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^{13}C Pathway Analysis of Biofilm Metabolism of Shewanella oneidensis MR-1†

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Biofilm metabolism of *Shewanella* was analyzed via ^{13}C tracing experiments for the first time. The activity of C1 metabolism in the biofilm cells was found to be interestingly higher than that in planktonic cells, which could be related to utilizing C1 metabolites as electron donors when growing *Shewanella* in biofilms.

The biofilms formed by *Shewanella oneidensis* MR-1 has been extensively studied[1-5] and found to play pivotal roles in bioremediation of heavy metals[6-10] and electric power generation[11, 12]. Despite recent discoveries of *Shewanella* metabolism under aerobic and anaerobic conditions using various analytical approaches[13-16], few studies have been accomplished to investigate the metabolic pathway usage in *Shewanella* biofilms. In this study, we applied the ^{13}C pathway analysis, a reliable and well-developed technology[17], to analyze the carbon metabolism of *S. oneidensis* cells derived from biofilm and planktonic growth, respectively. Through the comparison of isotopomer labeling patterns of key proteinogenic amino acids, we found that the C1 metabolism was much more active when growing *S. oneidensis* in biofilms compared to planktonic cells, which could be related to the utilization of C1 metabolites as electron donor by *S. oneidensis* when growing in biofilms. To our best knowledge, this is the first time that the biofilm metabolism of *S. oneidensis* was rigorously determined by isotopomer analysis.

S. oneidensis MR-1 was initially grown in shake flasks with minimal medium containing 3.7 mM [3-^{13}C] sodium L-lactate (Sigma-Aldrich) for two days at 100 rpm, 30ºC. It was then transferred to completely fill up a 140 mL sealed bottle reactor and cultivated under oxygen-limited condition (stirrer at the bottom) at 30ºC. To grow *S. oneidensis* MR-1 in a biofilm, a carbon cloth (2.5 cm×4.5 cm, Zoltek, Panex$^{®}$ 30 Fabric, PW06) was submerged in the medium of the sealed bottle reactor, with titanium mesh (McMaster) to support and titanium wire (Sigma-Aldrich) to bind the cloth tightly on mesh. Duplicate reactors were processed (n=2). When lactate was depleted (<0.01 mM), the minimal medium was refreshed by removing 10 mL medium from the sealed bottle reactor and injecting 10 mL fresh filter (0.22um pore size) sterilized medium. The final concentration of [3-^{13}C] sodium L-lactate was maintained at ~0.8 mM. The biomass of planktonic cells was monitored by OD_{600} using a plate reader (BioTek). The concentrations of lactate and acetate in the sealed bottle reactor were measured by high-performance liquid chromatography (HPLC, Shimadzu), following the method that has been previously developed[18] (Fig.1). It was found that lactate was consumed to produce acetate as the sole fermentation byproduct when growing *Shewanella* in the sealed bottle bioreactor. The declining OD_{600} of the planktonic cells in the reactor indicated that more cells would be grown in the biofilms with the replenishment of the medium.
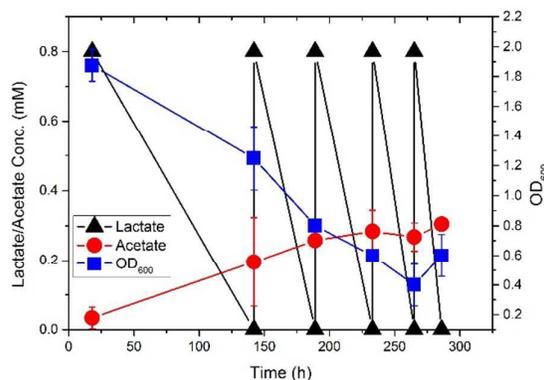
Fig. 1 Fermentation profile of *S. oneidensis* MR-1 in the sealed bottle reactor.

