_0452	<i>trxC</i>	Thioredoxin 2	1.6
SO_3738	<i>cysJ</i>	Sulfite reductase [NADPH] flavoprotein alpha-component	1.5
SO_1162	<i>lipB</i>	Octanoate-[acyl-carrier-protein]-protein-N-octanoyltransferase	1.5
SO_1126	<i>dnaK</i>	Chaperone protein	1.5
SO_2016	<i>htpG</i>	Chaperone protein	1.5
SO_4163	<i>hslU</i>	ATP-dependent protease ATP-binding subunit	1.4
SO_0703	<i>groES</i>	Heat shock protein 10 kDa family chaperone	1.4
SO_0053	<i>gpsA</i>	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	1.4
SO_0050	<i>yibN</i>	Rhodanese-related sulfurtransferase	1.3
SO_4652	<i>sbp</i>	Sulfate and thiosulfate binding protein	1.3
SO_0976	<i>ohr</i>	Organic hydroperoxide resistance protein	1.3
SO_4699	<i>prlC</i>	Oligopeptidase A	1.3

SO_0617	<i>argD</i>	Succinylornithine transaminase	1.3
SO_1796	<i>lon</i>	ATP-dependent protease La Type I	1.2
SO_3599	<i>cysP</i>	Sulfate and thiosulfate binding protein	1.2
SO_3737	<i>cysI</i>	Sulfite reductase [NADPH] hemoprotein beta-component	1.2
SO_1670	<i>fahA</i>	Fumarylacetoacetase	1.2
SO_3577	<i>clpB</i>	Chaperone protein ClpB (ATP-dependent unfoldase)	1.2
SO_0618	<i>astA</i>	Arginine N-succinyltransferase	1.2
SO_1127	<i>dnaJ</i>	Chaperone protein	1.2
SO_2017		Thioredoxin domain-containing protein EC-YbbN	1.2
SO_2903	<i>cysK</i>	Cysteine synthase	1.2
SO_2279	<i>ilvI</i>	Acetolactate synthase large subunit	1.1
SO_0406	<i>trxA</i>	Thioredoxin	1.1
SO_2728	<i>htpX</i>	Protease	1.0
SO_2018	<i>adk</i>	Adenylate kinase	1.0
SO_A0057		Uncharacterized	1.0
SO_0704	<i>groEL</i>	Heat shock protein 60 kDa family chaperone	1.0
Distinct, downregulated DEGs in WT in response to As			
SO_0278	<i>argG</i>	Argininosuccinate synthase	-1.8
SO_1560	<i>pstS</i>	Phosphate ABC transporter, substrate-binding protein PstS	-1.7
SO_3714		ABC transporter, substrate-binding protein (cluster 2, ribose/xylose/arabinose/galactose)	-1.6
SO_2251		Uncharacterized	-1.5
SO_2005		DksA-type zinc finger protein	-1.5
SO_4592		Uncharacterized	-1.5
SO_1753		Uncharacterized	-1.5
SO_2144		Putative NO-binding heme-dependent sensor protein	-1.4
SO_2124	<i>cheR-I</i>	Chemotaxis protein methyltransferase CheR	-1.3

SO_2067	<i>hisI</i>	Phosphoribosyl-AMP cyclohydrolase/ Phosphoribosyl-ATP pyrophosphatase	-1.3
SO_1813		Putative DNA competence protein, ComEA	-1.3
SO_1748		Putative monoheme cytochrome c	-1.2
SO_3629		Putative N-terminal BluF domain containing protein	-1.2
SO_3134	<i>dctP</i>	TRAP-type C4-dicarboxylate transport system, periplasmic component	-1.2
SO_0544		Sensory box histidine kinase	-1.2
SO_4714		Putative ABC-type amino acid uptake system substrate-binding component, PAAT family	-1.2
SO_2123		Methyl-accepting chemotaxis protein	-1.1
SO_2882		Uncharacterized protein, YeaG	-1.1
SO_1830		GGDEF domain protein	-1.1
SO_2795		27 kDa antigen Cfp30B	-1.1
SO_2366		Response regulator	-1.1
SO_3502		Conserved hypothetical protein 2001	-1.0
SO_3040		Mobile element protein	-1.0
SO_2109		L, D-transpeptidase	-1.0
Distinct, upregulated DEGs in $\Delta bpfA$ in response to As			$\Delta bpfA$+As vs $\Delta bpfA$
SO_3145	<i>etfB</i>	Electron transfer flavoprotein, beta subunit	1.3
SO_3021	<i>trpD</i>	Anthranilate phosphoribosyltransferase	1.2
Distinct, downregulated DEGs in $\Delta bpfA$ in response to As			
SO_1673		Outer membrane protein W precursor	-1.9
SO_0490		Transcriptional regulator	-1.6
SO_0581		Uncharacterized	-1.5
SO_2093	<i>hypB</i>	[NiFe] hydrogenase nickel incorporation-associated protein, HypB	-1.5
SO_A0155		Uncharacterized	-1.5

SO_4520		Putative oxygen-independent coproporphyrinogen III oxidase	-1.5
SO_2092	<i>hypC</i>	[NiFe] hydrogenase metallocenter assembly protein, HypC	-1.5
SO_2099	<i>hoxK</i>	Quinone-reactive Ni/Fe-hydrogenase small chain	-1.5
SO_1911		Oxidoreductase, short-chain dehydrogenase/reductase family	-1.4
SO_A0156		Uncharacterized	-1.4
SO_1415		Transcriptional regulator, AcrR family	-1.4
SO_0940		Transcriptional regulator	-1.4
SO_A0154		Copper/silver efflux RND transporter, membrane fusion protein, CusB	-1.4
SO_4053		Methyl-accepting chemotaxis sensor/transducer protein	-1.3
SO_1218	<i>deoA</i>	Thymidine phosphorylase	-1.3
SO_3549		Repair of Iron Centers di-iron protein	-1.3
SO_4157		Tetrathionate reductase two-component response regulator	-1.3
SO_0902	<i>nqrA-1</i>	Na(+)-translocating NADH-quinone reductase subunit A	-1.3
SO_3682		Uncharacterized	-1.2
SO_4730	<i>hemN</i>	Coproporphyrinogen III oxidase, oxygen-independent	-1.2
SO_2767	<i>asnB</i>	Asparagine synthetase [glutamine-hydrolysing]	-1.2
SO_0027		Protoporphyrinogen IX oxidase, oxygen-independent, HemG	-1.2
SO_3422	<i>yfiA-2</i>	Ribosome-associated inhibitor A	-1.2
SO_2098	<i>hyaB</i>	Quinone-reactive Ni/Fe-hydrogenase large chain	-1.1
SO_3538	<i>hlyU</i>	Transcriptional activator, HlyU	-1.1
SO_4155		Tetrathionate reductase sensory transduction histidine kinase	-1.1
SO_3059		Formate hydrogenlyase transcriptional activator	-1.1
SO_3681		Universal stress protein family	-1.1
SO_4805		Putative adhesin (Adhesin motif)	-1.1
SO_0843		LysR family regulatory protein Ssed_0896	-1.1
SO_3553		Putative sulfate permease	-1.1

SO_4591	<i>cymA</i>	Membrane anchored tetraheme cytochrome c, CymA	-1.1
SO_4452	<i>moaA</i>	GTP 3',8-cyclase	-1.1
SO_1428		Outer membrane protein	-1.1
SO_4062	<i>psrA</i>	Polysulfide reductase PsrA, molybdoperterin-binding subunit	-1.1
SO_0970		Periplasmic fumarate reductase, FccA	-1.1
SO_A0157		Uncharacterized	-1.1
SO_3787		Uncharacterized	-1.0

3.3.4 DEGs in $\Delta bpfA$ in comparison with WT

A total of 566 DEGs were observed in $\Delta bpfA$ in comparison with WT, of which 329 were upregulated and 237 were downregulated (Figure 3.3A). Interestingly, 48 out of the 50 most upregulated DEGs are megaplasmid-borne (Figure 3.3B, Table 3.3), suggesting potential *bpfA* interaction with chromosomal genes (Figure 3.2A). In $\Delta bpfA$, 6 DEGs (locus tags: SO_A0157, SO_A0156, SO_A0154, SO_A0158, SO_A0155, SO_A0153) form part of a gene cluster for the CzcA efflux system that controls the periplasmic metal homeostasis. The CzcA efflux system was termed in reference to its specificity to cobalt, zinc, and cadmium, but was also shown to interact with silver, nickel, and copper [224]. With the coverage of a wide range of metals by the CzcA efflux system, its upregulation in $\Delta bpfA$ implied the need to increase the management of metal content in the periplasm when BpfA is lacking. A group of 14 genes (locus tags: SO_A0095, SO_A0096, SO_A0141, SO_A0001, SO_A0170, SO_A0019, SO_A0016, SO_A0018, SO_A0017, SO_A0006, SO_A0169, SO_A0129, SO_A0171, SO_A0168) was upregulated and they have been annotated for functions in replication, recombination, and repair, including those encoding insertion sequence (IS) elements ISSo9 for resolvase, recombinase, transposase, and nucleotidyltransferase. The IS elements have been reported to play an important role in genetic transposition between the chromosome and megaplasmid in *S. oneidensis* for modulating its functional needs [225]. DEGs annotated for cellular defence roles (locus tags: SO_A0022, SO_A0023, SO_A0004, SO_A0003, SO_A0002, SO_A0122, SO_A0149), such as the type II restriction-modification system and the toxin-antitoxin system (*higA*, *higB*) were also upregulated, suggesting an induction of regulating genetic material exchanges, and cellular persistence during stress response in *S. oneidensis*. In general, these genes are asserting their recruitment for protective and adaptive roles when BpfA is lacking.

In contrast to the upregulated DEGs, all 50 most downregulated DEGs are chromosomal (Figure 3.3B, Table 3.3). Most are annotated for non-essential or energy-consuming functions. For example, genes *coxA* and *coxB* codes for aa3-type cytochrome c oxidases were downregulated. They have low affinity to oxygen and usually upregulated in aerobic conditions. Reports have documented their upregulation for enhancing energy production during nutrient-limited condition [226], or as a mechanism to mitigate oxidative stress [227]. Their downregulation in $\Delta bpfA$ indicate a probable a loss to engage this coping mechanism under BpfA deletion. It is also noted

that the TonB-dependent uptake system is downregulated. It is an energy-consuming uptake system for substrate-binding siderophores, which usually function to scavenge extracellular iron [228]. In this case, its downregulation may be needed to prevent excess introduction of free iron that can add to the already oxidative stressed cells [229]. In cases of As toxicity, managing cellular iron content is necessary because of As capability to release iron from iron-storage protein, ferritin [230]. Downregulation of these processes exhibited *S. oneidensis* competency in narrowing to essential scopes in order to retain cellular homeostasis.

Figure 3.3 DEGs associated with *bpfA* loss. (A) Total number of DEGs in $\Delta bpfA$ and As-treated $\Delta bpfA$ after comparison to respective untreated WT and As-treated WT controls. (B) Distribution of 50 most upregulated or downregulated DEGs from genomic or megaplasmid. (C) Venn diagram of overlapped upregulated (left) or downregulated (right) DEGs from $\Delta bpfA$ and As-treated $\Delta bpfA$ data.