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After five refreshment of the medium, both the planktonic cells and the biofilm cells were collected in duplicate from the liquid culture and the carbon cloth, respectively, followed by isotopomer analysis of proteiogenic amino acids using a previously developed protocol[19-21]. In general, the biomass was hydrolysed using 6 M HCl (24 h at 100 °C). The amino acids were derivatized in 20 µl of tetrahydrofuran and 20 µL of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich). A gas chromatograph (GC2010, Shimadzu) equipped with a SH-Rxi-5Sil column (Shimadzu) and a mass spectrometer (QP2010, Shimadzu) was used for analyzing the labeling profiles of metabolites. Three types of charged fragments were detected by GC-MS for Ala, Gly, Ser, Asp and Glu: the [M-57]$^+$ group (containing unfragmented amino acids); and the [M-159]$^+$ or [M-85]$^+$ group (containing amino acids that had lost an α-carboxyl group). For each type of fragments, the labeling patterns were represented by M0, M1, M2, etc., which were fractions of non-labeled, singly labeled, and doubly labeled amino acids. The effects of natural isotopes on isotopomer labeling patterns were corrected by previously reported algorithms[22].

By feeding [3-^{13}C] lactate as the sole carbon source to *S. oneidensis* under an oxygen-limited culture condition, most alanine molecules were expected to be singly labeled because pyruvate, the direct precursor of alanine, was believed to be mainly synthesized from [3-^{13}C] lactate (Fig 2 and Table 1). To our surprise, 34% alanine molecules were found to be non-labeled in the biofilm cells. As a result of the non-labeled pyruvate produced, we also found a significant amount of non-labeled aspartate and glutamate in the biofilm cells, which were synthesized from the futile cycle and the TCA cycle, respectively, by using the non-labeled pyruvate as the precursor. However, it remains unknown that how the non-labeled pyruvate was synthesized during *Shewanella* growth in biofilms.

Table 1 Mass distribution of detected metabolites for the ^{13}C tracing

Fig. 2 Central carbon metabolic pathways of *S. oneidensis* MR-1 from biofilm and planktonic growth. The ^{13}C and ^{12}C atoms were labeled as filled and empty dots, respectively. The lactate utilization pathways were colored as blue while the C1 metabolic pathways were colored as red. The number on the left side of each isotopomer (green) was the M-57 values of proteinogenic amino acids from the biofilm cells while the number on the right side of each isotopomer (blue) was the M-57 value of proteinogenic amino acids from the planktonic cells. Abbreviations: Ala, alanine; Asp, aspartate; C1, C1 molecule; CIT, citrate; Glu, glutamate; Gly, glycine; OAA, oxaloacetate; OXO, 2-oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; Ser, serine; TCA Cycle, tricarboxylic acid cycle.

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experiments ($n = 2$) [a]

	Biofilm samples		Planktonic samples	
	M-57	M-159/85	M-57	M-159/85
Ala				
M0	0.34	0.35	0.21	0.22
M1	0.59	0.58	0.68	0.70
M2	0.05	0.07	0.09	0.08
M3	0.01		0.02	
Gly				
M0	0.84	0.87	0.85	0.88
M1	0.13	0.13	0.13	0.12
M2	0.03		0.02	
Ser				
M0	0.33	0.39	0.28	0.29
M1	0.58	0.56	0.63	0.66
M2	0.08	0.06	0.08	0.05
M3	0.01		0.01	
Asp				
M0	0.31	0.33	0.23	0.23
M1	0.19	0.25	0.22	0.31
M2	0.22	0.28	0.31	0.31
M3	0.25	0.14	0.21	0.15
M4	0.02		0.02	
Glu				
M0	0.28	0.29	0.19	0.20
M1	0.14	0.16	0.13	0.16
M2	0.19	0.26	0.26	0.33
M3	0.26	0.27	0.31	0.30
M4	0.13	0.03	0.10	0.02
M5	0.01		0.00	

[a] The error in mass distribution from duplicates was <5%.