3.3.5 Impact of As on DEGs associated with *bpfA* loss

To evaluate the impact of As on the DEGs associated with *bpfA* loss, common DEGs in the untreated $\Delta bpfA$ and As-treated $\Delta bpfA$ were determined. Common DEGs between these groups consist of 46 upregulated and 30 downregulated DEGs (Figure 3.3C, Table 3.3). It is noteworthy that subjection of As stress on $\Delta bpfA$ resulted in repressive effect on the shared upregulated DEGs, with 35 of them displayed a lowered expression fold change by at least 2-fold. The CzcA efflux system components are repressed 3 to 7-fold when treated with As. The observed repression may be an avenue to rebalance between the need for metals such as copper, cobalt, zinc and nickel to serve as cofactors to enzymes such as the metallo-beta-lactamase protein (SO_A0149), a zinc-dependent hydrolase responsible for antibiotic degradation, and to avoid metal toxicity by their disruptive interaction with cellular thiol compounds such as beneficial antioxidant, glutathione [231]. Therefore, the CzcA efflux system repression reflected as a necessary recalibration of the stress response to the oxidative damage induced by As exposure. Similarly, *cusB*, encoding for a membrane fusion protein, CusB, in copper/silver efflux RND system was expressed 3-fold lesser when treated with As. CusB functions as a connector between a transmembrane protein (CusA) to an outer membrane protein (CusC) for the extrusion of free copper and silver ions. Unlike CusB, CusA expression is similar regardless of As treatment, and CusC is not differentially expressed. Reduction of *cusB* expression suggests a higher intracellular retention of copper ions, perhaps serving a similar purpose as the decreased expression of the CzcA efflux system.

Likewise, alleviation of 13 shared downregulated DEGs by at least 2-fold greater suggested the need for functions in maintaining cellular physiology. DNA polymerase III subunit epsilon (SO_2855), a proofreading 3'-5' exonuclease important for editing function, is expressed 5-fold greater in As-treated $\Delta bpfA$. Its elevated expression indicated a recruitment of its function in managing mismatch repairs in *S. oneidensis* genetic information.

Table 3.4 Comparison of 50 most upregulated or downregulated DEGs associated with *bpfA* loss, and As treatment. Data derived from comparative analyses between untreated $\Delta bpfA$ against untreated WT, and As-treated $\Delta bpfA$ against As-treated WT. DEGs selected based on fold-change expression difference ≥ 2 , with p-value ≤ 0.001 .

Upregulated DEGs in response to <i>bpfA</i> loss			Expression (Log ₂)	
Gene ID	Gene name	Gene function	$\Delta bpfA$ vs WT	$\Delta bpfA$ +As vs WT+As
SO_A0172		Site-specific recombinase, resolvase family	5.5	3.3
SO_A0007		Uncharacterized	4.8	3.0
SO_A0164		Alcohol dehydrogenase	4.6	3.2
SO_A0173		Uncharacterized	4.6	n.s.
SO_A0157		Putative component of CzcA efflux system gene cluster	9.8	7.6
SO_A0156		Putative component of CzcA efflux system gene cluster	9.5	7.4
SO_A0154	<i>cusB</i>	Copper/silver efflux RND transporter, membrane fusion protein	9.2	7.5
SO_A0110		Putative lipoprotein	9.2	7.5
SO_A0158		Putative periplasmic protein of CzcA efflux system gene cluster	8.9	7.2
SO_A0127		Uncharacterized	8.7	7.5
SO_A0155	<i>cusF</i>	Copper efflux pump periplasmic metallochaperone	8.6	5.9
SO_A0112		Putative lipoprotein	8.4	7.0
SO_A0115		Putative lipoprotein	8.4	8.0
SO_A0153	<i>cusA</i>	Copper/silver efflux RND transporter, transmembrane protein	8.3	8.3
SO_A0161		S-(hydroxymethyl)glutathione dehydrogenase	8.3	7.3
SO_A0095	<i>parA</i>	Chromosome (plasmid) partitioning protein	8.1	6.5
SO_A0096	<i>parB</i>	Chromosome (plasmid) partitioning protein	8.1	6.9
SO_A0100		Predicted metal-dependent hydrolase with the TIM-barrel fold	7.9	7.7
SO_A0022	<i>higB</i>	Toxin	7.9	6.6

SO_A0141		Uncharacterized	7.5	6.6
SO_A0004		Type II DNA modification methyltransferase	7.5	7.0
SO_A0160		S-formylglutathione hydrolase	7.4	6.6
SO_A0106		Methyl-accepting chemotaxis sensor/transducer protein	7.4	6.6
SO_A0023	<i>higA</i>	Antitoxin	7.2	6.7
SO_A0140		Rhodopsin domain-containing protein	7.1	6.2
SO_A0099		Amidohydrolase domain protein	7.1	6.5
SO_A0114		Outer membrane protein A precursor	7.0	5.9
SO_A0001	<i>repA</i>	Replication protein	6.7	6.4
SO_A0170		ISSod9, DNA-invertase	6.7	5.8
SO_A0122		Protein co-occurring with molybdenum cofactor biosynthesis protein B	6.7	5.2
SO_A0019		ISSod9, DNA-invertase	6.5	5.1
SO_A0150		Putative lipoprotein	6.4	5.5
SO_A0003		Putative type II restriction endonuclease	6.3	5.3
SO_A0006		ParA-like protein	6.0	5.0
SO_A0149		Metallo-beta-lactamase superfamily protein	5.7	4.5
SO_A0165		Transcriptional regulator, LysR family, in formaldehyde detoxification operon	5.6	4.8
SO_A0167		ISSod9, DNA-invertase	5.4	4.2
SO_A0016		ISSod9, DNA-invertase	5.3	4.2
SO_A0139		Uncharacterized	5.3	4.7
SO_A0018		ISSod9, DNA-invertase	5.2	3.8
SO_A0017		ISSo9, nucleotidyltransferase domain-containing protein	5.2	4.1
SO_A0182		Uncharacterized	5.1	3.9
SO_A0132		Transcriptional regulator Winged helix family	4.9	3.5
SO_A0169		ISSo9, nucleotidyltransferase domain-containing protein	4.9	4.0
SO_A0129		IS3 family transposase	4.8	3.7

SO_A0171		CEP19-like protein	4.7	3.6
SO_1519	<i>ykgF</i>	Predicted L-lactate dehydrogenase, Fe-S cluster-binding subunit	4.7	4.3
SO_1518		Predicted L-lactate dehydrogenase, hypothetical protein subunit SO1518	4.6	4.6
SO_A0168		ISSo9, nucleotidyltransferase domain-containing protein	4.6	4.2
SO_A0002		Putative type II restriction-modification system activator	4.5	3.9
SO_1520	<i>ykgE</i>	Predicted L-lactate dehydrogenase, Fe-S oxidoreductase subunit	4.3	4.2
SO_1048	<i>cidB</i>	CidA-associated membrane protein	2.6	3.9
SO_0761	<i>glnK</i>	Nitrogen regulatory protein P-II	4.3	3.6
SO_A0131		Uncharacterized	4.3	3.5
Downregulated DEGs in response to <i>bpfA</i> loss				
SO_1813	<i>comEA</i>	DNA competence protein	-3.5	-2.0
SO_3520	<i>fimT</i>	Type IV fimbrial biogenesis protein	-3.3	n.s.
SO_3373		Uncharacterized	-3.2	-2.0
SO_3134		TRAP-type C4-dicarboxylate transport periplasmic component	-3.2	n.s.
SO_2119		Protein phosphatase with response regulator receiver protein	-3.2	n.s.
SO_0115		Transposase	-3.1	-1.8
SO_3629		N-terminal BluF domain-containing protein	-2.9	-1.5
SO_0277		Ornithine carbamoyltransferase	-2.9	n.d.
SO_3447		Transposase	-2.9	-1.7
SO_2117		Methyl-accepting chemotaxis transducer domain protein	-2.8	-1.8
SO_0278		Argininosuccinate synthase	-2.8	n.d.
SO_0279		Argininosuccinate lyase	-2.8	n.d.
SO_0276		N-acetylglutamate kinase	-2.8	n.d.
SO_1361		3-deoxy-7-phosphoheptulonate synthase	-2.8	-1.9
SO_4530		Transposase	-2.7	-1.8
SO_3040		Transposase	-2.7	-1.7

SO_0116		Transposase	-2.7	n.s.
SO_0182		Uncharacterized	-2.6	-1.9
SO_1362		Chorismate mutase I	-2.6	-1.6
SO_4609		Cytochrome c oxidase polypeptide III	-2.6	-1.6
SO_4317	<i>bpfA</i>	T1SS secreted agglutinin RTX	-7.5	-6.9
SO_2857	<i>actP</i>	Acetate permease, cation/acetate symporter	-5.5	-4.5
SO_2858		Cation/acetate symporter	-5.1	-4.2
SO_2855		3'-5' exonuclease domain of the epsilon subunit of DNA polymerase III	-4.9	-2.5
SO_1900	<i>prpE</i>	Propionyl-CoA synthetase	-4.6	-3.4
SO_1971		AMP-dependent long-chain-fatty-acid--CoA ligase	-4.5	-3.6
SO_1484	<i>aceA</i>	Isocitrate lyase	-4.3	-4.2
SO_3704		PHA biosynthesis protein	-4.2	-4.3
SO_2856		Putative nucleotidyltransferase, CBS domain-containing protein	-4.1	-2.9
SO_1483	<i>aceB</i>	Malate synthase	-4.0	-4.0
SO_2743	<i>acsA</i>	Acetyl-CoA synthetase	-4.0	-3.1
SO_4466		Methyl-accepting chemotaxis sensor/transducer protein	-3.9	-2.6
SO_1748		Monoheme cytochrome c	-3.8	-2.8
SO_0349	<i>yifB</i>	ATP-dependent serine peptidase S16 family	-3.6	-2.2
SO_2054	<i>frmA</i>	S-(hydroxymethyl)glutathione dehydrogenase	-3.5	-3.6
SO_0180	<i>phyH</i>	Phytanoyl-CoA dioxygenase	-3.3	-2.2
SO_1510		Mobile element protein	-3.2	-2.9
SO_3106	<i>sapSH</i>	Extracellular serine alkaline protease	-3.2	-3.6
SO_0184		Microcystin-dependent MdpB family protein, Phage tail collar domain	-3.1	-2.8
SO_3331		ParA-like protein	-3.1	-3.8
SO_4606	<i>coxB</i>	aa3-type cytochrome c oxidase subunit II, periplasmic domain	-3.1	-2.2
SO_4607	<i>coxA</i>	aa3 type cytochrome c oxidase subunit I	-3.1	-2.1

SO_1241		Mobile element protein	-2.8	-2.5
SO_0185		Microcystin-dependent MdpB family protein, Phage tail collar domain	-2.8	-2.5
SO_0867		Collagenolytic serine protease	-2.7	-3.0
SO_0186		Microcystin-dependent MdpB family protein, Phage tail collar domain	-2.7	-2.6
SO_1822		TonB-dependent receptor	-2.6	-3.0
SO_4608	<i>ctaG</i>	Cytochrome c oxidase assembly protein, copper delivery to Cox1	-2.6	-2.2
SO_0187		Serine protease, subtilase family	-2.5	-2.1
SO_4480		Aldehyde dehydrogenase	-2.5	-2.4
SO_3332		Transcriptional regulator, CopG family	-2.3	-3.3
SO_0344		2-methylcitrate synthase	-2.3	-3.2
SO_0345		Methylisocitrate lyase	-1.6	-3.1
SO_2278		Acetolactate synthase small subunit	-2.1	-2.7
SO_2279		Acetolactate synthase large subunit	-1.7	-2.7
SO_1700		Putative lipoprotein	-1.9	-2.7
SO_0343	<i>acnD</i>	2-methyl citrate dehydratase Fe-S dependent	-2.2	-2.6
SO_2055	<i>frmB</i>	S-formylglutathione hydrolase	-1.8	-2.6
SO_1701		Uncharacterized	-1.5	-2.6
SO_0865	<i>csgA</i>	Major curlin subunit	-2.2	-2.5
SO_1770	<i>garK</i>	Glycerate kinase	n.s.	-2.5
SO_0762		Isochorismate hydrolase family protein	-2.4	-2.3
SO_0866	<i>csgB</i>	Minor curlin subunit	-2.1	-2.3
SO_1771	<i>grtP</i>	D-glycerate transporter	-2.3	-2.2
SO_3433	<i>nlpD</i>	Murein hydrolase activator	-1.7	-2.2
SO_0346	<i>prpR</i>	Propionate catabolism operon transcriptional regulator of GntR family	n.s.	-2.2
SO_2002		Uncharacterized	-1.7	-2.2
SO_2854		Uncharacterized	-1.9	-2.1

SO_4706		Malate synthase-related protein	-2.5	-2.1
SO_0342	<i>prpF</i>	2-methylaconitate isomerase	-2.0	-2.1

n.s. not significant (p-value >0.001)

3.4 Conclusions

Comparison of WT and $\Delta bpfA$ transcriptomes under arsenic stress (Figure 3.4) indicate that regardless of *bpfA* deletion, the core expression for As tolerance in *S. oneidensis* are mainly driven by the ars-detoxification system, and maintenance of cellular physiology by TyrR, a master regulator of metabolic processes in *S. oneidensis*. To cope with As toxicity, energy-consuming metabolic enzymes and low energy efficient metabolic pathways were repressed. With *bpfA* loss, *S. oneidensis* exhibited an inability to express an equivalent panel of genes observed in WT, which encodes for molecules with protective roles for cellular homeostasis. The $\Delta bpfA$ was limited to minimal capacity for metabolites biosynthesis and energy metabolism. Nevertheless, this inadequacy was compensated with alternative expression of megaplasmid-borne genes for controlling cellular metal content, genetic information modulation, genetic flux regulation, and cellular persistence. Downregulation of genes for unnecessary functions and energy-consuming systems exhibited *S. oneidensis* selective nature to maintain cellular preservation. The As toxicity in $\Delta bpfA$ displayed both repression on the upregulated DEGs and alleviation of the downregulated DEGs by at least 2-fold difference, indicating *S. oneidensis* capacity to recalibrate its stress response to suit a condition its subjected to. Henceforth, this study has shown the potential transcriptional influences that *bpfA* may have in *S. oneidensis* as an insight to how a key mediator for biofilm formation can facilitate microbial tolerance during a toxin stress.

Chapter 4 . Engineering *S. oneidensis* biofilm matrix to display ArsR for arsenic sorption

4.1 Introduction

S. oneidensis, an environmental organism [23] with no known pathogenicity is used for biofilm matrix engineering. BpfA, an EPS component of *S. oneidensis* biofilm matrix is chosen since it is the most abundant protein in the biofilm matrix [232] and the biofilm formation is shown to be dependent on it [24]. To test the modified biofilm matrix efficiency in contaminant removal, As is chosen as the model inorganic since it is a known contaminant in groundwater sources [186]. To pursue the interest in engineering *S. oneidensis* biofilm matrix to display As-binding modules, the *arsR* gene is selected for genetic fusion to chromosomal *bpfA* to aim for genomic expression of fused recombinant protein comprising of BpfA-ArsR. In comparison to other As-binding proteins, ArsR is a well-studied cytoplasmic As-responsive transcriptional repressor known to possess high specificity [233] and affinity for As(III) at concentration as low as 10^{-15} M [234]. The mode of As-binding by ArsR through As(III) interaction with the sulfhydryl groups of its cysteine residues Cys32, Cys34, and Cys37 [235], bypasses the prior need to oxidize As(III) into the more adsorptive As(V). Moreover, ArsR is a 13 kDa protein (117 residue), making it ideal for fusion to the intended protein carrier, BpfA. Several studies have utilized ArsR to develop As-sensitive whole cell-based biosensors [236] and cell surface display for As bioaccumulation [237, 238], but there is yet any study to display ArsR within a biofilm matrix. The ArsR presentation in the *S. oneidensis* biofilm matrix would be facilitated through the expression of BpfA during *S. oneidensis* transition into its biofilm state. Technically, when fused to BpfA, ArsR is expected to be transported together via the type 1 secretion system (T1SS) [24, 199] towards the extracellular space.

4.2 Materials and methods

4.2.1 In silico analyses of ArsR

To determine the *arsR* sequence to be used for this study, *in silico* analyses were performed. The number of *arsR* loci per bacterial species of interest were extracted from Joint Genome Institute Integrated Microbial Genome (JGI IMG) and NCBI Nucleotide databases of curated and annotated nucleic acids sequence data. Table 4.1 lists the reference points for the species

of interest that were selected for their *arsR* information. The nucleotide sequences were translated using freeware EMBOSS Transeq and the protein sequences were aligned using Clustal Omega Multiple Sequence Alignment.

Table 4.1 Reference point for species of interest selected for *arsR* information

Species name	Strain / Plasmid	IMG Genome ID	NCBI Accession Number
<i>Escherichia coli</i>	K-12	2513020041	NC_000913.3:3648528-3648881
<i>Staphylococcus aureus</i>	pI258	-	NC_013319.1:c17941-17627
<i>Bacillus subtilis</i>	168	-	NC_000964.3:579541-579876
<i>Pseudomonas aeruginosa</i>	PAO1	2636416113	NC_002516.2:2506614-2506964
<i>Shewanella oneidensis</i>	MR-1	637000258	NC_004347.2:553908-554252
<i>Comamonas testosteroni</i>	I2	2556793030	-
<i>Comamonas testosteroni</i>	WDL7	2648501372	-

4.2.2 Bacterial strains, culture conditions and plasmids

Bacterial strains and plasmid used for this study are listed in Table 4.2. Both *E. coli* (DH5 α , K-12) and *S. oneidensis* were cultured in Luria-Bertani Miller (Difco) (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) medium for approximately 16 h as overnight cultures at 37°C or 30°C, respectively. When required, an amount of 50 μ g/ml kanamycin was added to Luria-Bertani broth or agar to induce selection pressure. Every confirmed bacterial stock was stored in 25% glycerol at -80°C. Modified M1 medium (MM1) (30 mM HEPES, 7.5 mM NaOH, 28.04 mM NH₄Cl, 1.34 mM KCl, 1.09 mM NaH₂PO₄, 0.68 mM CaCl₂, trace amounts of mineral, vitamins, and amino acids, and supplemented with 10 mM sodium lactate) [195, 196] at pH 7 was used for biofilm growth of the *S. oneidensis*.

Table 4.2 List of bacterial strains and plasmids

Bacterial strain	Relevant characteristics	Reference
<i>E. coli</i> DH5 α	Strain for gene cloning procedure	[239]
<i>E. coli</i> K-12	Model organism for CXCXXC motif in ArsR	[240]
<i>S. oneidensis</i> MR-1	Environmental, facultative anaerobe Model host for BpfA-ArsR fusion	[195]
Plasmid		

pUC57	Amp ^R , Km ^R , <i>oriV</i> (ColE1), <i>bpfA</i> , <i>aggC</i>	[241]
pEX18ApGW	Amp ^R , <i>sacB</i> , <i>oriT</i> (ColE1)	[242]
pSB3C5- <i>cre</i>	Cp ^R , <i>lacI</i> , p15A ori	[243]

4.2.3 In-frame fusion of *bpfA* to *arsR*

To generate recombinant protein of fused BpfA-ArsR, an in-frame fusion of *bpfA* to *arsR* (Figure 4.1) was done using a 3' end portion (989 bp) of *S. oneidensis* genomic *bpfA* (NC_004347.2:4495162-4503468), with its stop codon removed before it is linked to the full length *arsR* of *E. coli* K-12. Overlapping PCR was performed to genetically fuse *bpfA* and *arsR* using the list of primers in Table 4.3. These primers were designed to include restriction enzyme cut sites EcoRI at the 5' end of *bpfA*, and SpeI at 3' end of *arsR* for subsequent ligation to pUC57. Both *bpfA* and *arsR* fragments were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the recommended manufacturer's PCR protocol.

Table 4.3 Primers for overlap PCR fusion of *bpfA*-*arsR*

Primers	5' → 3'
bpfA_F1	CCGGAATTCCCAGTAATCAGGATACAGCGCAAAGCACTGCTGACTTC CAAGTTACCGTTCT
bpfA- arsR_K12 _R1	GCAAGAATTTTGAACAATTGGATGGGTAACAGAAATGACATGATGG GTACAGGATCGACT
arsR_K12 _F2	ATCCTGTACCCATCATGTCATTTCTGTTACCCATCCAATTGTTCAAAA TTCTTGC
arsR_K12 _R2	GGACTAGTACTGCAAATGTTCTTACTGTCCCCGGAACAGTT

4.2.4 Preparation and transformation of fused *bpfA*-*arsR* into *S. oneidensis* for chromosomal integration via homologous recombination

The in-frame fused *bpfA*-*arsR* fragments were intended for chromosomal integration in *S. oneidensis* through homologous recombination process (Figure 4.2). A downstream gene of

the *S. oneidensis* genomic *bpfA*, *aggC* (NC_004347.2:4495162-4503468), was used as the second flanking homologous arm. The PCR fused product (*bpfA-arsR*) (Figure 4.1) was digested with *EcoRI* and *SpeI* at 37°C for 1 h before purified using Wizard® SV Gel and PCR Clean-Up System (Promega). It was then ligated to *EcoRI*/*SpeI* digested pUC57 that contains kanamycin resistance gene (*kmR*) and a 5' end portion (308 bp) of *aggC*. Overnight ligation using the T4 DNA ligase (Thermo Scientific) was done at 22°C for 2 h, followed by 16°C. The ligation product was later transformed into DH5α through heat shock at 42°C for 30 s, recovered at 37°C for 1 h in LB broth, before plated onto 50 µg/ml kanamycin supplemented LB agar for colony formation under 37°C.