To our best knowledge, there is only one pathway, C1 metabolism, which could lead to the synthesis of non-labeled pyruvate when growing with [3-^{13}C] lactate[3, 23-25]. Generally, pyruvate could be converted to 3-phosphoglycerate (PGA) through the gluconeogenesis, which is used to produce serine, and further converted into glycine and a C1 molecule (e.g., formate) via C1 metabolism. During the glycine synthesis from serine, the labeled carbon originated from the [3-^{13}C] lactate would get lost as C1 metabolites while glycine became mostly non-labeled, as found in this study (Fig 2). Glycine could be continuously divided into CO_2 and C1 molecule, both of which were non-labeled in this study.

Considering the high reversibility of the C1 metabolism, the non-labeled C1 metabolites could be incorporated with non-labeled glycine to synthesize non-labeled serine (i.e., reversed C1 metabolism), which would then be used to synthesize the non-labeled pyruvate via serine dehydratase (SDH, EC 4.3.1.17)[26, 27]. This is possible in the present study because we grew *Shewanella* in biofilms with very low concentration of [3-^{13}C] lactate (~0.8 mM), which was rapidly consumed for cell growth within 5~10 hours but also produced non-labeled C1 metabolites via the C1 metabolic pathway. Once lactate utilization stage was finished, *Shewanella* growing in biofilms would use the generated C1 metabolites as the secondary carbon source to continue the production of building blocks for cell growth. As a result of the combined effects of lactate utilization and C1 metabolites utilization, the proteinogenic amino acids, such as alanine, aspartate and glutamate, demonstrated a high percentage of non-labeled molecules (34%, 31%, and 28%, respectively). It is also worth noting that although most of detected glycine (84%) is non-labeled, there was 13% single-labeled glycine detected, which could be synthesized from glyoxylate shunt of TCA cycle as that has been discovered in previous studies.[3, 23, 25]

Compared to the isotopomer labeling patterns of the biofilm cells, the non-labeled proteinogenic amino acids (e.g., Ala, Glu, and Asp) were also detected in the planktonic cells of *S. oneidensis* MR-1, which could come from two sources. First, the C1 metabolism could be active in the planktonic cells and generate non-labeled amino acids. Secondly, considering the cohesiveness of the biofilm, some cells could be washed out from the biofilm and become the planktonic cells[28, 29], which brought non-labeled amino acids as detected in the planktonic cells. Indeed, at the end of the fifth medium replenishment, we did observe an increased OD_{600} of the planktonic cells (Fig. 1), which could be attributed to the washout of the biofilm cells. The washout was not observed in early stages since the cells attached on biofilms were not saturated, while in the late stages, the biofilm could no longer attach all the cells and the extra cells were washed out. We also found that the percentages of non-labeled proteinogenic amino acids in planktonic cells were much smaller compared to that in biofilm cells. For example, only 23% of alanine detected in the planktonic cells was non-labeled while 34% alanine was found to be non-labeled in biofilm cells. This is possible since the substrate (i.e., [3-^{13}C] lactate in this study) was more difficult to diffuse into the biofilms, more lactate would be used by the planktonic cells, which makes the lactate utilization metabolism more dominant in the planktonic cells and leads to smaller percentage of non-labeled pyruvate synthesized from C1 metabolism of planktonic cells.

The results of this study have important implications to use *S. oneidensis* MR-1 for energy production. For example, it has been well known that by forming the biofilms, *S. oneidensis* MR-1 could generate electricity from organic carbon substrates[12, 30]. The discovery from this study indicated that the C1 metabolite, especially formate, could serve as the electron donor and generate electricity during the C1 metabolism[24]. Considering the direct connection of biofilm cells and carbon cloth, the electron transfer of biofilm cells should be more active compared to planktonic cells, which could lead to active

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the C1 metabolism in the biofilm cells. The effects on C1 metabolites (e.g., formate) on bioelectricity generation and the exact pathway of carbon flux during bioelectricity generation are currently being investigated and expected to be reported in future. In summary, by applying ^{13}C pathway analysis to investigate microbial metabolism of *S. oneidensis* MR-1 growing in biofilms, we discovered that the activity of C1 metabolism was interestingly higher than that in planktonic cells, which could be related to the utilization of C1 metabolites as electron donor when growing *Shewanella* in biofilms.

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