Figure 4.1 Preparation of *bpfA-arsR-kmR-aggC* for homologous recombination in *S. oneidensis*. PCR products (A) *bpfA* and (B) *arsR* were fused (C) using overlapping primer pairs (Table 4.3) before it is ligated to pUC57 (D) for combination to *kmR* and flanking homology arm, *aggC* via *EcoRI*/*SpeI* sites. (E) The combined *bpfA-arsR-kmR-aggC* fragment is excised at *EcoRI*/*HindIII* sites for ligation to suicide plasmid, pEX18ApGW.

Colonies positive for the plasmid were selected through colony PCR using Taq polymerase (Thermo Scientific) and primer pairs for *bpfA-arsR* (*bpfA_F1*, *arsR-K12_F2*). PCR products were electrophorized on 1% agarose gel at 120 V for 45 min, and ethidium bromide stained for at least 15 min before viewed using Gel Doc XR+ Gel Documentation system (Bio-Rad). The positive DH5α recombinants were cultured for plasmid extraction (FavorPrep™ Plasmid DNA Extraction Mini Kit, Favorgen) and the complete fragment of interest (*bpfA-arsR-kmR-*

aggC) were obtained through EcoRI/HindIII digestion. The fragment is ligated to EcoRI/HindIII digested suicide plasmid pEX18ApGW, and subsequently transformed into DH5 α . The resulted *bpfA-arsR-kmR-aggC* sequence was verified by single pass DNA sequencing (1st BASE), and EcoRI/HindIII digestion for correct fragment size. Plasmid extraction (pEX18APGW-*bpfA-arsR-kmR-aggC*) was performed for eventual electroporation into *S. oneidensis* (Figure 4.2).

Figure 4.2 Homologous recombination in *S. oneidensis* for chromosomal insertion of fused *bpfA-arsR*. The prepared pEX18ApGW-*bpfA-arsR-kmR-aggC* was electroporated into *S. oneidensis* to obtain double-crossover recombinant through 2-step selection of kanamycin and sucrose pressures. The kanamycin resistance was removed via cre-lox system to generate final recombinant lacking antibiotic selection marker. Targeted chromosomal integration was assessed by PCR with primer pairs (*bpfA* chr_F, *aggC* chr_R) flanking the modified genomic region.

The extracted pEX18ApGW-*bpfA-arsR-kmR-aggC* was mixed in 0.2 cm gap cuvettes containing sucrose-prepared competent *S. oneidensis* for transformation using Gene Pulser Xcell™ electroporation system (Bio-Rad) set at 2.5 kV. The electroporated cells were immediately recovered with excess amount of LB and incubation at 30°C for at least 2 h before plated onto 50 μ g/ml kanamycin incorporated LB agar for colony formation under 30°C. Colonies positive for plasmid were then subjected to 15% sucrose in LB agar to exclude plasmid-harboring cells but selects those with chromosomally integrated *bpfA-arsR*-

kmR-aggC. Kanamycin-resistant colonies were picked for genomic DNA extraction using PureLink Genomic DNA Mini Kit (Thermo Scientific) and subsequently, PCR verified (Q5 polymerase, NEB) using primers *bpfA chr_F* (5'- ACGGTAGGCAAAGATGGCACTG -3') and *aggC chr_F* (5'- ACTGCACGGTCAGATTCGAGGTCAA -3') that are upstream and downstream, respectively, to the chromosomal integration site. Removal of the integrated *kmR* was performed via cre-lox system (pSB3C5-*cre*) through 0.2 mM IPTG induction. Colonies negative for *kmR* were selected through comparison of viability in LB with or without kanamycin. Recombinants negative for *kmR* were incubated for 8 passages at 37°C in MM1 to induce complete plasmid exclusion.

4.2.5 Growth and biofilm formation assessment

Overnight cultures of wild-type (WT) and recombinant (M) in LB were washed twice in MM1 before diluted into starting OD₆₀₀ 0.05 for both growth curve and biofilm formation assessment. All assessments were performed in at least triplicates, and in MM1 supplemented with 10 mM sodium lactate. Cultures for growth curve profiles were incubated in 30°C at 200 rpm, and the readings were measured at 600 nm using spectrophotometer (Shimadzu).

Static biofilm of WT and M were cultured in 96-well plate format in 30°C over 5 days in fed-batch mode by replenishing half of the used medium daily. Their biofilm amount was quantified using crystal violet (CV) staining. Briefly, the total growth was measured at 600 nm using microplate reader (Infinite M200 Pro, Tecan), the used medium was removed, and washed twice with distilled water to exclude non-adhering cells. The biofilms were stained with 0.1% CV for 20 min. After removal of the CV stain, the plates were washed 6 to 8 times with distilled water to remove excess stain. The remaining CV stain was dissolved in 100% absolute ethanol (Fisher Scientific) for 15 min, ensured homogenous mix, and transferred them into fresh plates before measuring the CV stain at 550 nm. The CV stain data at 550 nm was normalized against their respective growth data at 600 nm.

Biofilm formation was also tested under hydrodynamic condition at 2 ml/h flow rate controlled by peristaltic pump (Masterflex® L/S, Cole-Parmer). Inoculated cultures in 15 cm silicone tubing with inner diameter of 3 mm (96400-16, Cole-Parmer) was allowed attachment for 2 h prior subjecting it to the flow stress. Biofilm was grown for 7 days before removing the used medium within and flushed the remaining biofilm with 3 ml 0.1 M NaOH.

Harvested biofilm was heated at 80°C for 1 h, before measuring the total protein using Qubit Protein Assay (Invitrogen).

4.2.6 Gene expression analysis by reverse transcription PCR (RT-qPCR)

To determine the expression levels of *bpfA*, *bpfA-arsR*, and *arsR* in accordance with the analyzed growth curve (section 4.2.5), the WT and M cultures were sampled at their onset (4 h) and late (8 h) exponential phase. Harvested samples were immediately mixed in 2 volumes of RNeasy Protect Bacteria Reagent (Qiagen) before extracting their total RNA using QIAGEN RNeasy Mini Kit (Qiagen) according to the recommended manufacturer's protocol. Total RNA samples were quantified using Qubit™ RNA HS Assay kit (Invitrogen), and their quality ratios assessed using NanoDrop 2000 (Thermo Fisher Scientific). Equivalent amount of 63 ng total RNA of each sample was reverse transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad). An amount of 1 µl cDNA was used for qPCR in StepOnePlus Real-Time PCR System (Thermo Scientific) using KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems). Primers targeting *bpfA*, *bpfA-arsR*, *arsR* and reference gene are as listed in Table 4.4. Experiments were performed in three biological replicates. The relative gene expression data was derived as stated:

Normalization to reference gene: $Ct^{ref} - Ct^{sample} = \Delta Ct$

Relative expression against reference gene: $2^{\Delta Ct}$

Table 4.4 Primers for RT-qPCR analyses

Primers	5' → 3'
bpfA_F	GGAGCGACTAATAATGCGGGT
bpfA-R	AGGTCGACAGGGGTAGGTTG
bpfA-arsR_F	GTCGACCTTGAGGTGAAACTA
bpfA-arsR_R	CCTTCGCGCTGGATGAGTAATA
rpoB_F	AGCGAAGAAATCACGGCTGA
rpoB_R	CCCTTCACTTCTGCACCGAT

4.2.7 Extracellular protein analysis

To determine if the BpfA-ArsR is expressed extracellularly as part of the *S. oneidensis* biofilm matrix, total EPS was extracted for protein mass spectrophotometry analyses.

Similarly, the biofilms of WT and M were grown within 15 cm silicone tubing with inner diameter of 3 mm (96400-16, Cole-Parmer) for 7 days at 2 ml/h flow rate controlled using peristaltic pump (Masterflex® L/S, Cole-Parmer). The biofilms were flushed out gently into 15 ml tube, spun at 3000 rcf for 5 min at 4°C to remove used medium, and weighed for the approximate total biomass weight. Cation exchange resins (Amberlite® HPR1100, Sigma) were prepared in sterile conical flasks at 70 g per g of biomass, and washed thrice with 30 ml 0.9% NaCl on stirrer plate at 4°C. The biofilm samples were gently resuspended in 5 ml 0.9% NaCl before transferred to the washed resin resuspended in 25 ml 0.9% NaCl. Total EPS extraction was allowed for at least 1 h on stirrer plates at 4°C. The supernatant was decanted into fresh 50 ml tube for debris removal at 12000 rcf for 20 min, 4°C. Samples were passed through 0.2 µm filter (Millipore). Salt content was removed through dialysis tubing (Snakeskin™ 3.5K MWCO, Thermo Fisher Scientific) with 4 rounds of distilled water change over 24 h. The samples were frozen at -80°C before lyophilized in freeze-dryer system (FreeZone 4.5 Plus, Labconco) for 48 h, and then dissolved in 1.5 ml distilled water. The total protein was quantified using Qubit Protein Assay (Invitrogen). Protein samples were resolved on gradient SDS-PAGE, and later stained with Coomassie Blue. Gel portion of entire protein amount was excised and analyzed by protein mass spectrometry (School of Biological Sciences, Nanyang Technological University).

4.2.8 Quantification of As sorption

Sorption of As was assessed by two methods of which the biofilms were grown either on filter discs or in packed-bed biofilm reactors. Tests were performed in three biological replicates. Overnight cultures of WT and M in LB were washed twice in MM1 before diluted into final starting OD₆₀₀ 0.05 and 1 for filter disc and packed-bed biofilm reactor methods, respectively.

Inoculation of 50 ml culture volumes onto 47 mm mixed cellulose 0.45 µm filter discs (Millipore) were contained in 250 ml filter units (Nalgene™ Rapid-Flow™, Thermo Scientific) designed with its airflow outlets shut. The biofilms were grown for 7 days in fed-batch mode of replenishing half of its medium every 48 h. The used medium was then removed and washed once with either MM1 without carbon source at pH 5 or 7. To deprotonate potential sulfhydryl binding sites, all biofilm samples were reduced with 10mM DTT for 1 h, after which, they were washed twice with MM1 at the required pH for tests. The

As-sorption tests were performed with 10 ml MM1 spiked with 60 µg/l NaAsO₂ (Sigma-Aldrich), which was let dripped into 50 ml collection tube. Each filtrate was then diluted in 9 parts of distilled water for ICP-MS analysis of total As.

Biofilms inoculated in packed-bed biofilm reactors consisting of 5 sponge cubes (1x1 cm³) stacked in 5 ml syringe. Inoculations were allowed for 2 h attachment before supplying MM1 at a flow rate of 3 ml/h, which is controlled by peristaltic pump (Masterflex® L/S, Cole-Parmer). Biofilms were grown for 5 days before spiking the medium with 160 µg/l NaAsO₂ (Sigma-Aldrich). Effluents were collected over 70 h and passed through 0.2 µm filter (Millipore) before diluted in 9 parts of distilled water for ICP-MS analysis of total As.

The amount of removed As was calculated based on $C_i - C_t$, where C_i is the supplied initial As concentration, and C_t is the As concentration in the sampled effluent.

4.2.9 Statistics

All the experiments were carried out in triplicate. Statistical differences were analyzed via a Student t-test. P values below 0.05 suggest the differences are statistically significant.

4.3 Results and discussion

4.3.1 Different forms of As-binding motifs in ArsR

Comparison of the ArsR sequences from different sources revealed different forms of As-binding motifs (Figure 4.3). CXCXXC, the frequently reported motif for metal binding, and CXC, an alternative double Cys motif, were observed in 5 of the analyzed 7 bacterial species. In *C. testosteroni* I2 and WDL7, neither CXCXXC nor CXC was present. Binding of As to motifs without Cys residues might be because of the presence of hydroxyl-containing Ser residues [235].

The CXCXXC motif was presumed to provide better As binding than the CXC motif, as it forms stable triple-helix bundle when interacting with As(III) in aqueous solution [244]. The triple-helix bundle formed by CXCXXC in ArsR strengthens the metal-ligand bond through a trigonal-pyramidal thiolate coordination, which attributes its specificity for As(III) when challenged for displacement in presence of other metal(loid)s [245] within its hydrophobic interior of the trigonal thiolate geometry. The *arsR* sequence of *E. coli* K12 was selected for

fusion to *bpfA* of *S. oneidensis* because of its potential for optimal As-binding when displayed in the biofilm matrix.

E.coli_K-12	-----MSFLLPIQLFKILADETRLGIVLLLSSELGE--L CVC CDLC TALDQSQPKISRHLA 52
S.aureus	-----MSYKELSTILKILSDPSRLEILD-LSCGE--L CAC DLLEHFQFSQPTLSHHMK 51
B.subtilis_168	MDETKSELLRKYEQKFALADQKRLEIMYELCQRGK--T CVC DLTEIFEVTQSKLSYHLK 58
PAO1	-----MPSPAIEVFKCLADETRVRATLLIVDQGE--L CVC ELMCALADSQPKISRHLA 50
MR-1	-----MQPVTFFKALADETRLKCLLLIQREGE--L CVC ELMAALSEIQPKVSRHLA 49
I2	-----MQEAQVIRSLSALAHEARLRVFRALVVAGPEGLTPSALAQQLGIAPNALSFHLK 54
WDL7	-MYLKMQEANVIRSLSALAHEARLRVFRALVVAGPEGLTPSALAEQLGIAPNALSFHLK 59
E.coli_K-12	LLRESGLLLDRKQKGWVHYRLSPHIPAWAAKIIDEAWRCEQEKVQAIVRNRLARQNCSGDS 112
S.aureus	SLVDNELVTTTRKDGKHKWYQLNHAILEDIIQNLNIINTSNQR-----CVCKN----VK 100
B.subtilis_168	ILLDANLITKETKGTWSYYDLNDEEVNGLL-----SEELCCIFRK----KG 100
PAO1	QLRSAGLLDDRQGWVYRLNPALPAWIHEVLQVTLRANGDWLQADAARLRDMDGRPQR 110
MR-1	QLKKAGLLVDRRQGWVFYRINAELSPWCQQVLARTCDDNDIFLQDNLRLNLCQMGRPER 109
I2	ELFHAELVSQERQGRNVLYRAAFPIMNGLLGYLTENCCQGAA-----CTPDA 101
WDL7	ELFHADLVTQERQGRNVLYRAAFPVMNDLLAYLTENCCQGTV-----CTDDA 106
E.coli_K-12	KNICS- 117
S.aureus	SGDC-- 104
B.subtilis_168	EGDCC- 105
PAO1	ASACCQ 116
MR-1	AKACC- 114
I2	AALCHC 107
WDL7	VASCQC 112

Figure 4.3 Protein sequence alignment of ArsR across selected bacterial species. The highlighted As-binding motifs indicate the presence of triple Cys residues (CXCXXC) (yellow), double Cys (CXC) (blue) residues, or absence (not highlighted).

4.3.2 Growth analyses of WT and *bpfA-arsR* strain

Growth profiles of both the WT and the recombinant (M) are shown in Figure 4.4A. Both strains displayed similar growth latency and exponential phase until at the 6.5 h upon which the recombinant proceeded with longer exponential phase from 7.5 h onwards, but WT began to display growth deceleration. Their similarity in lag until mid-exponential phases indicate an equivalent capacity for their growth initiation. The difference in their exponential phase duration may be a result of the lengthened recombinant BpfA-ArsR, which may indicate perturbation to BpfA conformation crucial for its mediating biofilm formation. At 8 h onwards, a ring of aggregates was noted in the WT cultures but were absent in the recombinant cultures (Figure 4.4B). This suggests that the difference in recombinant having a longer exponential phase may not necessarily be due to it yielding higher biomass, but the probability of BpfA losing ability to mediate similar aggregates when fused with ArsR. Evaluation of their total protein at determining time points of their growth curves indicate

their comparable generation of biomass (Figure 4.4C). Unlike the 1.6-fold difference in their growth curves, their compared total protein amounts at 14 h reduced to a closer gap of 1.1-fold difference. Therefore, this affirmed the difference in the exponential phase period to be potentially associated with BpfA-dependent aggregate formation rather than biomass yield.

Figure 4.4 Growth curves and biomass yield of WT and recombinant (M). Comparisons between WT and M (A) growth curves. (B) Ring of biofilm (red arrow) formed in WT but absent in M from 8 h onwards. (C) Total protein amounts at selective time points (0, 3, 6, 8, 12, 14 h) during the growth curve analyses to represent biomass amount. Experiments performed in three biological replicates.

4.3.3 Expression of *bpfA*, *arsR*, *bpfA-arsR*

The RT-qPCR data showed the absence of the *bpfA-arsR* transcript for the WT and the positive expression of *bpfA-arsR* in the recombinant (M), confirming the transcriptional activity of the chromosomally integrated *bpfA-arsR* in the recombinant. (Figure 4.5). Comparable *bpfA* expression levels in WT were observed at the early- and late-exponential phases. The expression of *bpfA* was reported to be higher in the stationary phase [199]. The expression of *bpfA* in the recombinant is consistent with the previous report and the increased *arsR* expression at the late exponential phase suggests its co-expression with *bpfA*. The *bpfA* expression in the recombinant strain was about 1.5-fold of that in the WT. Expression of *bpfA* in *S. putrefaciens* was found to be regulated by the transcriptional repressor, FlrA [246]. The role of FlrA was found to be influenced by its interaction with c-di-GMP synthesized by oxygen-dependent diguanylate cyclase, DosD. Hence, under aerobic growth conditions, a high c-di-GMP level is expected to be available, which may increase its binding to FlrA, causing the disruption to FlrA blockage function at the *bpfA* promoter region. The presence

of hypoxic microenvironments in the WT aggregates could have resulted in a lower *bpfA* expression in the WT in comparison to the recombinant.

Figure 4.5 Expression of recombinant *bpfA-arsR* gene in WT and recombinant (M). Quantitative PCR analyses for *bpfA*, *arsR*, and fused *bpfA-arsR* expression in WT and M at their early- (4 h) and late- (8 h) exponential phase (Figure 4.4A). Experiments performed in three biological replicates. The symbol ‘*’ denotes p-value <0.05.

4.3.4 Expression of BpfA-ArsR in biofilm matrix

To support this transcriptional activity results, the total EPS of WT and the recombinant strain were extracted to determine the presence of BpfA-ArsR as exoproteins in their biofilms. Protein mass spectrometry analyses of their total bound EPS confirmed the presence of BpfA. Despite the high expression of *bpfA-arsR* shown using qPCR, there was a lack in ArsR detection. Protein mass spectrometry analyses involve trypsin digestion which could have reduced ArsR into small peptides sizes that may be missed out for detection. Alternatively, since BpfA was found to exist as the loosely-bound fraction of EPS [25], its abundance may be more in the supernatant than the extracted cell surface. Nevertheless, the results imply that the addition of ArsR to the C-terminal of BpfA does not affect the Type I secretion module needed for its extracellular translocation via T1SS. Taken together, these results signify positive transcriptional and translational activities of *bpfA-arsR*, and its translocation extracellularly to serve as a biofilm matrix constituent.

4.3.5 Biofilm formation

Biofilms grown in a fed-batch mode showed a lower biofilm amount in the recombinant strain than WT (p-value <0.05) (Figure 4.6A). Under hydrodynamic conditions with a continuous feed (Figure 4.6B), total protein evaluated from biofilms grown in silicone tubing also resulted in lower amount in recombinant than WT (p-value <0.05).

In the absence of agitation that was applied in the growth curve analyses (section 4.3.2), the recombinant strain displayed similar competency in forming biofilms like in WT, thus, suggesting its reduced ability to form aggregates when under agitation stress. This is likely resulted from the perturbation of BpfA folding to mediate biofilm formation. The proximity of ArsR fused to BpfA may have caused steric hindrance for its proper folding. Fusion of an amyloid protein constituent of *E. coli* biofilm, CsgA, to a metal-binding domain was shown to improve in Congo Red binding with increasing length of linker region in between the two protein modules [167]. Moreover, BpfA is a repeats-in-toxin (RTX) adhesin which consists of two domains (A and B) [24]. In RTX adhesin protein architecture, domain A contains tandem repeats believed to be responsible in facilitating the extension of domain B, which is a ligand-binding region that mediate its function as an adhesin [247]. Therefore, with insufficient spacing when fused to a passenger module at its C-terminal end, their proximity could have led to the inability of domain B in efficient adherence.

Figure 4.6 Biofilm evaluation in WT and recombinant (M). Biofilms amount grown under (A) fed-batch mode measured using crystal violet stain (OD₅₅₀) normalized against total growth (OD₆₀₀), and under (B) continuous feed in silicone tubing were measured for their total protein. Experiments performed in three biological replicates. The symbol ‘*’ denotes p-value <0.05.

4.3.6 As sorption analyses

Given that both WT and the recombinant strain displayed capability in forming biofilms, they were tested for As sorption. This work focused on As(III) removal from aqueous solution since As(III) is considered as more toxic and mobile than the precipitative As(V) [13]. In aqueous solution of pH < 9, As(III) exists as uncharged and undissociated species, H_3AsO_3 [248], hence, making it difficult for removal. Reports are contradictory on the pH influence for As sorption. Studies demonstrated optimal As sorption either at near neutral pH to high pH [249, 250] or at low pH [251, 252] since protonated surfaces can provide more positively charged sites for immobilizing the anionic As. Since the pH of groundwater systems is generally 6 to 8.5, As sorption in WT and recombinant were tested at pH 7. This is then compared to pH 5 to determine if higher protonated surfaces increase their As sorption capacities.

Figure 4.7 As sorption in WT and recombinant (M). As sorption capacity was tested in WT and M biofilms grown on (A) cellulose filter discs at pH 5 and 7, and in (B) continuous feed system in packed-bed biofilm reactors at pH 7. Experiments performed in three biological replicates. The symbol ‘*’ denotes p-value <0.05.

In Figure 4.7A, biofilms of WT and the recombinant strain grown on filter discs displayed comparable capability of As sorption at pH 5 and 7 (p-values >0.05). When compared with WT, the As sorption at pH 7 by the recombinant strain showed a higher As sorption capability (2.0±0.6-fold; p-value <0.05).

The pH of 7.0 was used to evaluate As sorption by the WT and the recombinant biofilms in a continuous feed system. The WT biofilm displayed a rapid As sorption in the first 8 h, after

which it reached a saturation point (Figure 4.7B). In contrast, the recombinant biofilm displayed a steady increase in As sorption up to the 22 h. Over 70 h, WT showed maximal As sorption of 11.7 ± 0.2 nmol/mg protein, while the recombinant achieved the As sorption capability of 28.7 ± 2.4 nmol/mg protein, which is approximately 2-fold higher (p-value <0.05) of that by the WT. These results suggest that displaying ArsR in the biofilm matrix is a promising approach to enhancing biofilm-mediated As sorption.

4.4 Conclusions

In summary, this chapter achieved to exemplify the feasibility of engineering biofilm matrix for bioremediation purposes. This was demonstrated through genetically modifying an EPS constituent of *S. oneidensis*, BpfA, to carry a functional peptide, ArsR, which is specific for binding to As(III). The aim was to generate a recombinant that expresses and translocate fused BpfA-ArsR extracellularly to form a part of its biofilm matrix for As(III) immobilization. The chromosomally integrated *bpfA-arsR* in the recombinant strain showed active transcriptional and translational expression. The recombinant biofilms displayed ~2-fold higher As sorption capability than the WT biofilms. This study demonstrated the potential of engineering biofilm matrix for bioremediation.

Chapter 5 . Development of synthetic biology toolkits for engineering biofilm matrix

5.1 Introduction

Chromosomal modification performed in Chapter 4 allows stable, plasmid-free expression of the recombinant protein, BpfA-ArsR, but expression of the modified BpfA is limited to only 1 chromosomal copy in *S. oneidensis*. Plasmid systems offer multiple copies expression of a recombinant gene. This Chapter aims to develop plasmid systems for the tunable BpfA-ArsR expression in biofilm matrix.

Plasmid construction to include *bpfA* (8.3 kbp) was reported to be challenging due to its large size, and its complex content of repeat domains proven to be an issue for cloning procedures [24, 199]. Specialized cloning vectors such as cosmid and bacterial artificial chromosome can be utilized for large inserts [253] , but these are mostly optimized for expression in the classical model organism, *E. coli*. For expression in *S. oneidensis*, a plasmid vector that is competent in countering its native restriction-modification system [254] is necessary for efficient expression of the introduced recombinant DNA.

With an aim to facilitate the molecular cloning procedures, this study explores the feasibility of truncating BpfA down to its essential genetic component that maintains the *S. oneidensis* biofilm formation. Smith et al. has demonstrated how a repeats-in-toxin (RTX) adhesin, LapA (520 kDa), can be truncated to act as a protein cargo to carry cadmium binding domain by retaining its N-terminal retention module and C-terminal secretion module [255]. Since LapA functions as an adhesin, exclusion of its large protein domains means a loss of the adhesive role. This Chapter aims to design a BpfA truncate that retain its primary role as an RTX adhesin, so as to display the As-binding protein, ArsR, as a part of the biofilm matrix. To date, there has yet any attempt in truncating BpfA, a similar RTX adhesin, thus, paving an avenue to concise this large protein for engineering biofilm matrix.

5.2 Materials and methods

5.2.1 Bacteria strains, culture conditions and plasmids

Bacterial strains and plasmids used for this study are as listed in Table 5.1. Both *E. coli* (DH5 α , K-12) and *S. oneidensis* (WT, $\Delta bpfA$) were cultured in Luria-Bertani Miller (Difco)

(tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) medium for approximately 16 h as overnight culture at 37°C or 30°C, respectively. When required, an amount of 50 µg/ml kanamycin or 30 µg/ml chloramphenicol was added to Luria-Bertani broth or agar to induce selection pressure. Every confirmed bacterial stock was stored in 25% glycerol at -80°C. Modified M1 medium (MM1) (30 mM HEPES, 7.5 mM NaOH, 28.04 mM NH₄Cl, 1.34 mM KCl, 1.09 mM NaH₂PO₄, 0.68 mM CaCl₂, trace amounts of mineral, vitamins, and amino acids, and supplemented with 10 mM sodium lactate) [195, 196] at pH 7 was used for biofilm growth of the *S. oneidensis*.

Table 5.1 List of bacterial strains and plasmids

Bacterial strain	Relevant characteristics	Reference
<i>E. coli</i> DH5α	Strain for gene cloning procedure	[239]
<i>E. coli</i> K-12	Model organism for CXCXXC motif in ArsR	[240]
<i>S. oneidensis</i> MR-1	Environmental, facultative anaerobe Model host for BpfA truncates-ArsR expression	[195]
<i>S. oneidensis</i> Δ <i>bpfA</i>	<i>bpfA</i> -deleted <i>S. oneidensis</i>	Section 3.2.1
<i>S. oneidensis</i> (M)	Chromosomally modified recombinant to express BpfA-ArsR	Section 4.2.4
Plasmid		
pUC57	Amp ^R , <i>oriV</i> (ColE1), <i>bpfA</i> truncates	[241]
pYYD	Neo ^R /Km ^R , <i>mob</i> , pBBR1 <i>oriV/Rep</i> ,	[256]
pSB3C5	Cp ^R , <i>lacI</i> , p15A ori	[243]

5.2.2 *In silico* analyses of BpfA and BpfA truncate designs

Designs of the BpfA truncates (SO_4317) were based on its genomic sequence (GenBank NC_004347.2:4495162-4503468) and protein data (UnitProtKB Q8E9G6) to select the specific domains of interest. In reference to BpfA finding in *S. oneidensis* MR-1 by Theunissen et al [24], analyses on its domains were cross-referenced using NCBI Conserved Domains Database (CDD), Protein Families (Pfam), and InterPro. To facilitate the translocation and the transmembrane nature of the BpfA truncates, all designs include both the N- and C-terminals, which serves as the retention and Type I secretion modules, respectively. A library of *bpfA* truncates were termed according to their selected domain components: (1) NC (N- and C-terminals only, 1665 bp), (2) DB (Domain B, 5717 bp), (3)

RF (RapA2/FrhA/Cadherin4, 5127 bp), (4) SR (Serralysin/RTX, 2253 bp). All sequences were processed through JCAT [257] to be codon optimized for expression in *S. oneidensis* MR-1 and re-modified to exclude restriction enzyme cut sites (EcoRI/XbaI/NdeI/SpeI/PstI) for cloning procedure. Confirmed sequences were synthesized by GenScript®, which was supplied as ligated to pUC57 for plasmid amplification in *E. coli* DH5α.

5.2.3 In-frame gene fusion of *bpfA* truncates to *arsR*, and plasmids construction

The library of *bpfA* truncates were initially ligated to 2 different plasmid expression systems before they were genetically fused to *arsR*: (1) pYYD (constitutive expressing system) through SpeI (5'-end) and PstI (3'-end); (2) pSB3C5 (IPTG-inducible expression system) through NdeI (5'-end) and SpeI (3'-end). pSB3C5 will generally be identified as pSB in this Chapter. Similarly, *arsR* of *E. coli* K12 (NC_000913.3:3648528-3648881) synthesized by GenScript® were codon optimized for expression in *S. oneidensis* MR-1, excluded of restriction enzyme cut sites (EcoRI/XbaI/NdeI/SpeI/PstI), and synthesized with an upstream 18 bp or 36 bp flexible linker (iGEM part: BBa_K2868018) consisting of Gly-Ser-Gly-Gly-Ser-Gly. The *arsR* was then ligated to the pSB3C5-*bpfA* truncates through SpeI (5'-end) and PstI (3'-end). To assess the constructs capability in biofilm formation, they were electro-transformed into *S. oneidensis* $\Delta bpfA$, which has its genomic *bpfA* deleted through a procedure that was described in thesis chapter 3 (section 3.2.1). For controls, the WT and $\Delta bpfA$ were electro-transformed with empty vectors, pYYD or pSB3C5. Positive transformants were validated through colony PCR using Taq polymerase (Thermo Fisher Scientific) and sequenced the extracted plasmids using primers: 5'-gcctcaggcatttgagaagcaca-3' (pYYD_F), 5'-agagcggtccaccgacaaacaacagataa-3' (pYYD_R), 5'-ggtcgtaaactactgcataattcgt-3' (pSB_F), 5'-gatggtactttcgaccaacatatcg-3' (pSB_F2), 5'-caatgataaccccaagggaagt-3' (pSB_R).

5.2.4 Growth and biofilm formation quantification

Overnight cultures of wild-type (WT), $\Delta bpfA$, and the recombinants expressing either pYYD or pSB systems grown in LB were washed twice in MM1 supplemented with 10 mM sodium lactate before prepared into final starting OD₆₀₀ 0.05 for both growth curve and biofilm formation assessment. All assessments were performed in at least three biological replicates. Cultures were prepared in MM1 and induced using 0.25 mM IPTG for the pSB group. Cultures for growth curve analyses were grown in 100 ml conical flasks and incubated at

30°C with shaking (200 rpm). The growth readings were measured at 600 nm using spectrophotometer (Shimadzu).

Static biofilm of WT, $\Delta bpfA$ and the recombinants were cultured in a 96-well plate format. Prepared plates were incubated at 30°C over 5 days in fed-batch mode, which involved replacing half of the used medium with fresh feed after every 24 h. Their biofilm amount was quantified using crystal violet (CV) staining. Briefly, the total growth was measured at 600 nm using microplate reader (Infinite M200 Pro, Tecan), the used medium was removed, and washed twice with distilled water before staining the remaining biofilms with 0.1% CV for 20 min. The CV stain was then removed, and the plates were washed 6 to 8 times with distilled water to remove the excess stain. The remaining CV stain was dissolved in 100% absolute ethanol (Fisher Scientific) for 15 min, ensured homogenous mix, and transferred them into fresh plates before measuring the CV stain at 550 nm. Every CV stain data at 550 nm was normalized against their growth data at 600 nm.

5.2.5 As sorption quantification

Sorption of As was assessed in cell suspension and biofilms grown in packed-bed biofilm reactor system. Tests were performed in three biological replicates.

For cell suspension tests, the overnight cultures of WT and the recombinants in LB were washed twice in MM1 supplemented with 10% LB before re-cultured overnight from starting OD₆₀₀ 0.01 and induced with 0.25 mM IPTG. The next day, the cultures were washed twice with MM1 without carbon source before resuspended into OD₆₀₀ 0.1. To deprotonate potential sulfhydryl binding sites, all suspensions were reduced with 10 mM DTT for 1 h, and then washed once with the same MM1 before spiked with 65 ug/l NaAsO₂ for 4 h. Supernatants were collected at 3000 g for 5 min, filtered using 0.2 µm filters and diluted with 9 parts of distilled water for ICP-MS analyses. The harvested cell pellets were boiled in 0.1 M NaOH at 80°C for 1 h, and subsequently quantified for total protein using Qubit Protein Assay (Invitrogen).

Biofilms inoculated in packed-bed biofilm reactors consisting of 5 sponge cubes (1x1 cm³) stacked in 5 ml syringe. Inoculations were allowed for 2 h attachment before supplying MM1 and 0.25 mM IPTG at a flow rate of 2 ml/h, which is controlled by peristaltic pump (Masterflex® L/S, Cole-Parmer). Biofilms were grown for 5 days before spiking the medium

with 160 µg/l NaAsO₂ (Sigma-Aldrich). Effluents were collected over 70 h and passed through 0.2 µm filter (Millipore) before diluted in 9 parts of distilled water for ICP-MS analysis of total As.

The amount of removed As was calculated based on $C_i - C_t$, where C_i is the supplied initial As concentration, and C_t is the As concentration in the sampled effluent.

5.2.6 Sorption competition assay

The As sorption performance of vector-expressed *bpfA-arsR* was tested using pSB DB-arsR expressed in *S. oneidensis* $\Delta bpfA$. Biofilms were grown on 0.45 µm cellulose membrane of 3 cm diameter (Millipore) that was placed in 10 cm diameter petri dish, for 5 days in fed-batch mode at 30°C in MM1 and 0.25 mM IPTG. Half of the used medium was replaced with fresh feed after each 24 h. The biofilm on the cellulose membrane was then transferred onto a 250-ml filter unit (Nalgene) and washed for 6 times with 15 ml 0.85% NaCl, pH 7, to remove extracellular phosphates. The As sorption performance using 150 µg/l NaAsO₂ (Sigma-Aldrich) was challenged with equivalent concentration of 150 µg/l Na₃PO₄ (Sigma Aldrich) dissolved in 0.85% NaCl, pH 7. Effluents were collected by gravity drip and passed through 0.2 µm filter (Millipore) before diluted in 9 parts of distilled water for ICP-MS analysis of total As. An effluent volume of 2 ml was used for phosphate sorption quantification using HACH assay kit for Phosphate (LCK349, HACH).

The amount of removed As was calculated based on $C_i - C_b$, where C_i is the supplied initial As or P concentration, and C_b is the As or P concentration in the sampled effluent.

5.2.7 Statistics

All the experiments were carried out in triplicate. Statistical differences were analyzed via a Student t-test. P values below 0.05 suggest the differences are statistically significant.

5.3 Results and discussion

5.3.1 Truncation of BpfA

Figure 5.1A outline BpfA as a type of RTX adhesin with a C-terminal Type I secretion module for its translocation into extracellular space via type I secretion system, and an N-terminal retention module for transmembrane anchoring as a loosely-bound protein. BpfA is hypothesized to be dependent on the c-di-GMP levels [199] that dictate its fate as either a cell

surface bound exoprotein or cleaved from the cell surface. In reference to the protein databases (section 5.2.2), BpfA comprises of 2 domains (domain A and B). Domain A is largely rich in tandem repeats of bacterial immunoglobulin-like (BIg) domains. Domain B is collectively known as the ligand-binding domain in an RTX adhesin architecture. Closer to the C-terminal, the serralyisin-like metalloprotease and the RTX domains overlap. Both are also calcium-binding domains. While the serralyisin-like metalloprotease is annotated as a potential virulence factor [258, 259], its overlapping with the RTX domain may highly be more functional for proper protein folding. At a lower intracellular calcium concentration, RTX proteins are maintained in unfolded form to facilitate its transportation through the T1SS, after which, upon binding with the higher concentration of extracellular calcium, they fold into their proper conformation [260, 261].

Based on the sequence analyses, BpfA was truncated into 4 designs (Figure 5.1B-E) to determine the domains necessary to maintain *S. oneidensis* biofilm formation. All BpfA truncates were designed to possess the N- and C-terminals. The 4 BpfA truncate designs interplayed the combinations of RapA2, FrhA, Cadherin 4 and RTX since they are annotated for attachment, aggregation, and adhesion. The 4 BpfA truncates are subsequently abbreviated as the following: (1) NC (N- and C-terminals only), (2) DB (the entire of Domain B), (3) RF (RapA2 and FrhA domains), and (4) SR (serralyisin-like metalloprotease and RTX domains). Uncharacterized protein regions located upstream of each selected domain were included as they may have unknown roles or as possible linker regions to separate them for their eventual folding. Domain A was primarily excluded due to their high repeat content that is difficult to be replicated accurately and reviewed to not contribute adhesin properties as domain B [247].

Figure 5.1 Schematics of BpfA truncate designs. The BpfA truncates were designed based on the (A) Full length of BpfA (re-illustrated in reference to Theunissen et al [24]). Protein regions a, b, c, d that are highlighted in grey are uncharacterized. The BpfA truncates are termed as (B) NC, which contains N- and C-terminals, (C) DB, which contains the entire Domain B, (D) RF, which contains Cadherin domains of RapA2 and FrhA, (E) SR, which contains the serralyisin-like metalloprotease and RTX domains. All designs include their upstream uncharacterized protein regions to serve as the spacer between domains.

5.3.2 Growth and biofilm formation of the strains expressing BpfA truncates

Both pYYD and pSB3C5 are vector systems of low to medium copy number and of broad host range plasmids that were proven to express efficiently in *S. oneidensis* [256, 262]. The $\Delta bpfA$ expressing *bpfA* truncates in the constitutive expression vector system (pYYD) exhibited poor biofilm formation capability (Figure 5.2A), suggesting that the constitutive expression of *bpfA-arsR* may be incompatible for high expression in *S. oneidensis*. When expressed in an inducible vector system (pSB3C5), the maximal expression (at least 2-fold higher than $\Delta bpfA$ pSB) was achieved in the presence of increasing IPTG concentration (Figure 5.2B). The declined expression at higher IPTG concentrations (0.5 mM and 1 mM) provide support to the analyzed expression in pYYD, in which the attempt to increase their expression resulted in reduced biofilm yield. Therefore, suggesting a negative regulation on their translation in *S. oneidensis*. Despite the positive validation of the pSB RF construct, no colonies were generated when electro-transformed into *S. oneidensis*, suggesting that

expression of this recombinant gene may have toxicity to the model host. Upon induction by IPTG, pSB NC yielded minimal biofilm that is comparable to $\Delta bpfA$ pSB. This suggests the inability of the pSB NC construct to generate substantial amount of biofilm in $\Delta bpfA$. Therefore, both pSB RF and pSB NC were subsequently excluded from the genetic fusion to *arsR* and the downstream analyses. The pSB SR and pSB DB constructs displayed comparable biofilm quantity to the WT pSB when fused to *ArsR* (Figure 5.2C). This may be attributed to the presence of the flexible linker, which could have improved the protein folding for biofilm formation. Doubling the linker length (2L) and increasing the IPTG concentration did not result in an increase to the biofilm quantity. Therefore, IPTG concentration of 0.25 mM and linker length of 6 amino acid (1L) were confirmed sufficient for the following experiments.

Figure 5.2 Biofilm evaluation in recombinants expressing BpfA truncates. Biofilms were quantified using crystal violet staining in recombinants expressing BpfA truncates in expression vector systems that are (A) constitutive (pYYD) and (B) IPTG-inducible (pSB). (C) Biofilm quantification of selected BpfA truncates (SR & DB) that were fused to *ArsR* with flexible linkers of either 6 amino acids (1L) or 12 amino acids (2L). Crystal violet stains were measured at OD₅₅₀ and normalized against the total growth measurement at OD₆₀₀. Experiments performed in at triplicates.

Since expressing BpfA truncates in inducible vector system is positive for biofilm formation, their growth curves were then compared to the empty vector controls, WT pSB and $\Delta bpfA$ pSB (Figure 5.3) under induction of 0.25 mM IPTG in MM1. Both pSB SR-arsR and pSB DB-arsR displayed similar growth curves to WT pSB that has exponential phase lasted until they began their stationary phase from the 14 h onwards. This significantly differ from the $\Delta bpfA$ pSB, which has 5 h longer exponential phase before reaching its stationary phase from the 19 h onwards. This suggests a higher biomass yield in $\Delta bpfA$ pSB, but with a loss of the adhesin, BpfA, there could be more biofilm or microbial aggregates, which may have resulted in a lower end OD₆₀₀ measurement in WT pSB, pSB SR-arsR and pSB DB-arsR. Recombinants' comparable growth curves to WT are therefore indicative of the BpfA truncates expression, which would require similar metabolic expense as the native BpfA in WT.

Figure 5.3 Growth curves of recombinants expressing BpfA truncates. Growth curves of recombinants expressing SR-arsR and DB-arsR in inducible vector system (pSB) compared to the empty vector controls (WT pSB, $\Delta bpfA$ pSB) under induction of 0.25 mM IPTG in MM1 over 22h. Experiments performed in three biological replicates.

5.3.3 As sorption by BpfA truncate-ArsR-expressing strains

With the shown ability to form biofilms, pSB SR-arsR and pSB DB-arsR were further tested for their As sorption capacity. The biofilms of the BpfA truncate-expressing strains achieved 1.5 to 2.2-fold higher in As sorption than the WT pSB, but modestly 1.3 to 1.7-fold higher than the $\Delta bpfA$ pSB (Figure 5.4A). *S. oneidensis* with stripped off EPS (extracellular polymeric substances) layer was reported to present more sulfhydryl binding sites on its

cellular surface [263]. This could be the reason for the observed high As sorption by $\Delta bpfA$ pSB in comparison to WT pSB. Since the aim of this Chapter is to demonstrate that increasing BpfA-ArsR expression through vector systems can result in higher As sorption, the As sorption in biofilms of the vector-expressed BpfA truncate-ArsR recombinants were then compared to the biofilms of the chromosomally modified recombinant (M) that expresses BpfA-ArsR (Figure 5.4B). In comparison to the WT, the As sorption in the biofilms of the vector-expressed BpfA-ArsR showed an average of 3.6-fold higher (pSB SR-arsR: 45.1 ± 4.1 nmol/mg protein; pSB DB-arsR: 40.1 ± 1.6 nmol/mg protein, p-values <0.05) while the biofilms of recombinant expressing BpfA-ArsR in the chromosome achieved 2.4-fold higher (28.7 ± 2.4 nmol/mg protein, p-value <0.05). Therefore, indicating that the vector-expressed BpfA-ArsR can result in a higher capacity for As sorption in the biofilm matrix than the chromosomally expressed recombinant. In addition, biofilms of the vector-expressed BpfA-ArsR strains exhibited higher As sorption at pH 7 than pH 5, with ~ 1.3 -fold capacity for As sorption than the empty vector controls (Figure 5.4C), thus, this suggests their applicability in water matrices that are generally near neutral pH.

Figure 5.4 As sorption capacity by BpfA truncates. Recombinants expressing SR-arsR or DB-arsR in inducible vector system (pSB) were tested for As sorption capacity (A) and compared to the recombinant expressing BpfA-ArsR in the chromosome (B). (C) pH

influence on As sorption in biofilms were evaluated at pH 5 and 7. Experiments performed in three biological replicates. The symbol ‘*’ denotes p-value <0.05.

5.3.4 Selectivity for As sorption by BpfA truncate-ArsR-expressing strains

The As sorption performances in water sources can be challenged by the presence of interfering factors such as organic matters or other adsorbing anions [264, 265]. Phosphates which is a known chemical analog to As, can bind to the same transport proteins like Pit and PstS [266]. Phosphates can also reduce As sorption in natural water systems through complexation with metal hydroxides like iron(III) hydroxides [267], which is needed at an adequate Fe:As ratio of at least 50 for effective As precipitation and removal.

Theoretically, when a biofilm matrix is engineered to possess functional displays specific to a contaminant, it would function at a high selectivity for that contaminant even in presence of competing adsorbing elements. To test this, pSB DB-arsR, which displayed highest As sorption as biofilms (Figure 5.4B) was chosen to have its As sorption performance challenged with equivalent concentration of phosphate. Despite in presence of phosphate, the resulted As sorption in the biofilms of pSB DB-arsR (13.9 ± 2.1 nmol/mg protein, p-value <0.05) is similar to the biofilms exposed to only As (Figure 5.5), and the P sorption was negligible. Therefore, indicating that a biofilm matrix can have high selectivity for an element when engineered to display binding sites specific for the targeted element.

Figure 5.5 Selectivity for As in biofilms of BpfA truncate-ArsR-expressing strain. The As sorption in the pSB DB-arsR biofilm grown on cellulose membrane were challenged with equivalent concentration of P (150 μ g/l). Experiments performed in three biological replicates. The symbol ‘*’ denotes p-value <0.05.

Conclusions

BpfA was truncated into domains deemed essential to retain its mediation for biofilm formation in *S. oneidensis*. Reducing *bpfA* size enabled its expression in an expression vector of an acceptable size for efficient transformation in *S. oneidensis* that possesses obstructive native restriction-modification system. Constitutive and high induction of BpfA truncates expression resulted in low yield in biofilm formation. Substantial biofilm formed by IPTG-inducible, BpfA truncate-expressing strains allowed a further engineering of it to carry ArsR, As(III)-specific binding module, at its C-terminal end to be displayed as a component in the biofilm matrix for As sorption. When compared to WT pSB, the As sorption by the BpfA truncate-expressing strains were 1.5- to 2.2-fold higher (Figure 5.4A). When compared to the WT, the As sorption by the BpfA truncate-expressing strains were 3.6-fold higher, while the recombinant expressing BpfA-ArsR in the chromosome was 2.4-fold higher (Figure 5.4B). Therefore, implicating that vector-expressed BpfA-ArsR can result higher As sorption than when expressed in the chromosome that is limited to only 1 copy number for BpfA. The biofilms of BpfA-truncate-ArsR-expressing strain were also shown to have high selectivity for As even when challenged with competing element, P (Figure 5.5). This demonstrates how a biofilm matrix engineered to display specific binding modules can exhibit significant affinity for the targeted element, thus, beneficial for application in contaminant removal.

Chapter 6 . Summary and future recommendations

6.1 Summary

Biofilm matrix are naturally sorptive and this thesis hypothesized that it can be engineered for enhanced sorption of a targeted contaminant. Engineering biofilm matrix for environmental application is still minimal in comparison to the extensive efforts in engineering microbial cell surface that focused on transmembrane proteins as anchorage for display of functional peptides. This thesis utilized *S. oneidensis*, an environmentally relevant organism as the model host and its key mediator for biofilm formation, BpfA, as the EPS component for biofilm matrix modification. To test this hypothesis, As was used as the target contaminant since As-contaminated water sources remains a current crisis. Key findings from this thesis work include:

(1) BpfA is involved in the modulation of cellular responses of *S. oneidensis* to As.

Biofilm matrix are generally noted for their physical attributes that contribute protective roles while encapsulating the microbes within the biological structure [27]. For example, its gel-like composition is sorptive to beneficial elements, yet it creates a diffusion barrier against the potential biocidal compounds [103]. Furthermore, key mediators to biofilm formation were often studied in relation to their physical support in providing structure integrity [247]. Instead of focusing on their physical contributions, this Chapter unravels the potential molecular influences that a biofilm matrix may have when the expression of its key mediator is disrupted and subjected to a toxicant stress. In this case, BpfA expression for biofilm formation in *S. oneidensis* and arsenic stress. The global transcriptomic profile comparison between WT and *bpfA*-deleted *S. oneidensis* indicate BpfA to have influences in *S. oneidensis* transcriptional activities on its chromosomal and extrachromosomal (megaplasmid) genetic materials when responding to arsenic stress. With BpfA expression, the transcriptional responses to arsenic stress were observed to be chromosome-borne, but with the loss of BpfA expression, this shifted significantly towards megaplasmid-borne (Figure 3.4). Therefore, implicating BpfA to function more than a key mediator for biofilm matrix structure, but also being involved in modulating the cellular responses of *S. oneidensis* under a toxicant stress.

(2) Modification of chromosomal BpfA to co-express As-binding protein (ArsR) enhances As sorption in *S. oneidensis*.

Majority of the functional displays for specific binding to a targeted element were limited to cellular surface through manipulation of fixed

transmembrane proteins serving as the anchorage to the binding modules displayed in the extracellular space [268]. These design concepts often overlooked the tendency for microbes to form biofilms, thus, cellular surface expression of such engineered proteins may experience lesser contact with the targeted element when embedded in the biofilm matrix. Although biofilm matrix is known to be naturally sorptive [6, 103] due to the attractive charges presented by the EPS (extracellular polymeric substances) components, it is not specific. In this Chapter, a biofilm matrix component such as BpfA, which is an abundant protein in *S. oneidensis* biofilm matrix, was modified to carry a functional protein, ArsR, to be displayed as the As-binding modules in the biofilm matrix. Theoretically, biofilm matrix modification through its abundant component would not only be expressed on the cell surface but also be distributed in the biofilm matrix if they were to be shed off the cellular surface, thus, presenting a higher number of binding sites for the targeted element. Here, the recombinant expressing BpfA-ArsR in the chromosome achieved a ~2-fold higher As sorption than the WT (Figure 4.7B). Hence, demonstrating how a biofilm matrix can increase its sorption for a targeted element when engineered to express specific functional displays.

(3) BpfA truncation facilitates its higher copy number expression in a vector system and the in-frame fusion of BpfA truncates to ArsR results in enhanced As sorption capacity in *bpfA*-deleted *S. oneidensis*. Biofilm matrix modification through manipulation of a gene at the chromosomal level results in a stable expression, but this limits the expression amount due to dependency on the available copy number of the gene in the chromosome. To counter this limitation, higher expression of BpfA-ArsR was attempted through using vector systems that gives multiple copies. To date, expression of full length BpfA in vector systems is a challenge due to its large size (>8 kb) and the high number of repeats, which complicates the accuracy of molecular cloning procedure [199]. This Chapter has successfully expressed BpfA in vector systems through its truncation into domains that retain its function to form biofilms. Similar strategy was reported for LapA truncation to serve as cargo vehicle for functional modules, but the work did not report if the 90% exclusion of LapA domains retain its role as adhesin [172]. BpfA truncation not only facilitated its expression in vector systems, but it also allowed a co-expression with a functional module, ArsR, for specific binding to As. When compared to the WT, the biofilms of vector-expressed BpfA-ArsR recombinants achieved ~3.6-fold higher As sorption, while the recombinant expressing BpfA-ArsR in the chromosome achieved 2.4-fold higher. Hence, demonstrating that vector-expressed BpfA-ArsR can improve the As sorption.

In summary, this work demonstrated that biofilm matrix modification can serve as a biological avenue for enhancement in biofilm-mediated bioremediation. There is an advantage in utilizing biofilm feature for biofilm-mediated contaminant removal since microbes have the tendency to form biofilms in nature. The sorptive nature of biofilm matrix can be engineered into a specific biological tool to target an element. This work has demonstrated that biofilm matrix modification can be carried out through manipulation of its known abundant component such as BpfA in *S. oneidensis*, to co-express a functional passenger protein like ArsR to bind As. Biofilm matrix that is engineered to high specificity to an element can increase its sorption selectivity and reduce sorption interferences from competing adsorbing factors. The significance of this work is that a platform for biofilm matrix modification is developed and its application is not limited to contaminant removal, but also for resources recovery such as metals from the used electronic gadgets or for limited resources such as P.

6.2 Future recommendations

6.2.1 Characterizing BpfA-ArsR in biofilm matrix

In Chapter 4, the protein, BpfA-ArsR, was confirmed for positive expression via RT-qPCR analysis, but the mass spectrometry detected BpfA but not ArsR in the biofilm matrix. The lack of ArsR detection may be due to the cation exchange resin method that focuses on harvesting cell surface bound proteins. This method may have excluded BpfA-ArsR that exists in the loosely-bound EPS fraction, which could mostly be shed into the supernatant during the preparation steps for protein mass spectrometry. Therefore, it is necessary to include both the loosely associated and tightly bound EPS fractions of the biofilm matrix. Similar approach is needed to characterize matrix-associated BpfA-ArsR for the biofilms of the BpfA truncate-expressing strains.

6.2.2 Optimizing expression of BpfA-ArsR

The chromosomal BpfA-ArsR (Chapter 4) has only 1 codon spacing between BpfA and ArsR. The close proximity of them could have interfered with the optimal folding of BpfA, affecting biofilm formation of the engineered strains. This can be addressed by optimizing the linker to provide ample spacing to prevent steric hindrances that may affect their proper folding. Typically, an 18 bp flexible linker consisting of Gly-Ser-Gly-Gly-Ser-Gly is utilized (section 5.2.3), but this may vary depending on protein sizes.

The inducible vector system developed in Chapter 5 is dependent on IPTG. For bioremediation purposes, this is deemed an unattractive vector expression inducer. It is more sensible to construct a vector system that is inducible upon sensing the targeted contaminant itself. In this case, the *arsR* promoter (*Pars*) of *E. coli* K12 or *S. oneidensis* could be the upstream regulator to *bpfA-arsR* expression. Since ArsR is the repressor of *Pars*, upon binding to As(III), the release of *Pars* permits the transcription of *bpfA-arsR*.

6.2.3 Assessing As sorption by engineered biofilms in natural water matrices

The performance of the engineered biofilms displaying ArsR in the biofilm matrix for As sorption was examined in minimal medium to mimic environmental condition such as groundwater. However, it still differed from natural waters in terms of salinity, nutrient, and mineral content. In addition, although ArsR has been reported to be highly specific in binding to As(III), the specificity of the engineered biofilm matrix for As removal needs experimental validation. Determining the host robustness to express the engineered biofilm matrix and evaluating its As sorption in groundwater matrices with the presence of other metal(loid)s would provide a clearer affirmation on the engineered biofilm matrix efficacy and specificity for As removal for real applications.

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